Elevated serum endostatin levels is associated with favorable outcome in acute myeloid leukemia

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Abstract

Introduction Endostatin is the C-terminal antiangiogenic fragment of the extracellular matrix protein collagen XVIII, and is generated by tumor-derived proteases. The levels and the prognostic relevance of serum endostatin in AML patient is not fully clear.

Aim To evaluate serum levels of endostatin in acute myeloid leukemia patients before chemotherapy and after achieving complete remission and to correlate endostatin levels with patients outcome.

Materials and methods Serum samples from 30 adult patients (22 males and 8 females, median age 37, range 19–66 years) with AML had been taken before chemotherapy was administered. In addition 20 out of 30 patients were reinvestigated again at complete remission (CR). Ten samples from healthy normal persons of matched age and sex were evaluated as a reference control group. Serum endostatin levels were determined using enzyme linked immune sorbent assay (ELISA).

Results Endostatin serum levels were not significantly different in the pretreatment AML patients as compared to that in normal controls ($P > 0.05$). In AML patients the baseline endostatin levels were significantly lower than at CR ($P = 0.001$). No significant correlation were detected between pretreatment serum endostatin levels and age, peripheral blood white cell counts, platelet counts, bone marrow blast cell counts, blast cell distribution ratio. The prognostic value of sE was also evaluated by dividing AML patients into high and low sE groups using the 75 percentile sE levels of the patients group as cutoff. The authors found that patients group in the high sE group survived for significantly longer time than those patients in the low sE group.

Conclusions Elevated endostatin levels at AML diagnosis is a good prognostic marker for patients' outcome. Wide scale study is recommended in order to establish the clinical value of this study.

Keywords AML · Endostatin · Prognosis · Angiogenesis

Introduction

Angiogenesis is the growth of new blood vessel from pre-existing one. It is an important natural process occurring in the body both in health and disease [1]. The ability to form new blood vessels provides malignant cells with survival advantage over their normal counterparts and facilitates the malignant cell dissemination. Several angiogenic molecules have been identified, including both positive and negative angiogenic factors.

Initiation of angiogenesis, the angiogenic switch is dependent on dynamic balance between proangiogenic factors

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and angiogenic inhibitors in the immediate environment of endothelial cells. A positive balance in favor of angiogenic factors, leads to new vessel formation, whereas the prevalence of antiangiogenic factors shifts the equilibrium to vessel quiescence, or under particular circumstances, even to vessels regression [2].

In addition to angiogenic factors, endogenous inhibitors of angiogenic phenotype have been identified. It has been proposed that the angiogenic phenotype of solid tumors is the result of a balance between positive and negative regulators of new vascularization. Endostatin is one of the better characterized endogenously-produced angiogenesis inhibitors [3]. Endostatin has been shown to be highly effective in inhibiting angiogenesis and tumor growth [4, 5]. It may mediate these biological effects by inhibiting proliferation and inducing apoptosis in endothelial cells [6]. In an animal model, endostatin has been shown to induce regression of metastatic tumor. The data about endostatin levels in AML patients are controversial and the relation between its levels and patients outcome is scarce [7, 8].

The aim of this study was to evaluate serum levels of endostatin in Acute myeloid leukemia patients before chemotherapy and after achieving complete remission and to correlate endostatin levels with patients outcome

Materials and methods

Patients

Serum samples from 30 adult patients with AML (22 male, 8 female) had been taken before chemotherapy was administered. In addition 25 out of the 30 AML patients were reanalyzed again after achieving complete remission. Ten samples from healthy normal persons were evaluated as control group. The patients were selected serially from both Mansoura and Cairo Cancer Institutes.

In each case the diagnosis of acute myeloid leukemia was based on a combination of morphology, cytochemistry, immunophenotyping, and cytogenetic data in some situation.

The studied groups of persons were divided into the following:

Group I (AML pretreatment) This group comprised 30 patients recently diagnosed as acute myeloid leukemia before starting induction chemotherapy. They were 22 males and 8 females, their ages range from 19 to 65 years with a median age of 37 years. Cases were followed up after induction chemotherapy therapy for up to one year.

Group II (AML post induction) This group comprised 25 out of the 30 patients with AML at day 30 post induction chemotherapy.

Group III: This group comprised 10 normal healthy persons of matched age and sex to act as reference control group. They were 4 males and 6 females; their ages range from 19 to 55 years. They were subjected to the same examination and laboratory investigations

Samples collection and storage

Seven milliliters of venous blood sample were collected from each case by a clean venipuncture using plastic disposable syringes between May 2005 to May 2006. Blood was withdrawn slowly without venous stasis and care was taken to avoid frothing, then each blood sample was distributed into tubes as follows:

- (1) One and half ml of blood was delivered into plastic specimen tube containing 50 mL Di-K EDTA solution (1.0 g/dL) for performing complete blood picture (CBC), and immunophenotyping of acute leukemias.
- (2) Four ml of blood were left on polypropylene tube for 30 min. to clot before centrifugation for 15 min. at approximately 1000 ×g to obtain the serum samples for determination of Endostatin (sE) were putted on ependorff tubes and stored frozen at −70°C until the time of assay with avoidance of repeated freeze-thaw cycles.

It was done to all cases before and after remission and controls, enzyme linked immunoassay was performed to measure endostatin level in human serum using the Accucyte Human Endostatin kit total quantitative ELISA kit supplied by Cytimmune sciences.

Statistical methods

The statistical analysis was performed on an IBM compatible personal computer using the Statistical Package for Social Scientists (SPSS) program version 10. The data were tested for normality of distribution by using Kolmogorov-Smirnov (K-S) test. The data were at non parametric numeric distribution, so non parametric statistical methods were used to analyze the numerical data. Mann-Whitney rank test was used to compare each two groups and Kruskal wallis (ANOVA) test was used for analyzing overall differences among the three groups. Frequency, mean, median, range and standard deviation were used to describe data. Spearman's correlation co-efficient was used to evaluate the correlation between non parametric variables. Chi-Square test was used to find the relation between row and column variables. Kaplan-Meier test was used for survival analysis and the statistical significance of differences among curves was determined by Log-Rank analyzed values. The

75 percentile was used as cut off value to test the effect of baseline serum endostatin levels on the AML patient's outcome using discontinuous Cox regression analysis.

Results

Serum endostatin levels in AM

The pre-treatment serum endostatin (sE) level ranges from 3 to 70 ng/ml and median of 14.8 ng/ml, post-treatment sE level ranges from 15.8 to 78 ng/ml with median of 35 ng/ml and the control group endostatin level ranges from 5 to 20 ng/ml with median of 11.8 ng/ml. The post-treatment endostatin level was statistically significantly higher than the level in pre-treatment and controls ($P=0.001$ and 0.000 respectively). On the other hand, no statistically significant

difference was found between the pretreatment sE level and that in normal controls ($P>0.05$) (Fig. 1).

The relation between sE and achievement of CR was then evaluated. Of the 30 AML patients 20 AML patients (66.6%) achieved CR. Wilcoxon test showed that patients who achieved CR had significantly higher sE levels ($P=0.041$).

SE levels at time of diagnosis compared in died, survived AML patients and control groups showed marked increase of sE level in survived group (range 9.6–70 ng/ml and median 38.15) as compared to that levels in the died group (range 3–25.5 ng/ml and median 14.8) ($P=0.04$) and control group (range 5–20 ng/ml and median 11.8) ($P=0.026$) (Table 1).

Baseline serum endostatin level was not significantly correlated to age, hemoglobin level, peripheral WBCs counts, platelet counts, blast cells percentage in bone marrow, and BCDR ($P>0.05$) (Table 2).

Fig. 1 Box-plot diagram representing the range of serum endostatin levels in pretreatment and posttreatment AML patients versus controls. Upper and lower lines of each box indicate the 25th and 75th percentiles. The line through each box indicates the median. Pretreatment versus control ($p>0.05$); posttreatment versus control ($P=0.000$); pretreatment versus posttreatment ($P=0.001$)

Survival study

Using the Cox proportional hazard regression model, we found that low sE had an adverse influence on survival, it was entered as a factor into multivariate analysis together with some other prognostic factors (relative risk 17; 95% confidence interval, 1.81–36.06, $P=0.024$) (Table 3).

The prognostic value of sE was then evaluated by dividing AML patients into low and high sE groups using the 75 percentile level of AML group (i.e. 20.5 ng/ml) as the

cut-off. As illustrated in table 4, and fig. 2, high sE patients survived for a significantly longer time than low sE patients ($P = 0.02$).

Discussion

Acute myeloid leukemia is a life threatening problem with overall disease free survival below 80 %. Accumulating evidences suggest the role of angiogenesis in pathogenesis

Table 1 Baseline serum endostatin levels in the died AML patients as compared to survived one as well as normal controls

Group	Mean SD	Median	Range	P value
Died (n=19)	12.87 +/- 6.07	14.5	(3–25.5)	
Survived (n=6)	56.58 +/- 60.39	38.15	(9.6–70)	0.026
Control (n=10)	11.73 +/- 4.28	11.8	(5–20)	

P1 = 0.04 (died vs survived)

P2 > 0.05 (died vs control)

P3 = 0.026 (survived vs control)

Table 2 Correlation between baseline endostatin levels and some clinical and laboratory parameters

Features	Endostatin	
	r	P
Age (years)	0.166	0.444
Hemoglobin g/dl	0.068	0.745
WBCs $\times 10^3/\text{cmm}$	0.112	0.593
Platelet count $\times 10^3/\text{cmm}$	0.101	0.630
Bone Marrow Blast cell %	0.029	0.889
BCDR	−0.01	0.98

BCDR (blast cell distribution ratio) = peripheral blast cell % / Bone marrow blast cell %

Table 3 Influence of serum endostatin levels and some prognostic clinicopathological parameters on survival in multivariate analysis

Factors	Died (n = 19)	Survived (n = 6)	P Value	Odds Ratio
WBCs ($\times 10^3$)				
≤50	5 (26.3%)	2 (33.3%)	1.00	1.4
>50	14 (73.7%)	4 (66.7%)		(0.19–10.15)
Blast Cells (%)				
≤50	12 (63.2%)	5 (83.3%)	0.624	2.92
>50	7 (36.8%)	1 (16.7%)		(0.28–30.3)
No. L.N groups				
≤3	12 (63.2%)	4 (66.7%)	1.00	1.17
>3	7 (36.8%)	2 (33.8%)		(0.17–8.09)
Platelets ($\times 10^3$)				
>60	12 (63.2%)	4 (66.7%)	1.00	1.17
<60	7 (36.8%)	2 (33.3%)		(0.17–8.09)
Pre-treatment Endostatin				
>75%	2 (10.5%)	4 (66.7%)	0.024	17
≤75%	17 (89.5%)	2 (33.3%)		(1.81–36.06)
FAB				
1,2,3	6 (31.6%)	4 (66.7%)	0.175	4.33
0,4,5	13 (68.4%)	2 (33.3%)		(0.61–30.57)
Hepatomegally				
Absent	6 (31.5%)	4 (66.6%)	0.01	3.00
Present	13 (68.4%)	2 (33.3%)		(0.44–20.44)
Splenomegally				
Absent	8 (42.1%)	3 (50%)	0.73	1.38
Present	11 (57.8%)	3 (50%)		(0.22–8.67)

Fig. 2 The overall survival of AML patients according to pretreatment concentrations of serum endostatin. The 75% concentration level of serum endostatin was used as cut off value

Table 4 Forty eight weeks disease free survival of the studied cases in relation to the pre-treatment serum endostatin level

Endostatin level	Total	Died	% censored	Mean survival time	P value
Below 75%	19	17	10.53%	18.84	0.02
Above 75%	6	8	66.67%	36.33	

and progression of AML [9, 10]. Angiogenesis is controlled by a balance between positive and negative angiogenic factors. Among the inhibitors of angiogenesis is endostatin. The value of using recombinant endostatin in the treatment of AML should be based on a clear data regarding the level of endostatin in AML and its relation to patient outcome; which are up till now is controversial. Lai et al [7] did not find significant differences in the median plasma endostatin levels between AML/MDS patients and normal controls. On the other hand Gelenjen et al [11] and Wrobel et al [8] observed increased serum endostatin levels in untreated AML. Furthermore endostatin remains high even after induction chemotherapy. In chronic lymphocytic leukemia and NHL no difference was found between endostatin plasma levels in complete remission and progressive disease. Moreover endostatin serum levels did not correlate with disease free survival [2–16].

In the current study serum levels of endostatin in the untreated AML patients were insignificantly higher as compared to that in healthy controls. Furthermore endostatin levels express more elevation after induction chemotherapy. Elevated baseline endostatin levels in AML group may be a result of enhanced angiogenesis in bone marrow of AML. This is associated with increased of both pro and antiangiogenic factors. The more elevation of endostatin levels at AML remission might represent a rebound effect after reduction of the tumor mass and subsequently reduction in the production of the secretion of proangiogenic factors while the production of inhibitors continue. The theory proposed that direct release of endostatin by the malignant cells is less likely due to (i) the lack of correlation between serum levels and release by in vitro cultured AML blasts; and (ii) the persistently increased levels even after massive reduction of the AML cell burden by induction therapy [11]. Wrobel et al [8] suggested that chemotherapy can modulate the systemic components of angiogenic regulation. In solid cancer endostatin levels were raised after surgical removal of the primary tumors [17].

Inhibitors of angiogenesis are more often fragment of extra cellular matrix. Endostatin a C-terminal proteolytic fragment of type XVIII collagen, specifically inhibit proliferation of endothelial cells. Administration of recombinant

endostatin resulted in regression of various tumors in xenograft model. Both systemic and local endostatin therapy is now investigated for treatment of the malignant disorders. Systemic therapy with endostatin has been shown to suppress tumor induced angiogenesis. However in vitro endostatin is able to inhibit growth of the new vessels only in high concentration [3, 15, 18, 19, 20].

Based on our data plasma endostatin is a prognostic factor for AML, and high plasma endostatin levels correlate with good clinical outcome. The predictive value of plasma endostatin levels appears to be independent of several known prognostic value. The mechanism underlying the association between the clinical outcome and PE is unclear. The good outcome in AML patients expressing high endostatin levels at diagnosis could be attributed to its hindering effect on angiogenesis process. This finding raises the possibility that endostatin may be used in AML therapy. In support of this opinion is the finding reported by Schuch et al [21] who stated that endostatin microbeads significantly inhibits growth of subcutaneous chorionomas in SCID mice as compared to controls. In a leukemia model using M1 cells the concomitant treatment of mice with EEs microbeads prolonged median survival significantly.

Conclusions

Elevated endostatin levels at AML diagnosis is a good prognostic marker for patients' outcome. Wide scale study is recommended in order to establish the clinical value of this study.

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