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Differential CD95 expression and function in T and B lineage acute lymphoblastic leukemia cells

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CD95 (Fas/APO-1) is a cell surface receptor able to trigger apoptosis in a variety of cell types. The expression and function of the CD95 antigen on leukemic blasts from 42 patients with B lineage and 53 patients with T lineage acute lymphoblastic leukemia (ALL) were investigated using immunofluorescence staining and apoptosis assays. The CD95 surface antigen was expressed in most ALL cases, with the T lineage ALL usually showing a higher intensity of surface CD95 expression as compared with the B lineage ALL cells (relative fluorescence intensity, RFI: 4.8 ± 0.47 *vs* 2.2 ± 0.23, respectively, $P < 0.01$). Functional studies disclosed that upon oligomerization by anti-CD95 monoclonal antibodies the CD95 protein was either not able to initiate apoptosis of leukemic cells (75% of cases) or induced low rates of apoptosis (20% of cases). Only in 5% of cases did the apoptosis rate exceed the 20% level of the CD95-specific apoptosis. Most of the CD95-sensitive cases were found among T lineage ALLs (38% of T lineage *vs* 10% of B lineage ALLs). Overall, the extent of CD95-induced apoptosis did not correlate with the expression level of CD95. Similarly, no significant correlation between expression level and functionality of CD95 in human leukemia cell lines of B and T cell origin could be observed. Bcl-2 protein has been associated with prolonged cell survival and has been shown to block partially CD95-mediated apoptosis, but for ALL cells no correlation between bcl-2 expression and spontaneous or CD95-mediated apoptosis could be found. The results obtained in this study indicate that, despite constitutive expression of CD95, the ALL cells are mainly resistant to CD95-triggering. More detailed investigations of the molecular mechanisms involved in the intracellular apoptotic signal transduction, such as interactions of the bcl-2 and the other members of the bcl-2 family, and functionality of the interleukin-1β converting enzyme (ICE) like-proteases, may give new insights into key events responsible for the resistance or sensitivity to the induction of apoptosis in acute leukemia.

Keywords: leukemia; CD95; bcl-2; apoptosis

Introduction

Regulation of tissue development and cellular homeostasis is controlled by cell growth, differentiation and death. Programmed cell death as the regulatory form of homeostasis occurs mainly by apoptosis which is characterized by DNA fragmentation between nucleosomes resulting in 200 bp DNA ladder and specific morphological changes, including chromatin condensation, membrane blebbing and appearance of apoptotic bodies.[1–3] In contrast to apoptosis, necrosis occurs during pathological cell death as a result of tissue injury, complement attack, severe hypoxia, hyperthemia, lytic viral infection and exposition to various toxins.[2,4]

Apoptosis may develop following removal of growth factors or by triggering of specific cell surface molecules, such as CD120a (TNFR p60) or CD95 (Fas/APO-1) (reviewed in Ref. 5). The 48 kDa CD95 surface antigen can mediate apoptosis upon crosslinking with monoclonal antibodies (anti-Fas, anti-APO-1) or natural cognate, CD95 ligand (CD95L/FasL).[6–8] The CD95 antigen belongs to the tumor necrosis factor (TNF)/nerve growth factor (NGF) receptor superfamily.[6–10] CD95 expression is found on a broad range of cells, including activated T and B cells, virally transformed lymphoblastoid cells (eg HTLV-1, HTLV-2, HIV, EBV) or various types of tumor cells including lymphomas and leukemias (eg adult T cell leukemias (ATL), immature T lineage ALL, B cell chronic lymphocytic leukemias (B-CLL), hairy cell leukemias, AML and some follicular/diffuse non-Hodgkin lymphomas (NHL) (reviewed in Ref. 11). However, surface expression of CD95 is not always sufficient to transduce apoptotic signals, particularly for primary human cells.[12] In addition, CD95 is capable of mediating co-stimulatory proliferative signals for freshly activated T cells and has mitogenic potential for human diploid fibroblasts, indicating a dual role of CD95 with proliferative or apoptotic effects depending on the cell type and activation stage.[13,14] Similarly, a few cases of B-CLL or B cell NHLs showed growth enhancement after treatment with CD95 moAbs under co-stimulatory conditions (eg IL-2).[15,16]

The bcl-2 gene was cloned from the breakpoint region of the chromosomal translocation t(14;18) frequently detectable in follicular center B cell NHLs.[17] The 26 Kda bcl-2 proto-oncogene has been demonstrated to prolong the survival of various hematopoietic cells including B and T cells (reviewed in Ref. 18). The bcl-2 expression seems to be restricted to tissues which are able to undergo apoptosis.[19] In addition, recently, several bcl-2-related genes were cloned (summarized in Ref. 20). The bcl-x gene gives rise to short (bcl-X_S) and long (bcl-X_L) splice variants capable either of accelerating or inhibiting apoptosis, respectively.[21] The bax protein promotes apoptosis due to its ability to form heterodimers with bcl-2.[22] The ability of bcl-2 to suppress apoptosis may also influence the treatment outcome of cancer patients. High-level expression of bcl-2 by leukemia cells is associated with resistance to various apoptosis-inducing chemotherapy regimens or radiotherapy.[23,24] Most primary leukemia/lymphoma tumor cells are resistant to anti-CD95-mediated apoptosis and the resistance to apoptosis has been linked with the expression of the bcl-2 protein.[25] Experimental overexpression of bcl-2 interferes with CD95-transduced apoptotic signals.[26] Furthermore, in primary leukemia cells a correlation between bcl-2 expression and resistance to CD95-mediated apoptosis was observed.[15,27] In other studies, however, no correlation has been found for bcl-2 expression and resistance to CD95-mediated cytotoxicity.[28,29] Resistance to apoptosis might be involved in the development of leukemias/lymphomas by withstanding elimination of tumor cells through endogenous apoptosis pathways or chemotherapy.[30]

In our study, we analyzed a large series of newly diagnosed B and T lineage ALL for expression of CD95 protein and for its functionality as an apoptosis-inducing molecule upon *in*

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vitro crosslinking with anti-CD95 antibodies. In addition, the relevance of bcl-2 expression for the apoptosis behavior of ALL cells was investigated.

Materials and methods

Cell samples

Leukemic blasts from 95 patients with newly diagnosed ALL of either B lineage ($n = 42$) or T lineage ($n = 53$) affiliation were included. The diagnosis was based on bone marrow (BM) aspirates, and the smears were classified as ALL according to the FAB criteria using light microscopy and cytochemistry.[31] Immunophenotyping was carried out on leukemic blasts isolated by standard Ficoll–Hypaque density gradient centrifugation, and cell-surface as well as cytoplasmic antigens were detected using direct or indirect immunofluorescence (IF) techniques as described elsewhere.[32,33] The criteria for marker positivity as well as the subclassification of B and T lineage ALL followed the guidelines of the 'European Group for the Immunological Characterization of Leukemias' (EGIL) with B lineage ALL being classified into pro-B, common, pre-B and mature B-ALL, and T lineage ALL into pro-T-, pre-T-, cortical and mature T-ALL.[34]

Human leukemia cell lines of T (MOLT-3, RPMI-8402, P12/Ichikawa, PF-382) and B (207, 697, BV-173) lineage were taken from the DSM Cell Culture Bank (Braunschweig, Germany). All cultures were free of mycoplasma contaminations.

Cell purification and culture

Leukemic cells recovered from either fresh or cryopreserved patient samples were purified by density gradient centrifugation using Ficoll–Hypaque separation (Pharmacia, Uppsala, Sweden). Viability of cells was always more than 90% as determined by trypan blue or propidium iodide (PI) (Sigma, Deisenhofen, Germany) exclusion. All samples contained more than 90% leukemic cells based on morphological criterias. Functional assays with freshly isolated or thawed cells of the same patient gave similar results. Leukemic cells were maintained in RPMI 1640 (Biochrom, Berlin, Germany) standard medium (SM) containing 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL, Paisley, UK). Cell lines were grown in SM, supplemented with 10–20% heat-inactivated fetal calf serum (Gibco BRL) at 37°C in an atmosphere of 7% CO_2 in air.

To assess spontaneous apoptosis, 0.5×10^6 cells/well were cultured in 96-well microtiter plates (Nunc, Roskilde, Denmark) using SM for 20 h at 37°C in a humidified atmosphere of 7% CO_2 in air. To investigate the CD95-mediated effects, leukemic cells were preincubated with 1 μg/10^6 cells of anti-CD95 monoclonal antibody (moAb) DX2 (IgG_1; Pharmingen, San Diego, CA, USA) for 30 min, washed twice with SM and cultured for an additional 20 h in the presence of sheep anti-mouse immunoglobulin (0.5 μg/well) used to crosslink the DX2 antibody bound to CD95 protein on the surface of the cells. Alternatively, the cells were cultured for 20 h in the presence of the anti-CD95 moAb CH-11 (IgM; Immunotech, Marseille, France; 1 μg/10^6 cells) which was shown to crosslink the CD95 protein on the cell surface directly.[35] As a negative control, cells were preincubated with irrelevant control antibodies. In selected experiments, the antagonistic anti-CD95 moAb, clone ZB4 (IgG_1; Immunotech), which has been shown to block CD95/CD95L-mediated apoptosis, was used at concentrations of 0.1, 1.0 and 10 μg/10^6 leukemic cells.[35]

Assessment of CD95 and bcl-2 expression

CD95 antigen was detected by conventional immunofluorescence staining of intact cells using phycoerythrine (PE)-conjugated anti-CD95 antibody (clone DX2; Pharmingen).[36] To evaluate the expression of the intracellular bcl-2 protein, leukemic cells were fixed and permeabilized using the fixation-permeabilization kit (Fix & Perm; An-der Grub, Kaumberg, Austria) as recommended by the manufacturer. Bcl-2 antigen was detected by the FITC-conjugated anti-bcl-2 antibody 124 (Dako, Glostrup, Denmark). FITC- and PE- conjugated irrelevant mouse antibodies of the appropriate subclasses (Immunotech) were used as negative controls to determine background fluorescence. Immunofluorescence analysis was performed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) using LYSYS II or CellQuest software (Becton Dickinson). Expression of antigens was quantified as relative fluorescence intensity (RFI), determined by the ratio of mean fluorescence intensity of cells stained for either CD95 or bcl-2 to mean fluorescence intensities of the corresponding negative controls.

Apoptosis assay

Apoptotic cells were detected by flow cytometric analysis of fixed, permeabilized and PI-stained samples as described elsewhere.[37] Briefly, a pellet of 0.5×10^6 leukemic cells was fixed by addition of 2 ml of ice-cold 70% ethanol for 1 h at 4°C. After washing the cells were resuspended in 0.5 ml PBS containing 1 mM EDTA, 0.05% Triton X-100 (Serva, Heidelberg, Germany), and 50 μg/ml PI, pH 7.5. Following the treatment with 0.2 mg/ml RNase (type I-A; Boehringer Mannheim, Mannheim, Germany) for 30 min at room temperature in the dark, the cells were stored at 4°C until analyzed. Cells undergoing apoptosis were identified by flow cytometric analysis of DNA histograms or dot plots of DNA content *vs* forward scatter using the LYSYS II or CellQuest software (Becton Dickinson) and quantified as percentage of cells with subdiploid DNA content.[37]

The extent of the specific CD95-mediated apoptosis (%) was assessed by a formula as described elsewhere:[28]

$$\frac{(\text{CD95-mediated apoptosis} - \text{spontaneous apoptosis}) \times 100}{(100 - \text{spontaneous apoptosis})}$$

The CD95-mediated apoptosis is the percentage of apoptotic cells in the presence of the anti-CD95 moAb, and the spontaneous apoptosis is the percentage of apoptotic cells in the presence of the irrelevant control moAb or medium alone.

Morphology of apoptotic cells

For morphological analysis, apoptotic leukemia cells were first separated from viable cells by flow sorting using a FACS Vantage cell sorter (Becton Dickinson). Morphology of the sorted cells was assessed by a MultiProbe 2010 confocal laser microscope (Molecular Dynamics, Sunnyvale, CA, USA), equipped

with a krypton-argon laser. Cell nuclei were stained with PI, excited at 568 nm wavelength and the emitted light was detected with a 590 nm long-pass filter. The size of the basic element of the fluorescence data collected (three-dimensional pixel or voxel) was $0.11 \times 0.11 \times 0.29\ \mu m^3$. The elaboration of the three-dimensional images and their reconstruction as surface-shaded projections was performed on a Silicon Graphics Indy workstation (Silicon Graphics, Mountain View, CA, USA) using the ImageSpace software (Molecular Dynamics).

Statistical analysis

Results are mean ± s.e.m. as indicated. Differences were evaluated using the two-tailed, non-parametric Mann–Whitney *U*-test for unpaired samples. *P* value of <0.05 was considered significant.

Results

Surface expression of the CD95 antigen on T and B lineage ALL

By using direct IF staining we analyzed the surface expression of the CD95 antigen in 42 cases of B lineage and 53 cases of T lineage ALL. As summarized in Figure 1, all cases of B and T lineage ALL expressed at least weakly the CD95 antigen on the cell surface. Overall, the CD95 surface expression on the B lineage was significantly lower ($P < 0.01$) as compared with the T lineage cases (2.2 ± 0.23 RFI *vs* 4.8 ± 0.47 RFI, respectively). The range of relative CD95 surface expression for the B lineage cases was between 1.07 and 5.36 RFI while that for the T lineage cases with 1.1 to 16 RFI was considerably broader. The surface expression of the CD95 antigen, analyzed in different subtypes of B lineage ALL (pro-B-ALL $n = 9$, common-ALL $n = 24$, pre-B-ALL $n = 9$) did not show any significant differences in the CD95 surface expression (2.8 ± 0.5 *vs* 2.1 ± 0.3 *vs* 1.9 ± 0.5 RFI, respectively) (Figure 1). Similarly, the pro-T-/pre-T-ALL ($n = 15$), cortical T-ALL ($n = 23$) and mature T-ALL ($n = 15$) cases did not disclose different intensity of CD95 staining (4.4 ± 1.1 *vs* 5.4 ± 0.7 *vs* 4.1 ± 0.7 RFI, respectively) (Figure 1). Representative examples of CD95 expression on B and T lineage ALL cells are shown in Figure 2.

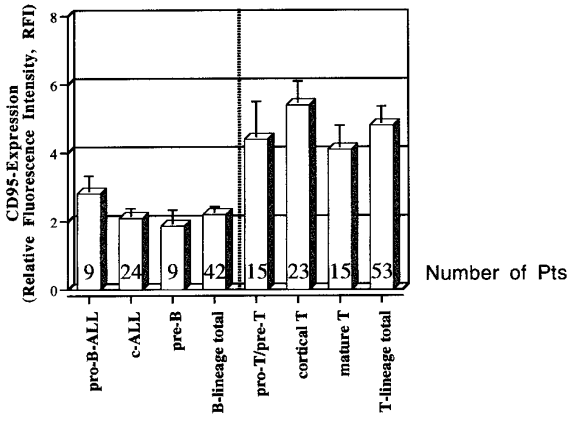

Figure 1 Surface expression of CD95 on leukemia cells from patients with T and B lineage ALL evaluated by flow cytometry. Average values (mean ± s.e.m.) of RFI estimated for the two different ALL groups and several immunophenotypic subgroups are presented. RFI is the ratio of mean fluorescence intensity of cells stained for CD95 to mean fluorescence intensity of the corresponding negative control.

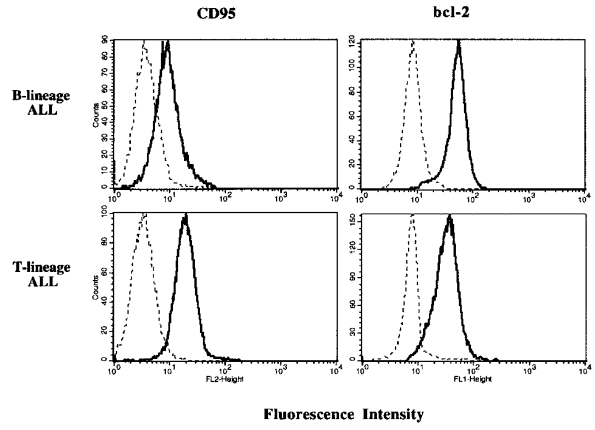

Figure 2 Fluorescence histograms of two ALL samples which were stained for CD95 and bcl-2 protein expression. The cells were stained by direct immunofluorescence using either PE-conjugated anti-CD95 and FITC-conjugated bcl-2 moAbs (solid lines) or isotype-matched irrelevant control antibodies (dotted lines). Representative samples of a B lineage ALL (common ALL) and a T lineage ALL (cortical ALL) cases are shown. The relative fluorescence intensities of CD95 and bcl-2 expression were 2.4 and 5.8 for the B lineage ALL case, but 5.4 and 4.0 for the T lineage ALL sample, respectively.

Expression of the bcl-2 protein in B and T lineage ALL

To investigate the correlation between CD95 and bcl-2 expression, we stained the ALL samples for the intracellular bcl-2 (Figure 2). All B-lineage cases were bcl-2 positive with a mean RFI of 5.9 ± 0.7 (range: 1.38–18.72 RFI) (Figure 3). Similarly, in all the T lineage cases bcl-2 protein could be detected with a mean RFI of 5.4 ± 0.5 (range: 1.1–11.33 RFI) (Figure 3). There were no significant differences between the

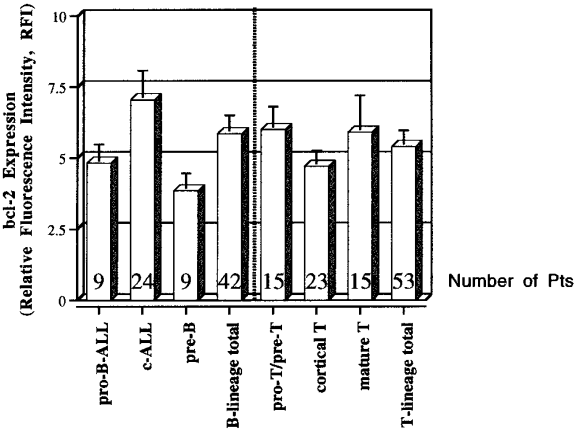

Figure 3 Intracellular expression of the bcl-2 protein in leukemia cells from patients with T and B lineage ALL. The leukemic cells were fixed, permeabilized and stained with the FITC-conjugated anti-bcl-2 moAb. Relative fluorescence intensities (RFI) of the samples were measured by flow cytometry and average values (mean ± s.e.m.) of RFI for the two different ALL groups and their immunophenotypic subgroups were estimated. RFI is the ratio of mean fluorescence intensity of cells stained for bcl-2 to mean fluorescence intensity of the corresponding negative control.

T and B lineage cases ($P > 0.05$). In addition, B lineage and T lineage ALL subgroups did not differ significantly as to bcl-2 antigen expression ($P > 0.05$) (Figure 3).

B and T lineage ALL cells undergo spontaneous apoptosis in culture

In the next set of experiments, we were interested in determining the spontaneous apoptosis of ALL cells. The leukemia cells were cultured for 20 h in SM after either thawing or fresh preparation. Spontaneous apoptosis was determined using flow cytometric detection of cells with subdiploid DNA content in ethanol-fixed and PI-stained cell samples. The spontaneous apoptosis lower than 10% was rated as negative. Spontaneous apoptosis was observed in 33 of the 42 B lineage (79%) and 49 of 53 T lineage (92%) ALL cases (Figure 4). There was a significant difference ($P < 0.05$) between the mean values of spontaneous apoptosis when comparing T and B lineage ALL (mean: $31.8 \pm 2.4\%$ *vs* 26.4 ± 2.5, respectively). As shown in Figure 4, most of the T lineage and B lineage cases showed either a low (10–30%) or moderate (30–50%) degree of spontaneous apoptosis. Some cases failed to demonstrate spontaneous apoptosis (Figure 4). Subgrouping the T and B lineage cases according to their maturational stage did not reveal any significant differences in the spontaneous apoptosis rate (data not shown).

To test a possible involvement of autocrine CD95–CD95L interactions in the spontaneous apoptosis, several leukemia samples (12 of T and eight of B lineage ALL) were incubated in the presence of the soluble antagonistic anti-CD95 moAb (clone ZB4) which has been demonstrated to block CD95L-induced apoptosis.[35] No differences were found for cells incubated with medium alone or with the antagonistic CD95 moAb at concentrations between 0.1 μg and 10 μg of antibody per 10^6 leukemic cells (data not shown). These data indicate that the spontaneous apoptosis of most ALL cells is not triggered by an autocrine CD95/CD95L interaction.

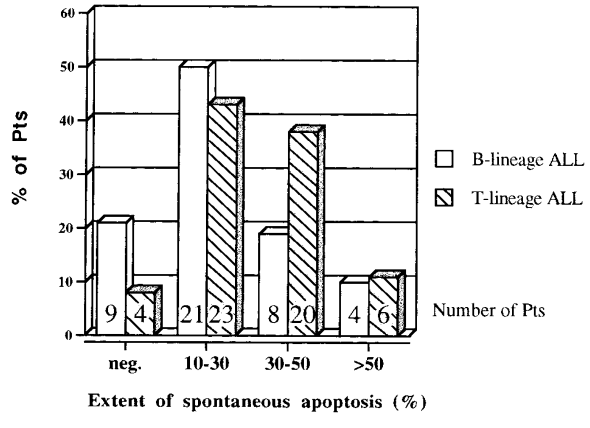

Figure 4 Distribution of spontaneous apoptosis rates associated with the B and T lineage ALLs. The rate of spontaneous apoptosis was determined as a percentage of apoptotic cells in the sample cultured in standard medium for 20 h at 37°C. Apoptotic cells were detected by flow cytometric analysis of fixed, permeabilized and PI-stained cells. Experiments were repeated at least twice.

CD95-mediated apoptosis in B and T lineage ALL cells

To assess the CD95-mediated specific apoptosis, the ALL cells were treated with anti-CD95 moAb (either DX2 or CH11), and the apoptosis was detected by flow cytometry. As shown in Table 1, only in five cases did the extent of CD95-triggered specific apoptosis exceed the value of 20%, and all of these cases were of T lineage (9%). However, we observed a substantial number of cases revealing a low but distinct susceptibility to crosslinking of CD95, especially in T lineage ALL (28%) but also in B lineage ALL (10%). As summarized in Table 1, the anti-CD95 moAb CH11 (IgM subclass) was more effective in induction of CD95-mediated apoptosis of leukemic cells than the DX2 anti-CD95 moAb (IgG_1 subclass) crosslinked with a sheep-anti-mouse Ig. Subgrouping of the ALL cases according to their maturational stage did not reveal any significant differences in their susceptibility to CD95 triggering. To analyze whether CD95-induced apoptosis depends on the intensity of CD95 cell surface expression by leukemic cells, the specific CD95 RFI surface expression was correlated with the extent of CD95-mediated apoptosis. We were unable to find a correlation between the level of CD95 surface expression and the CD95-mediated effects (data not shown). A representative example of the spontaneous and CD95-mediated apoptosis using the CH11 anti-CD95 moAb is shown in Figure 5. The apoptotic cell fraction (subset 'a') can be seen as a subpopulation of cells with lower forward scatter and lower DNA content compared with normal cells (subset 'n'). We further separated these subpopulations by flow cytometry and analyzed their morphological appearance by confocal laser microscopy (Figure 6). The microscopic analysis showed characteristic morphological changes of the PI-stained nuclei of the cells from the apoptotic but not from the viable cell fraction (subset 'a' *vs* subset 'n', Figure 6).

Expression of CD95 and bcl-2 and CD95-mediated apoptosis in human leukemia T and B cell lines

We additionally investigated expression of bcl-2 and CD95 as well as CD95-mediated apoptosis in several leukemia B and T cell lines. As shown in Table 2, leukemia cell lines revealed a broader range of CD95 expression as compared with that of leukemic samples of ALL patients (1.9–29 RFI *vs* 1.07–16

Table 1 CD95-mediated apoptosis for T and B lineage ALL cells

Effect of anti-CD95[a]	*T-ALL*		*B-ALL*	
	CH11 clone (%)	*DX2 clone(%)*	*CH11 clone (%)*	*DX2 clone (%)*
<10%	9/18 (50)[b]	24/35 (68)	17/19 (89)	21/23 (91)
10–20%	6/18 (33)	9/35 (26)	2/19 (11)	2/23 (9)
>20%	3/18 (17)[c]	2/35 (6)[d]	0/19 (0)	0/23 (0)

[a]Cells were incubated with the indicated anti-CD95 moAb for 20 h at 37°C, assessed for apoptosis by flow cytometry and the specific CD95-mediated apoptosis was calculated as described in Materials and methods. Experiments were performed at least twice for each leukemia sample.
[b]Number of cases with the indicated apoptosis rate/number of cases tested (percent of cases).
[c]Rates of specific apoptosis were 91, 26 and 37%.
[d]Rates of specific apoptosis were 27 and 37%.

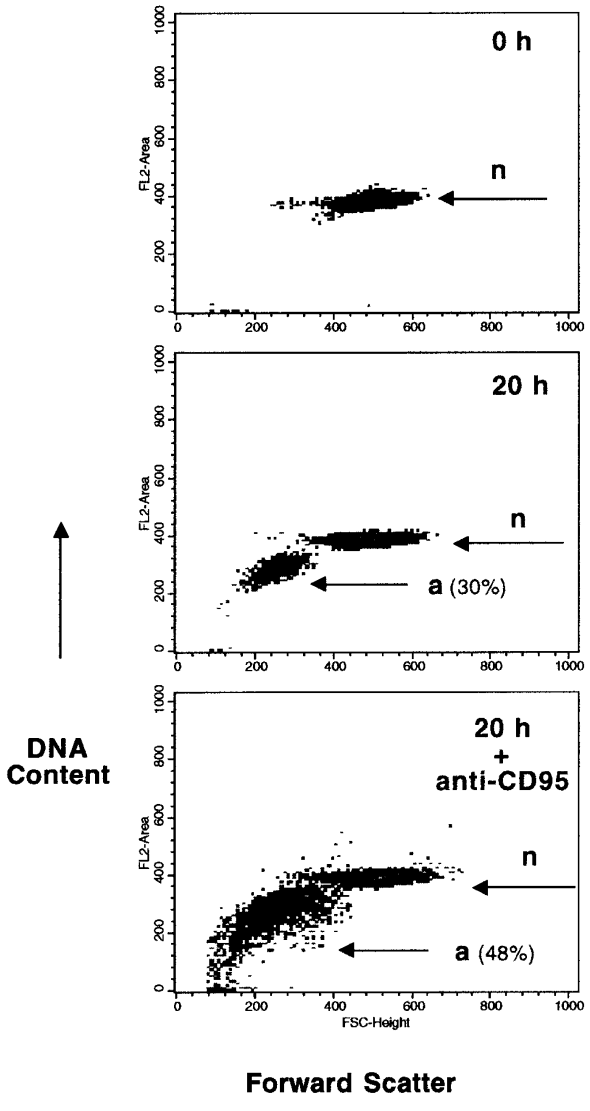

Figure 5 Spontaneous and CD95-mediated apoptosis of T lineage ALL (mature T-ALL) cells detected by flow cytometry. The cells were cultured in standard medium with the absence or presence of anti-CD95 (clone CH11) moAb for 20 h at 37°C. Subpopulation of apoptotic cells ('a') can be readily distinguished from normal, nonapoptotic cells ('n') in the density plot of linear DNA content *vs* forward scatter. In the example shown, the spontaneous apoptosis rate was 30% and the detected CD95-specific apoptosis 26%.

RFI, respectively). By contrast, bcl-2 levels did not vary significantly and, generally, were lower than in ALL samples (mean RFI = 2.8 *vs* 5.6, respectively).

Discussion

In the present study, we investigated the expression and function of the CD95 (Fas/APO-1) antigen on leukemic blasts from 95 patients with B or T lineage ALL. All samples studied were found to express surface CD95 with expression levels, however, varying considerably between very low (RFI = 1.07) and high (RFI = 16). Despite its constitutive expression, the CD95 protein was either unable to initiate apoptosis of leukemic cells upon the oligomerization by anti-CD95 antibodies (75% of cases) or induced low rates of apoptosis (20% of cases).

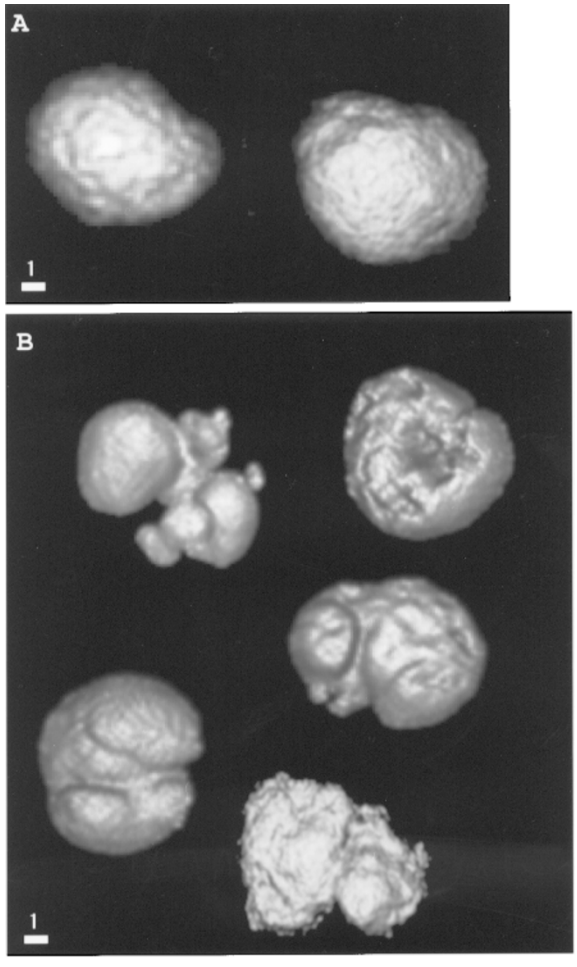

Figure 6 Confocal microscopy of nuclei from non-apoptotic (A) or apoptotic (B) leukemia cells. Apoptotic and non-apoptotic T lineage ALL cells were separated by flow sorting after 20 h of incubation with the anti-CD95 moAb (subsets 'a' and 'n' of Figure 5). Fluorescence from PI-stained nuclei was collected as a three-dimensional sequence of fluorescence intensities of volume elements (voxels) with a $0.11 \times 0.11 \times 0.29\ \mu m^3$ size and reconstructed as a surface-shaded projection using the ImageSpace software (Molecular Dynamics). Scale bar = 1 μm.

Only in 5% of cases did the apoptosis rate exceed the 20% level of specific apoptosis. Overall, the rate of CD95-induced apoptosis did not correlate with the expression level of CD95. Similarly, the CD95-induced apoptosis rate was not associated with the expression level of the bcl-2 protein, despite its constitutive expression in leukemic cells. Compared with leukemic cell samples from ALL patients, CD95 expression and sensitivity to CD95 triggering in human leukemia cell lines were found to be extremely variable, with some of them showing high levels of both parameters. However, no clear-cut correlation between these parameters could be found. In fact, the cell lines with different levels of CD95 expression, eg BV-173 (RFI = 4) and 207 (RFI = 22), exhibited comparable extents of CD95-mediated apotosis. By contrast, two cell lines with similar levels of CD95 expression, MOLT-3 and PF-382, were either resistant (PF-382) or highly sensitive (MOLT-3) to CD95-triggering. These data are consistent with recent studies[28,38] which did not reveal a significant association of expression and functionality of CD95 in leukemia cell lines.

A comparison of B and T lineage ALL revealed significant

Table 2 CD95-sensitivity of T and B leukemia cell lines in relation to expression levels of CD95 and bcl-2

Cell line	*Origin*	*CD95 (RFI)*[a]	*bcl-2 (RFI)*[a]	*CD95-mediated apoptosis (%)*[b]
MOLT-3	T-ALL	19	2.6	75
RPMI-8402	T-ALL	1.9	1.9	0
P12/Ichikawa	T-ALL	29	2.8	30
PF-382	T-ALL	18	2.3	0
207	pre-B-ALL	22	2.6	16
697	pre-B-ALL	4.1	3.4	0
BV-173	CML (BC)	4.2	3.7	15

[a]Cells were stained by direct immunofluorescence using either anti-CD95 or bcl-2 moAbs. RFI is the ratio of mean fluorescence intensity of cells stained for antigen to mean fluorescence intensity of corresponding negative controls.
[b]Cells were incubated with anti-CD95 IgM moAb for 20 h at 37°C, assessed for apoptosis by flow cytometry and the extent of specific CD95-mediated apoptosis was calculated as described in Materials and methods. Experiments were performed in triplets for each sample.

differences in the expression levels of CD95 but not of the bcl-2 protein. T lineage ALL cells expressed significantly more CD95, and most cases sensitive to CD95-mediated apoptosis were observed among T lineage ALL (38 *vs* 10%). Interestingly, the rate of spontaneous apoptosis of the leukemic cells upon culturing *in vitro* was significantly higher in T than in B lineage ALL samples. However, further subclassification of T and B lineage ALL according to their maturational stage did not reveal significant differences as to the expression of CD95 and bcl-2 or the apoptotic behavior of the cells.

Our findings indicating that all ALL samples studied expressed, at least very weakly, the CD95 antigen are in line with recent reports on CD95 expression in malignant hematopoietic cells. Thus, CD95 was detected on the majority of myeloma cells,[39,40] in adult T cell leukemias,[41] acute myeloid leukemias[42] and B or T lineage ALL.[28] It should be noted that the antigen expression in these studies has been assessed by flow cytometry, a method which is more sensitive than a semiquantitative assessment of immunofluorescence using fluorescence microscopy. This fact may explain a significantly lower number of CD95 positive cases (10%) in acute leukemia investigated by the latter method in another recent study.[43]

Although CD95 is broadly expressed on normal and malignant blood cells, its functional importance for triggering of apoptosis could be observed only in a limited number of cell lineages. Thus, the crosslinking of CD95 by antibody or by its natural APO-1/Fas ligand was demonstrated to be highly effective in activated peripheral T and B lymphocytes,[44–46] mature neutrophils[47] and, with regard to malignant cells, in adult T cell leukemia,[41,48] as well as partially in AML cells.[42] Most malignant cells such as myeloma cells,[39,40] AML[42] and B and T lineage ALL cells[28] failed to undergo CD95-mediated apoptosis, and additional treatment of the cells was necessary to overcome their refractoriness to CD95 triggering.[15,28,40,42] Our results obtained by analyzing a large series of ALL samples demonstrate a low susceptibility of ALL cells to CD95 triggering, thus supporting previous observations that the expression of CD95 on the surface of leukemic cells alone is not sufficient for CD95-induced apoptosis.[28] Moreover, there was no quantitative correlation between CD95 expression and sensitivity to CD95 in individual samples, although, by comparing mean values, T lineage ALL were found to express more CD95 protein and to be more sensitive to CD95 triggering compared with B lineage ALL. The latter finding indicates that the relationship between the expression and sensitivity to CD95 is not straightforward, and may imply additional molecular events. In fact, there is accumulating evidence that, after signalling by extracellular factors, the decision of a cell to die depends on various endogeneous gene products, especially the members of the Bcl-2 gene family.[20,21,49] Among these genes, the expression of the bcl-2 proto-oncogene is the most broadly investigated, and its apoptosis-inhibiting function has been well documented in systems which were forced to overexpress the bcl-2.[18,20] In acute leukemias, the bcl-2 protein was detected both in AML[50,51] and B and T lineage ALL.[52–54] Additionally, its expression level was found to be increased in AML[55] and in ALL[56] cells when compared with that of normal progenitor counterparts. Interestingly, comparable with our data, Coustan-Smith *et al*[56] did not find any significant difference in expression levels between B and T lineage ALL. The increased bcl-2 expression was shown to be associated with resistance of AML cells and leukemic cell lines to various apoptosis-inducing cytotoxic drugs, as well as with a low complete remission rate after intensive chemotherapy of AML patients.[24,50,57] In contrast, an influence of the bcl-2 expression on the outcome was not yet observed in ALL patients treated with intensive chemotherapy.[52]

In accordance with the central role of bcl-2 in the inhibition of apoptosis, its expression has been shown to correlate reciprocally with the susceptibility of cells to CD95-mediated apoptosis. Thus, resting T lymphocytes express high levels of bcl-2 and are highly resistant to CD95 triggering, while activated T cells express low amounts of bcl-2 and are CD95-sensitive.[58] A similar relationship was found in B cells, monocytes and neutrophils.[47] Despite this correlation between bcl-2 expression and CD95-susceptibility, a direct interference of bcl-2 with the apoptotic signalling triggered by the CD95 system has not been shown unequivocally. Thus, although a transfection with human bcl-2 gene resulted in an inhibition of anti-Fas-mediated cell death in a murine cell line, this inhibition was only partial.[26] Furthermore, the negative selection during the thymocyte development was found to use two different pathways, one being Fas-mediated but bcl-2 independent and the other Fas-independent and bcl-2-sensitive.[59]

The resistance or low susceptibility to CD95 in leukemic cells could be ascribed to the increased bcl-2 presence in leukemic cells if compared with their normal counterparts.[55,56] However, the absence of quantitative correlation between expression of bcl-2 and sensitivity to CD95 triggering in our study as well as in other reports[28,39] suggests that the expression and complex interactions of other members of the bcl-2 family, such as bak, bax, bad, mcl-1, bcl-X_L and bcl-X_S, being involved in the intracellular apoptotic signalling process, have to be taken into consideration.[49] The expression of these members of the bcl-2 family in ALL is presently under investigation.

Considering the resistance to CD95-mediated apoptosis, defects in the cytoplasmic domain of the CD95 molecule ('death domain'), homologous to that of TNF receptor, cannot be excluded. Thus, mutations of the 'death domain' were found to prevent cytotoxicity mediated by CD95L or TNF.[60] In addition, the observed resistance to apoptosis may be due to defects on the level of cysteine proteases of ICE family involved in execution of apoptosis.[61] However, the CD95 refractory ALL cells were observed to undergo spontaneous

apoptosis accompanied by the characteristic DNA degradation and morphologic appearance, thus indicating that at least part of a complex proteolytic cascade shared by both spontaneous and CD95-triggered apoptosis, should remain intact.[61,62] The spontaneous apoptosis observed, to different extents, in ALL cells *in vitro*[28,53,63] may be due to the depletion of leukemic cells from growth factors. Interestingly, this kind of apoptosis, induced by growth factor depletion, was previously shown, at least in leukemic cell lines, to be bcl-2 dependent.[26] In our study, however, no correlation was observed between bcl-2 expression and rate of spontaneous apoptosis. The reason for spontaneous apoptosis, therefore, remains unclear, but seems to be independent of an autocrine CD95/CD95L interaction because an antagonistic anti-CD95 moAb failed to block the spontaneous apoptosis.

In conclusion, our results show that in most cases of ALL the CD95-mediated signalling cascade for apoptosis cannot be functionally activated by CD95 crosslinking alone. Identification of the molecular pathways maintaining resistance to the induction of apoptosis, and of the molecular events responsible for susceptibility to spontaneous apoptotic cell death may point to therapeutic strategies useful in overcoming cell death resistance mechanisms of leukemic blasts *in vivo*, and may provide new insights into the functional characteristics of acute leukemias.

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