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THE Bax α :Bcl-2 RATIO MODULATES THE RESPONSE TO DEXAMETHASONE IN LEUKAEMIC CELLS AND IS HIGHLY VARIABLE IN CHILDHOOD ACUTE LEUKAEMIA

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Bcl-2 over-expression has been shown to inhibit apoptosis induced by a variety of stimuli, whereas a predominance of Bax α to Bcl-2 accelerates apoptosis upon apoptotic stimuli. We sought to study the relevance of these apoptotic regulating gene products in leukaemia. In a panel of leukaemia and lymphoma cell lines (HL60, DoHH2, CEM C7, L1210 and S49), the Bax α -to-Bcl-2 ratio as assessed by Western-blot analysis correlated with sensitivity to dexamethasone treatment. In addition, in HA-Bax α -transfected CEM C7 clones, a similar correlation was found for dexamethasone and thapsigargin sensitivity. In bone-marrow aspirates from patients with childhood acute lymphoblastic or myelocytic leukaemia (ALL, $n = 48$; AML, $n = 8$), the Bcl-2 and Bax α levels were highly variable, but well within the range found in the Bax α transfectants and in the established cell lines. Bcl-2 levels were lower in T- than in B-lineage ALL, which could be ascribed to simultaneous inverse relation between Bcl-2 and WBC. By contrast, Bax α :Bcl-2 was independent of any presenting feature and was largely dependent on Bax α levels. Results suggest that Bax α :Bcl-2, rather than Bcl-2 alone is important for the survival of drug-induced apoptosis in leukaemic cell lines and ALL. *Int. J. Cancer* 71:959–965, 1997.

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Bcl-2 was initially identified because of its involvement in the chromosomal translocation t(14;18), resulting in high levels of the 26-kDa Bcl-2 protein. Bcl-2 over-expression was subsequently shown to inhibit apoptosis (for review, see Reed, 1995). Moreover, over-expression of Bcl-2 has been implicated in the insensitivity of cells to a variety of anti-cancer drugs, especially those used in the chemotherapy of childhood ALL, notably glucocorticoids (GC) (Miyashita and Reed, 1992; Sentman *et al.*, 1991; Smets *et al.*, 1994). Bcl-2 may, therefore, define a new category of drug-resistance genes regulating the physiological cell-death pathway (Reed, 1995). Accordingly, increased levels of Bcl-2 may be associated with poor prognosis in leukaemia by a dual action. First, it could expand the population of circulating blasts by promoting their survival and, second, it could antagonize response to chemotherapy by inhibiting steroid hormone- and cytostatic-drug-induced apoptosis. Attesting to this view, high expression of Bcl-2 has been associated with high WBC and poor prognosis in adult AML (Campos *et al.*, 1993).

In childhood ALL, the role of Bcl-2 is not clear yet. A number of studies (Campana *et al.*, 1993; Gala *et al.*, 1994; Coustan-Smith *et al.*, 1996) agree that high Bcl-2 expression is the rule in ALL blasts, compared with their normal precursors, while no study found an association between Bcl-2 levels and the presenting features immunophenotype, ploidy and WBC. Increased expression of Bcl-2 was not found to correlate with prognosis in one study (Gala *et al.*, 1994), but correlated with poor initial response to chemotherapy in another report (Maung *et al.*, 1994). By contrast, Coustan-Smith *et al.* (1996) found that elevated levels of Bcl-2 were associated with improved survival and the absence of chromosomal abnormalities. These conflicting results may be due to the small size of patient populations studied and the limitations in determining quantities of Bcl-2 by cytochemistry. On the other hand, it is

becoming clear that Bcl-2 and related anti-apoptotic proteins (Bcl_L, Mcl1, Bag) interact with apoptosis-promoting proteins (Bax, Bcl_{x_s}, Bak) to form heterodimers (reviewed by Reed, 1995) and that the amount of Bcl-2 relative to Bax α is more important than Bcl-2 alone (Oltvai *et al.*, 1993). For instance, a predominance of Bax α over Bcl-2 accelerates apoptosis in response to IL-3 withdrawal in FL5.12 cells, while in the reverse situation, Bcl-2 greatly diminishes apoptosis on removal of the cytokine (Oltvai *et al.*, 1993). Moreover, in primary thymocytes from transgenic mice, a relative increase of Bax α sensitized these thymocytes to dexamethasone(DEX)-induced apoptosis (Brady *et al.*, 1996). Accordingly, the clinical relevance of Bcl-2 levels in ALL may be obscured by simultaneous variations in Bax α . Moreover, it is not known whether elevated levels of Bax α or Bcl-2 in clinical samples are comparable with those in transfectant cells.

We have earlier described an inverse correlation between Bcl-2 levels and sensitivity to DEX in a panel of leukaemic cell lines (Smets *et al.*, 1994). We have now investigated whether the Bax α -to-Bcl-2 ratio also determines the DEX response. To more precisely determine the Bax α :Bcl-2 ratio critical for DEX sensitivity, we transfected Bax α into the human T-leukaemia-CEM C7 cells of intermediate DEX sensitivity. Expression of the Bax α transgene was directed by the human CD2 promoter and locus control region. These cells were tested for the induction of apoptosis by DEX and thapsigargin (TG). TG was tested because of its capacity to increase Ca²⁺ levels, a central signalling event in apoptosis that is inhibitable by Bcl-2 (Lam *et al.*, 1994). Finally, we have assayed the Bax α :Bcl-2 levels in bone-marrow aspirates of 48 ALL and 8 AML patients by Western-blot analysis and compared these with the results obtained with cell lines and Bax α -transfected CEM C7 cells. Our data show large inter-patient variation in expression levels of Bcl-2, Bax α and in the Bax α :Bcl-2 ratio. These values were subsequently compared with the clinically presenting features WBC, immunophenotype, hyperdiploidy and percentage of S-phase cells.

MATERIAL AND METHODS

Cell culture

The human T-cell leukaemia CEM C7 cell line was obtained from Dr. T. Schmidt (Iowa City, IA). Transfected CEM C7 cells were grown at 37°C in 5% CO₂ in RPMI-1640 medium, supplemented with 10% fetal bovine serum (GIBCO BRL, Paisley, UK),

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100 U/ml penicillin, 100 µg/ml streptomycin and 1 mg/ml G418 (Geneticin, GIBCO BRL). HL60 (human myeloid leukaemia), DoHH2 (human non-Hodgkin's B-cell lymphoma), and the murine T-leukaemia cells L1210 and S49 were cultured as described (Smets *et al.*, 1994). All drug treatments were started 24 hr after sub-culture in fresh culture medium at a density of 2×10^5 cells/ml. DEX was added from 1000-fold ethanol concentrates to a final concentration of 1 µM. Stock solutions of 2 mM TG (Sigma, St. Louis, MO) in dimethyl sulfoxide (DMSO) were first diluted in culture medium into 100-fold concentrates (0.01% DMSO), which were added to final concentrations of 100, 250 and 500 nM.

Transfections and characterization of CEM C7 transfectants

CEM C7 stable transfectants were generated after electroporation of 8×10^7 cells in serum-free medium at 960 µF 300 V with 20 µg of linearized CD2HABaxα DNA (Brady *et al.*, 1996) + 2 µg pRcCMVNeo DNA. At 48 hr after electroporation, the cells were diluted 1:5 and selected for 2 weeks in RPMI + 1 mg/ml G418. Single-cell clones were isolated by limiting dilution, followed by expansion in medium including 1 mg/ml G418. Individual clones were then examined by Southern-blot analysis for the presence of CD2HABaxα and/or Neo. Following this, CD2HABaxα-containing clones were analyzed for HABaxα expression and DEX sensitivity. The number of specific DEX-binding sites was determined in conventional whole-cell ³H-DEX binding assays as described (Smets *et al.*, 1994) and were not significantly different from the parental cell line. Cell-cycle times were calculated from the exponential part of the growth curves and were similar to those in the parental cells. p53-dependent inducers were not studied, because the functional analysis of separated alleles in yeast (FASAY) proved that p53 is functionally mutant in all the CEM clones.

Quantitation of apoptosis by flow-cytometric analysis

The nuclei of CEM HABaxα and Neo-transfected cells were stained with propidium iodide and analyzed by flow cytometry for hypodiploid DNA content as described by Brady *et al.* (1996). Briefly, 10^6 cells were pelleted, washed once in PBS and then re-suspended in 300 µl hypotonic propidium-iodide solution (propidium iodide 50 µg/ml in 0.1% sodium citrate, 0.1% Triton X-100). The nuclei were incubated overnight at 4°C in the dark before the propidium-iodide fluorescence of 10,000 nuclei was analyzed in a FACScan flow cytometer. The percentage of apoptotic nuclei was determined as the percentage of nuclei with hypodiploid DNA content (below the G₀G₁ peak) and was normalized to that found in control cultures.

Detection of (HA)Baxα and Bcl-2 by Western-blot analysis

Bcl-2, HABaxα and Baxα levels were determined in samples of 10^6 cells boiled in Laemmli sample buffer. Protein lysates were separated on a 15% SDS-PAGE and transferred to Immobilon

PVDF membranes (Millipore, Bedford, MA). To detect Bcl-2 in blots containing only human samples, a mouse monoclonal antibody (MAb) against human Bcl-2 (clone 124, DAKO, Glostrup, Denmark) was used. To detect Bcl-2 and Baxα levels in cells of human and of murine origin, rabbit polyclonal antibodies recognizing human Bcl-2 (amino acids 4-21) and Baxα (amino acids 11-30) epitopes that are identical to the corresponding domains in the murine proteins (antibodies Sc-492 and Sc-493, respectively, from Santa Cruz Biotechnology, Santa Cruz, CA). A mouse MAb to actin (clone C4, Boehringer, Mannheim, Germany) was used as a loading control. Secondary antibodies were ¹²⁵I sheep anti-whole-mouse Ig and ¹²⁵I donkey anti-whole-rabbit Ig (Amersham, Aylesbury, UK). All antibody incubations were performed in blocking buffer (0.15% BSA, 0.5 mM EDTA, 0.5% vol/vol Tween-20 in PBS) overnight at 4°C. When the Baxα, Bcl-2 and actin levels were studied in the same blot, the blot was cut into 2 sections. The section containing proteins <30 kDa was first used to detect Bcl-2, followed by quantitation, and then re-probed to detect the Baxα levels, while the section containing proteins of >30 kDa was used to detect actin for normalization. Protein levels were quantitated with a phosphor imager. In all samples, Baxα and Bcl-2 levels were compared in each blot with those in the reference DoHH2 cells, a follicular non-Hodgkin's B-cell lymphoma that harbours the t(14;18), resulting in high Bcl-2 levels. The Baxα and Bcl-2 levels and their ratio in DoHH2 cells were all arbitrarily set at 1.0. Levels were calculated from at least 2 independent determinations. Variation between duplicates was usually less than 20%; if higher, a third analysis was performed. Reproducibility in Bcl-2 levels in DoHH2 cells was established in separate experiments with ethanol-fixed, cryopreserved or fresh cells, yielding a SEM of ±10%. Aliquots of 10^6 DoHH2 cells were frozen at the beginning of this study and always included in each experiment for calibration.

Patient material

Clinical specimens represented archival bone-marrow(BM) aspirates derived from untreated patients with childhood ALL (age 0–15 years), collected in the course of a national study ALL-V (non-high risk, 1979–1984) by the Working Party on Childhood Tumours Amsterdam (Emma Children's Hospital and Central Laboratory of the Netherlands Red Cross Blood Transfusion Service) after confirmed consent by the parents. Samples also included non-study patients with high-risk features, according to the criteria applied in ALL-V: WBC ≥50 and/or mediastinal mass or CNS involvement. In addition, some samples were obtained from patients of study ALL-VI (1984–88), collected at the Sophia Children's Hospital for a comparison of centralized vs. local determination of cell kinetic parameters, as reported (Smets *et al.*, 1995). Patients with high WBC are over-represented in the present analysis with a relative paucity of patients with hyperdiploid disease and other markers of low risk, because of the limited

FIGURE 1 – Western-blot analysis of Bcl-2 and Baxα levels in a panel of cell lines with increasing sensitivity to DEX (HL60 < DoHH2 < L1210 < S49). Protein lysates (100 µg) were separated by SDS-PAGE and transferred to PVDF membranes. The membranes were probed with a rabbit polyclonal antibody against human and murine Bcl-2 (a) or against Baxα (b), followed by the appropriate ¹²⁵I-linked secondary antibody and autoradiography. The membranes were also probed with a MAb against actin as a loading control. Band intensities were calculated by phosphor imaging.

availability of archival material from non-high-risk patients. BM cells were centrifuged on Ficoll-Isopaque density gradients to obtain the mononuclear-cell fractions, and subsequently either cryopreserved at the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service or fixed in 70% ethanol at the hospital. Morphologic examination before preservation of the aspirates showed that over 80% of the cells were blasts.

Statistical analysis

The relation between Bcl-2, Bax, Bax α :Bcl-2 and features at presentation were assessed by the Pierson correlation coefficient (r). For this analysis, a natural logarithmic transformation of the protein levels and the quantitative features at presentation was performed. The relationship between Bcl-2 and B- vs. T-lineage was assessed by the Students t -test. A similar analysis was performed for the relationship between Bcl-2 levels and pre-B vs. common ALL. The relation between WBC and B- vs. T-lineage ALL was established with the same test after adjustment for unequal variance. The simultaneous relation between immunophenotype, WBC and Bcl-2 was analyzed by analysis of co-variance (ANCOVA). In the Tables, the geometric mean and coefficient of variation (CoV) are provided, rather than the arithmetic mean and (absolute) SD, because of non-normality of the data and unequal variances. The geometric mean is derived from the anti-log of the mean of the logarithmical transformed data. The CoV equals the SD of the natural logarithmical transformed values.

All calculations were performed using the SPSS statistical program for MS WINDOWS release 6.1.

RESULTS

Bax α :Bcl-2 ratio correlates with DEX sensitivity in cell lines

We earlier described an inverse correlation between Bcl-2 levels and sensitivity to DEX in a panel of leukaemic cell lines (Smets *et al.*, 1994). This panel included (in increasing order of DEX

sensitivity): HL60 (human myeloid leukaemia); DoHH2 [human non-Hodgkin's B-cell lymphoma containing the t(14;18)]; CEM C7 (human T leukaemia); L1210 and S49 (murine T leukaemia) cells. Because the ratio of Bax α to Bcl-2 rather than the absolute levels of Bcl-2 is thought to be important for DEX-induced apoptosis, we have now investigated whether DEX sensitivity in this cell panel was also determined by the Bax α :Bcl-2 ratio. The Bcl-2 levels in the very sensitive S49 cells could hardly be detected

FIGURE 2 – Western-blot analysis of CEM C7 clones transfected with *HABaxα* and *Neo* (CEM 1, 7, 16, 18, 29) or with the *Neo* vector alone (neo). The DoHH2 cells were used as a reference, and the Bax α :Bcl-2 ratio was set at 1.0. Total lysates of 10^6 cells were separated on a 15% SDS PAGE and transferred to PVDF membranes. The membrane section containing the proteins <30 kDa were probed subsequently with antibodies against Bcl-2 (*b*) on HA-tag (*c*) or with an antibody against Bax α (*d*). The membrane section containing the proteins >30 kDa was probed with an antibody against actin as a loading control (*a*). The proteins were detected and quantitated as described in Figure 1.

A

B

FIGURE 3 – Induction of apoptosis by DEX (*a*) or TG (*b*) treatment in CEM NEO (black bars) and CEM 18 cells. Cells were harvested at the indicated times and prepared for FACS analysis to measure the DNA content of nuclei by propidium-iodide staining, as described in "Material and Methods". FACS analysis allowed apoptotic nuclei to be identified as a broad hypodiploid DNA peak, easily separable from the narrow peak of nuclei with a normal diploid DNA content. The percentage of apoptotic cells shown is normalized to the amount of apoptotic cells found in untreated CEM NEO and CEM 18 cells at the corresponding time points. Results similar to those shown were observed in 3 independent experiments. Each value represents the mean $\pm$ range of duplicate determinations in 10,000 cells each.

using an antibody specific for identical epitopes in human and mouse Bcl-2 followed by a ^{125}I -linked secondary antibody (Fig. 1). In this analysis we ran duplicate gels, because the antibody used cross-reacts with lower-molecular-weight proteins, affecting Bax α detection. These low Bcl-2 levels in S49 compared with the other cell lines studied are in accordance with the results obtained by Northern blotting using a mouse *bcl-2* probe (Smets *et al.*, 1994). The results confirmed that decreasing Bcl-2 levels correlate with increasing sensitivity to DEX. Moreover, DEX sensitivity was proportional to increasing Bax α levels as determined by Western blotting using antibodies that recognizes epitopes of Bax α and Bcl-2 that are identical both in human and in murine species (Fig. 1). The Bax α :Bcl-2 ratio was determined by phosphor-image analysis (see "Material and Methods") and amounted to 0.4 in HL60, 1.0 in DoHH2, 1.4 in CEM C7, 4.6 in L1210 and to 8.6 in S49 cells.

Effect of HABax α transfection on the rate of DEX- and TG-induced apoptosis

To specifically analyze the contribution of Bax α to DEX sensitivity, the relatively insensitive CEM C7 cells were stably transfected with a human CD2 cassette driving expression of HA-tagged *bax α* and with a *Neo* expression vector. Following selection in G418, clones were isolated containing both the HABax α and *Neo* expression vectors or the *Neo* vector alone. The HABax α protein could be distinguished from endogenous Bax α protein because the addition of the HA tag results in a protein that migrates as a 22-kDa protein, that is approximately 1.1 kDa larger than the endogenous Bax α of 21 kDa (Oltvai *et al.*, 1993). The expression levels of HABax α , Bax α and Bcl-2 in 5 HABax α - and *Neo*-transfected clones and the negative controls DoHH2 and a CEM clone which was transfected with only the *Neo* vector, were assessed by Western-blot analysis (Fig. 2). The Bax α :Bcl-2 ratio in the *Neo* control was 1.4 as compared with the DoHH2 (1.0) and amounted to 2.4 (CEM 1 and 7), 1.3 (CEM 16 and 29) and 2.8 (CEM 18). A representative control (CEM NEO) and a HABax α transfected clone (CEM 18) were selected for further studies.

FIGURE 4 – Representative Western-blot analysis of lysates of bone-marrow blasts from 6 untreated children with acute lymphoblastic leukaemia (lanes 1–6). Total lysates of 1×10^6 bone-marrow cells were separated on a 15% SDS PAGE. Bcl-2 and Bax α levels were determined essentially as described in Figure 2. DoHH2 cells [t(14;18) positive follicular non-Hodgkin's B-cell lymphoma] were used as a reference throughout. The values of Bax α -to-Bcl-2 ratios are depicted in the figure (bottom), with that of DoHH2 cells arbitrarily set at 1.0.

The effect of the moderate increase in Bax α :Bcl-2 in CEM 18 compared with CEM NEO on DEX sensitivity is visualized in Figure 3. Incubation with 1 μM DEX induced apoptosis with the kinetics shown in Figure 3a. A clear differential (approximately 2.5-fold increase in apoptotic nuclei) was seen between the CEM NEO and CEM 18 cells. Similar results were obtained for 2 other clones with an increased Bax α :Bcl-2 ratio (CEM 1 and 7), while the response of the clones with a Bax α :Bcl-2 ratio of 1.3 (CEM 16 and 29) was not different from the parental CEM C7 cells. In addition, we examined the sensitivity of CEM 18 and CEM NEO cells to the apoptotic effect of TG. Continuous incubation of CEM 18 and CEM NEO cells with TG (100–500 nM) induced apoptosis, as shown for 500 nM in Figure 3b. These results demonstrate that a moderate increase in the Bax α :Bcl-2 ratio sensitizes CEM C7 cells not only to DEX- but also to TG-induced apoptosis.

Bax α and Bcl-2 levels in childhood acute leukaemia

Morphologic examination of all aspirates showed that over 80% of the cells were blasts. Bcl-2 and Bax α levels were determined using the same methods as described for the cell lines. Figure 4 shows a representative Western-blot analysis of 6 bone-marrow samples of ALL patients and the DoHH2-lymphoma reference cells, arbitrarily set at 1.0 (see "Material and Methods"). It is apparent that the Bax α and Bcl-2 levels in the leukaemic samples were both above and below those in the reference cells. Table I summarizes the results obtained for bone-marrow aspirates of 48 ALL and 8 AML patients and of 4 peripheral-blood-lymphocyte (PBL) samples. In ALL blasts, Bcl-2 levels (geometric mean 0.6) were higher than in normal PBL (0.3), as reported by Coustan-Smith *et al.* (1996), but with considerable inter-patient variation (CoV 96%). In 35% (17/48), the Bcl-2 levels were equal to or lower than in normal PBL. Over-expression was observed in 40% (19/48), *i.e.*, patients with Bcl-2 levels similar to, or even higher than, those in the t(14;18)-harbouring DoHH2 cells (≥ 1.0). The Bax α levels (geometric mean 0.7) were also higher than in normal PBL (0.4). In 27% (11/41) of the ALL samples, the Bax α levels were equal to, or lower than, in PBL, and in 34% (15/41) this protein was highly expressed, *i.e.*, greater than 1.0. Although Bcl-2 levels correlated with those of Bax α ($p < 0.001$, $r = 0.60$), the Bax α :Bcl-2 ratio also varied widely (CoV 118%), as did the individual proteins. The values of Bax α :Bcl-2 ratio correlated with Bax α ($p < 0.001$, $r = 0.74$) but not with Bcl-2 ($p = 0.78$, $r = 0.04$), indicating the importance of Bax α in determining this ratio. Furthermore, we investigated 8 bone-marrow aspirates of childhood AML patients. Though the sample was rather small, the range of Bcl-2 and Bax α levels was similar to that in lymphatic leukaemia (Table I).

The use of identical methods and the standard inclusion of reference samples allowed direct comparison of the Bax α :Bcl-2 settings in clinical samples with the experimental values found in *in vitro* models associated with DEX sensitivity. Table II shows that patient values were distributed over all the settings of Bax α :Bcl-2 as found in cell lines. In 25 out of 41 ALL patients, the ratio of Bax α to Bcl-2 was similar to or lower than in the relatively insensitive CEM NEO cells, indicating its possible clinical relevance.

TABLE I – Bcl-2 AND Bax α LEVELS IN CHILDHOOD ACUTE LEUKAEMIA (ALL, AML) AND NORMAL PERIPHERAL-BLOOD LYMPHOCYTES (PBL)

	Number	Bcl-2 level		Number	Bax α level		Bax α :Bcl-2	
		Mean ¹	Range		Mean	Range	Mean	Range
ALL	48	0.6 (96%)	0.1–6.6	41	0.7 (120%)	0–14.7	1.1 (118%)	0–10
AML	8	0.5 (87%)	0.1–1.4	6	1.2 (58%)	0.5–2.4	1.9 (106%)	0.5–12
PBL	4	0.3 (9%)	0.2–0.4	6	0.4 (6%)	0.4–0.4	1.4 (5%)	1.3–1.5

¹The geometric mean (and CoV) rather than the arithmetic mean and SD, are presented in arbitrary units, calibrated against reference DoHH2 cells (=1.0; see "Material and Methods").

Relation with presenting features

Bcl-2 and Bax α levels were compared with various presenting features of ALL patients (Table III). Bcl-2 levels were lower in T-ALL than in B-lineage ALL ($p = 0.014$). Moreover, Bcl-2 levels were higher in common ALL than in pre-B ALL ($p = 0.017$).

Bcl-2 expression levels in ALL were also inversely associated with WBC ($p = 0.023$, $r = -0.33$). Figure 5 illustrates the distribution of WBC values in T- and in B-lineage ALL as a function of Bcl-2-expression level. The generalized correlation of high WBC with the T-cell marker was also apparent in this patient sample ($p < 0.001$). Analysis of co-variance showed that the association between WBC and the T-cell marker was not explained by Bcl-2 values ($p = 0.008$ controlled for Bcl-2). On the other hand, after controlling for WBC, the association between Bcl-2 and the T-cell marker disappeared ($p = 0.11$, controlled for WBC). The difference between common ALL and pre-B ALL remained after controlling for WBC ($p = 0.036$). By contrast, similar analyses for Bax α and Bax α :Bcl-2 showed no correlation between Bax α and immunophenotype or WBC.

No correlation was found between absolute values of %S-phase cells and Bcl-2, Bax α or Bax α :Bcl-2. Correlations with DNA ploidy could not be established because of the low number of patients with hyperdiploid DNA content in our study.

DISCUSSION

Studies of the prognostic value of Bcl-2 in ALL have yielded conflicting results (Campana *et al.*, 1993; Gala *et al.*, 1994; Coustan-Smith *et al.*, 1996; Maung *et al.*, 1994). Unlike in AML (Campos *et al.*, 1993), and contrary to the suspected multidrug-resistant role of Bcl-2, high Bcl-2 levels were not found to be associated with known predictors of poor response in childhood ALL: high WBC, T-cell marker and %S-phase cells. In fact, the results of Coustan-Smith *et al.* (1996) and the data of the present

report suggest that Bcl-2 levels may be higher in patients with a favourable response to treatment. Because the Bax α -to-Bcl-2 ratio, rather than the Bcl-2 levels alone, determines the apoptotic response in primary T cells of transgenic mice (Brady *et al.*, 1996), we sought to investigate which Bax α :Bcl-2 ratio is critical in DEX and TG sensitivity of leukaemic cell lines. The relevance of the Bax α :Bcl-2 levels determined in clinical leukaemia was subsequently assessed by comparing them with those found in cell lines and with various features at presentation.

We have described an inverse correlation between Bcl-2 levels and sensitivity to DEX in a panel of leukaemic cell lines (Smets *et al.*, 1994). By including the Bax α levels in that comparison, the scale of Bax α :Bcl-2 ranged from 0.4 to 8.6, for fully resistant HL60 cells and for the very sensitive S49 cells respectively. To investigate the crucial role of Bax α , we transfected HAbax α into the human T-leukaemia CEM C7 cells, using a CD2 cassette to drive expression. The CEM C7 cells were selected for these studies because they express intermediate Bcl-2 levels and are relatively insensitive to DEX treatment. A critical increase of Bax α sensitized CEM C7 cells to DEX- and TG-induced apoptosis by approximately 2.5-fold (Figs. 2, 3). Increased sensitivity was achieved by a moderate change of the Bax α -to-Bcl-2 ratio from 1.4 in CEM NEO cells to 2.4 in HAbax α -transfected CEM C7 clones (CEM 1 and 7) or 2.8 in CEM 18, confirming observations in primary T cells from bax α transgenic mice (Brady *et al.*, 1996).

The present study in ALL confirmed the large inter-patient variation in Bcl-2 levels, similar to those reported by Coustan-Smith *et al.* (1996). Here we report large inter-patient variation also in Bax α levels and in the functionally more important Bax α :Bcl-2 ratios in ALL. In fact, statistical analysis revealed that Bax α levels dominated the Bax α :Bcl-2 setting. Moreover, comparison of these ratios with those in leukaemic cell lines and manipulated CEM C7 cells revealed that the range of Bax α :Bcl-2 values in ALL samples corresponded with that in cell lines with different sensitivity to DEX (Table I). Thus, the biological variation in ALL in the expression levels of these proteins correlates with their function in the response to glucocorticoid treatment in leukaemic cell lines and after genetic enhancement of Bax α expression. Important in this analysis is that over 80% of the cells were blasts, excluding a significant contribution of PBL. Moreover, Bax α and Bcl-2 levels were simultaneously assessed in at least 2 independent Western blots, with a variation of less than 20%, using DoHH2 cells as a reference in each analysis.

The molecular basis for the high range in expression levels of both Bcl-2 and Bax α in ALL is unknown. The classic cause of over-expression of Bcl-2 in follicular NHL the translocation t(14;18) is very rare in childhood leukaemia (Campos *et al.*, 1993). The fact that high levels of Bcl-2 are also observed in follicular

TABLE II – Bax α :TO-Bcl-2 RATIO IN LEUKAEMIC BLASTS FROM CHILDHOOD ALL CLASSIFIED ACCORDING TO THE VALUES FOUND IN LEUKAEMIC CELL LINES WITH DIFFERENT SENSITIVITIES TO DEX TREATMENT¹

Bax α :Bcl-2	DEX response in cell lines	Number of patients (total, 41)
≤ 1.0	resistant (DoHH2)	20
> 1.0 and ≤ 1.4	relatively insensitive (parental CEM C7, CEM NEO, CEM 16, 29)	5
> 1.4 and ≤ 2.8	sensitive (CEM 1, 7, 18)	11
> 2.8	very sensitive (L1210, S49)	5

¹The response to DEX treatment in CEM 18 and CEM NEO cells is shown in Figure 3 and that of DoHH2, CEM C7, L1210 and S49 cells has been described (Smets *et al.*, 1994).

TABLE III – ASSOCIATION OF Bcl-2 AND Bax α PROTEIN LEVELS WITH VARIOUS PRESENTING CLINICAL FEATURES IN CHILDHOOD ALL

Presenting feature	Number	Mean ¹ Bcl-2	Number	Mean Bax α	Mean Bax α :Bcl-2
WBC ²					
< 50	22	0.8* (104%)	17	0.7 (126%)	0.8 (122%)
≥ 50	26	0.5 (82%)	24	0.7 (118%)	1.3 (112%)
Immunology					
B-lineage ALL ³	32	0.8* (85%)	26	0.8 (118%)	1.1 (103%)
common ALL ³	21	1.0* (81%)	17	0.9 (120%)	1.2 (86%)
pre-B ALL	11	0.5 (72%)	9	0.5 (110%)	0.9 (133%)
T-lineage ALL	16	0.4 (100%)	15	0.5 (124%)	1.1 (144%)
DNA content ⁴					
diploid	41	0.6 (101%)	35	0.7 (130%)	1.1 (124%)
hyperdiploid	5	0.8 (48%)	4	0.8 (21%)	1.2 (68%)
%S-phase ²					
≤ 6	24	0.7 (101%)	21	0.7 (101%)	1.1 (105%)
> 6	22	0.5 (92%)	18	0.7 (92%)	1.1 (137%)

¹Geometric mean (and CoV) are presented. ²Correlations were tested by comparing absolute values in the Pierson test. ³Correlations were tested with the *t*-test. ⁴Not tested.

* $p < 0.05$.

FIGURE 5 – Distribution of WBC values in patients ($n = 48$) with T- (triangles) and B-lineage (dots) ALL classified according to Bcl-2 content “low” (<1.0) or “over-expressed” (≥ 1.0) as described in Figure 4.

lymphomas in the absence of t(14;18) indicates the existence of other mechanisms that de-regulate *bcl-2* (Pezzella *et al.*, 1990). The high range of Bcl-2 and Bax α expression levels in ALL might reflect the various differentiation stages in B- and T-cell development. For instance, most primitive haematopoietic precursors express Bcl-x, but not Bcl-2 (Park *et al.*, 1995). We found that the Bcl-2 levels were significantly higher in common ALL than in pre-B ALL, originating from pre-pre-B cells and pre-B cells respectively, suggesting effects of differentiation on Bcl-2 levels. However, Bcl-2 levels in pre-B ALL are much higher than in normal pre-B cells (Campana *et al.*, 1993). Differentiation stage is, therefore, probably not the sole determinant of Bcl-2 levels in ALL.

Another candidate for the de-regulation of Bcl-2 and Bax α is *p53*. *p53*-knockout mice show elevated Bcl-2 expression in at least some tissues, suggesting de-regulation of the gene *in vivo*. Furthermore, *p53* is able to induce expression of *bax* α (Miyashita and Reed, 1995). However, in childhood ALL, inactivation of *p53* by mutation or *mdm2* over-expression is not frequently found, al-

though a high incidence of potential *p53* inactivation at diagnosis in poor-outcome leukaemia has been reported (Marks *et al.*, 1996). In conclusion, the mechanism of Bcl-2 and Bax α over-expression, encountered in approximately 40 and 34% of patients respectively, remains unclear.

In contrast to the results of Coustan-Smith *et al.* (1996), we show that the T-cell marker is associated with low Bcl-2. This discrepancy may be due to a larger fraction of T-ALL patients in our population; but an influence of the different methods used, *e.g.*, immunocytochemistry *vs.* Western blotting, combined with statistical analysis using cut-off points *vs.* absolute levels, cannot be excluded. In addition, our data differ from other reports (Campana *et al.*, 1993; Coustan-Smith *et al.*, 1996) in that we observed an inverse correlation between WBC and Bcl-2 levels in ALL. T-lineage ALL is frequently associated with elevated WBC values, which was confirmed in this study. Consequently, the apparent correlation between immunophenotype and Bcl-2 level (Fig. 5) could be attributed to a major impact of WBC on (low) Bcl-2 level.

The inverse correlation between Bcl-2 and WBC classes ALL among other malignant disorders in which high levels of Bcl-2 are associated with slowly progressing, indolent disease (Pezzella *et al.*, 1993; Sinicrope *et al.*, 1995). This conclusion is not in conflict with the lack of correlation with percentage S (Table III), since the latter parameter has no relationship with WBC in ALL (Smets *et al.*, 1995). It agrees, however, with an apparent proliferation-suppressing effect of Bcl-2 observed in some model systems (Smets *et al.*, 1994; Pietenpol *et al.*, 1994).

Although non-homogeneous treatment regimes of high-risk patients prevented a meaningful comparison between Bcl-2 and follow-up data, such an analysis would most likely reveal a worse outcome for patients with low Bcl-2, in agreement with the report of Coustan-Smith *et al.* (1996). Clear over-expression of Bcl-2 was most frequently found (Fig. 5) in patients with B-lineage ALL and WBC <50 , who respond most favourably to treatment. However, because of the inverse relationship with WBC, an important prognostic factor in ALL, it remains to be established whether Bcl-2 level can be an independent predictor of response. The discrepancy between the prognostic value of Bcl-2 in AML (Campos *et al.*, 1993) and ALL is remarkable, and, according to our results (Table I), cannot be explained by large differences in Bax α and Bax α :Bcl-2. It is possible that Bcl-2-antagonizing proteins, other than Bax α , as well as post-translational modifications (Gajewski and Thompson, 1996) are involved.

The large variation in Bax α :Bcl-2 in ALL, independent of any prognostic feature (Table III), suggests that the Bax α :Bcl-2 ratio may be an independent marker of potential prognostic value. Bax α :Bcl-2 in clinical samples spanned the full range of levels relevant for DEX response (Table II) and was largely determined by Bax α , not by Bcl-2. Model studies with primary thymocytes (Brady *et al.*, 1996) further confirmed the critical role of Bax α in the response of various inducers of apoptosis. Indeed, in B-CLL cells a relation was found with the *bcl-2/bax* ratio and drug-induced apoptosis (Thomas *et al.*, 1996). Moreover, a study of 14 AML patients showed a correlation between the Bcl-2/Bax ratio and the clinical response after induction therapy (Stoetzer *et al.*, 1996). Because of over-representation of patients with high-risk features in the present study, the large fraction of patients (61%) with a Bax α :Bcl-2 ratio corresponding with decreased sensitivity to DEX (Table II) is not necessarily in conflict with the overall higher response rate in this disease. Collectively, our results suggest that Bax α :Bcl-2, rather than Bcl-2 alone, is important for the survival of ALL cells after cytotoxic treatment.

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