

Membrane and Soluble Apo-1 as a Marker of Apoptosis in Patients with Acute Leukaemia

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ABSTRACT

Purpose: We have planned this work to evaluate the significance and prognostic values of both membrane and soluble APO-1 as markers of apoptosis in patients with acute leukaemia before and after chemotherapy.

Methods and Materials: For that, 30 patients suffering from acute leukaemia (15 patients with ALL and 15 patients with AML) and 10 apparently healthy individuals serving as control group, were selected and subjected to the following: thorough history and clinical examination, routine investigations including: complete blood picture, bone marrow examination, cytochemistry, immunophenotyping of the blast cells and specific investigations including: detection of mAPO-1 (CD95) on surface of blast cells by flow cytometry, detection of DNA fragmentation by agarose gel electrophoresis and measurement of soluble APO-1 by ELISA technique before and after chemotherapy.

Results: Surface membrane CD95 was found to be expressed on the majority of ALL blast cells (86.6%) and in only 60% of AML blast cells. The degree of surface membrane expression was variable ranging from 23-86% in ALL and from 43-89% in AML. In both ALL and AML patients, a significant relationship was detected between surface CD95 expression and response to initial induction chemotherapy. Ninety-one percent of ALL patients and 84% of AML patients who had surface CD95 expression > 20% on their blast cells showed complete hematological remission after initial induction chemotherapy. This was confirmed by finding that DNA extracted from patients under chemotherapy, whose blast cells CD95 expression was > 20%, showed DNA fragmentation (DNA laddering) by agarose gel electrophoresis (characteristic of apoptosis). As regards soluble CD95 (SCD95) before starting chemotherapy, no statistically significant difference was observed between the level of soluble CD95 in both ALL and AML patients and the control group ($p > 0.05$). But, in AML patients, the level of soluble CD95 tended to be elevated (not significantly) in comparison with normal control. After initial induction chemotherapy, the level of soluble CD95 was found to be significantly decreased in both ALL and AML patients in comparison to its level before

therapy ($p < 0.001$ and < 0.01 , respectively). By following up patients who were resistant to chemotherapy, it was observed that patients who did not achieve complete remission after induction chemotherapy had relatively higher levels of sAPO-1.

Conclusion: From these results we can conclude that, since there is a significant relationship between surface CD95 expression in both ALL and AML patients and response to chemotherapy, the expression of surface CD95 could serve as a new prognostic marker as it is helpful in predicting the outcome of therapy. In addition, because soluble APO-1 was found to be relatively high in patients resistant to anti-leukaemic therapy, so measurement of s-APO-1 in sera of acute leukaemia patients could serve as a putative marker for an active persisting leukaemia.

Key Words: Acute Leukaemia - Apoptosis - Apo-1 - CD 95 - S-APO-1

INTRODUCTION

Apoptosis is an active death process genetically encoded to eliminate abnormal unwanted cells. It has been considered a programmed cell death, since the cell activity participates in its own destruction [6]. During apoptosis, the nucleus of the affected cell condenses and becomes fragmented, the cytoplasm also condenses. At the final stage of apoptosis, the cell itself becomes fragmented forming apoptotic bodies, which are rapidly eliminated by phagocytes, thus restricting the disruption of living tissues [9].

Membrane APO-1 (CD95) is a cell surface protein, belonging to the tumor necrosis factor (TNF) / nerve growth factor (NGF) receptor superfamily. It is expressed on a variety of human B and T cell lines, on various normal human

tissues and on many different tumor cells. Triggering of mAPO-1 by its ligand or by monoclonal Ab anti-APO-1 results in rapid induction of programmed cell death. The tissue distribution of mAPO-1 and of the APO-1 ligand suggested that this APO-1 receptor ligand system plays an important role in the homeostasis of the immune system [27].

A soluble form of APO-1 (s-APO-1) was identified and characterized by Cascino et al. [2]. It was found that its elevation in the serum prevents binding of the ligand to cell surface receptor (mAPO-1) and thus preventing cells from undergoing APO-1 ligand-induced apoptosis [23]. In 1994, Cheng et al. [3] found elevated levels of sAPO-1 in sera from SLE patients. Therefore, it is conceivable that changes in sAPO-1 level are associated with abnormal growth regulation of lymphoid cells and the secretion of sAPO-1 may provide a mechanism for cells to escape immunosurveillance and may be involved in leukemogenesis [21].

Induction of apoptosis is considered to be the underlying mechanism that accounts for the efficiency of chemotherapeutic drugs [25]. Releasing apoptogenic factor from mitochondria and caspase activation seems to be a key event in apoptosis induced by cytotoxic drugs. In addition the CD95 L receptor system is involved in drug-induced apoptosis in various cell types [7]. In 2000, Fulda et al. [8] found that increased CD95 expression and induced CD95 L on treatment with anticancer drugs contributed to apoptosis by activating the CD95 death-signaling pathway.

The aim of this work is to evaluate the significance and prognostic values of both membrane and soluble APO-1 as markers of apoptosis in acute leukaemia patients before and after chemotherapy.

PATIENTS AND METHODS

This study included 40 subjects, 30 patients suffering from acute leukaemia and 10 apparently healthy individuals who served as control group. They were 4 females and 6 males. The 30 patients were further classified according to morphology, cytochemistry & immunophenotyping into 2 groups (Table 1).

Table (1): Patients characteristics.

	Group (1) ALL patients (N = 15)	Group (2) AML patients (N = 15)
Age range	2-32 years	2.5-54 years
Mean±SD	12.5±9.6	14.9±11.9
Sex: F/M	7/8	6/9
FAB subtypes	7L1, 6L2, 2L3	4M2, 3M3 5M4, 3M5
Immunophenotyping	6 precursor B ALL 7 T ALL 2 B ALL	1 M0, 3 M2 3 M3, 5 M4 and 3 M5

The patients were receiving the following protocols:

For precursor B and T-ALL: Induction chemotherapy consisted of prednisone, vincristine, daunorubicin with or without L-asparaginase with high dose methotrexate for B-ALL.

For AML: Induction chemotherapy consisted of daunorubicin and cytarabine with or without etoposide.

All the members of the study were subjected to the following investigations:

- A- Complete clinical history and clinical examination.
- B- Routine laboratory investigations:
 - 1- Complete blood picture (Sysmex S.F. 3000).
 - 2- Bone marrow examination [4].
 - 3- Peroxidase stain: to differentiate ALL from AML [10].
 - 4- Immunophenotyping of the blast cells by flow cytometry [14].
- C- Specific investigations:
 - 1- Detection of Fas antigen (CD95) on blast cells by flow cytometric (FCM) analysis [14].
 - 2- Qualitative assessment of DNA damage by agarose gel electrophoresis [24].
 - 3- Determination of soluble Apo-1/Fas (sCD95) in sera of ALL and AML patients before and after chemotherapy by cytoscreenTH human Apo-1/Fas (solid phase sandwich ELISA technique). The kit was supplied from Biosource International Inc., USA [15].

Immunophenotyping and detection of Fas antigen on the blast cells by flow cytometric (FCM) analysis.

Fluorochrome labeled monoclonal Abs (Becton Dickinson-Immunocytometry systems, USA):

The panel contained 13 vials of monoclonal antibodies against surface antigens and one vial for CD95. CD45 (leucogate), IgG2a (control), CD10, CD20, CD3, CD7, HLADR, CD34 and CD95 were labeled by fluorescence isothiocyanate (FITC), while CD14 (leucogate), control IgG1, CD19, CD5, CD22, CD33 and CD13 were labeled by phycoerythrin (PE).

Double labelling was performed as follows: CD45/CD14, control, CD10/CD19, CD20/CD5, CD3 / CD22, CD7 / CD33, HLA - DR / CD13, while CD95 FITC labeled was evaluated as a single color.

Technique:

Peripheral blood or bone marrow samples were prepared and analyzed within 24 hours after collection using whole blood staining method [14]. Samples were then analyzed using FAC Scan flowcytometer Becton Dickinson "FCM". Data acquisition was done using cell Quest program. Gating was performed using forward-scatter (FSC) and side scatter (SSC) and % positivity was calculated.

The minimum value for the lymphoid, lineage-associated antigens (CD10, CD19, CD20, CD22, CD5, CD7, CD3 and CD95) should be greater than 20% to be considered positive in the quadrant, while for the myeloid antigens (CD33, CD13) the percentage of positive events in a quadrant must be greater than 30%.

Qualitative assessment of DNA damage by agarose gel electrophoresis:

A- Sample preparation (extraction):

Genomic DNA was extracted from patient's whole blood (during initial induction chemotherapy) with the Bio-Rad Instagene™ whole blood kit.

B- Analysis of nucleosomal DNA fragments by agarose gel electrophoresis:

DNA was separated on 1.6% agarose gel containing 10 µl of 10 mg/ml ethidium bromide to allow subsequent visualization of the DNA. The gel, including 1 KB DNA ladder as a mo-

lecular size standard (2 µl/lane), was run at 70 volts for 2 hours. The bands were visualized by UV fluorescence transilluminator and the sizes of the bands were compared to the ladder (Figs. 3,4).

Statistical methods:

Data were entered and analyzed using EPI-info version 6.02 computer package [5]. For qualitative variables, Fisher Exact results were recommended as the expected cell value was less than 5. For paired observation, Friedman test was used. *p*-value was considered significant at 5% level ($p \leq 0.05$).

RESULTS

In ALL patients, the surface membrane CD95 was found to be expressed on the majority of their leukaemic blast cells. Thirteen out of 15 patients (86.6%) showed variable degree of surface expression ranging from 23% to 86% (Fig. 1). Also, it was observed that all T lineage ALL cases were CD95 +ve and showed significantly higher percentage of cells with CD95 expression as compared to CD10 +ve precursor B ALL cases ($p < 0.01$) (Table 2). In AML patients, only 9 out of 15 patients (60%) showed variable intensity of CD95 expression on their blast cells, ranging from 43% to 89% (Fig. 2). In both ALL and AML patients, a significant relationship was detected between surface CD95 expression and response to initial induction chemotherapy. Eight out of 9 ALL patients (91%) and 5 out of 6 AML patients (84%) who had surface CD95 expression > 20% on their blast cells, showed complete hematological remission after initial induction chemotherapy [Tables (3 & 4), respectively].

One biochemical feature of apoptotic cell death is digestion of chromosomal DNA into an oligonucleosomal ladder made up of DNA fragments that are multiples of 180 base pairs in size. DNA fragmentation was detected clearly in cells from acute leukaemia patients under chemotherapy, whose blast cells showed CD95 expression > 20%. No DNA fragmentation was observed in control cells or cells from patients with blast cells with CD95 expression < 20% (Figs. 3,4).

The mean \pm SD of soluble CD95 in ALL before and after chemotherapy were $11.78 \pm 4.5\%$, $9.32 \pm 5.99\%$, in AML $13.5 \pm 8.3\%$, $11.29 \pm 7.6\%$, respectively, while in the control group it was

9.18±4.1%. The level of soluble CD95 in ALL and AML patients was not statistically different from its level in the control group, but in AML patients before chemotherapy, the level of soluble CD95 tended to be elevated (not significantly) in comparison with normal control. No significant correlation was found between surface and soluble CD95 in both ALL and AML patients (r 0.36, $p > 0.05$ and r 0.34, $p > 0.05$ respectively), also the level of sCD95 was not significantly correlated with standard disease

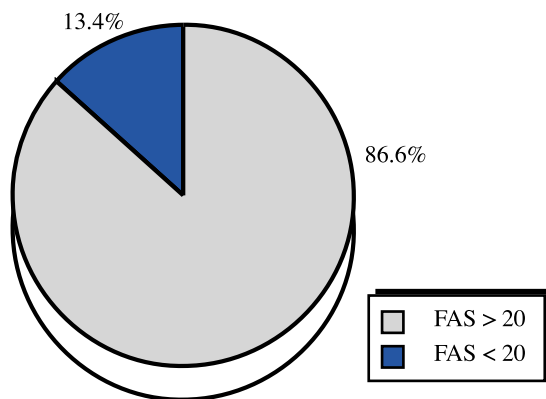


Fig. (1): % of surface CD95 in ALL cases.

parameters such as TLC and blast cell count in both ALL and AML patients.

After initial induction chemotherapy, the difference between soluble CD95 before and after chemotherapy was found to be highly significant in ALL patients ($p < 0.001$) and only significantly different in AML patients ($p < 0.01$) Table (5). Finally, no significant difference was detected between sCD95 in both ALL and AML patients and the control group after chemotherapy ($p > 0.05$).

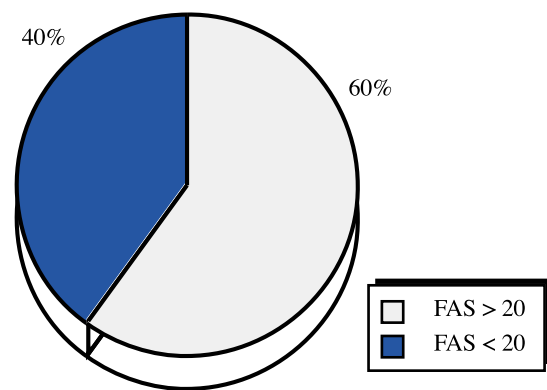


Fig. (2): % of surface CD95 in AML cases.

Table (2): CD95 expression in relation to immunophenotype in ALL cases.

	Precursor B ALL (N=5 out of 6 cases)	T ALL (N=7 out of 7 cases)	<i>t</i>	<i>p</i>
Mean ± SD	36.4±11.6	69.7±14.2	4.29	< 0.01
Range	23%-51%	48%-86%		(sig.)

Table (3): Number and percent of remission and resistant cases in ALL cases after initial induction chemotherapy.

	FAS < 20% N=2		FAS > 20% N=9		<i>p</i>
	No.	%	No.	%	
Remission cases	0	0	8	91	0.05
Resistant cases	2	100	1	9	

Table (4): Shows number and percent of remission and resistant cases in AML cases after initial induction chemotherapy.

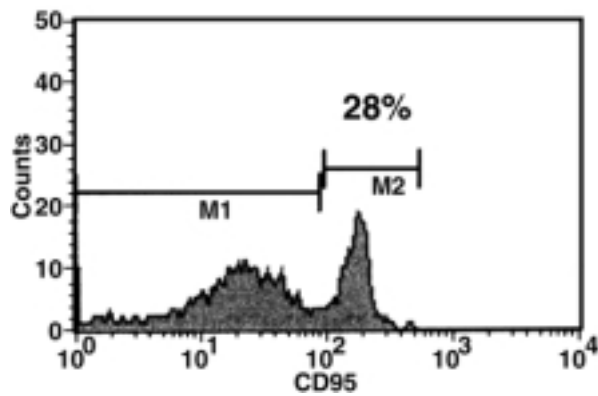
	FAS < 20% N=4		FAS > 20% N=6		<i>p</i>
	No.	%	No.	%	
Remission cases	0	0	5	84	0.023 S
Resistant cases	3	75	1	16	0.11 NS

S: Significant NS: Not Significant

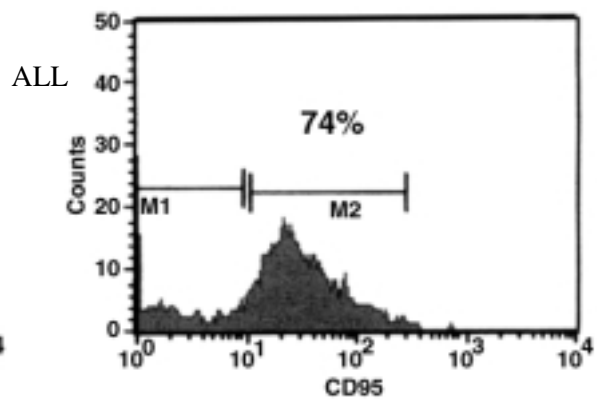
Table (5): Fas change before and after chemotherapy in ALL and AML cases.

	Before therapy X±SD (Range)	After therapy X±SD (Range)	<i>p</i>
ALL	11.78±4.5 (5.3-22.5)	9.3±5.99 (2.9-22)	< 0.001 (HS)
AML	13.5±8.3 (2.1-25.6)	11.3±7.75 (4.3-24)	< 0.01 (Sig.)

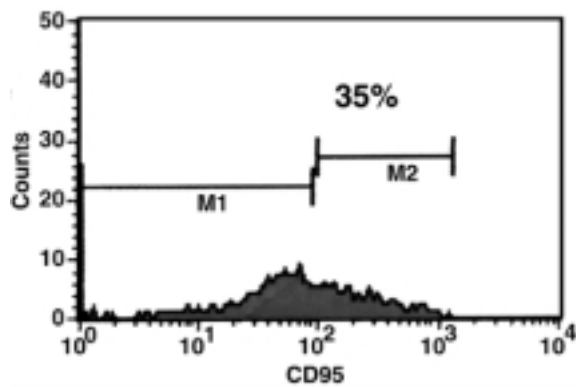
* Freedman test was used for analysis of repeated measurement. HS: Highly Significant



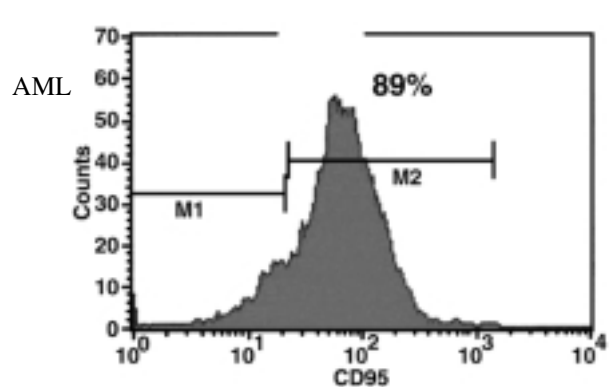
FCM analysis showing low CD95% expression (28%).



FCM analysis showing high CD95% expression (74%).



FCM analysis showing low CD95% expression (35%).



FCM analysis showing high CD95% expression (89%).

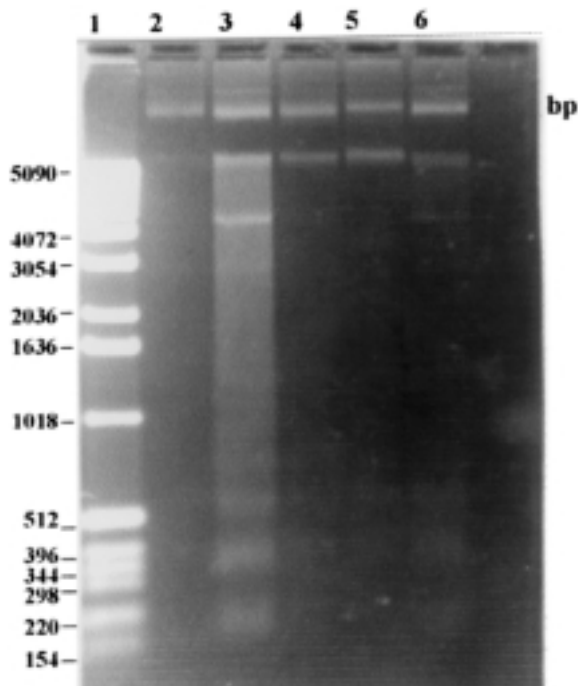


Fig. (3): DNA fragmentation into multiples of 180-200 base pairs by 1.6% agarose gel electrophoresis (ethidium bromide staining).

- 1: Molecular size standard.
- 3: ALL patient with CD95 expression > 20%.
- 2,4,5,6: ALL patients with CD95 expression < 20%.

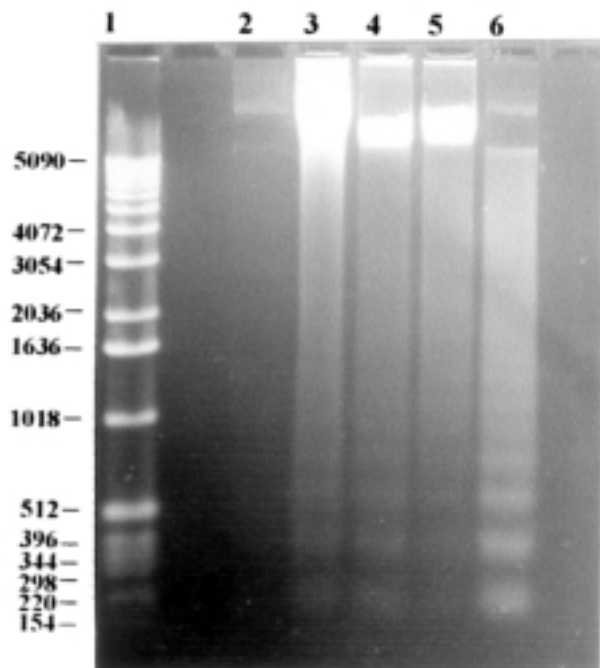


Fig. (4): DNA fragmentation into multiples of 180-200 base pairs by 1.6% agarose gel electrophoresis (ethidium bromide).

- 1: Molecular size standard.
- 2: AML patients with CD95 expression < 20%.
- 3,4,5,6: AML patients with CD95 expression > 20%.

DISCUSSION

In the current study, we found that the surface membrane CD95 was expressed on the majority of ALL blast cells (13 out of 15 cases), with variable degree of surface expression ranging from 23-86%. Our results in this respect are similar to those reported by Karawajew et al. [11] and Beltinger et al. [1]. On the other hand, Wang et al. [26] found that CD95 was expressed in only one of 10 cases of ALL. This discrepancy could be due to the difference in the selected ALL cases; they had done their work only on B-ALL cases, while in our study 7 out of 15 cases were T-ALL. All of them were CD95 +ve and showed significantly higher percentage of surface CD95 expression as compared to precursor B ALL cases. These findings were documented by Karawajew et al. [11].

In AML patients, only 9 out of 15 of our patients (60%) showed variable positivity of CD95 expression on their blast cells. This result is nearly consistent with that observed by Min et al. [18] and Mekawy et al. [17]. Our results are in contrast with Munker et al. [19] who found that Fas receptor was expressed on only 28% of AML cases. In their study, samples were analyzed by the indirect immunofluorescence assay using fluorescence microscope whereas expression of Fas receptor was assessed by flow cytometry in our study. We assumed that this discrepancy could be due to difference in the detection methods. Concerning the variability of CD95 expression on AML blast cells, Lijima et al. [16] reported that this might reflect the heterogeneity of AML. Fas expression becomes enhanced with the maturation pathway of myeloid series. So, variable expression of CD95 in AML may reflect only difference in the maturation stage of the leukaemic cells. They demonstrated that the immature subtype AML-M1 showed significantly weaker expression than the other subtypes.

Regarding the relation between CD95 positivity and the outcome of patients after induction chemotherapy, our results revealed that in both ALL and AML patients, a significant relationship was detected between surface CD95 expression and response to initial induction chemotherapy. It was found that 8 out of 9 ALL patients and 5 out of 6 AML patients who had surface CD95 expression > 20% on their blast cells showed complete remission after induc-

tion chemotherapy. Similar results were found by Min et al. [18] who demonstrated that the patient outcome after induction chemotherapy is accurately predicted by Fas expression. The complete remission rate was significantly lower in the Fas -ve cases compared to Fas +ve cases. They hypothesized that Fas antigen in leukaemic cells is implicated in sensitivity to chemotherapy: cross-linking of Fas protein by chemotherapy may trigger inhibition of leukaemic cell growth. Finally, they concluded that the quantitation of Fas expression can be predictive of treatment outcome in acute leukaemia patients.

Also, similar results were observed by Lijima et al. [16]. They suggested that there is synergistic effect of Fas mediated apoptosis and anti-leukaemic agents on apoptosis. They concluded that the analysis of the expression and function of Fas on leukaemic cells will shed light on the mechanism of eradication of leukaemic cells and the prognosis and response to several kinds of strategies such as chemotherapy, radiotherapy, immunotherapy and cytokine therapy.

In 1995, Komada et al. [13] demonstrated that the blast cells in patients with AML are susceptible to the induction of apoptosis by anti-Fas MoAb. Thus, the use of anti-Fas Ab to induce selectively the endogenous suicide program in leukaemic cells should be considered as a potential strategy for the treatment of acute leukaemia. However, the use of anti-Fas Ab in therapy is only feasible if the Ab can be targeted specifically to leukaemic cells while vital normal tissues remain unaffected [22].

In our study, before starting chemotherapy, no statistically significant difference was observed between soluble CD95 levels in ALL patients and the control group. In contrast to our result, Knipping et al. [12] found that sera from patients with B and T ALL contained increased levels of sAPO-1. This discrepancy might be attributed to technical differences in the ELISA technique. They had established and prepared their own sandwich ELISA technique. Another cause may be due to the fact that the sera of their ALL cases were obtained from pediatric patients only. In AML patients before therapy, we found that the level of soluble CD95 tended to be elevated in comparison with normal control, but this elevation was not statistically significant. Similar results were reported by Munker et al. [19].

Correlation studies revealed that no statistically significant correlation was detected between surface and soluble CD95 in both ALL and AML patients. This finding goes hand in hand with that demonstrated by Knipping et al. [12]. They confirmed this finding by analyzing sera of HIV infected children for sAPO-1. They found that these sera contained no detectable levels of sAPO-1 despite the fact that mAPO-1 expression on T cells in HIV-1 infected children is greatly increased. This finding shows that the presence of high numbers of mAPO-1 positive cells is not always correlated with elevated sAPO-1 levels. Thus, sAPO-1 in sera of acute leukaemia patients may not always be derived from leukaemic cells, another putative source of sAPO-1 may be stromal cells, reactive T and B cells or other cell populations reflecting the activation status of these cells [20].

To assess the relation between sAPO-1 levels and standard disease parameters, we compared factors indicating tumor cell burden such as white blood cell count and risk factors as defined by Berlin Frankfurt leukaemia protocols, such as blast cell count, with sAPO-1 levels in both ALL and AML patients. No significant correlation was found. This result is consistent with Knipping et al. [12] who assumed that sAPO-1 levels are not determined by tumor load but rather by the tumor biology.

In our study, the difference between soluble CD95 before and after chemotherapy was found to be highly significant in ALL patients and only significantly different in AML patients. On other hand, no significant difference was detected between soluble CD95 in both ALL and AML patients after chemotherapy and the control group. Similar results were reported by Knipping et al. [12] and Munker et al. [20]. Also, we observed that patients who did not achieve complete remission after initial induction chemotherapy had relatively high levels of sAPO-1. But, in order to confirm this finding larger number of patients should be studied and followed up, if possible for longer periods of time. Similarly, Munker et al. [19] reported that sAPO-1 was found to be constantly high in patients relatively resistant to anti-leukaemic therapy.

So, we can conclude that the expression of surface CD95 on the blast cells could serve as a new prognostic factor as it is helpful in monitoring the outcome of therapy. Also, sAPO-1

detection in acute leukaemia patients could serve as a putative marker for an active persisting leukaemia.

Finally, we recommend that the surface CD95 marker should be included in the immunophenotyping panel of acute leukaemia and that the sCD95 must be measured in acute leukaemia patients resistant to anti-leukaemic therapy. Patients with elevated sCD95 may require more intense chemotherapy to achieve complete remission.

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