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Serum Matrix Metalloproteinase-9 and Transforming Growth Factor $ß_1$ in Patients with Acute Leukemia With and Without Extramedullary Involvement: A Comparative Study

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ABSTRACT

A novel functional relationship between matrix metalloproteinase-9 (MMP-9) and the multifunctional cytokine transforming growth factor ß (TGF-$ß_1$) in the control of tumor-associated tissue remodeling has been recently uncovered. The aim of this work was to estimate the serum levels of MMP-9 and TGF-$ß_1$ in patients with acute leukemia (AL) with and without extramedullary involvement (EMI). Twenty seven adult patients with newly diagnosed AL were enrolled: Group I; 10 patients with clinically evident EMI (mean age: 24.6±14.6 years) and Group II; 17 patients without EMI (mean age: 30.7±14.54 years). Ten healthy age-and sex-matched individuals were included as a control group (Group III).

In addition to routine diagnostic work-up and imaging studies, serum MMP-9 and TGF-$ß_1$ were estimated for all patients and controls using the ELISA technique.

The results showed that the mean values of serum MMP-9 and TGF-$ß_1$ were significantly higher in group I compared to group III. However, the mean serum MMP-9 did not differ significantly between group II and group III, although the mean serum TGF-$ß_1$ was significantly higher in group II compared to group III. In the mean time, group I showed significantly higher mean values of MMP-9 and TGF-$ß_1$ compared to group II. In conclusion, serum MMP-9 and/or TGF-$ß_1$ can be added as new biological markers that indicate the presence of EMI in adult patients with acute leukemia. However, the possible roles of these markers in the follow-up and in evaluating patients' response to therapy should be addressed in future studies.

Key Words: *Serum MMP-9 – TGF-$ß_1$ – AL.*

INTRODUCTION

A major hallmark of acute leukemia is the uncontrolled capacity of hematopoietic cells to proliferate and breakdown cell-stroma interactions leading to egress of immature blood cells from the bone marrow (BM) to the peripheral blood (PB) **[1]**. Extracellular matrix (ECM) degrading enzymes are required to breakdown these structural barriers. Extensive evidence indicates that several members of the matrix metalloproteinase (MMP) family of enzymes play a key role in this regard **[2]**.

MMP-9 (Gelatinase B), a member of MMP family, is capable of degrading type IV, V and VI collagens **[1]**. Thus, MMP-9 is assumed to play a key role during the metastasizing process through the disruption of basement membranes. Although the role of MMP-9 is widely studied in solid tumors, little is known about its role in hematological malignancies **[2]**. Moreover, little is known about the application of tissue inhibitors of MMPs (TIMMPs) in the treatment of leukemia **[3]**.

Transforming growth factor beta (TGF-ß) is a pleiotropic cytokine involved in a variety of biological processes in both normal and transformed cells. The TGF-ß signaling pathway is an essential regulator of cellular processes, including proliferation, differentiation, migration, and cell survival. During hematopoiesis, the TGF-ß signaling pathway is a potent negative regulator of proliferation while stimulating differentiation and apoptosis when appropriate **[4]**.

Deregulated TGF-ß signaling is known to be involved in a variety of human cancers including those of the colon, pancreas, breast and prostate. Recently, evidence demonstrating deregulated TGF-ß signaling in leukemogenesis,

has started to emerge **[5]** thus defining a tumor suppressor role for the TGF-ß pathway in human hematologic malignancies **[4,5]**. On the other hand, elevated levels of TGF-ß can promote myelofibrosis and the pathogenesis of some hematologic malignancies through their effects on the stroma and immune system **[4]**.

TGF-ß has three isoforms (TGF-ß$_1$, ß$_2$, and ß$_3$). The first recognized growth factor is TGF-ß$_1$. It belongs to a family of dimeric 25kDa polypeptides that are ubiquitously distributed in the tissues and synthesized by many different cells **[6]**.

A novel functional relationship between MMP-9 and the multifunctional cytokine TGF-ß in the control of tumor-associated tissue remodeling has been recently uncovered. The hyaluronan receptor CD44 provides a cell surface docking receptor for proteolytically active MMP-9. Cell surface localization of MMP-9 is an important factor in its ability to promote not only tumor invasion, but angiogenesis and growth as well. MMP-9 proteolytically cleaves latent TGF-ß thus providing a novel and potentially important mechanism for TGF-ß activation. It is tempting to speculate that latent TGF-ß activation may constitute a part of the mechanisms whereby MMP-9 activity induce or promote angiogenesis **[7]**.

The aim of this work was to estimate the levels of MMP-9 and TGF-ß$_1$ in the sera of patients with acute leukemia with and without extramedullary involvement. This could add new biological markers for this disease and possibly help to determine the patients who develop extramedullary infiltration.

PATIENTS AND METHODS

Twenty seven patients with newly diagnosed acute leukemia, 16 males and 11 females, were enrolled in the study. The patients were divided into two groups:

Group I : 10 patients with clinically evident extramedullary leukemic infiltration. Their ages ranged from 18 to 60 years with a mean age of 24.6±14.6 years.

Group II: 17 patients without extramedullary disease. Their ages ranged from 19 to 54 years with a mean of 30.7± 14.54 years.

In addition, 10 healthy age- and sex-matched individuals were included as a control group (group III). A written informed consent was obtained from all patients and controls before enrollment in the study. All patients were subjected to full history taking, thorough clinical examination and routine laboratory investigations including CBC, renal and liver function tests and serum uric acid. Imaging studies including CT scans and/or MRI were done whenever indicated.

The diagnosis of AML and ALL was made by standard morphology and cytochemistry of peripheral blood (PB) and bone marrow (BM) films according to the French-American-British (FAB) criteria **[8]** and the immunophenotyping using a comprehensive panel of monoclonal antibodies (mAbs) against myeloid and lymphoid associated antigens as proposed by the EGIL group **[9]**.

Immunophenotypic analysis: **[10]**

It was performed on fresh PB or BM samples taken at the time of diagnosis. Samples were analyzed using FACScan analyzer (Becton and Dickinson, San Jose, CA). Data were processed using Cell Quest Software (Becton and Dickinson, San Jose, CA).

A wide panel of mAbs was used. It included common leukocyte antigen CD45, myeloid markers; MPO, CD117, CD13, CD33, CD14, CD15, T cell markers; CD1, CD2, CD3, CD4, CD5, CD8, B cell markers; CD19, CD20, CD22, CD10, IgM, Kappa and Lambda light chains, stem cell marker CD34 and the erythroid marker glycophorin A. Double marker labeling was performed including proper isotype controls. All mAbs and isotype controls were supplied from Dako Cytomatiom (Denmark), and Immunotech (France).

A membrane surface marker is considered positive when over 20% of the gated population expressed it, and an intracellular marker (MPO and IgM) was considered positive when over 10% of the gated cells expressed it. In all experiments, a minimum of 10,000 cells were analyzed.

Determination of serum concentration of MMP-9: **[11]**

It employed the enzyme linked immunosorbent assay technique (ELISA). MMP-9 assay kit was purchased from quanti kine.

Standards and samples were pipetted into microwells precoated with anti MMP-9. Thus, any MMP-9 present in the standards or samples was bound by the immobilized antibody. After washing away unbound substances, an enzyme linked polyclonal antibody specific for MMP-9 was added to the wells. Following a wash to remove antibody-enzyme reagent, a substrate solution was added to the wells and a colour developed in proportion to the amount of total MMP-9 bound in the initial step. The colour development was stopped and the absorbance of each well was measured using a microplate reader set at 450nm. A standard curve was plotted from the seven MMP-9 standard dilutions and the concentration of each sample was determined.

Determination of serum concentration of TGF-ß1: **[11]**

It was done by enzyme linked immunosorbent assay (ELISA). TGF-$ß_1$ assay kit was purchased (from Bendermed systems).

Standards and samples were pipetted into microwells precoated with anti TGF-$ß_1$. Consequently, any TGF-$ß_1$ present in standards or samples was bound to the antibodies adsorbed to the wells; a Horse Radish Peroxidase (HRP) conjugated monoclonal anti TGF-$ß_1$ antibody was added and bound to TGF-$ß_1$ captured by the immobilized antibody.

After removal of the enzyme-antibody by aspiration and washing, a substrate solution reactive with HRP was added to the wells. A colour was formed in proportion to the amount of TGF-$ß_1$ present in the standards and samples. Finally, the reaction was terminated by addition of an acid and absorbance was measured using a microplate ELISA reader set at 450nm. A standard curve was plotted from seven TGF-$ß_1$ standard dilutions and the concentration of each sample was determined.

Induction chemotherapy was instituted for all patients. For ALL patients; standard induction included prednisone, vincristine, anthracyclines and L-asparaginase **[12]**. For AML patients, the 7 and 3 protocol was given i.e., doxorubicin 45mg/m^2/day for 3 days and cytosine arabinoside 100-200mg/m^2/day for 7 days **[13]**.

Patients were considered in complete remission (CR) when they have a morphologically normal BM containing <5% blasts and no Auer rods; absence of extramedullary leukemia and normalization of neutrophils ($\geq 1.5x10^9$/L) and platelet counts ($>100x10^9$L). These criteria should be maintained for at least 4 weeks or until initiation of intensification therapy if earlier than 4 weeks. Partial remission (PR) was defined by 5 to 25% BM blasts. Patients was considered refractory when their BM contained ≥25% blasts **[12,13]**. All patients were followed-up for at least 6 months to assess their response to chemotherapy.

Statistical analysis was done using SPSS package. Data parameters were described in the form of mean ± standard deviation. For comparative studies, student '*t*' test was used for comparing the means of two continuous variables. The probability (*p* value) was considered significant when $p<0.05$ **[14]**.

RESULTS

The results of this study are presented in tables (1) through (3) and Figs. (1) through (3). Table (1) illustrates the clinical data in the two studied patients groups and their response to induction chemotherapy. Extrameduallary involvement was in the form of meningeal leukemia with CSF positive for blasts in 5 patients (4 patients had right facial palsy and one had bilateral facial palsy). One patient developed spinal compression and paraplegia, one had intracerebral mass (Chloroma), one had right proptosis, one had right kidney infiltration (diagnosed by renal biopsy) and one had testicular leukemia. No significant differences were found between groups I and II regarding the clinical data except for the response to induction chemotherapy which was significantly inferior in group I patients (with EMI) compared to group II (without EMI) (Pearson Chi-Square = 16.815, $p=<0.001$).

Table (2) shows the important hematological findings in the two studied patients groups and their statistical comparison. No significant difference was found between them except for the platelet count which was significantly lower in group I patients compared to group II.

The mean values of serum MMP-9 and TGF-$ß_1$ in the 3 studied groups are shown in Table (3), while Figs. (1,2,3) illustrate the statistical comparison as regards these two parameters

between group I and group III, group II and group III, and group I and group II, respectively.

The mean values of serum MMP-9 and TGF-ß$_1$ were significantly higher in group I compared to group III (p=0.002 and 0.004 respectively). However, the mean serum MMP-9 did not differ significantly between group II and group III, although the mean serum TGF-ß$_1$ was significantly higher in group II compared to group III (p=<0.001).

On comparing group I and group II, we found that the former showed significantly higher mean values of MMP-9 and TGF-ß$_1$ compared to the latter (p=<0.001 and 0.007 respectively).

Table (1): Clinical data in the two studied patients groups.

Parameter	Group I (n=10)		Group II (n=17)		p* value
	No.	%	No.	%	
Fever	7	70	8	47	0.424
Pallor	8	80	12	70.6	0.363
Hepatomegaly	5	50	7	41.1	0.656
Splenomegaly	7	70	7	41.1	0.236
Lymphadenopathy	4	40	6	35.3	1.000
Diagnosis:					
B-ALL	3	30	6	35.3	
T-ALL	1	10	3	17.6	0.777
AML	6	60	8	47	
Response to induction chemotherapy:					
CR	–	–	13	76.5	
PR	3	30	3	17.6	<0.001
Refractory	7	70	1	5.9	

Abbreviations:
CR = Complete remission. PR = Partial remission.
*Fisher's Exact Test and Person Chi-Square Test are used.

Table (2): Important hematological data in the two studied patients groups.

Parameter	Group I (Mean±SD)	Group II (Mean±SD)	t test	p value
Hb (g/dl)	7.03±1.27	7.14±1.96	–0.178	0.86
TLC (x10^9/L)	21.75±26.38	18.62±18.51	0.362	0.72
PLT (x10^{9}L)	35.4±30.78	71.29±59.6	–2.06	0.05*
BM blasts (%)	65.7±24.76	75.29±18.96	–1.134	0.27

Abbreviations:
Hb = Hemoglobin concentration. PLT = Platelet count.
TLC = Total leucocytic count. BM = Bone marrow.

Table (3): Mean values of serum MMP-9 and TGF-ß$_1$ in the three studied groups.

Parameter (Mean±SD)	Group I (n=10)	Group II (n=17)	Group III (n=15)
Serum MMP-9 (ng/ml)	2.63±0.99	0.55±0.39	1.08±0.91
Serum TGF-ß$_1$ (ng/ml)	137.96±104.74	23.58±11.79	8.6±2.17

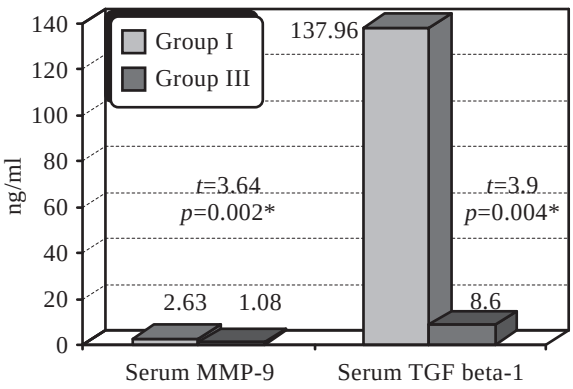

Fig. (1): Comparison between group I and group III as regards serum MMP-9 and TGF-ß$_1$.

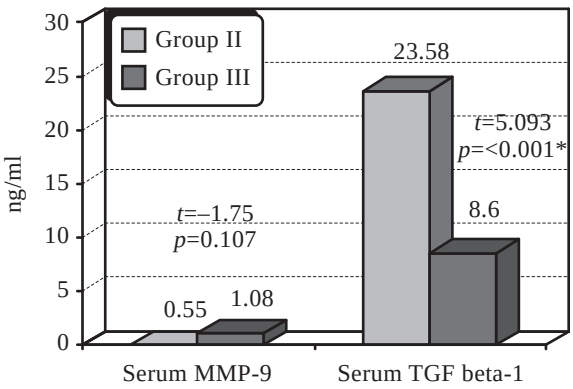

Fig. (2): Comparison between group II and group III as regards serum MMP-9 and TGF-ß$_1$.

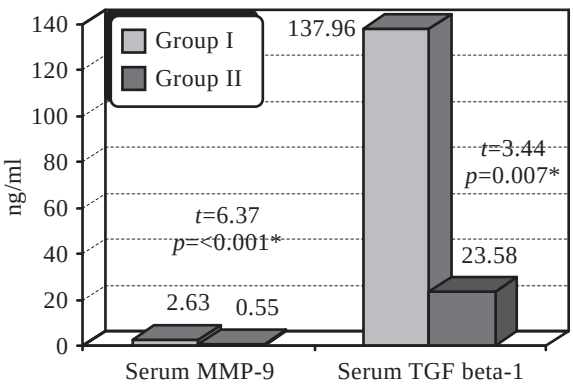

Fig. (3): Comparison between group I and group II as regards serum MMP-9 and TGF-ß$_1$.

DISCUSSION

The role of angiogenesis in solid tumors is well recognized, but its importance in hematological malignancies is less well understood. In leukemia, mainly the determination of microvascular density utilizing immunohistochemistry in the BM trephines and the measurement of soluble angiogenic factors have led to the recognition that angiogenesis may be important in leukemia as well **[15]**.

In the present study, leukemic patients without EMI exhibited lower (but not significant) serum level of MMP-9 compared to the controls. This is in accordance with Lin et al. (2002) **[1]** who reported a significantly lower MMP-9 level in the BM of patients with ALL or AML than in normal controls. Also, Ries et al. (1999) **[16]** reported that MMP-9 gene transcription level was lower in patients with AML and MDS than in healthy individuals. They explained this finding by the presence of MMP-9-releasing mononuclear phagocytes and lymphocytes in the BM-mononuclear cell fraction of healthy individuals compared to that of AML patients consisting predominantly of leukemic blasts. The same result has been more recently reported by Aref et al. (2003) **[17]**.

On the other hand, patients with acute leukemia and EMI had significantly higher level of MMP-9 compared to the controls and to patients without EMI. This result could be explained by the fact that MMP-9 which is involved in mobilization of normal hematopoietic cells from the BM to the PB, also plays a key role in tumor invasion by digestion of ECM. Extramedullary tissue involvement necessitates the excessive egress of leukemic cells from the BM into PB, followed by infiltration of various organs such as lymph nodes, liver, spleen, lungs, intestinal tract, skin or mucous membranes. This means that the cells have to cross, matrix barriers and penetrate blood vessel walls, depending on the catalytic modification of ECM and basement membrane. MMPs, including MMP-9 are capable of digesting almost all components of the ECM **[18]**.

In accordance with our results, Aref et al. (2003) **[17]** reported significantly higher MMP-9 levels in AML patients with EMI compared to those without. They suggested that MMP-9 is involved in the mobilization of leukemic cells. Blast cells purified from PB of AML patients with EMI have been documented to continuously release MMP-9 **[19]**.

In a very recent study, Yang et al. (2006) **[20]** found significant positive correlation between the expression of MMP-9 and vascular endothelial growth factor (VEGF) m-RNA or protein levels in AML patients. Moreover, significant higher expression was noted in patients with EMI. They suggested that VEGF and MMP-9 may participate in the extramedullary leukemic invasion in AML patients.

Kuittinen et al. (2001) **[21]** found significant positive correlation between MMP-9 expression and EMI in adult ALL, but not in pediatric patients indicating basic biological differences between adult and childhood ALL.

As regards TGF-$ß_1$, we observed significantly higher serum levels in group I and group II patients, when each group was compared with the controls. Also, group I exhibited significantly higher TGF-$ß_1$, level than group II. Our results are in agreement with Albitar (2001) **[21]** who reported increased levels of various angiogenic factors including TGF-*ß* in patients with AML and MDS. Moreover, these findings support the recent speculation that elevated TGF-*ß* is involved in the pathogenesis of some hematologic malignancies including leukemias **[4,5]**.

This seems to be in contrast to the results reported by Al-Mowalled et al. (2006) **[15]** who found no significant difference between TGF-$ß_1$ levels in children with ALL compared to controls. However, this could be explained by the fact that the majority of the cases included in the present study were adults and there are basic biological differences between adult and childhood ALL as proposed by Kuittinen et al. (2001) **[22]**.

In the present study, the response to induction chemotherapy was significantly inferior in patients with EMI compared to those without (p=<0.001). Complete remission was achieved in 13 out of 17 patients without EMI while none of those with EMI achieved CR. The former group exhibited significantly lower serum levels of MMP-9 and TGF-$ß_1$ compared to the latter. These data could delineate a strong association between serum MMP-9 and TGF-$ß_1$ and response of patients with acute leukemia

to induction chromotherapy. Similar results were reported by Lin et al. (2002) **[1]** and Aref et al. (2003) **[17]** who found that MMP-9 levels were significantly lower in AML patients who achieved CR compared to those who did not.

It can be concluded from the present study that estimation of MMP-9 and/or TGF-ß$_1$ in the sera of adults' patients with acute leukemia can be used as new biological markers that indicate the presence of extramedullary involvement. However, the possible roles of these markers in the follow-up and in evaluating patients' response to therapy should be addressed in future studies.

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