Dexamethasone resistance in B-cell precursor childhood acute lymphoblastic leukemia occurs downstream of ligand-induced nuclear translocation of the glucocorticoid receptor

Petra S. Bachmann, Rosemary Gorman, Karen L. MacKenzie, Louise Lutze-Mann, and Richard B. Lock

Glucocorticoids are among the most effective agents used in the treatment of childhood acute lymphoblastic leukemia (ALL), and patient response to treatment is an important determinant of long-term outcome. Despite its clinical significance, the molecular basis of glucocorticoid resistance in lymphoid malignancies is still poorly understood. We have recently developed a highly clinically relevant experimental model of childhood ALL, in which primary childhood ALL biopsies were established as xenografts in nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice. The in vivo and in vitro responses of a panel of these xenografts to the glucocorticoid, dexamethasone, reflected the outcome of the patients from whom they were derived. In this report we show that glucocorticoid resistance in B-cell precursor (BCP) ALL xenografts was not due to down-regulation of the glucocorticoid receptor (GR) nor to defective ligand binding of the GR. Moreover, dexamethasone-induced GR translocation from the cytoplasm to the nucleus was comparable in all xenografts. However, glucocorticoid resistance was associated with profoundly attenuated induction of the BH3-only proapoptotic protein, Bim, when xenograft cells were exposed to dexamethasone. These results show that dexamethasone resistance in BCP ALL xenografts occurs downstream of ligand-induced nuclear translocation of the GR, but upstream of Bim induction. (Blood. 2005;105:2519-2526)

Introduction

Glucocorticoids have occupied a central role in the treatment of hematologic malignancies for more than 50 years, due to their ability to induce apoptosis in normal and neoplastic lymphoid cells.[1] Glucocorticoid therapy plays a crucial role in the treatment of childhood acute lymphoblastic leukemia (ALL), and patient response to treatment is an important determinant of clinical outcome.[2] Prolonged use can result in the development of resistance in leukemia cells, although the mechanisms by which resistance develops remain poorly defined.[3,4]

Molecular studies, largely using cultured cells, have established several basic facts about the action of glucocorticoids on leukemia cells, although the pathway remains incompletely defined. Glucocorticoids are steroid hormones that act on their target cell by signaling through a specific, cytosolic glucocorticoid receptor (GR) to induce apoptosis.[5] The GR is a member of the nuclear receptor family of ligand-dependent transcription factors and exists in 2 major forms, α and β, and the β may exert dominant-negative effects over the α.[6] A large number of studies using patient biopsy material have attempted to establish a relationship between cellular GR content and glucocorticoid sensitivity, and while a low number of receptors are frequently associated with resistance, high receptor number does not necessarily predict sensitivity.[7-9]

Upon ligand binding, the GR dissociates from the large protein complex that maintains it in an inactive conformation in the cytoplasm.[10,11] Nuclear translocation and activation of the GR results in (1) transactivation of target genes via direct interaction with specific palindromic DNA sequences known as glucocorticoid response elements (GREs),[12] (2) repression of gene activation via interaction with DNA sequences called negative GREs,[13] (3) enhancement or repression of gene transcription via interaction with composite GREs, which specify binding sites for the GR and 1 or more nonreceptor factors,[12,14] and (4) repression of gene activation via DNA-independent interactions between the GR and other transcription factor complexes such as activator protein-1 (AP-1) and nuclear factor κB (NF-κB).[12,15-17] This complex protein interplay results in activation or repression of gene transcription, leading to caspase and endonuclease activation, and apoptosis.[3,12] However, it is still unclear which specific glucocorticoid-regulated genes are involved in transducing the apoptotic signal, upstream of caspase activation.

The Bcl-2 family of proteins is critical in regulating apoptosis induced by numerous cellular stresses, including exposure to glucocorticoids, which proceeds via breakdown of mitochondrial transmembrane potential and release of proapoptotic molecules into the cytosol, the "intrinsic" pathway.[12,18] Bcl-2 family proteins possess either proapoptotic or antiapoptotic function and are related through conserved sequence motifs, Bcl-2 homology (BH) domains. Bcl-2 family members related by a single region of homology, BH3-only, are proapoptotic via binding to and antagonizing antiapoptotic members such as Bcl-2, Bcl-x_L, Bcl-w, and, to a lesser extent, Mcl-1 and A1.[19] The only Bcl-2 family member consistently shown in microarray analysis of gene expression

studies to be up-regulated by glucocorticoids in lymphoid cells is the BH3-only protein, Bim (Bcl-2L11).[20-22] Alternative splicing of *bim* mRNA gives rise to 3 isoforms, Bim_S, Bim_L, and Bim_{EL}, all of which potently induce apoptosis, with Bim_S being the most cytotoxic.[19] The larger isoforms, Bim_L and Bim_{EL}, contain additional regulatory regions that interact with the dynein light chain LC8, which modulates their proapoptotic activity.[19]

The study of leukemia cell lines has shown glucocorticoid resistance to be almost invariably associated with receptor defects leading to impaired ligand-receptor interactions.[23-28] However, such mechanisms are rarely observed in primary patient material,[26-31] the only report being the original biopsy material from which the CEM cell line was derived.[32] To date, the apparent discrepancy between clinical findings and those using cell line model systems remains unexplained.

We have previously shown that the in vivo and in vitro responses to the glucocorticoid, dexamethasone, of childhood ALL biopsy specimens established as xenografts in nonobese diabetic/ severe combined immunodeficient (NOD/SCID) mice closely reflect the clinical outcome of the patients from whom they were derived.[33] Consequently, mechanisms governing the differential glucocorticoid responses exhibited by these xenografts are likely to be highly relevant to the clinical disease. In this study, we show that dexamethasone resistance in a subset of xenografts occurs downstream of ligand binding and GR translocation to the nucleus, in contrast with almost all cell line studies, but upstream of induction of the proapoptotic protein Bim. These findings are likely to be important in understanding mechanisms of glucocorticoid resistance in lymphoid malignancies.

Materials and methods

In vitro culture and drug treatments

The development, characterization, and in vivo and in vitro dexamethasone responses of a series of childhood ALL xenografts derived from patient biopsies have been described previously.[33,34] All children were treated at the Centre for Children's Cancer and Blood Disorders, Sydney Children's Hospital. Approval was obtained from the University of New South Wales Institutional Review Board for these studies. Informed consent was provided according to the Declaration of Helsinki. For all experiments described in this study, xenograft cells were retrieved from cryostorage and resuspended at a density of 2×10^6 cells/mL in QBSF-60 medium (Quality Biological, Gaithersburg, MD) supplemented with Flt-3 ligand (20 ng/mL; Amgen, Thousand Oaks, CA), penicillin (100 U/mL), streptomycin (100 μg/mL), and L-glutamine (2 mM) (QBSF-60/F). Viability was determined by the exclusion of 0.2% trypan blue. For drug treatments, cells were equilibrated at 37°C, 5% CO_2, for at least 4 hours prior to the addition of dexamethasone to a final concentration of 1 μM in QBSF-60/F. An equivalent volume of media only was added to control cells. Cells were harvested at the appropriate time points by centrifugation at 490g for 10 minutes, aspirating media, and washing cells twice with sterile calcium- and magnesium-free phosphate-buffered saline (PBS).

The human T-lineage ALL cell line, CEM-WT, and its dexamethasone-resistant subline, MTX-R3,[35] were maintained as static suspensions at 37°C, 5% CO_2, in RPMI-1640 medium (Invitrogen Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS; Invitrogen Life Technologies), penicillin (100 U/mL), streptomycin (100 μg/mL), and L-glutamine (2 mM) (complete RPMI). All procedures using CEM cell lines have been described previously.[35]

In vitro cytotoxicity assays

In vitro drug sensitivity was assessed using the colorimetric methyl-thiazolyl-tetrazolium (MTT) assay, which measures a combination of inhibition of proliferation and cell death. One day prior to drug treatment, xenograft cells were retrieved from cryostorage and resuspended in QBSF-60/F at a cell concentration previously optimized for each xenograft ($2\text{-}5 \times 10^6$ cells/ mL), and 100 μL was seeded per well in 96-well U-bottomed plates. Dexamethasone was added in QBSF-60/F to a range of final concentrations (10^{-5} to 10^{-12} M) in triplicate wells. Following an additional 48 hours of incubation at 37°C, 5% CO_2, MTT (Sigma, St Louis, MO) was added (final concentration 0.5 mg/mL). The formazan crystals formed after 6 hours were dissolved in 2 volumes of 10% sodium dodecyl sulfate (SDS) in 0.01 M HCl, and the optical density was measured at 570 nm, with reference to 655 nm. Cell viability was calculated as a percentage of untreated controls. Results presented are the mean ± standard error of the mean (SEM) of at least 3 independent experiments, and IC_{50} (inhibitory concentration 50%) values were calculated from cumulative survival curves. The CEM-WT and MTX-R3 cell lines were similarly studied by this method, with the exception that complete RPMI was used throughout, and the initial seeding density was 5×10^4 cells/mL.[35]

Analysis of protein expression

Whole-cell protein extracts were prepared by lysis of cells (10^8 cells/mL) in 50 mM Tris (tris(hydroxymethyl)aminomethane)–Cl pH 7.4, 150 mM NaCl, 0.2% NP-40, 5 mM EDTA (ethylenediaminetetraacetic acid), 50 mM sodium fluoride, 0.1 mM sodium orthovanadate supplemented with protease inhibitor cocktail (Sigma). Insoluble materials were removed by centrifugation at 10 000g for 10 minutes at 4°C, and supernatants were stored at −80°C. Total protein concentration was quantified by the bicinchoninic acid (BCA) assay method (Pierce, Rockford, IL) using bovine serum albumin standard. Cytoplasmic and nuclear fractions were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Pierce) according to the manufacturer's instructions. Equivalent amounts of protein (30-100 μg) from whole-cell lysates, or cytoplasmic and nuclear fractions, were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA). Proteins were detected by immunoblotting followed by chemiluminescence detection (SuperSignal West Extended Duration Substrate; Pierce). The following polyclonal antibodies were used: GR for detection of GRα and GRβ (Santa Cruz Biotechnology, Santa Cruz, CA), actin and Bim (Sigma), Bax and Bcl-2 (BD Biosciences Pharmingen, San Diego, CA), poly(ADP-ribose) polymerase (PARP; Affinity BioReagents, Golden, CO), and DNA topoisomerase I (topo I, clone C-1; kindly provided by Dr Y-C Cheng, Yale University School of Medicine, New Haven, CT). Secondary antibodies used were horseradish peroxidase conjugates of either anti–mouse or anti–rabbit immunoglobulin G (IgG; Amersham Biosciences, Buckinghamshire, England). Results were visualized by autoradiography, and signals were quantified by phosphoimaging using a VersaDoc 5000 Imaging System. Data were analyzed using QuantityOne software (Version 4.00; BioRad, Hercules, CA).

Radioligand binding assays

Specific glucocorticoid receptor binding sites were measured in whole-cell binding assays as we have previously described.[35] Xenograft cells were resuspended in QBSF-60/F media at a final concentration of 5×10^6 cells/mL. Aliquots of this cell suspension (980 μL) were allowed to equilibrate at 37°C for 1 hour. Cells were then incubated an additional 1 hour at 37°C with 10 μL [³H]-dexamethasone (0.1-7.5 μM) plus either 10 μL 100% ethanol or 10 μL of 1 mM unlabeled dexamethasone in 100% ethanol. Following incubation, cells were washed 3 times with Hanks Balanced Saline Solution (HBSS; Gibco, Carlsbad, CA) at 37°C before being resuspended in 1 mL HBSS. Radioactivity was determined by using a Tri-Carb 2100TR Liquid Scintillation Counter (Packard BioScience, Boston, MA). Where stated, some experiments were performed using cells freshly harvested from the spleens of engrafted mice, as we have described.[33,34] Specific binding sites per cell were calculated by subtracting the nonspecific binding (samples with excess unlabeled ligand) from the total binding and graphed as saturation curves. Data shown are cumulative from 2 independent experiments. The number of binding sites per cell (B_{max}) and the affinity of the receptors for dexamethasone (K_D) were

determined using GraphPad Prism (Version 4.00; GraphPad Software, San Diego, CA).

Immunofluorescence microscopy

Xenograft cells were resuspended in QBSF-60/F at a density of 5×10^5 cells/mL and allowed to equilibrate at 37°C, 5% CO_2, for 2 hours. Dexamethasone was diluted in QBSF-60/F media and added to a final concentration of 1 μM. At appropriate time points thereafter, cells were centrifuged for 5 minutes at 490g and finally resuspended in 1 mL 5% (vol/vol) FBS/PBS. An aliquot of 5% FBS/PBS was prespun onto glass microscope slides at 139g for 5 minutes, followed by cytocentrifugation of 100 μL xenograft cell suspension. Cells were fixed in 100% methanol at −20°C for 7 minutes and rehydrated in PBS for 5 minutes, and nonspecific binding sites were blocked by incubating cells with 10% FBS/PBS for 20 minutes at room temperature. Cells were rinsed with PBS, then incubated with anti-GR antibody in 5% FBS/PBS (1:100; Santa Cruz Biotechnology) for 50 minutes at 37°C in a humidified chamber. Secondary only controls were incubated with 5% FBS/PBS only. All slides were subsequently incubated with carbocyanine 3 (Cy3)–conjugated goat anti–rabbit secondary antibody (1:1000; Amersham Biosciences) for 45 minutes at room temperature. Slides were mounted with antifade mounting solution containing 1 μg/mL 4′,6-diamidino-2-phenylindole (DAPI; Vysis, Downers Grove, IL). Epifluorescence was imaged using a 12-bit cooled charge coupled device (CCD) camera and a 63 ×/1.40 oil immersion objective lens (Zeiss Axioplan 2; Carl Zeiss, Oberkochen, Germany), in conjunction with Image Pro-Plus software (Version 4.0; Media Cybernetics, Silver Spring, MD).

Apoptosis assays

Loss of mitochondrial transmembrane potential was assessed by using the cationic dye JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolyl-carbocyanine iodide; Molecular Probes, Eugene, OR). Following dexamethasone treatment (1 μM up to 24 hours) cells were harvested and incubated in PBS containing 0.5 g/mL JC-1 for 10 minutes at 37°C. Cells were washed twice in 2 mL PBS, and the proportion of cells appearing in the viable region by forward- and side-scatter characteristics that exhibited a shift from red fluorescent J-aggregates to green fluorescent JC-1 monomers was determined by using a FACSCalibur flow cytometer (BD Immunocytometry Systems, San Jose, CA). The proportion of cells with active caspase-3 or caspase-7 (caspase-3/7) following treatment with dexamethasone (1 μM up to 48 hours) was assessed by flow cytometry using the cell-permeable inhibitor, carboxyfluorescein-labeled fluoromethyl ketone (FAM-DEVD-FMK, CaspaTag; Chemicon International, Temecula, CA), according to the manufacturer's instructions.

Statistical comparisons

Quantitative variables were compared by using the nonparametric Mann-Whitney U test (GraphPad Prism, Version 4.00; GraphPad Software). The level of significance was set to .05.

Results

Sensitivity of childhood ALL xenograft cells to dexamethasone in vitro

We have previously described the development, characterization, and in vivo and in vitro dexamethasone responses of a series of childhood ALL xenografts established in NOD/SCID mice.[33,34] The relevant disease-specific details of the patients from whom the xenografts were derived are shown in Table 1. To study glucocorticoid resistance mechanisms in these xenografts, their responses to dexamethasone were compared using the MTT colorimetric assay. Figure 1 clearly demonstrates a broad spectrum of xenograft sensitivity to dexamethasone, with ALL-3, -8, -11, -16, and -17 all responding to short-term drug exposure, in contrast to ALL-2, -4, -7, -10, -18, and -19, which were resistant. Interestingly, 5 of 6 of the dexamethasone-resistant xenografts were derived from patients either at relapse or who have died of their disease (closed symbols, dashed lines), while 4 of 5 sensitive xenografts were derived from long-term survivors (open symbols, solid lines). IC$_{50}$ values from the mean of 3 independent experiments are shown in Table 1 and indicate that a subset of 6 xenografts were highly inherently resistant to dexamethasone, with IC$_{50}$ values more than 10 μM compared with 20 to 30 nM for ALL-8 and -17, and less than 10 nM for the most sensitive xenografts (ALL-3, -11, and -16). The difference between the most resistant and sensitive xenografts represents more than 1000-fold level of resistance. Cell viability by MTT assay showed an excellent correlation with our previously documented flow cytometric enumeration of viable cells at 10 nM ($r = 0.87$; $P = .0004$), 100 nM ($r = 0.87$; $P = .0006$), and 1 μM ($r = 0.84$; $P = .0012$) dexamethasone for all 11 xenografts.[33] The IC$_{50}$ values for CEM-WT and MTX-R3 cells exposed to dexamethasone for 72 hours have previously been reported to be 51 nM and more than 1 mM, respectively.[35]

### GR protein expression and functionality in xenograft cells

To determine whether resistance to dexamethasone correlated with down-regulation of GR expression, basal levels of GR protein were compared between xenografts by immunoblot analysis of whole-cell lysates. Figure 2A, which shows a representative immunoblot and cumulative quantitative data, indicates that there was a marked decrease in receptor expression in only 1 xenograft, ALL-8 ($P = .04$ compared with CEM-WT cells). All other xenografts expressed at least equivalent quantities of receptor protein to the

Table 1. Patient clinical data

Xenograft	Age at diagnosis, mo/sex	ALL subtype	Disease status at biopsy	Length of CR1, mo	Site of relapse	Survival after first relapse, mo	Current clinical status	DEX IC$_{50}$
ALL-2	65/F	c-ALL	Relapse 3	30	BM/CNS	46	DOD	> 10 μM
ALL-3	154/M	Pre-B	Diagnosis	38	BM	98*	CR2	4.7 nM
ALL-4	105/M	Ph+/B-ALL	Diagnosis	10	BM	1	DOD	> 10 μM
ALL-7	88/M	Biphen	Diagnosis	7	BM	6	DOD	> 10 μM
ALL-8	152/M	T-ALL	Relapse 1	17	BM	1	DOD	24.1 nM
ALL-10	48/M	c-ALL	Diagnosis	62*	No relapse	NA	CR1	> 10 μM
ALL-11	37/F	c-ALL	Diagnosis	125*	No relapse	NA	CR1	6.5 nM
ALL-16	122/F	T-ALL	Diagnosis	108*	No relapse	NA	CR1	1.2 nM
ALL-17	107/F	c-ALL	Diagnosis	25	CNS	36*	CR2	20.1 nM
ALL-18	30/F	c-ALL	Relapse 1	43	BM	57*	CR2	> 10 μM
ALL-19	194/M	c-ALL	Relapse 1	4	BM	7	DOD	> 10 μM

CR1 indicates alive in first complete remission; DEX, dexamethasone; c-ALL, common (CD10+) ALL; BM, bone marrow; CNS, central nervous system; DOD, dead of disease; CR2, alive in second complete remission; Ph+, Philadelphia chromosome-positive; Biphen, biphenotypic; NA, not applicable.
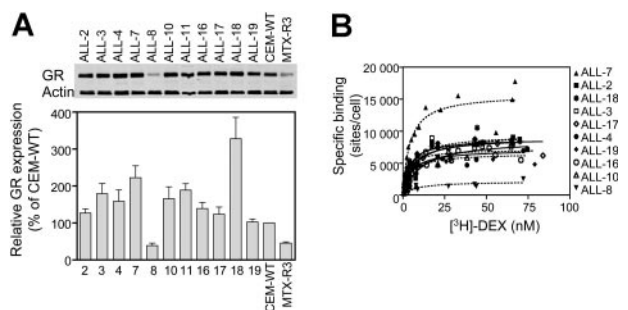
*No event.
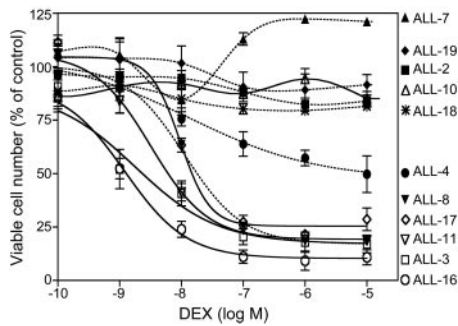
Figure 1. Responses of xenografts to dexamethasone in vitro. Xenograft cells were retrieved from cryostorage, and sensitivity to dexamethasone was assessed by MTT assay following a 48-hour drug exposure. The viable cell number at each drug concentration was calculated relative to untreated controls. Xenografts were stratified into good (solid lines, open symbols) or poor (dashed lines, closed symbols) clinical outcome subgroups, as defined in "Sensitivity of childhood ALL xenograft cells to dexamethasone in vitro." Each data point represents the mean ± SEM of 3 independent experiments.

Table 2. Specific GR binding sites/cell (B_{max}) and receptor affinity for ligand (K_D) values for ALL xenografts

Xenograft	B_{max} (sites/cell)	K_D (nM)
ALL-2	9 762 ± 446	8.4 ± 1.3
ALL-3	8 931 ± 314	5.9 ± 0.7
ALL-4	6 555 ± 222	4.6 ± 0.6
ALL-7	15 794 ± 543	4.1 ± 0.6
ALL-8	1 752 ± 140	5.5 ± 1.6
ALL-10	8 713 ± 435	11.0 ± 1.8
ALL-16	7 117 ± 247	4.9 ± 0.7
ALL-17	8 772 ± 236	3.9 ± 0.5
ALL-18	9 432 ± 296	5.3 ± 0.6
ALL-19	7 149 ± 294	2.7 ± 0.5

dexamethasone-sensitive CEM-WT cell line, with 4 xenografts (ALL-3, -7, -11, and -18) expressing significantly more GR protein ($P < .05$). CEM-WT and MTX-R3 cell lines were used as controls, as the MTX-R3 cell line has previously been shown to express approximately 50% of GR protein compared with CEM-WT cells.[35] These results indicate that GR down-regulation does not account for the high-level dexamethasone resistance observed in the B-lineage xenografts, ALL-2, -4, -7, -10, -18, and -19.

Previous reports have almost invariably associated glucocorticoid resistance in leukemia cell lines with receptor defects resulting in impaired ligand binding.[23-28] Since analysis of GR protein expression alone could not exclude the presence of mutations in the GR that might affect ligand-receptor interactions in the resistant xenografts, radioligand binding studies were carried out. Results from these studies indicate that only 1 xenograft, ALL-8, exhibited defective ligand binding (Figure 2B; Table 2). The reduced number of specific binding sites per cell observed in ALL-8 (1752 ± 140) correlated with the reduced expression of receptor protein evident in the immunoblot analysis (Figure 2A). Similarly, the higher number of binding sites present in ALL-7 (15 794 ± 543) corresponded with higher GR protein expression in this xenograft, although the same relationship was not apparent for ALL-18 (compare GR protein expression in Figure 2A with ligand binding characteristics in Figure 2B and Table 2). Overall, these studies

revealed that, with the exception of the T-lineage ALL-8, defects at the level of receptor-ligand binding do not play a role in dexamethasone resistance of these xenografts. Radioligand binding assays using cells harvested from the spleens of animals engrafted with ALL-3, -7, and -19, and placed immediately into culture, confirmed that the high-level dexamethasone resistance in ALL-7 and -19 was not due to defective ligand binding (P.S.B. and R.B.L., unpublished observations, June 26, 2003).

Nuclear translocation of the GR following dexamethasone treatment

Since the glucocorticoid-induced apoptotic response is dependent on mRNA and protein synthesis, translocation of the receptor-ligand complex to the nucleus is a critical step in this pathway.[12] To determine whether dexamethasone resistance was associated with inhibition of GR nuclear translocation, GR protein was visualized by immunofluorescence labeling. Two glucocorticoid-sensitive (ALL-3 and ALL-16) and 2 glucocorticoid-resistant (ALL-7 and ALL-19) xenografts were studied in detail. All 4 xenografts had been shown in the previous section to contain GR protein that was functional in binding ligand. The GR was localized predominantly to the cytoplasm in untreated cells (Figure 3). However, in the xenografts studied comparable amounts of GR protein colocalized with the DAPI-stained nuclei at 2 and 4 hours of dexamethasone treatment, independent of sensitivity to dexamethasone.

Figure 2. GR protein expression and ligand binding in xenograft cells. GR protein expression in xenografts was assessed by immunoblot (A). Band intensities were quantified by phosphoimage analysis and normalized to actin. GR expression in all xenografts was also normalized to the CEM-WT cell line. Quantified data revealed a significant deficit in GR protein expression in a single xenograft, ALL-8, compared with CEM-WT cells ($P = .04$). (B) Saturation curves were plotted for each xenograft following radioligand binding assays. The respective B_{max} and K_D values are given in Table 2. Specific binding sites per cell were plotted over a range of dexamethasone concentrations. Results shown are cumulative data from 2 independent experiments.
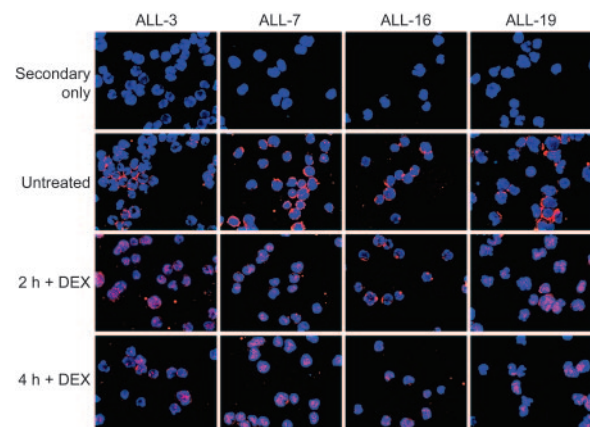

Figure 3. Immunofluorescence detection of nuclear translocation of the GR in xenografts ALL-3, ALL-7, ALL-16, and ALL-19. Xenograft cells were retrieved from cryostorage and exposed to dexamethasone (1 μM) for up to 4 hours prior to cytocentrifugation onto microscope slides and staining with GR antibody. GR was detected using Cy3-labeled secondary antibody (red), and slides were counterstained with DAPI to indicate nuclei (blue). Images were captured using a fluorescent microscope equipped with a cooled CCD camera, under a 63 × oil-immersion objective. Fields shown are representative of at least 5 imaged fields.
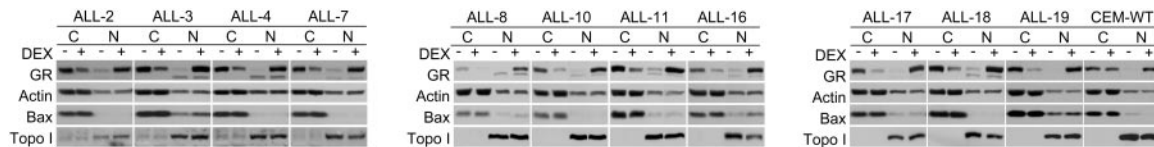
Figure 4. Subcellular fractionation to detect nuclear translocation of GR protein in xenograft cells. Nuclear translocation of GR protein was confirmed in our entire panel of xenografts by subcellular fractionation followed by immunoblot analysis. Xenograft cells were retrieved from cryostorage, exposed to dexamethasone (1 µM, 5 hours), and subjected to subcellular fractionation. Equal amounts of protein (30 µg) from cytoplasmic (C) and nuclear (N) fractions were separated by SDS-PAGE. Membranes were probed with antibodies against GR, actin (loading control), Bax (cytoplasmic control), and topo I (nuclear control).

To confirm these results using an independent technique, xenograft cells were separated into cytoplasmic and nuclear fractions, which were subjected to immunoblot analysis. Figure 4 clearly shows that in untreated cells the GR was almost exclusively cytoplasmic in all xenografts. However, following exposure to dexamethasone (1 µM, 5 hours), translocation of the GR into the nucleus was comparable in all xenografts, regardless of their relative sensitivity to dexamethasone, including ALL-8 in which GR expression was down-regulated. Relevant protein controls for nuclear (topo I) and cytoplasmic (Bax) fractions, along with actin control, are also represented in Figure 4. These results indicate that glucocorticoid resistance in B-cell precursor (BCP) ALL xenograft cells is not due to cytoplasmic retention of the GR following ligand binding and suggest that the lesion(s) underlying the resistant phenotype occur downstream of GR nuclear translocation.

Modulation of GR, Bcl-2, Bax, and Bim protein expression by dexamethasone in ALL xenograft cells and cell lines

Independent studies have documented increased expression of the GR and Bim proteins in glucocorticoid-treated CEM cells.[20,36] These results were confirmed in our CEM-WT cells. Figure 5A clearly shows induction of GR protein and all 3 Bim isoforms within 8 hours and continuing up to 24 hours of exposure to 1 µM dexamethasone. This effect was not observed in the dexamethasone-resistant MTX-R3 subline (Figure 5A), suggesting that this is a response to dexamethasone that requires a functional receptor.[35] Xenograft cells were then exposed to 1 µM dexamethasone, and changes in expression of specific proteins were investigated by immunoblotting of whole-cell lysates. In contrast to findings with the CEM-WT cell line, dexamethasone treatment caused down-regulation of GR protein expression in 4 of 4 xenografts over the time course (Figure 5B-C). This down-regulation of GR protein was independent of sensitivity to dexamethasone, as ALL-3 and -16 are highly sensitive and ALL-7 and -19 highly resistant. Cell viability was dramatically reduced in the sensitive xenografts (ALL-3 and -16) at the 48-hour time point (Figure 5D), leading to reduced protein recovery.

Dexamethasone-induced changes in expression of the proapoptotic Bax and Bim proteins, as well as the antiapoptotic Bcl-2, were also investigated. Figure 6 shows that, while expression of Bcl-2 and Bax remained essentially unaltered, increased Bim expression was evident within 8 hours of exposure of ALL-3 and -16 to dexamethasone. This effect was dramatically attenuated in the resistant xenografts ALL-7 and -19 (Figure 6A-B), although some induction of the smaller isoforms of Bim, Bim_L and Bim_S, was observed in ALL-7 (Figure 6A). Quantitative data shown in Figure 6B represent the cumulative expression of all 3 major isoforms of Bim averaged from 3 independent experiments and indicate that the magnitude of Bim induction was markedly attenuated in ALL-7 and -19 compared with ALL-3 and -16. Degradation of PARP in ALL-3 and -16 cells (and Bax protein in

ALL-16 cells) was evident within 16 hours of dexamethasone exposure, consistent with the induction of apoptosis.[37]

Analysis of all 11 xenografts following exposure to dexamethasone (1 µM, 16 hours) revealed a robust induction of Bim in the 3 highly sensitive ($IC_{50} < 10$ nM) xenografts, ALL-3, -11, and -16 (Figures 6-7). The 2 xenografts of intermediate sensitivity (IC_{50}, 20-30 nM), ALL-8 and -17, failed to induce Bim at this time point (Figure 7A-B). Compared with the 3 most sensitive xenografts (ALL-3, -11, and -16), induction of Bim was significantly attenuated in 4 of the 6 highly resistant xenografts ($IC_{50} > 10 µM$), ALL-2, -7, -10, and -19 ($P < .01$). Bim induction in the highly resistant xenograft, ALL-4, was moderately attenuated ($P = .05$), while its induction in ALL-18 was comparable to the 3 highly sensitive xenografts ($P = .86$). While Bax and Bcl-2 protein expression remained unaffected by dexamethasone treatment (Figures 6-7), the Bcl-2/Bax ratio appeared notably higher in ALL-18 than all other xenografts (Figure 7A).
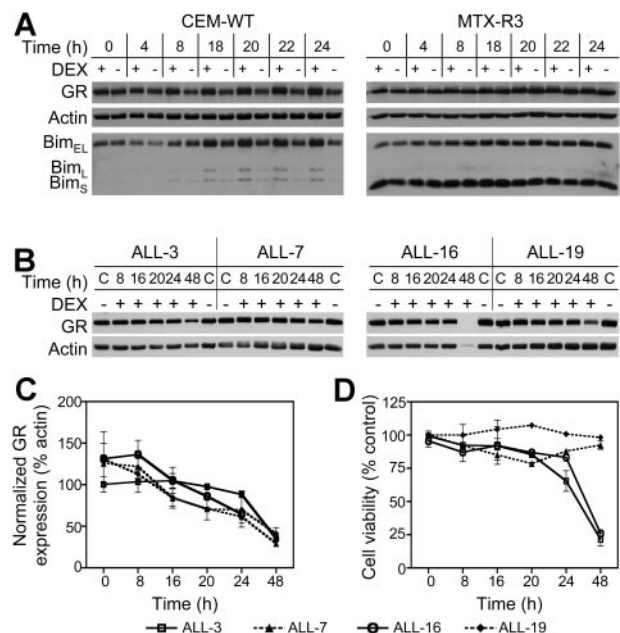
Figure 5. Time course of GR and Bim protein expression in CEM-WT and MTX-R3 cells and changes in GR expression and effects on cell viability in xenograft cells exposed to dexamethasone. (A) CEM-WT and MTX-R3 cells were exposed to dexamethasone (1 µM) and harvested at the indicated time points. Total cellular protein (75 µg) was separated by SDS-PAGE and immunoblotted for GR, actin, and Bim protein expression. Data shown are representative of 2 independent experiments. (B-D) Xenograft cells were retrieved from cryostorage and exposed to dexamethasone (1 µM), and whole cell lysates were prepared at appropriate time points thereafter (0, 8, 16, 20, 24, and 48 hours). (B) Total cellular protein (50 µg) was separated by SDS-PAGE and immunoblotted for GR and actin. *C* indicates solvent-treated controls. (C) Quantitative data showing GR down-regulation with respect to actin in all 4 xenografts. (D) Cell viability was assessed by trypan blue exclusion at each time point. □ represents ALL-3; ▲, ALL-7; ○, ALL-16; and ◆, ALL-19. In panels C and D each data point represents the mean ± SEM of 3 independent experiments.
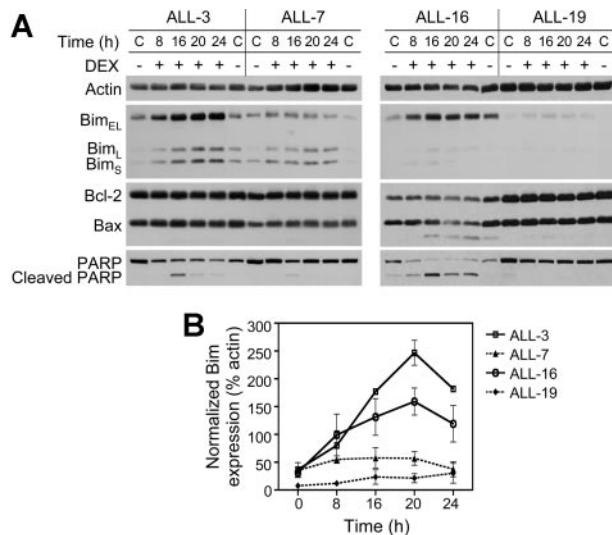
Figure 6. Effects of dexamethasone on expression of Bcl-2 family members and PARP cleavage in xenografts ALL-3, ALL-7, ALL-16, and ALL-19. Xenograft cells were exposed to dexamethasone (1 μM) as indicated in the legend to Figure 5. (A) Total cellular protein (50 μg) was separated by SDS-PAGE and immunoblotted for actin, Bim, Bcl-2, Bax, and PARP. C indicates solvent-treated controls. (B) Quantified expression of the 3 major isoforms of Bim normalized as a percentage of actin controls. Symbols indicate same information as in Figure 5D. Data presented are the mean ± SEM of 3 independent experiments.

Bim induction in cells from a dexamethasone-sensitive xenograft (ALL-16) exposed to dexamethasone was followed by loss of mitochondrial transmembrane potential and an increase in caspase-3/7 activity, all of which were suppressed in a resistant xenograft (ALL-19) (Figure 7C-D). Overall, these results indicate that dexamethasone resistance in xenografts ALL-2, -4, -7, -10, and -19 occurs at a point downstream of ligand-induced GR nuclear translocation, but upstream of Bim induction, loss of mitochondrial transmembrane potential, and caspase activation.

Discussion

We have previously reported that the in vivo and in vitro responses to dexamethasone of childhood ALL xenografts established in NOD/SCID mice significantly correlated with the clinical outcome of the patients from whom the xenografts were derived.[33,34] In the present study, the relative in vitro sensitivities of xenografts to dexamethasone differed by more than 1000-fold, with 5 of 6 highly resistant xenografts (IC$_{50}$ > 10 μM) being derived from patients at relapse or who have died from their disease. Therefore, it is highly likely that the mechanisms of resistance expressed by these xenografts will be relevant to those occurring in patients who present with glucocorticoid-resistant leukemia. Moreover, and in contrast with almost all studies using leukemia cell lines, no defects in receptor-ligand interactions or in dexamethasone-induced nuclear translocation of the GR were apparent in any of the BCP-ALL xenografts. Further investigation revealed that the resistance mechanisms were associated with, at least in part, the failure to induce expression of the proapoptotic Bcl-2 family member, Bim.

Studies that have attempted to associate the number of GR molecules per leukemia cell with clinical outcome have met with limited success. While low numbers of receptors per cell are generally associated with resistance, high receptor expression shows less of a correlation with sensitivity.[7,9] Furthermore, the correlation between receptor content and response to glucocorti-

coids is generally poor.[8,38] Evaluation of our panel of xenografts revealed no correlation between GR protein expression and sensitivity to dexamethasone. Only one xenograft, ALL-8, derived from a patient with T-lineage ALL at relapse, showed a significant deficit in GR expression when compared with other xenografts and the CEM-WT cell line.

Radioligand binding studies subsequently confirmed that, with the exception of ALL-8, glucocorticoid resistance in our xenografts could not be attributed to defects at the level of receptor-ligand interactions. The highly resistant xenograft, ALL-7, contained significantly higher numbers of GR binding sites per cell, consistent with the higher expression levels of GR protein evident by immunoblot analysis. These results were validated by radioligand binding studies using leukemia cells freshly harvested from the spleens of engrafted mice, which confirmed the comparable binding properties of 2 resistant xenografts (ALL-7 and -19) with the sensitive (ALL-3). These findings support the conclusion that, while common in laboratory-derived cell lines,[25,39,40] defects at the level of receptor-ligand interactions are unlikely to account for most instances of resistance observed in the primary disease.

Ligand-induced translocation of GR from the cytoplasm to the nucleus is a critical step in the pathway of glucocorticoid action, prior to activation/repression of gene transcription.[41,42] Expression of several proteins that function to tether the GR in the cytoplasm has previously been associated with glucocorticoid resistance in cell line models. Such proteins include the hsp90 protein that forms the major component of the multiprotein complex that retains the GR in its inactive conformation in the cytoplasm.[10,11] In addition, interaction between the GR and 14-3-3 family proteins may also affect glucocorticoid signaling pathways.[43] In our entire panel of xenografts, no defects in ligand-induced GR nuclear translocation were apparent, and this was confirmed by 2 independent methods.
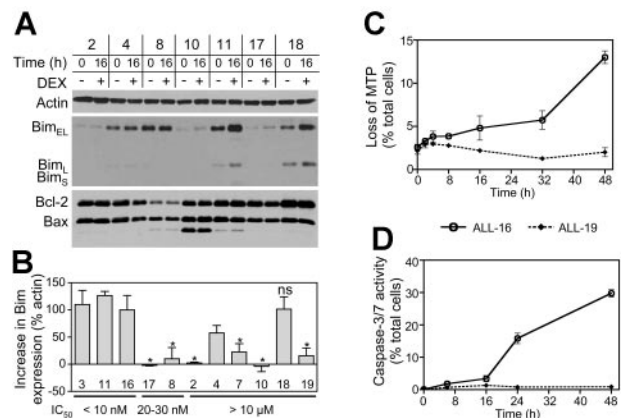
Figure 7. Effect of dexamethasone on Bim, Bcl-2, and Bax expression, loss of mitochondrial transmembrane potential, and caspase activation in ALL xenografts. (A) Bim expression in xenografts cells was determined by immunoblotting of cell lysates prepared at a single time point of treatment with dexamethasone (1 μM, 16 hours), as described in the legend to Figure 5. Immunoblots were also probed with antibodies against Bax, Bcl-2, and actin. (B) Quantitative data illustrating the increase in Bim expression in drug-treated cells over nontreated controls. Expression of the 3 major Bim isoforms was normalized as a percentage of actin controls, and Bim expression in nontreated control cells was subtracted from dexamethasone-treated cells. Data presented are the mean ± SEM of 3 independent experiments. *Significantly different from ALL-3, -11, and -16 (P < .01); ns, not significantly different from ALL-3, -11, and -16. The difference between ALL-4 and ALL-3, -11, and -16 approached significance (P = .05). Dexamethasone IC$_{50}$ values of xenografts are indicated. The proportion of ALL-16 (○) and ALL-19 cells (◆) that exhibited loss of mitochondrial transmembrane potential (MTP) (C) or active caspase-3/7 (D) following exposure to dexamethasone (1 μM). Values for nontreated control cells were subtracted from the respective dexamethasone-treated samples, and data presented are the mean ± SEM of 2 independent experiments.

These results indicate that the failure of the highly resistant xenografts to undergo apoptosis in response to dexamethasone is not a result of cytoplasmic retention of the GR. These observations are consistent with previous findings that failed to detect any correlation between hsp90 expression and glucocorticoid resistance in primary patient material.[44]

Increased GR expression in response to glucocorticoid treatment has been documented in a number of cell lines and has been hypothesized to be necessary for induction of apoptosis in leukemia cells.[36] In nonlymphoid cell lines and lymphoblasts from healthy volunteers, GR down-regulation is observed in response to glucocorticoid treatment.[45] It has been proposed that there is a T-cell specific promoter in the human GR gene that explains why T-lineage ALL cell lines, such as CEM cells, up-regulate GR in response to glucocorticoids, while BCP-ALL cell lines undergo ligand-induced GR down-regulation.[46,47] However, in the present study, the GR was down-regulated in response to dexamethasone in 4 of 4 xenografts tested, one of which was a dexamethasone-sensitive T-ALL. Thus, basal receptor levels appear to be sufficient to induce an apoptotic response in these childhood ALL xenograft cells, and glucocorticoid resistance mechanisms appear to lie downstream of this point in our xenografts.

Glucocorticoid-induced apoptosis is mediated by the intrinsic apoptotic pathway, which involves loss of mitochondrial transmembrane potential, release of apoptotic factors from the mitochondria, and subsequent activation of effector caspases. The Bcl-2 family of proteins are key regulators of these mitochondrial-mediated apoptotic events.[3] A number of recent microarray-based studies have identified *bim* as the only proapoptotic Bcl-2 family member to be consistently up-regulated by glucocorticoids in leukemia and lymphoma cell lines.[20-22] The most profound difference observed between sensitive and resistant xenografts in this study was the lack of Bim induction in 7 of 8 xenografts of intermediate or high-level resistance. ALL-18, the highly resistant xenograft which did induce Bim in the presence of dexamethasone, was also found to have the highest Bcl-2/Bax ratio (R.G. and R.B.L., unpublished observations, November 25, 2003). These results suggest that the mechanism of resistance exhibited by ALL-18 lies downstream of Bim induction. Although the role of Bim in glucocorticoid-induced apoptosis has not been clearly defined, and it is unlikely that *bim* is the only gene that is differentially regulated between sensitive and resistant xenografts, our results and those of others indicate that it is likely to be a critical mediator of this process in childhood ALL cells.

The mechanism by which glucocorticoids regulate Bim expression in lymphoid cells remains unclear. Bim has been shown to be regulated by FOXO3a, a member of the forkhead family of winged helix transcription factors, in a number of systems, including hematopoietic cells.[48,49] Interestingly, FOXO3a was also shown to be induced by glucocorticoids in microarray studies.[20,21] Alternatively, signaling mediated by the Jun N-terminal kinase (JNK) group of mitogen activated protein (MAP) kinases may result in induction of Bim.[50] MAP kinase phosphatase-1 (MKP-1) expression was shown to be down-regulated in response to dexamethasone in a pre-B–leukemic cell line.[21] Down-regulation of MKP-1 resulted in a concomitant increase in JNK activity that induced BH3-only proteins, including Bim, by signaling through c-Jun.[50] Investigations of the role of protein-protein interactions between the GR and other transcription factors such as NF-κB and AP-1 may also provide insight to the molecular mechanisms of glucocorticoid action and associated resistance mechanisms in our childhood ALL xenografts.

In conclusion, this study provides novel insight into the mechanisms of glucocorticoid resistance that may occur in childhood ALL patients. Previous studies using cell lines have implicated defects at the level of receptor-ligand interactions to be responsible for the development of glucocorticoid resistance. In contrast with such reports, we have used our unique and clinically relevant experimental model to show that the lesions underlying glucocorticoid resistance in childhood ALL cells occur downstream of ligand binding and nuclear translocation of the GR. Moreover, the most notable difference between sensitive and resistant xenografts was the failure of resistant xenografts to induce the proapoptotic BH3-only protein, Bim, when exposed to dexamethasone. Lack of Bim induction was accompanied by maintenance of mitochondrial transmembrane potential and failure to activate effector caspases. Precise delineation of this resistance pathway is likely to identify novel targets for the development of new therapeutic strategies for the management of glucocorticoid-resistant lymphoid malignancies.

Acknowledgments

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