

Significance of tumor necrosis factor- α and B₂ microglobulin in patients having chronic lymphocytic leukemia

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Abstract

Background and objectives: Tumor necrosis factor- α (TNF- α) is important for the growth and survival of the leukemic cells in B-cell chronic lymphocytic leukemia (B-CLL). B₂ microglobulin (B2MG) is elevated in tumors, it is clinically used for lymphoproliferative diseases, where serum B2MG is related to tumor cell load, prognosis, and disease activity. The aim of this study was to investigate the serum levels of TNF- α and B2MG in B-CLL patients with correlation to relevant haematological data and disease characteristics. **Patients, materials and methods:** The study included 15 newly diagnosed untreated B-CLL patients obtained from the outpatient clinic at the National Cancer Institute in Cairo and 15 ages and sex matched controls. Venous blood samples were obtained from B-CLL and control groups for complete blood count (CBC). Serum was separated for measurement of TNF- α and B2MG levels by ELISA. Bone marrow (BM) aspiration was done to all B-CLL cases. **Results:** The studied B-CLL group consisted of 7 females and 8 males with mean \pm SD age (54.9 ± 11.6 years). The clinical staging according to Rai classification was: 66.7% cases in stage 0-II and 33.3% cases in stage III-IV. There was highly significant elevation of white blood cell count (WBC), absolute lymphocytes in peripheral blood (PB), serum TNF- α and serum B2MG with high significant reduction of haemoglobin (Hb) in B-CLL group when compared with the control ($P < 0.001$). There was significant reduction of platelets and significant elevation of absolute monocytes in PB in B-CLL group ($P < 0.01$ & $P < 0.02$ respectively). There was significant elevation of TNF- α in B-CLL patients with anaemia and B-CLL patients with thrombocytopenia in comparison with B-CLL patients without anaemia and B-CLL patients without thrombocytopenia ($P = 0.02$ and $P < 0.05$ respectively). There was highly significant positive correlation between TNF- α and both absolute monocytes in PB & serum B2MG with inverse highly significant correlation with Hb. A significant positive correlation was found between TNF- α and: WBC, BM lymphocytes and Rai III-IV disease stage with inverse significant correlation with platelets. A significant positive correlation was detected between B2MG and both WBC and absolute peripheral blood lymphocytes ($P = 0.01$) with highly significant positive correlation with Rai III-IV disease stage ($P < 0.001$), while no correlations were demonstrated with the other parameters. **Conclusion:** TNF- α and B2MG are important for the process of leukemogenesis and progression and may serve as bad prognostic markers for B-CLL. On the basis of these observations, therapeutic inhibition of TNF- α and B2MG could be a new strategy of importance in the treatment of B-CLL.

Introduction

Tumor necrosis factor- α (TNF- α) is a cytokine having a peptide structure that possesses pleiotropic biological activities. The active form of TNF- α is a homotrimer having a molecular mass of 53 kd (Le and Vilcek, 1987). The gene for TNF- α has

been localized to the short arm of chromosome 6 (Wingfield *et al*, 1987). TNF- α plays a physiological role in host defense, inflammation, and cell differentiation and a pathological association with diverse conditions such as fever, cachexia,

Significance of tumor necrosis factor- α

septic shock, rheumatoid arthritis, and inflammatory bowel disease (Pisetsky, 2000). Monocytes and macrophages are the main source of TNF- α , although other cell types, in particular, T and B lymphocytes and large granular lymphocytes, have been shown to produce TNF- α upon stimulation (Pawelec et al, 1989). di Celle et al(1996) reported that cytokines produced and released directly by CLL cells such as TNF- α participate in autocrine or paracrine loops and affect CLL cell survival and proliferation.

B-2 Microglobulin (B2MG) is an 11.8 kD protein identical to the light chain of the HLA-A, -B, and -C antigen. B2MG is expressed on nucleated cells, and is found at low levels in the serum and urine of normal individuals. B2MG concentrations are increased in inflammatory diseases, some viral diseases, renal dysfunction, and autoimmune diseases (Evrin and Wibell 1972).

About 40%-60% of patients with CLL are diagnosed in the absence of disease-related symptoms, even with very high numbers of circulating lymphocytes $>100 \times 10^9/l$. Frequently, the presence of lymphadenopathy or an abnormal CBC performed during a routine medical examination is the only reason to consider the diagnosis (Rai and Patel, 1995). The remaining patients may present with weakness, fatigue, night sweats, fever, and may be with or without infections or autoimmune diseases (Sthoeger *et al*, 1993). Physical examination generally reveals non tender, painless, and mobile lymphadenopathy, splenomegaly, or hepatomegaly. Metabolic abnormalities (e.g., hyperuricemia) or mechanical disorders (e.g., airway obstruction) related to the tumor burden, may also be present. Any part of the body, including skin and meninges may be infiltrated by CLL cells; however, such findings are uncommon. Manifestations of bone marrow (BM) involvement, particularly significant anemia (Hb $\leq 11\text{gm/dl}$) or thrombocytopenia (platelets count $<150 \times 10^9/L$), are noted at presentation in 15% of CLL patients (Dighiero, 1993).

In recent years, the biological features classically ascribed to the leukemic B cells of B-CLL have changed. B-CLL cells were

once assumed to derive from immature immunologically incompetent B lymphocytes and to behave as inert cells that divided minimally, died rarely, and thereby passively accumulated to numbers that eventually compromised the patient. It now appears that B-CLL cells derive from mature, antigenically experienced, immunologically competent B lymphocytes and that the leukemic cells from most patients turn over at definable rates suggesting that a genetic abnormality in apoptosis that cannot be overridden by an exogenous signal may not exist. These findings implicate that B-CLL is a dynamic, not passive, accumulative disease (Chiorazzi and Ferrarini, 2003).

The etiology of CLL is unknown. Several lines of evidence suggest a genetic component, such as the increased prevalence of CLL among first-degree relatives, the phenomenon of anticipation, where there is an increased severity and earlier age of onset with each generation and the increased frequency of autoimmune disorders in relatives of CLL patients (Catovsky *et al*, 1997). Environmental factors, such as ionizing radiation, chemicals (benzene and solvents from the rubber industry), and drugs have shown no apparent relationship. There is no single specific cytogenetic abnormality in CLL (Inskip et al, 1993).

Three main phenotypic features present on B-CLL cells to differentiate CLL from PLL (prolymphocytic leukemia) and HCL (hairy cell leukemia). B-CLL cells are positive for B-cell antigens, CD19, CD20, and CD23, and they coexpress CD5 in the absence of T-cell markers. The cells are monoclonal with respect to their expression of either kappa or lambda light chains. Surface immunoglobulin (sIg) is of low density. Other markers more characteristic of NHL (non Hodgkin Lymphoma) or HCL, such as CD10 and CD103, are absent (Kalil and Cheson, 1999).

Rai and Binet staging systems are the most commonly used staging systems in CLL. However, neither system accurately identifies in early stages those patients who will progress from those who will remain indolent. Lymphocytosis alone is not classified by the Binet system, and neither

system includes splenomegaly alone (Zweibel and Cheson, 1998).

Patients, materials and methods

Peripheral venous blood samples collected in sterile test tubes were obtained from newly diagnosed untreated 15 patients having B-CLL during routine diagnostic evaluation at The National Cancer Institute in Cairo. CBC was done by automated cell counter (Sysmex SE 9000). The diagnosis of B-CLL was established based on morphology, the peripheral blood should exhibit an increase in the number of small mature-appearing lymphocytes to $>5,000/\mu\text{l}$ according to the National Cancer Institute-Sponsored Working Group (NCI-WG) published guidelines for the diagnosis for CLL (Cheson et al, 1996) and flow cytometric analysis (CD5+, CD19+, and CD23+). Patient characteristics are illustrated in Table (1). Peripheral blood samples obtained from 15 normal-age and sex matched-healthy donors were used as controls (Samples were collected from control subjects only if the subjects had not had fever within 1 week, were not receiving any medications, were not known to be pregnant, and did not have a history of any chronic or acute illnesses). Serum was separated from each specimen and stored at -20°C until the time of TNF- α & B2MG analyses.

TNF- α levels were assayed using enzyme-linked immunosorbent assay (ELISA) (Quantikine Human TNF- α Immunoassay; R&D Systems, Minneapolis, MN). This assay used a solid-phase monoclonal anti-TNF- α antibody bound to microtiter plates. Unbound protein was removed by washing; a polyclonal anti-TNF- α antibody conjugated to horseradish peroxidase was then added, with excess conjugated antibody removed by further washing. Next, a substrate solution was added; color developed in proportion to the amount of TNF- α bound in the initial step. The reaction was stopped, and the color intensity was quantified by means of a microtiter plate reader at a wavelength of 450 nm. A standard curve was generated

with the use of known concentrations of human TNF- α . The concentration of TNF- α in both patient and control serum samples was obtained from the standard curve. The ELISA has been reported by the manufacturer to measure the total amount of TNF- α present, free TNF- α and TNF- α bound to the soluble receptors (Range of detection: 0.195 ng/ml to 200.0 ng/ml).

The B2MG ELISA test is based on the principle of a solid phase enzyme-linked immunosorbent assay (R&D Systems). Mouse monoclonal anti-B2MG antibody was used for solid phase immobilization (on the microtiter wells). A sheep anti-B2MG antibody was in the antibody-enzyme (horseradish peroxidase) conjugate solution. The diluted test sample was allowed to react first with the immobilized antibody. The sheep anti-B2MG-HRPO conjugate was then added and reacted with the immobilized antigen, resulting in the B2MG molecules being sandwiched between the solid phase and enzyme-linked antibodies. After washing a substrate solution was added, resulting in the development of a blue color. The color development was stopped by Stop Solution, changing the color to yellow. The concentration of B2MG is directly proportional to the color intensity of the test sample. Absorbance was measured spectrophotometrically at 450 nm. Healthy individuals are expected to have B2MG serum or plasma values 0 - 2.0 $\mu\text{g/ml}$.

BM aspirate was done to all B-CLL cases (smear must show $>30\%$ of all nucleated cells to be lymphoid). Although a BM examination is rarely required to make the diagnosis of CLL in general practice, it may be indicated primarily to evaluate response to treatment or to assess normal elements if there is an unexplained anemia or thrombocytopenia (Cheson et al, 1996).

Statistical analysis

Data were expressed as mean \pm SD. Student's t test used to detect the difference. Spearman correlation was tested, a probability (P) value <0.05 was considered significant.

Significance of tumor necrosis factor- α

Results

The studied B-CLL group consisted of 7 females (46.7%) and 8 males (53.3%) with mean \pm SD age (54.9 \pm 11.6

years). The clinical staging according to Rai classification was: 10(66.7%) cases in stage 0-II and 5(33.3%) cases in stage III-IV (table 1).

Table (1): B-CLL patients' characteristics.

Characteristic		
Age (years)	mean \pm SD	54.9 \pm 11.6
Sex: female	n (%)	7 (46.7)
male		8 (53.3)
Rai stage: 0-II	n (%)	10 (66.7)
III-IV		5 (33.3)

Table (2): statistical differences between B-CLL group and control group as regard all studied parameters.

Item	CLL (n=15) Mean \pm SD	Control(n=15) Mean \pm SD	t	P	Significance
WBC $\times 10^9/L$	106.4 \pm 69.1	6.8 \pm 1.2	5.6	<0.001	HS
Hb gm/dl	9.6 \pm 2.99	13.8 \pm 1.6	4.8	<0.001	HS
Platelets $\times 10^9/L$	185.7 \pm 115.6	385.9 \pm 80.7	2.8	<0.01	S
Absol Lymph PB $\times 10^9/L$	98.97 \pm 66.3	2.3 \pm 0.6	5.6	<0.001	HS
Absol Mono PB $\times 10^9/L$	1.02 \pm 1.07	0.3 \pm 0.01	2.6	<0.02	S
BM Lymph (%)	77.2 \pm 17.4	—	—	—	—
Serum TNF- α pg/ml	36 \pm 5	4.3 \pm 1	24.4	0.000	HS
Serum B2MG ug/ml	6.5 \pm 3.8	0.5 \pm 0.5	6.6	<0.001	HS

S: significant

HS: highly significant

WBC: white blood cell count

Hb: haemoglobin

Absol: absolute

Lymph: lymphocytes

Mono: monocytes

PB: peripheral blood

BM: bone marrow

There was highly significant elevation of WBC, absolute PB lymphocytes, serum TNF- α and serum B2MG with high significant reduction of Hb in B-CLL group when compared with the control. There was significant reduction of platelets and significant elevation of absolute PB monocytes in B-CLL group (table 2).

There was significant elevation of TNF- α in B-CLL patients with anaemia (Hb \leq 11gm/dl) (n=10) and B-CLL patients with thrombocytopenia (platelets $<$ 150 $\times 10^9/L$) (n=6) in comparison with B-CLL patients without anaemia (Hb $>$ 11gm/dl) (n=5) and B-CLL patients without thrombocytopenia (platelets \geq 150 $\times 10^9/L$) (n=9) (P=0.02 and P<0.05 respectively).

Table (3): correlation study between TNF- α and all other studied parameters in B-CLL group.

	r	P	significance
WBC $\times 10^9/L$	0.51	<0.05	S
HB gm/dl	-0.8	<0.001	HS
Platelets $\times 10^9/L$	-0.54	<0.02	S
Absol Lymph PB $\times 10^9/L$	0.1	>0.05	NS
Absol Mono PB $\times 10^9/L$	0.69	<0.002	HS
BM Lymph (%)	0.51	<0.05	S
Serum B2MG ug/ml	0.76	<0.001	HS
Rai III-IV	0.54	<0.02	S

There was highly significant positive correlation between TNF- α and both absolute monocytes PB & serum B2MG with inverse highly significant correlation with Hb. A significant positive correlation

was found between TNF- α and: WBC, BM lymphocytes and Rai III-IV disease stage with inverse significant correlation with platelets (table 3).

Table (4): correlation study between B2MG and all other studied parameters in B-CLL group.

		r	P	significance
WBC	x10⁹/L	0.6	0.01	S
HB	gm/dl	-0.2	>0.05	NS
Platelets	x10⁹/L	-0.4	>0.05	NS
Absol Lymph PB	x10⁹/L	0.6	0.01	S
Absol Mono PB	x10⁹/L	-0.3	>0.05	NS
BM Lymph	(%)	-0.1	>0.05	NS
Serum TNF-α	ug/ml	0.76	<0.001	HS
Rai III-IV		0.7	<0.001	HS

Significant correlation was detected between B2MG and both WBC and absolute peripheral blood lymphocytes with highly significant correlation with Rai III-IV disease stage. No correlation was demonstrated with the other parameters (table 4).

Discussion

The results of this study indicated that the TNF- α serum level was increased in patients having B-CLL when compared with the control group (P=0.000). Ferrajoli *et al* (2002) reported significant elevation of plasma TNF- α in B-CLL patients in comparison with normal control. They suggested that TNF- α was involved in the progression of CLL. Mainou-Fowler *et al* (2001) reported significant increases in the TNF- α and IL4 mean levels for B-CLL lymphocytes compared to normal control cells. There was significant variability in the literature regarding the effect of TNF- α on CLL cells. This may be a consequence of the complexity of the TNF- α system in vivo. TNF- α can be active in soluble and cell-bound form and the 2 are in continuous equilibrium (Eugster *et al*, 1996). TNF- α was shown to stimulate, inhibit, or have no effect on CLL cell proliferation. This is not surprising since TNF- α activates cell

survival and cell death mechanisms simultaneously and can influence cell growth by apoptotic, nonapoptotic, and antiapoptotic mechanisms (Alvarez-Mon *et al*, 1993).

Also TNF- α was significantly elevated in both B-CLL patients with anaemia and those with thrombocytopenia (P=0.02 and P<0.05 respectively) when compared with both B-CLL patients without anaemia and those without thrombocytopenia. The anemia and thrombocytopenia in these patients may have been due to extensive bone marrow replacement by CLL but may also be attributed to the direct suppressive effect of TNF- α on the erythroid and thrombopoietic lineages. This is in agreement with the potent inhibitory effect of TNF- α on hematopoiesis in vitro described by (Ferrajoli *et al*, 1993) and with the development of progressive anemia reported in patients who received TNF- α for prolonged time (Ulich *et al*, 1993). Furthermore, Michalevecz *et al* (1991) were able to restore in vitro normal hematopoiesis in 11 out of 15 patients having CLL using anti-TNF- α antibodies. Capalbo *et al* (2002) reported that in B-CLL patients with anemia, the serum levels of TNF- α were significantly higher than in those without anemia and TNF- α serum levels

Significance of tumor necrosis factor- α

had an inverse correlation with hemoglobin levels.

B₂-Microglobulin is a MHC type-1 molecule, and its levels may be regulated, at least in part, by endogenous cytokines. B2MG levels may, therefore, reflect the convergence of several cytokines, as well as other pathways (Fayad *et al*, 2001). The data of this study revealed significant elevation of B2MG in B-CLL patients when compared with normal control (P <0.001). Keating *et al* (1995) reported that B-CLL patients having an elevated TNF- α level also had an increased concentration of B2MG, a poor prognostic factor in lymphoid malignancies. Di Giovanni *et al* (1989) found that B2MG mean value in the CLL group was significantly higher than in the control group. The elevated B2MG may be secondary to increased proliferation of lymphoma cells because of stimulation by cytokines causing an increase in the turnover of the membrane-bound B2MG (Yee *et al*, 1989). Ibrahim *et al* (2001) reported that serum levels of B2MG greater than 4.0 mg/L are adverse prognostic factors in a wide range of lymphoid malignancies, including B-CLL. Fayad *et al* (2001) reported that elevated Rai stage and elevated B2MG correlated with shorter survival in CLL patients.

On the other hand the results of this study showed significant elevation of absolute monocytes in the peripheral blood of B-CLL cases when compared with the control (P <0.02). Monocytosis may be a result and / or a cause which may result in dysregulated cytokine production in B-CLL. These data may suggest that in addition to the B-CLL neoplastic cells, the peripheral blood monocytes may be involved in the release of TNF- α and also, may be involved in the process of leukemogenesis or progression of B-CLL. Anand *et al* (1998) reported dysregulated cytokine production by monocytes from CLL patients. They concluded that the observed defect in cytokine production by functionally impaired monocytes may have an important implication for the immune system of the CLL patients.

In the present study there was highly significant positive correlation between

TNF- α and both serum B2MG & absolute peripheral blood monocytes with inverse highly significant correlation with Hb. A significant positive correlation was found between TNF- α and: WBC, BM lymphocytes and Rai stage III-IV with inverse significant correlation with platelets. B2MG is an important prognostic factor in low and intermediate grade lymphoma and multiple myeloma. Ferrajoli *et al* (2002) reported that TNF- α plasma levels correlated significantly with stage of the disease. Patients with Rai stage III or IV disease had higher TNF- α level. They also reported that TNF- α plasma concentration in the patients with CLL correlated strongly and directly with the serum B2MG levels; mildly with CD38 expression; and slightly with WBC. They also found inverse correlation between TNF- α levels and both HB and platelets values. Adami *et al* (1994) found an increase of TNF- α serum levels in all stages of CLL including stage 0, with a progressive increase in relation to the stage of the disease. In contrast, they reported no correlation between TNF- α levels and WBC counts. The results of the present study may indicate that TNF- α has relevant biological activity and its level can serve as a prognostic factor in patients with B-CLL.

Conclusion:

Tumor necrosis factor- α and B2MG may be involved in leukemogenesis and progression of B-CLL. A better understanding of the molecular biology and immunology of this disease will lead to the rational development of strategies targeted at the underlying mechanisms of this disease. On the basis of these observations, it is possible that inhibition of TNF- α and B2MG in B-CLL patients could have therapeutic importance.

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Significance of tumor necrosis factor- α

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أهمية معامل نخر الورم- α و الجلوبيين الدقيق ب₂ فى مرضى سرطان الدم الليمفاوى المزمن

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يعتبر معامل نخر الورم- α ذى أهمية لنمو و حياة الخلايا السرطانية فى سرطان الدم الليمفاوى المزمن للخلية- ب.

هدف البحث

يهدف البحث إلى فحص وتقييم مستويات معامل نخر الورم- α و الجلوبيين الدقيق ب₂ فى مصل مرضى سرطان الدم الليمفاوى المزمن للخلية – ب مع إضاح علاقتهما ببيانات أمراض الدم المختصة و سمات المرض.

طرق البحث

إشتملت الدراسة خمسة عشر من من مرضى سرطان الدم الليمفاوى المزمن للخلية – ب حديثى التشخيص و لم يسبق علاجهم و المترددى على العيادة الخارجية لمعهد الأورام القومى بالقاهرة. و كذلك إشتملت الدراسة خمسة عشر من الأصحاء و المتوافقين فى السن و النوع كمجموعة ضابطة. و تم سحب عينات من الدم الوريدي من المجموعتين لعمل صورة دم كاملة كما تم فصل المصل لقياس معامل نخر الورم- α و كذلك قياس الجلوبيين الدقيق ب₂ بواسطة تقنية قياس الإمتصاص المناعى للإنزيم المتصل. كذلك تم إجراء بذل لنخاع العظم وفحصه للمجموعة المرضية فقط.

النتائج

إشتملت مجموعة مرضى سرطان الدم الليمفاوى المزمن للخلية – ب على عدد 7 من الإناث و عدد 8 من الذكور وبلغ متوسط أعمارهم 54,9. وقد كانت مراحلهم الإكلينيكية طبقاً لتقسيمه راي كالأتى: 66,7% فى المرحلة II-0 و 33,3% فى المرحلة III-IV. وقد كان هناك زيادة إحصائية هامة فى عدد كرات الدم البيضاء و العدد المطلق للخلايا الليمفاوية و مستويات معامل نخر الورم- α فى المصل و مستويات الجلوبيين الدقيق ب₂ وكذلك العدد المطلق للخلايا وحيدة النواة مع وجود نقص فى الهيموجلوبين و عدد الصفائح فى مرضى سرطان الدم الليمفاوى المزمن للخلية – ب عند مقارنتهم بالمجموعة الضابطة. كذلك أثبت البحث وجود زيادة فى معامل نخر الورم- α فى مرضى سرطان الدم الليمفاوى المزمن للخلية – ب الذين يعانون من فقر الدم و كذلك الذين يعانون من نقص عدد الصفائح الدموية. كما أظهر معامل نخر الورم- α علاقة إيجابية قوية مع العدد المطلق للخلية وحيدة النواة وكذلك الجلوبيين الدقيق ب₂ بينما كانت العلاقة إيجابية مع كل من: عدد كرات الدم البيضاء و عدد الخلايا الليمفاوية فى نخاع العظم و المرحلة المرضية III-IV من تقسيم راي. وعلى الجانب الأخر أظهر معامل نخر الورم- α علاقة سالبة قوية مع الهيموجلوبين. كما أثبت البحث وجود علاقة موجبة بين مستويات الجلوبيين الدقيق ب₂ و كل من عدد كرات الدم البيضاء و العدد المطلق للخلايا الليمفاوية فى الدم.

Significance of tumor necrosis factor- α

الإستنتاج والتوصيات

أظهر كل من معامل نخر الورم α - فى المصل و مستويات الجلوبيين الدقيق ب₂ أهمية فى عملية السرطنة و تقدمها مما قد يجعلهما علامات سيئة لتقييم مرضى سرطان الدم الليمفاوى المزمن للخلية - ب. و بناء على هذه النتائج فإن الإثباط العلاجى لكل من معامل نخر الورم α - فى المصل و مستويات الجلوبيين الدقيق ب₂ قد يكون إستراتيجية جديدة ذات أهمية لعلاج مرضى سرطان الدم الليمفاوى المزمن للخلية - ب.