Dexamethasone-induced apoptosis in acute lymphoblastic leukemia involves differential regulation of Bcl-2 family members

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ABSTRACT

Background and Objectives

The mechanism of glucocorticoid -induced apoptosis is not fully understood and early predictive assays based on apoptotic markers for clinical outcome in acute lymphoblastic leukemia (ALL) are scarce. The aim of this study was to characterize the involvement of Bcl-2 family members and caspase activation in dexamethasone(Dex)-induced apoptosis in ALL.

Design and Methods

Primary childhood ALL samples, the pre-B ALL cell line RS(4;11), and the T-ALL cell line CCRF-CEM were used. The involvement of Bcl-2 family members was evaluated by flow cytometry, immunocytochemistry, and western and northern blotting. Apoptosis was analyzed by annexin V and TMRE staining. Caspase activity was evaluated by a fluorometric assay.

Results

Dex induced significant down-regulation of the anti-apoptotic Bcl-2 family members Bcl-2 and Bcl-xL, differential activation of the pro-apoptotic Bak and Bax, loss of $\Delta\psi m$ and cytochrome c release. Dex-induced apoptosis also involved early activation of caspases 2 and -3. Inhibition of caspase activity did not, however, protect against Dex-induced Bak/Bax activation, loss of $\Delta\psi m$ or cell death. In 12 primary ALL samples Dex-induced apoptosis was associated with activation of Bax (p=0.045) and down-regulation of Bcl-2 (p=0.016) and/or Bcl-xL (p=0.004). Furthermore, *ex vivo* Dex-sensitivity was associated with an early treatment response to polychemotherapy (p=0.026).

Interpretation and Conclusions

The differential regulation of pro- and anti-apoptotic Bcl-2 family members appears to be a key event in the execution of Dex-induced apoptosis in ALL cell lines, and also indicates a role for these proteins in primary ALL cells.

Key words: glucocorticoids, apoptosis, Bcl-2 family, caspases, acute lymphoblastic leukemia

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Glucocorticoids (GC) are involved in many biological processes. Due to their potent lethal effect on lymphoid cells, GC are used as a backbone drug in most treatment protocols for various lymphoid malignancies, including acute lymphoblastic leukemia (ALL).[1,2] Sensitivity to GC-treatment *in vivo* is also a major prognostic factor in childhood ALL.[3] The *in vitro* effect of GC on lymphoid cells is dramatic and includes the induction of G_0/G_1 cell cycle arrest and apoptosis. Although these events seem to be mediated through GC binding to its intracellular receptor, a detailed molecular understanding of GC sensitivity and resistance in ALL is lacking.[4] A diminshed expression of glucocorticoid receptor (GR) mRNA levels has been found to be associated with some degree of *in vitro* resistance to prednisolone.[5] However, other studies have failed to show an association between the levels of GR protein expression and *in vivo* GC resistance, indicating that the overall levels of GR expression may be of minor importance for *in vivo* GC resistance in childhood ALL.[6]

Two major apoptotic pathways, the mitochondrial and the death receptor-mediated pathways, have been described. The death receptor-mediated pathway is not critically involved in the apoptotic response to GC.[7] GC seem to utilize the mitochondrial-pathway by inducing the disruption of the mitochondrial membrane potential ($\Delta\psi m$) and the release of several apoptogenic factors including cytochrome c leading to the activation of caspase-9.[8] Release of cytochrome c from the mitochondrial intermembrane space to the cytoplasm is commonly mediated by the pro-apoptotic Bcl-2 family proteins Bak and Bax, which in apoptotic cells are suggested to either oligomerize and form pores in the mitochondrial outer membrane,[9,10] or to interact with the proteins of the mitochondrial megapore.[11,12] Although the activation of Bak and Bax has been detected in leukemic cells treated with dexamethasone (Dex),[13,14] the roles of Bak and Bax in relation to Bcl-2 and Bcl-xL in Dex-induced apoptosis have not been investigated in detail. Activation of caspases is one of the hallmarks of apoptosis. However, conflicting results exist concerning the role of caspases in GC-induced apoptosis.[15–17] Therefore, in this study, we analyzed the kinetics of modulation and the interrelation between the Bcl-2 family members as well as the dependence on caspase activation during Dex-induced apoptosis in ALL cells. We also investigated the correlation between Dex-induced cell death *ex vivo* and the early treatment response in childhood ALL as well as the role of Dex-induced alterations in the pro- and anti-apoptotic Bcl-2 family members in this respect.

Design and Methods

Patients

The study included leukemic cells from 12 children (5 boys and 7 girls) with ALL (9 B-precursor ALL and 3 T-precursor ALL) who were treated according to the NOPHO-ALL 2000 protocol.[18,19] Their median age at diagnosis was 7 (3-15) years. The diagnosis was established according to the World Health Organization classification.[20] Minimal residual disease (MRD) was defined as the presence of residual leukemic cells in the bone marrow at the 0.01% level. MRD data were acquired and analyzed as previously described.[21] All the patients' parents were informed of the investigational nature of this study, and informed consent was obtained from each parent in accordance with the conditions of the approval of the study by the local ethics committee (Stockholm, Sweden).

Isolation and culture of primary cells

Mononuclear cells were isolated from bone marrow samples by centrifugation on a Ficoll/Hypaque (Lymphoprep, Oslo, Norway) gradient and cryopreserved in liquid nitrogen in the presence of 10% dimethyl sulfoxide. After thawing, lymphoblasts were cultured at a cell concentration of $2.5x10^6$/mL in RPMI 1640 culture medium supplemented with 10% heat-inactivated fetal calf serum (GIBCO, Berlin, Germany), 2 mM L-glutamine, 50 μg/mL streptomycin, 50 μg/mL penicillin and maintained in a humidified incubator in 5% CO_2 at 37°C. Cells were incubated for up to 24 hours with 200 nM of Dex (Oradexon 5mg/mL, Oy Organon AB, Finland).

Cell lines, culture conditions and treatment

Two pre-B ALL cell lines, RS4;11 (RS4) cells (ATCC, Manassas, USA) and Reh-6 (Reh) cells and T-ALL CCRF-CEM cells (*F. Albertioni, Karolinska Institute, Stockholm, Sweden*) were used in these experiments. RS4 cells were cultured in RPMI 1640 (GIBCO) supplemented with 10% (v/v) heat-inactivated fetal calf serum (GIBCO), 2 mM L-glutamine, 50 μg/mL streptomycin, 50 μg/mL penicillin and for RS4 cells also 25 mM Hepes buffer and maintained in a humidified incubator in 5% CO_2 at 37°C. The cells were treated with the indicated concentrations of Dex for up to 72 h. Doxorubicin (adriamycin from Pharmacia and Upjohn, Stockholm, Sweden) was used at a concentration of 60 ng/mL for 24 h. All experiments using cell lines were performed at least three times except the caspase-6 and –8 assays, which were performed twice with similar outcomes. Error bars represent the standard deviation of the repetitions in the graphic presentations (Figure 1B,C, 5A,B and *Supplementary Figure 4*).

Inhibitors and antibodies

The pan-caspase inhibitor z-VAD-FMK (z-Val-Ala-Asp(OMe)-fluoromethylketone) (50 μM and 10 μM), caspase-3, -7 and -10 inhibitor z-DEVD-FMK (z-Asp(Ome)-Glu(Ome)-Val-Asp-FMK) (5 μM), and caspase-2 inhibitor z-VDVAD-FMK (Z-Val-Asp(Ome)-Val-Ala-Asp(OMe)-FMK) (25μM) were obtained from Enzyme System Products (Livermore, CA, USA). The GR antagonist RU38486 (RU486) was used at 100 nM and obtained from Sigma-Aldrich Sweden AB (Stockholm, Sweden). The antibody against active Bak (AM03, clone TC100) was from Oncogene Research Products (San Diego, CA, USA), and

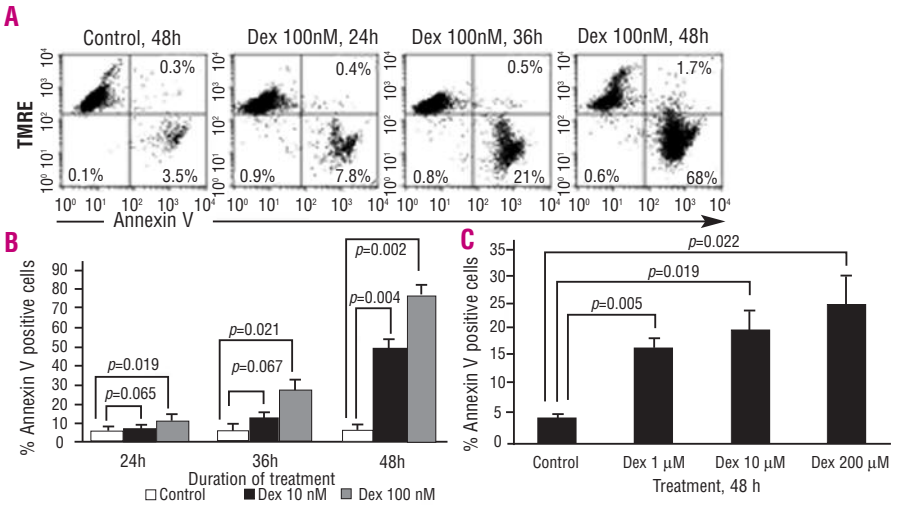

Figure 1. Dex induces cell death in RS4 and CCRF-CEM cells. RS4 cells were cultured in the presence or absence of the indicated doses of Dex for up to 48 h. To assess cell death, cells were stained for annexin V/TMRE (A) and analyzed for annexin V positivity (B). CCRF-CEM cells were cultured for 48 h and treated with 1-200 μM of Dex (C).

the antibody against active Bax (clone 6A7) was from Pharmingen (San Diego, CA, USA). The antibodies against Bcl-2 (cat. 554160) and Bcl-xL (cat. 610212) were purchased from Pharmingen. The cytochrome c antibody (6H2B4) was from Pharmingen. The β-tubulin antibody (T 4026) was from SIGMA (Saint Louis, MO, USA), and the mouse IgG1 isotype (X 0931) from Dako (Glostrup, Denmark). The allophycocyanin (APC)-conjugated goat anti-mouse Ig (cat. 550826) was from Pharmingen and flourescein isothiocyanate-(FITC)-conjugated swine anti-rabbit Ig (code nr. F0206) and FITC-conjugated rabbit anti-mouse Ig (code nr. F0261) from Dako.

Assessment of apoptosis

Redistribution of plasma membrane phosphatidyl serine (PS) was assessed using annexin V FLUOS (Roche Diagnostics Gmbh, Mannheim, Germany) according to the manufacturer's protocol as previously described.[22] Stainings were evaluated in a FACS Calibur flow cytometer (Becton Dickinson, San José, CA, USA) using the Cell Quest software. To detect Dex-induced changes in mitochondrial membrane potential, $\Delta\psi m$, cells were stained with tetramethylrhodamine ethyl ester perchlorate (TMRE; Molecular Probes, Inc., Eugene, OR, USA) as previously described.[22] For double annexin V/TMRE stainings, TMRE-labeled cells were then stained with annexin V FLUOS and analyzed as described elsewhere.[23]

Flow cytometric analysis of Bcl-2 family members

Staining for active Bak and Bax, and also Bcl-2, Bcl-xL was performed as previously described.[24] The amount of cells with activated Bak and Bax was defined by the percentage of cells within the M1 gate for activated Bak and Bax, as shown in *Online Supplementary Figure 1*. The amount of cells showing down-regulation of Bcl-2 and Bcl-xL was defined by the percentage of cells within the M1 gate showing low Bcl-2 and Bcl-xL staining, as illustrated in *Online Supplementary Figure 2*.

Immunocytochemistry

Cells were incubated for 30 min at 37°C in normal growth medium containing 10 μM MitoTracker (Molecular Probes). Cells were then cytospun on glass slides, fixed in 3% PFA for 20 min, permeabilized in digitonin (100 mg/mL) in PBS for 10 min and stained with anti-Bax (1:100) or anti-Bak (1:100) or cytochrome c (1:300) antibodies for 1 hr at room temperature, followed by a FITC-conjugated rabbit-anti-mouse Ig. The slides were mounted using Vectashield with DAPI for the staining of nuclei (Vector Laboratory, Inc.). The images were acquired on a Zeiss Axioplan 2 imaging microscope using AxioVision Rel. 4.5 and further processed using Adobe Photoshop software.

In vitro caspase assay

Caspase activity was measured by cleavage of the following substrates: Ac-VDVAD-AMC (caspase-2), Ac-DEVD-AMC (caspase-3 like), Ac-VEID-AMC (caspase-6), Ac-IETD-AMC (caspase-8); and Ac-LEHD-AMC (caspase-9) in a fluorometric assay as described elsewhere.[23] All substrates were obtained from Peptide Institute Inc. (Osaka, Japan). The experiments were performed in duplicate and repeated at least three times except the caspase-6 and -8 assays, which were performed twice with similar outcomes.

Western and northern blot analyses

For western blot analysis $5x10^6$ cells were lysed and the protein concentration was quantitated as described elsewhere.[22] The isolation of cytoplasmic RNA and northern blot analysis were carried out as previously described.[25]

Statistical analysis

The statistical analysis of Dex-induced changes in Bak, Bax, caspase activation, and Bcl-2, Bcl-xL down-regulation was performed using Student's *t* test for paired data. The non-parametric Mann-Whitney U test was used for the comparison of *ex vivo* Dex sensitivity with early treatment response as well as for comparisons of Dex-induced

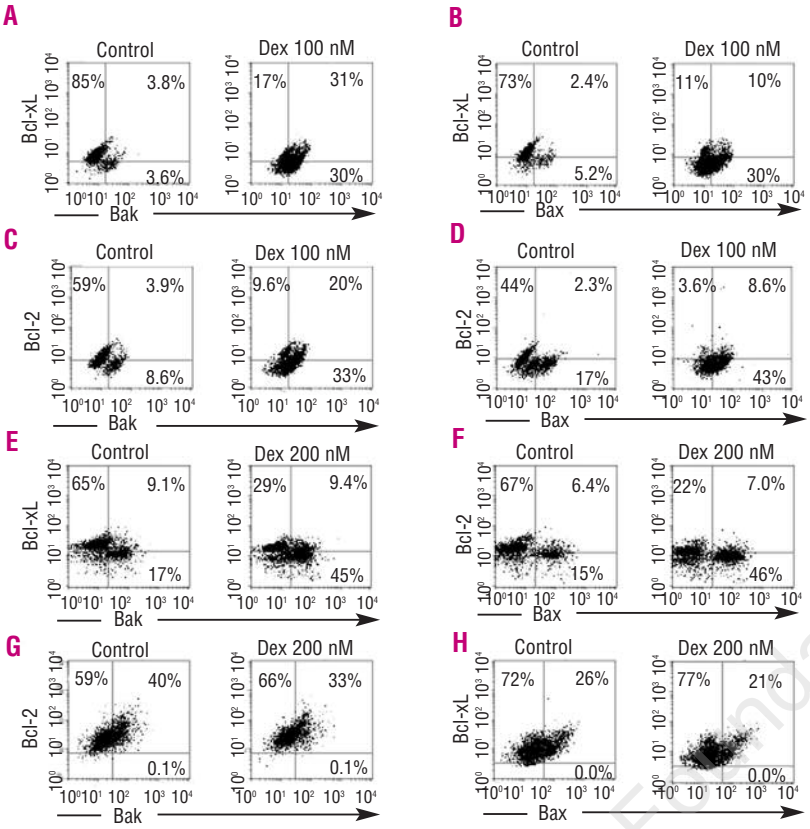

Figure 2. Effects of Dex on Bak and Bax in correlation to Bcl-2 and Bcl-xL. Cell Quest analysis of RS4 cells treated with Dex 100 nM for 48 h demonstrating (A) activation of Bak (cells in upper right and lower right quadrants) in relation to the down-regulation of the Bcl-xL protein levels (cells in lower left and lower right quadrants), (B) activation of Bax in relation to the down-regulation of Bcl-xL, (C) activation of Bak (cells in upper right and lower right quadrants) in relation to the down-regulation of the Bcl-2 protein levels (cells in lower left and lower right quadrants) and (D) activation of Bax in relation to the down-regulation of Bcl-2. Treatment of cells from an *ex vivo* Dex-sensitive patient with Dex 200 nM for 24 h increases the population of cells with (E) higher Bak and (F) Bax (cells in upper right and lower right quadrants), and (E) lower Bcl-xL and (F) lower Bcl-2 (cells in lower left and lower right quadrant). The population of cells with (G) higher Bcl-2 and lower Bak, and (H) higher Bcl-xL and lower Bax was not affected after treatment with Dex 200 nM for 24 h in ALL cells from a Dex-resistant patient.

changes in Bak, Bax activation, and Bcl-2, Bcl-xL down-regulation, between *ex vivo* Dex-sensitive and Dex-resistant samples from patients. *p*-values <0.05 were considered statistically significant. All reported *p*-values are two-sided. All calculations were performed using the SPSS 12.0 (SPSS, Chicago, IL, USA) software.

Results

Dex induces apoptosis in ALL cell lines

RS4, Reh and CCRF-CEM cells were treated with various doses of Dex and apoptosis was detected after 24-48 h as simultaneous annexin V positivity and mitochondrial depolarization (loss of $\Delta\psi m$) (Figure 1A-B, *not shown*). In RS4 cells, a time- and dose-dependent increase in the population of apoptotic cells was observed (Figure 1A-B). Similar data were recorded with annexin V/ propidium iodide (PI) double staining, to ensure that necrosis was not induced (*data not shown*). CCRF-CEM cells were less responsive to Dex and only started to die after 48 h (Figure 1C). No increase in apoptosis was observed in Reh cells treated with Dex (*data not shown*).

Dex induces the activation of Bak and Bax in ALL cell lines

Upon induction of apoptosis the pro-apoptotic Bak and Bax proteins undergo conformational changes that expose otherwise inaccessible N-terminal epitopes. We used two antibodies that specifically recognize these epitopes and that can be exploited in flow cytometry or immunocytochemistry to monitor the activation state of Bak and Bax in individual cells.[13,26] Treatment of RS4 cells with Dex for 24 h and 48 h induced time- and dose-dependent increases in the amount of cells showing activated Bak and Bax (*Supplementary Figure 1A, a-d*). Both Bak and Bax were activated already 24 h after exposure to dexamethasone 100 nM ($p< 0.001$ for Bak and $p=0.027$ for Bax). At 24 h, 36 h and 48 h time-points, 10.9±2.2% SD, 36.2±4.3% SD ($p=0.001$) and 68.5±5.0% SD ($p<0.001$) of cells treated with Dex 100 nM showed activated Bak, respectively. At the corresponding time-points, Bax was activated in 8.6±2.0% SD, 27.1±7.1% SD ($p=0.005$) and 44.7±3.4% SD ($p<0.001$) of cells treated with Dex 100 nM. The increased amounts of activated Bak and Bax detected by flow cytometry were clearly not due to an increase in the protein levels of Bak or Bax as assessed by immunoblotting (*Online Supplementary Figure 1B*). In the Bak immunoblots, we observed an additional band of higher molecular weight, especially in extracts from Dex-treated samples. The nature of this band is unclear. In Dex-treated CCRF-CEM cells we also noted Bak activation after 48 h, at the time when CCRF-CEM cells started to die (*Online Supplementary Figure 1C*). This activation of Bak in CCRF-CEM cells was most clearly observed when very high doses of Dex (1-200 μM) were used. Bax was not activated in CCRF-CEM cells. The reason for this may be that Bax is mutated in this cell line, as previously reported.[27] Due to the relative insensitivity of CCRF-CEM cells to Dex, the significance of the data for the normal *in vivo* situation should be interpreted with some caution. In Reh cells Dex treatment did not induce either Bak or Bax activation (*data not shown*).

Dex induces down-regulation of Bcl-2 and Bcl-xL in ALL cell lines

The effects of Dex treatment on the two major anti-apoptotic Bcl-2 family members, Bcl-2 and Bcl-xL were investigated. In RS4 cells Bcl-2 was found to be gradually down-regulated up to the 48 h time-point (*Online Supplementary Figure 2A, a and b*). Bcl-xL levels were also affected by Dex, with similar kinetics as Bcl-2 (*Online Supplementary Figure 2A, c and d*), which suggests their involvement in Dex-induced apoptosis. At the 24 h, 36 h and 48 h time-points Bcl-2 was down-regulated in 18.2±5.5% SD (p=0.051), 36.6±13.5% SD (p=0.016) and 59.8±8.6% SD (p=0.001) of cells treated with Dex 100 nM and Bcl-xL was down-regulated in, respectively, 16.7±6.3% SD (p=0.043), 35.6±12.5% SD (p=0.014) and 67.3±14.3% SD (p=0.004) of such cells. To exclude the possibility that the observed down-regulation of Bcl-2 and Bcl-xL was due to non-specific down-regulation of proteins during cell death, the cells were also analyzed for the expression of a control protein, tubulin. As can be seen in *Online Supplementary Figure 2A e and f*, no Dex-induced changes of tubulin expression could be observed. Moreover, when apoptosis was induced in RS4 cells by the chemotherapeutic compound doxorubicin instead, no changes in either Bcl-2 or Bcl-xL levels were detected (*data not shown*) further excluding that these proteins are non-specifically down-regulated during apoptosis. In order to investigate whether the Bcl-2 and Bcl-xL down-regulation occurs at the transcriptional level, northern blot analysis was performed. The mRNA level of Bcl-2 was down-regulated following treatment with Dex by as early as 12 h, whereas the Bcl-xL mRNA down-regulation was observed later, at 24 h following Dex treatment (*Online Supplementary Figure 2B*). We searched for putative GC-regulated elements by BLAST analysis using the GRE consensus sequence, AGAACAnnnTGTTCT, within the 1 kb promoter region of the Bcl-2 gene. An element GGAAGAgggGGTCCA, with partial homology to the consensus was found 487 bp upstream of the transcription start site of Bcl-2 (NM_000633). This block of nucleotides is conserved in mice, suggesting that this GR binding site may be functional, although this needs to be confirmed experimentally in future studies.

Similar down-regulation of Bcl-2 and Bcl-xL was observed at the 48 h time-point in Dex-treated CCRF-CEM cells (*Online Supplementary Figure 2C*). Finally in Dex-resistant Reh cells, the levels of Bcl-2 and Bcl-xL did not change following Dex treatment (*data not shown*).

In order to investigate how changes in the levels of active Bak or Bax correlate with down-regulation of the anti-apoptotic Bcl-2 family members, we performed double stainings. In RS4 cells treated for 48 h with 100 nM Dex, cell populations with activated Bak had either low or high levels of Bcl-2 and Bcl-xL (Figures 2A and 2C). This indicates that a decrease in Bcl-2 levels is not a prerequisite for Bak activation. On the other hand, Dex-treated cells that displayed high levels of active Bax were found only among cells with lower protein levels of Bcl-2 and Bcl-xL (Figures 2B and 2D). Thus, in contrast to Bak, activation of Bax seems to require down-regulation of Bcl-2 and Bcl-xL proteins levels.

Effect of Dex on Bcl-2 family members in primary ALL cells

We examined Dex-induced apoptosis in diagnostic samples from 12 patients subsequently treated for ALL with combination chemotherapy, consisting of the GC prednisolone, vincristine, doxorubicin and intrathecal methotrexate.[19] Eight patients were defined as *in vivo* sensitive and four as *in vivo* resistant to the GC according to the presence of <4% or ≥ 4% blasts, respectively, in bone marrow, as detected by flow cytometry on treatment day 15, i.e. at the assessment of early treatment response (Table 1). Lymphoblasts were treated *ex vivo* with 200 nM Dex for 16-24 h and assayed for cell death by annexin V positivity and mitochondrial depolarization (loss of $\Delta\psi$m). All four *in vivo* resistant patients were also resistant *ex vivo* to Dex treatment whereas only two *in vivo* sensitive patients were resistant to Dex *ex vivo* (Table 1). With the exception of one patient, we did not observe activation of Bax/Bak in the lymphoblasts from the *ex vivo* Dex-resistant patients (Figures 2G and 2H), whereas Bax and Bak were clearly activated in four of six *ex vivo* Dex-sensitive patients. In two *ex vivo* Dex-sensitive patients no Bax activation was found, but both Bcl-2 and Bcl-xL were down-regulated (Table 1). The Bcl-2/Bcl-xL levels were down-regulated in all *ex vivo* sensitive patients. Only one of the *ex vivo* Dex-resistant patients showed slight Bcl-xL down-regulation (Table 1). Furthermore, as in our cell line system, Bax activation seemed to require down-regulation of Bcl-2 and Bcl-xL (Figure 2F and *data not shown*). Statistical analysis showed a significant correlation between *ex vivo* Dex-sensitivity and an early treatment response (p=0.026) (Figure 3A). Additionally, *ex vivo* Dex-sensitive patients had significantly higher Bax activation (Figure 3B) and greater Bcl-2/Bcl-xL down-regulation (Figure 3Bc,d). *Ex vivo* Dex-sensitive patients also had higher Bak activation (Figure 3Ba), but this change was not statistically significant, probably because of the low number of patients.

Effect of the GR antagonist RU486 on Bcl-2 family members

In RS4 cells, addition of RU486 prior to Dex completely blocked the cell death induced by 100 nM of Dex at 48 h of treatment (*Online Supplementary Figure 3A*) and also blocked the Dex-induced activation of Bak and Bcl-2 down-regulation (*Online Supplementary Figure 3B*). RU486 also completely inhibited the Dex-mediated regulation of Bax and Bcl-xL (*data not shown*). Furthermore, we noticed that cell death was completely blocked even when RU486 was added 12 h after Dex treatment. Addition of RU486 as late as 24 h after initiation of Dex-treatment led to an attenuated apoptosis detected at 48 h with a cell death rate corresponding to that after 24 h of Dex-treatment (*data not*

Table 1. Correlation of *ex vivo* sensitivity to dexamethasone with Bak/Bax activation, Bcl-2/Bcl-xL down-regulation and *in vivo* early treatment response.

Pt. nr	Diagnosis	Control*	Dex 200 nM*	Ex vivo	Ex vivo cat	MRD %	In vivo cat	Bak Control	Bak D200	Bak ratio	Bax Control	Bax D200	Bax ratio	Bcl-2 Control	Bcl-2 D200	Bcl-2 ratio	Bcl-xL Control	Bcl-xL D200	Bcl-xL ratio
1	preB-ALL	58.52	57.98	1.00	R	5	R	Nd	Nd	Nd	8.51	9.37	1.10	9.14	6.95	0.76	27.54	15.77	0.57
2	T-ALL	68.21	84.62	1.24	S	<0.01	S	Nd	Nd	Nd	47.45	54.51	1.15	61.39	78.04	1.27	67.84	81.64	1.2
3	T-ALL	69.84	95.47	1.37	S	<0.01	S	Nd	Nd	Nd	32.54	34.12	1.05	19.49	39.98	2.05	41.88	73.4	1.75
4	preB-ALL	40.79	50.62	1.24	S	<0.01	S	Nd	Nd	Nd	12.32	16.04	1.30	11.76	24.65	2.10	8.53	16.83	1.97
5	preB-ALL	4.87	4.54	0.93	R	85	R	Nd	Nd	Nd	2.76	2.60	0.94	4.41	4.70	1.07	7.81	5.79	0.74
6	preB-ALL	63.23	52.75	0.83	R	<0.01	S	Nd	Nd	Nd	68.53	72.10	1.05	Nd	Nd	Nd	75.4	62.67	0.83
7	preB-ALL	36.77	29.74	0.81	R	4	R	41.71	36.93	0.89	35.31	38.31	1.08	1.36	1.52	1.12	2.82	2.56	0.91
8	T-ALL	44.22	73.35	1.66	S	0.04	S	28.78	58.09	2.02	23.76	55.69	2.34	26.02	90.1	3.46	34.51	68.55	1.99
9	preB-ALL	37.66	46.97	1.25	S	3	S	15.59	24.43	1.57	9.55	19.46	2.04	8.50	27.77	3.27	18.54	27.48	1.48
10	preB-ALL	60.65	65.27	1.08	R	<0.01	S	49.15	59.04	1.20	25.58	43.40	1.70	Nd	Nd	Nd	7.59	9.16	1.21
11	preB-ALL	68.91	70.29	1.02	R	5.8	R	22.15	18.05	0.81	28.89	22.30	0.77	8.82	8.88	1.01	2.84	3.16	1.11
12	preB-ALL	32.62	39.98	1.23	S	2.5	S	5.79	13.00	2.25	8.54	16.85	1.97	Nd	N d	Nd	7.04	19.02	2.7

**denotes percentage of apoptotic (TMRE–/annexinV+) cells among cells cultured in the absensce (control) or presence (Dex 200 nM) of Dex for 24 h. preB-ALL: B-precursor acute lymphoblastic leukemia; T-ALL: T-precursor acute lymphoblastic leukemia; ex vivo: ex vivo sensitivity index to Dex defined as a ratio of TMRE–/annexin V+ cells between Dex 200 nM and control cells; Ex vivo cat, Ex vivo Dex-sensitive cases are defined as sensitivity index >1.20; MRD: minimal residual disease; in vivo cat: in vivo sensitive cases to an early treatment response are defined as MRD <4%; Bak ratio, Bak positive population in Dex 200 nM divided by Bak positive population in control cells; Bax ratio, Bax positive population in Dex 200 nM divided by Bax positive population in control cells; Bcl-2 ratio, Bcl-2 negative population in Dex 200 nM divided by Bcl-2 negative population in control cells; Bcl-xL ratio, Bcl-xL negative population in Dex 200 nM divided by Bcl-xL negative population in control cells; D200: Dex 200 nM; R: resistant; S: sensitive; Nd: not done. Bak and Bax activation was defined as a ratio of Bak or Bax positive cells in Dex 200 nM to control of ≥1.2. Bcl-2 and Bcl-xL down-regulation was defined as a ratio of Bcl-2 or Bcl-xL negative cells in Dex 200 nM to control of ≥1.2.*

shown). These findings suggest that continuous receptor activation is needed for Dex-induced apoptosis.

Dex induces Bak activation prior to the loss of $\Delta\psi m$ and Bax activation

In order to investigate the sequence of events regarding cytochrome c release from mitochondria and activation of Bak and Bax in RS4 cells we used immunocytochemistry. Prior to cytospin preparation, cells were incubated with Mitotracker to monitor the integrity of mitochondria. Activated Bak was found in the vast majority of apoptotic cells (Figure 4A, arrows). However, it was activated relatively early before the cells lost their mitochondrial membrane potential and DNA fragmentation had occurred (Figure 4A, c and d, cell marked*; note a distinct mitochondrial staining pattern). In contrast, activation of Bax was only observed in cells that had lost their mitochondrial membrane potential and had condensed nuclei (Figure 4B c and d, arrows; note the disrupted and diffuse mitochondrial pattern), indicating that Bax is involved in the later stages of apoptosis (compare Figure 4A and B). Furthermore, the release of cytochrome c (diffuse staining pattern compared to distinct mitochondria-localized pattern in control cells), although found mostly in cells with fragmented nuclei and diffuse mitochondrial staining (arrows), occurred before the complete disruption of mitochondrial membrane potential in some of the cells (Figure 4C, c and d, cells marked with *). Thus, we concluded that Bak is probably activated concomitantly with cytochrome c release, but prior to the loss of mitochondrial membrane potential and prior to Bax activation. Furthermore, activation of Bax was a relatively later event in the course of Dex-induced apoptosis of RS4 cells. These data further strengthen the notion that Bak and Bax are differentially regulated in this system.

Involvement of caspases in Dex-induced Bak and Bax activation

Using a fluorometric assay for caspase activity, caspases-2 and -3 were found to be activated in RS4 cells already after 12 h of exposure to dexamethasone and their activity continued to increase up to 36 h (*Online Supplementary Figure 4*). Activation of caspase-9 was first noted at 24 h, although a statistically significant activation of caspase-9 was only seen at 36 h. Caspase-8 and caspase-6 showed their first signs of activity 36 h after Dex treatment.

Effects of caspase inhibitors on Dex-induced cell death

With the aim of investigating the relative importance of different caspases we initially performed blocking experiments using the pan-caspase inhibitor ZVAD-fmk at a concentration of 10 μM, which completely blocked Dex-induced activation of caspases-2, -3 and -9 after 36 h of culture (*data not shown*). Since caspase-2 is the first initiator caspase to be activated following Dex treatment, we also examined the effect of the caspase-2 inhibitor, zVDVAD-fmk on caspase-2 and the effector caspases-3 and -9. The Dex-induced activation of caspases-2, -3 and 9 was potently inhibited by zVDVAD-fmk (Figure 5A,B, *data not shown*). Next, we analyzed the effect of zVDVAD-fmk and zVAD-fmk on Dex-induced cell death (Figure 5C). Surprisingly, both caspase inhibitors had a marginal inhibitory effect on annexin V positivity, and did not protect the cells from loss of $\Delta\psi m$. Furthermore, zVAD-fmk and zVDVAD-fmk did not protect against Dex-induced Bak and Bax activation (Figure 5D). The lack of protection from Dex-induced cell death was also observed by counting living and apoptotic cells under a light microscope using Türk staining (*data not shown*). Finally, Dex-induced cell death in the presence of caspase inhibition was not due to necrosis, since an early apoptotic non-necrotic cell population (annexin V^+/PI^-) could still be observed in flow cytometry (*data not shown*).

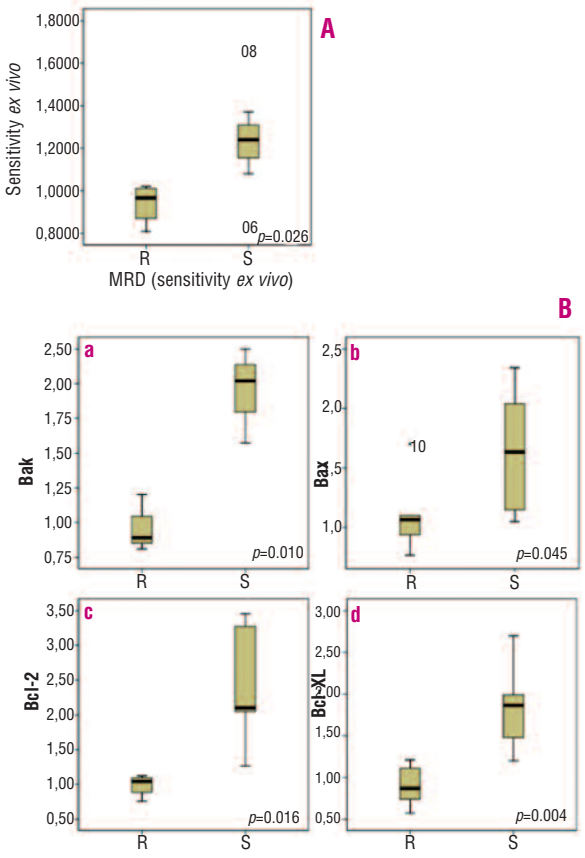

Figure 3. Comparison of the *ex vivo* Dex sensitivity index in *in vivo* sensitive and *in vivo* resistant samples from patients and modulation of Bcl-2 family members in *ex vivo* Dex-sensitive and Dex-resistant patients group. (A) *In vivo* sensitive patients demonstrated a significantly higher Dex-sensitivity index compared to that of *in vivo* resistant patients. *In vivo* sensitivity was defined as indicated in the results section. The *ex vivo* sensitivity index to Dex was defined as the ratio of apoptotic ($TMRE^-$/$annexinV^+$) cells in cells exposed to Dex 200 nM and control cells. *Ex vivo* Dex-sensitive cases are defined as having a sensitivity index >1.2. (B) Patients, who were *ex vivo* sensitive to Dex had higher activation of Bak (a) and significantly higher activation of Bax (b) and down-regulation of Bcl-2 (c) and Bcl-xL (d).

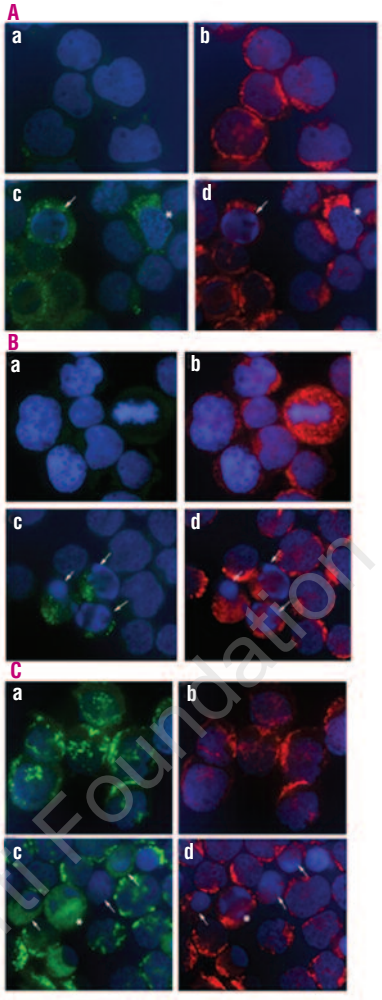

Figure 4. Bak activation occurs prior to the loss of mitochondrial membrane potential and Bax activation. RS4 cells were cultured in the absence or presence of 100 nM Dex for 24 h, incubated with 10 nM of MitoTracker for 30 min at 37°C and double stained for active Bak (A,a,c - green), active Bax (B, a,c - green) and cytochrome c (C, a,c - green). Red: MitoTracker; blue: DNA. In A, B and C: a and b – represent the same fields of control cells, c and d – the same fields of Dex-treated cells.

Discussion

In the present study we analyzed the relationship between various components regulating the intrinsic apoptotic pathway in GC-induced apoptosis in ALL blasts from patients and in cell lines. We identified the differential regulation of Bcl-2 family members including Bak, Bax and Bcl-2, Bcl-xL, as key events in Dex-induced apoptosis.

The Bcl-2 family members have been suggested to play an important role in the execution of the apoptotic program induced by GC.[28,29] The anti-apoptotic Bcl-2 was able to protect against apoptosis, but not against the anti-proliferative responses induced by GC.[30] Furthermore, inhibition of the anti-apoptotic Bcl-xL sensitized T-ALL cells to Dex-induced apoptosis.[31] Several pieces of our data point to the differential regulation of Bak and Bax in Dex-induced cell death in ALL cells. To study activation of Bak and Bax, we used antibodies recognizing active conformations of these proteins[13,26] and flow cytometry analysis. Live and early apoptotic cells were gated based on forward and side scatter properties prior to analysis to exclude the non-specific binding of antibodies to the partially denatured proteins in late apoptotic cells. Immunocytochemistry on glass slides was also used to visualize the cells and to monitor the kinetics of the events at the level of single cells. Thus, based on all our data, Bak activation was present in cells with retained mitochondrial membrane potential and intact nuclei while Bax was activated at a later stage of cell death in cells with fragmented nuclei. It is noteworthy that this type of regulation is analogous to that occuring following the use of other apoptosis-inducing agents such as doxorubicin and interferon-α.[22,24] Furthermore, Bak activation was observed in cells that still maintained certain levels of Bcl-2 or Bcl-xL while Bax activation could only be detected in cells with low levels of Bcl-2 and Bcl-xL. Thus Bcl-2 and Bcl-xL down-regulation is not necessary for the activation of Bak but seems to be important for the later activation of Bax. Further studies will be necessary to determine the mechanism of the down-regulation of Bcl-2 and Bcl-xL. Bcl-2 mRNA was down-regulated already after 12 h of Dex treatment suggesting that this down-regulation might occur at the transcriptional level. Dex activates a GR that binds to specific glucocorticoid receptor element (GRE) sequences in the promoters of the target genes resulting in the activation or repression

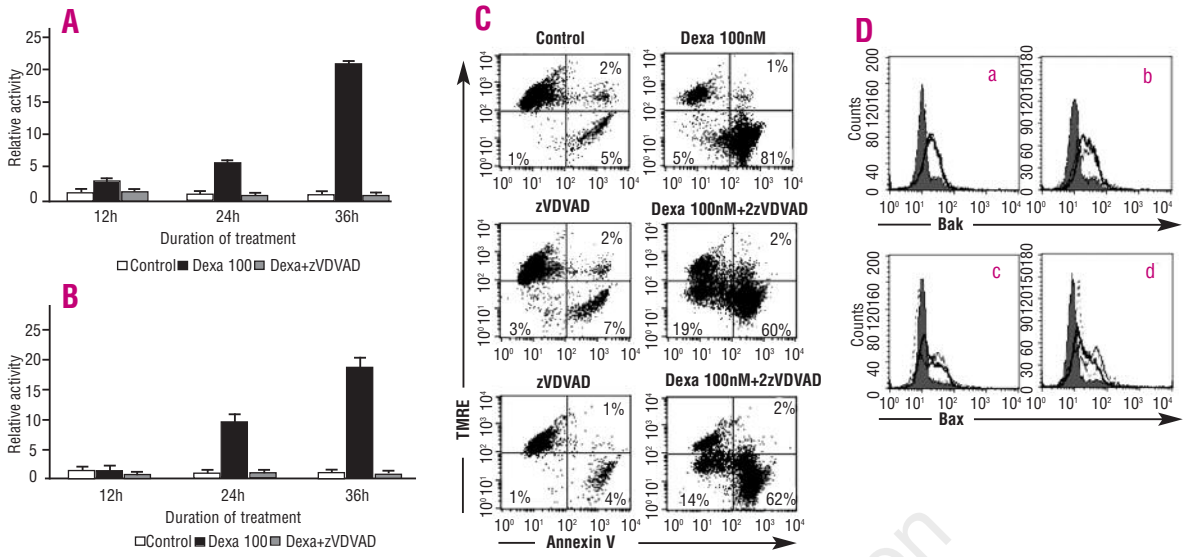

Figure 5. Effect of the caspase-2 inhibitor, zVDVAD-fmk, and the pan-caspase inhibitor, zVAD-fmk, on Dex-induced apoptosis, the activity of casapses-2 and -3 and Bak, and Bax activation. RS4 cells were treated with Dex 100 nM for 48 h in the presence or absence of zVDVAD-fmk or zVAD-fmk. The effect of the inhibitors was assessed as their effect on the activity of caspase-2 (A) and caspse-3 (B), as well as alterations in annexin V/TMRE (C). (D) RS4 cells were treated with Dex 100 nM for 48 h in the presence or absence of zVDVAD-fmk (a and c) or zVAD-fmk (b and d) and analyzed for activation of Bak and Bax. Gray histogram: control cells; black bold line: Dex-treated cells; gray dashed line: zVDVAD-fmk (a and c) or zVAD-fmk (b and d) treated cells; black thin line: Dex + zVDVAD-fmk (a and c) Dex + zVAD-fmk (b and d).

of gene transcription.[27] We attempted to search for the GRE and found a potential GRE site in the Bcl-2 promoter and, although this was not perfect, it was conserved in the mouse. Thus, it is conceivable that inhibition of Bcl-2 gene transcription might occur via GR-induced repression. Several published microarray studies have been conducted to identify Dex-specific targets involved in the apoptosis of ALL cells *ex vivo* and *in vivo*. Neither the Bcl-2 nor the Bcl-xL mRNA was found to be down-regulated by GC in these studies.[31,32] Although we are confident that the expression of these genes was down-regulated at the mRNA level by Dex in RS4 cells, we cannot be certain that they are direct, and not indirect GR targets. It is also possible that the microarray technique might underestimate some down-regulated targets with a low basal level of expression, because normalization procedures could conceal any differences. Bcl-2 and Bcl-xL protein expression was down-regulated in both primary cells treated with Dex *ex vivo* and in ALL cell lines; however, the mechanism of the down-regulation of Bcl-2 and Bcl-xL *in vivo* might be different. Further studies on ALL cells from patients treated with dexamethasone will determine whether Bcl-2/Bcl-xL mRNA down-regulation occurs in Dex-sensitive ALL cells *in vivo*. According to the Berlin-Frankfurt-Münster (BFM) ALL studies, lack of an early response to the GC prednisone, defined as persistence of more than $1x10^9$/L lymphoblasts in the peripheral blood after 7 days of exposure to prednisone and one intrathecal dose of methotrexate, defines about 10% of patients with a very high risk of relapse.[3] Furthermore, in a recently published randomized trial, children with ALL who did not receive dexamethasone treatment before chemotherapy had a significantly higher blast count in the bone marrow at day 14 and showed a trend to worse disease-free survival at 40 months compared to children pretreated with Dex.[34] One mechanism behind this stratifying nature of the initial clinical GC-response may be the down-regulation of Bcl-2 and/or Bcl-xL. Since cell death induced by other components of the induction treatment in ALL, such as doxorubicin, is highly dependent on the Bcl-2 family of proteins,[22] the *pre-down-regulation* of Bcl-2 and Bcl-xL by Dex may facilitate their killing ability during ALL induction therapy. Several studies have investigated the predictive value of the expression level of various Bcl-2 family members in ALL at diagnosis.[35-45] The majority of these studies indicate that steady-state levels of individual Bcl-2 family members are not optimal predictors of outcome[35,36,38,39] or *ex vivo* GC-sensitivity.[37, 40, 42] On the other hand, several studies using cell viability assays have demonstrated that *ex vivo* GC-sensitivity has a predictive value in relation to the short-term response to systemic GC monotherapy and to the long-term clinical outcome in response to combination chemotherapy.[46-50] Therefore, analysis of GC-mediated changes of several Bcl-2 family members might provide an even more reliable predictive assay. Our data on Dex-induced changes in the levels and activation status of Bcl-2 family members in a limited set of samples from patients show a good correlation between the *ex vivo* response to Dex and an early response to treatment *in vivo*. Accordingly, in *ex vivo* samples from Dex-resistant ALL patients, Dex was not able to activate Bax and/or Bak or down-regulate Bcl-2 and/or Bcl-xL. In summary, these data indicate that Dex-induced apoptosis requires activation of

either Bax or Bak in combination with down-regulation of at least one of the anti-apoptotic Bcl-2 family members. However, our analysis was performed on thawed samples, often with reduced viability, and the data should, therefore, be interpreted with some caution.

Our data should constitute a solid basis for future comprehensive research aimed at resolving whether this type of analysis, using apoptotic markers, could represent an optimal predictive assay for *in vivo* treatment response and clinical outcome. The classical apoptotic mitochondrial pathway requires caspase activation to complete the cell death execution program. We detected activation of several caspases in the course of Dex-induced cell death. Interestingly, caspase-2, which was activated relatively early following Dex-treatment, seemed to be the most apical caspase. There are conflicting results concerning the exact role of caspases in GC-induced cell death. Some studies have indicated that inhibition of caspases protects against GC-induced apoptosis,[15,17,30,51,52] whereas other studies have shown opposite results.[16,53] In the present analysis, pan-caspase inhibition by zVAD-fmk clearly showed that Bak and Bax activation, mitochondrial depolarization and cell death induced by Dex could not be rescued. Collectively, these data indicate that Dex might induce an alternative, caspase-independent mode of cell death, which also involves Bak and Bax activation as well as mitochondrial depolarization. Such alternative cell death pathways are presently under investigation in our laboratory.

In conclusion, our data indicate that Dex induces apoptosis through the classical mitochondrial apoptotic pathway. Importantly, the key events in the execution of apoptosis, such as activation of Bak and Bax and down-regulation of Bcl-2 and Bcl-xL by Dex, occur in cells from sensitive but not resistant ALL cell lines and samples from patients. These data not only strengthen the notion of the importance of the modulation of Bcl-2 family members in the apoptotic response but also provide a basis for further molecular, as well as clinical, experimental investigations of the action of GC in lymphoid malignancies. Studies pinpointing the mechanism of resistance to drugs such as Dex may aid the pre-selection of responsive patients, and may also pave the way for future targeted therapies aimed at sensitizing resistant leukemic cells. Analysis of the *ex vivo* sensitivity of leukemic blasts to Dex, in combination with subsequent MRD detection, could become a powerful new strategy for improving risk-adjusted therapy in children with ALL.

Authors' Contributions

EL: experimental work, collection, organization and analysis of all data, manuscript writing; TP: project planning, experimental work and analysis of all data, manuscript writing; KP: experimental work, analysis of data, review of manuscript; EB: experimental work, review of manuscript; MC: analysis of data, review of manuscript; SS: collection of clinical data, data interpretation, review of manuscript; MH: collection of clinical data, data interpretation, review of manuscript; JM: statistical analysis, data interpretation; BZ: experimental work, analysis of data, review of manuscript; AP: project planning, design of the study, data interpretation, review of manuscript; DG: project planning, design of the study, data analysis, data interpretation, writing of manuscript. All authors approved the final version of the manuscript.

Conflict of Interest

The authors reported no potential conflcits of interest.

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