Resistance to Glucocorticoid-induced Apoptosis in Human T-Cell Acute Lymphoblastic Leukemia CEM-C1 Cells Is Due to Insufficient Glucocorticoid Receptor Expression

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ABSTRACT

The ability of glucocorticoids (GCs) to induce death in lymphoid-origin cells is the basis for their frequent use in the therapy of various human hematological malignancies. However, the occurrence of primary or secondary GC resistance limits their clinical usefulness. Prior investigations into the mechanism of GC resistance in established human leukemic cell lines revealed loss-of-function mutations in the GC receptor (GR) gene. In this study, we analyzed the GC-resistant human acute T-cell leukemia line CEM-C1, which has been reported to express biochemically functional GR and, thus, was thought to owe its GC resistance to signal transduction changes distal from the GR. Radioligand binding assays revealed a 2-3-fold lower expression of GR in CEM-C1 than in the GC-sensitive sister cell line CEM-C7H2. Analysis of transcriptional activity using mouse mammary tumor virus-long terminal repeat-controlled chloramphenicol acetyltransferase expression in transient transfection assays confirmed the expression of functional GR in CEM-C1 but at levels lower than those in CEM-C7H2 cells. Upon molecular analyses of the GR gene and its transcripts, we found that CEM-C1 cells were heterozygous for the ligand binding domain L753F point mutation in exon 9, which is also present in GC-sensitive CEM-C7H2. No mutations, however, were found on the second GR allele of CEM-C1. To test the possibility that resistance in CEM-C1 cells might be caused by insufficient expression of GR, we established several cell lines stably transfected with rat GR expression vectors. These cell lines differed in exogenous GR expression as determined by Northern blotting and radioligand binding assays. The GR expression level in individual lines correlated well with their sensitivity to GC-induced apoptosis. Thus, GC resistance of CEM-C1 cells might be due to subthreshold expression of functional GR rather than defects in signal transduction pathways distal from the GR. Since several clinical investigations showed a correlation between reduced GR expression and poor response to GC-containing treatment, the CEM-C1 line may represent a valid model for GC resistance in human acute T-cell leukemia.

INTRODUCTION

GCs are among the most potent agents used in the treatment of childhood acute lymphoblastic leukemia and are included in every standard remission induction regimen (1, 2). Their beneficial effect is based on the cytotoxic potency of GC on cells of lymphoid origin (3, 4). This cell death exhibits the classical hallmarks of apoptosis, i.e., cell shrinkage, nuclear condensation, and DNA fragmentation. Apoptosis is considered a form of programmed cell death that is actively carried out by cells after reception of appropriate death signals (5). GCs induce apoptosis in immature thymocytes (6) and bone marrow pre-B cells (7) and, under certain conditions, in peripheral mature lymphocytes in vitro and in vivo (8). The molecular mechanisms by which GCs induce apoptosis are, however, still poorly understood, and seem to differ between cell types. In murine thymocytes, for example, GC-induced apoptosis apparently depends on protein biosynthesis (9, 10), whereas GC-induced cell death in murine pre-B cells and mature T cells from the spleen seems to be independent of the induction of newly synthesized proteins (11). GCs bind to, and activate their specific cytoplasmic receptors (GR), which causes translocation of the activated GR to the nucleus, with ensuing transcriptional or transcriptional repression of gene expression (12-15). Induction of presumptive “death genes” by transactivation and/or transcriptional repression of “survival genes” might lead to the final steps of apoptosis. Although evidence for the induction of “death genes” has been provided (16-19), recent data suggest that, at least in human leukemic T cells, transcriptional repression underlies GC-induced apoptosis (20, 21).

Although a significant number of acute lymphoblastic leukemia patients respond well to GCs, some reveal primary GC resistance, and those sensitive to GCs almost exclusively develop secondary resistance after prolonged GC therapy (2). Large clinical surveys suggested a correlation between GC expression levels and primary GC sensitivity and prognosis, but other mechanisms, such as regulation of intracellular substrate availability or alterations distal of the GR involving apoptosis effector mechanisms, should also be considered (2). Possible mechanisms of secondary resistance have been addressed in several laboratories in the last few years using in vitro established mouse and human cell lines (reviewed in Refs. 4 and 22). In the human, the CCRF-CEM acute T-lymphoblastic cell line (23), isolated from a 4-year-old girl after initial GC-including therapy, has been studied in great detail. Several clones have been derived from the parental line, such as the GC-sensitive CEM-C7 and several GC-resistant cell lines, including CEM-C1 and other GR-resistant CEM derivatives. Essentially all of the resistant lines investigated failed to express appreciable levels of GR due to loss-of-function mutations in the GR gene (24-27). The relevance of this finding for the in vivo situation, however, is questionable, at least for chronic lymphoblastic leukemia, since no GR mutations were identified in 22 patients (28). GC-resistant CEM-C1 cells, in contrast, have been reported to express biochemically functional GR at levels only moderately lower than the sensitive sister line CEM-C7 (29). Hence, this line was considered a human model for GC resistance caused by an alteration distal of the GR.

To further investigate whether CEM-C1 cells indeed represent a model for post-receptor defects, we subjected the GR of CEM-C1 cells to detailed functional and molecular analyses. Radioligand binding and transient MMTV-CAT transfection assays confirmed that CEM-C1 expressed functional GR molecules. However, CEM-C1 cells showed substantially lower responses than GC-sensitive CEM-C7H2 cells (a subclone of the CEM-C7 line), suggesting that CEM-C1...
cells either express less GR than their GC-sensitive counterpart or
mutant GR molecules with reduced activity. Sequence analysis of GR
cDNA precluded the latter possibility, thereby arguing for reduced
expression of functional GR molecules. Overexpression of exogenous
rat GR by stable transfection into CEM-C1 cells induced GC sen-
sitivity, further supporting the concept that CEM-C1 cells owe their
resistance to GC-induced apoptosis to reduced GR expression levels
rather than to a defect in the death pathway distal of the GR.

MATERIALS AND METHODS

Cell Lines and Cell Culture. The GC-sensitive cell line CEM-C7H2 was
obtained by limiting dilution cloning of CEM-C7 cells (23), kindly provided by
A. Cserdas (University of Innsbruck, Innsbruck, Austria). The GC-resistant
cell line CEM-C1 (23) was obtained from E. B. Thompson (Galveston, TX).
The cell line CEM-R6 has been described recently (27). Cell lines were grown
in 5% CO₂, saturated humidity, at 37°C in RPMI 1640 supplemented with 10%
bovine calf serum (HyClone, Logan, UT), 100 units/ml penicillin, 100 μg/ml
streptomycin, and 2 mM l-glutamine.

Proliferation, Apoptosis, and Radioligand Binding Assays. Proliferation
(27), apoptosis (27), and GC binding (30) were determined as detailed previ-
ously. Briefly, to analyze proliferation and apoptosis, cells were cultured in the
presence or absence of dexamethasone for 24, 48, and 72 h. Proliferation was
determined in aliquots incubated for a pulse period of 6 additional h with
[^3]H]thymidine, and the uptake was counted by liquid scintillation. To estimate the
degree of apoptosis, the cells were lysed in Triton X-100 and stained with propidium
iodide; the fluorescence was determined by flow cytometry. For whole-cell radioligand binding assays, approximately 5 × 10⁶ cells were incubated in triplicate with increasing amounts of[^3]H]triamcinolone acetonide in the presence or absence of a 500-fold molar excess of unlabeled triamcinolone acetonide at 37°C for 1 h, washed, resuspended in scintillation cocktail, and counted in a scintillation counter. Specific triamcinolone binding sites were determined by Scatchard analyses (31).

MMTV-CAT Assay. Approximately 1 × 10⁵ logarithmically growing
CEM-C7H2, CEM-C1, and CEM-R6 cells were washed, resuspended in 400 μl of PBS, mixed with 80 μg of plasmid MMTV-CAT (kindly provided by S. Mayr, University of Innsbruck, Innsbruck, Austria), incubated on ice for 10 min, and transfected by electroporation (Bio-Rad Laboratories, Vienna, Austria) with an instrument setup of 960 microfarads at 300 V. After electroporation, cells were kept on ice for an additional 10 min, diluted in 5 ml of growth medium, and seeded into one well of a six-well plate. After a 24 h incubation, another 5 ml of growth medium were added, and the cells were divided into two wells with the addition of dexamethasone into one well to a final concentration of 0.1 μM. Cells were harvested after 36 h and washed once in PBS; the pellets were lysed in 100 μl of reporter lysis buffer (Promega Corp., Madison, WI) and incubated at 50°C for 10 min. After Bradford determination of the protein content, 20–50 μg of protein were diluted in 40 μl of reporter lysis buffer and incubated with 2 μl of biotinylated chloramphenicol (50 μM in 25% methanol; Amersham Corp., Aylesbury, United Kingdom), 6 μl of 1 M Tris/HCl (pH 7.8), and 2 μl of[^3]H]acetate-CoA (250 μCi/ml; DuPont NEN, Boston, MA) at 37°C for 30 min. Biotinylated chloramphenicol was bound by the addition of 10 μl of streptavidin-coated polystyrene beads (100 mg/ml wash buffer) and pelleted by brief centrifugation. Unconverted[^3]H]acetate-CoA was washed away by two consecutive washings in wash buffer, and the washed pellet was resuspended in 1 ml scintillation cocktail and counted in an automatic liquid scintillation counter.

DNA Dot Blot Analysis, cDNA Cloning, and Sequencing. The L753F
mutation (24) was detected on PCR-amplified cDNA surrounding codon 753
using mutation-specific oligonucleotides in DNA dot blot analyses, as
described previously (27). GR cDNA cloning and sequence analyses have also
been detailed (27). Briefly, poly(A)+ RNA was transcribed into cDNA using
Moloney murine leukemia virus reverse transcriptase and random nucleo-
clotides. GR cDNA was amplified by PCR using oligonucleotide primers for the
α-form of the GR, ligated into T-vector (Promega), and introduced into
Escherichia coli; transformed colonies were screened using GR-specific oligo-
nucleotides discriminating between the L753F mutant and wild-type GR
alleles. The GR cDNA insert corresponding to the L753 wild-type allele was
sequenced by the chain termination method.

Stable Transfection. Logarithmically growing CEM-C1 cells were
washed in PBS, pelleted at 300 g, and resuspended at a density of 1 × 10⁶ cells/400 μl of PBS. Cells were mixed with 20 μg of PvuI-linearized plasmid
pGR-cat, a CMV promoter/enhancer-driven rat GR expression vector (21),
or pRC-batGR, a chicken β-actin promoter-driven rat GR expression vector (21),
icubated for 10 min on ice, and electroporated with the electroporator set
at 960 microfarads and 300 V. After electroporation, cells were again placed on
ice for 10 min, diluted in 20 ml of growth medium, and seeded on 96-well,
flat-bottomed plates. Selection of stably transfected cells was initiated 48 h
after electroporation using 1 mg/ml G418 (bioactivity, 70%; Life Technolo-
gies, Inc., Detroit, WI).

Northern Analysis. Total RNA was extracted from 1 × 10⁷ cells by a
single-step extraction procedure (32) using Trisolve. Approximately 10 μg of
total RNA per lane were loaded on denaturing 1% agarose gels containing
formaldehyde in 1 X 4-morpholinopropanesulfonic acid buffer (0.2 M morph-
olinopropanesulfonic acid, 50 mM sodium acetate, and 5 mM EDTA), electro-
phoresed at 0.5 V/cm, blotted overnight onto Hybond-N membranes (Amer-
sham Corp.) in 20× SSPE (1 × SSPE = 150 mM NaCl, 10 mM NaH₂PO₄, 1
mM EDTA, pH 7.4), and baked at 80°C for 2 h. Filters were prehybridized in
5× SSPE, 5× Denhardt’s solution (33), 0.1% SDS, and 50 μg/ml denatured sheared salmon sperm DNA at 65°C for 3 h and hybridized for an additional
12 h to heat-denatured ratGR (XbaI fragment of pGR-cat) or α-tubulin
cDNA probes (1 × 10⁶ cpm/ml) labeled by random priming to a specific activity of approximately 4 × 10⁶ cpm/μg. After hybridization, blots were washed in 2× SSPE/0.1% SDS at room temperature and in 0.2× SSPE/0.1% SDS at 65°C and exposed to an Agfa Curix X-ray film with an amplifying screen at ~90°C for 3–24 h. Between hybridization with the ratGR and the α-tubulin probes, the blot was stripped by boiling in 0.1% SDS.

RESULTS

Phenotypic Characterization of CEM-C1. To determine the degree of
GC resistance in CEM-C1 cells, the effect of dexamethasone on proliferation
and apoptosis was assessed by[^3]H]thymidine uptake and FACS analyses in comparison with GC-resistant CEM-R6 and GC-sensitive CEM-C7H2 cells. As shown in Table 1, CEM-C1 cells were resistant to the antiproliferative effect of GC similar to the
GR-defective CEM-R6 line. In contrast, CEM-C7H2 cells showed
marginally reduced[^3]H]thymidine uptake at 24 h and essentially no
uptake at 72 h. Over the incubation period, a marked increase in
hypodiploidic nuclei, characteristic of apoptotic cell death, was de-
Table 1 Effect of dexamethasone on proliferation of CCRF-CEM derivatives
[^3]H]Thymidine uptake (cpm × 1000/culture) of GC-sensitive CEM-C7H2, GC-resistant, receptorless CEM-R6, and GC-resistant CEM-C1 cells cultured in the absence (0) or
presence (Dex) of 1 μM dexamethasone for the indicated time. Shown is the mean of 2 assays performed in triplicate, SD values were <9% of mean values.

<table>
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<th>Cell Line</th>
<th>0 h</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
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<tr>
<td></td>
<td>24 h</td>
<td>48 h</td>
<td>72 h</td>
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<td>146.6</td>
<td>157.8</td>
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<td>140.4</td>
<td>138.4</td>
<td>159.5</td>
<td>155.7</td>
</tr>
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*Dex, dexamethasone.*
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Fig. 1. Representative example of flow cytometric apoptosis analysis of 0.1 μM dexamethasone-treated GC-sensitive CEM-C7H2 cells (A, 24 h; B, 72 h) and GC-resistant CEM-C1 cells (C, 24 h; D, 72 h). Nuclei from apoptotic cells appear between the vertical bars, and the first and second peaks correspond to nuclei from cells in the G1 and G2-M phases of the cell cycle, respectively. Whereas CEM-C7 cells underwent significant apoptosis (10% and 82% after 24 and 72 h, respectively), CEM-C1 appeared essentially resistant. Similar results were obtained in several additional experiments.

Fig. 2. Whole-cell radioligand binding assay of GC-sensitive CEM-C7H2 (○), GC-resistant, receptorless CEM-R6 (△), and GC-resistant CEM-C1 (□) cells. Shown are means of specifically bound radioactivity from an assay performed in triplicate; bars, SD. Similar results were obtained in several additional experiments.

Functional Analyses of GR Molecules in CEM-C1. To investigate whether GC resistance in CEM-C1 might be due to defective GR expression, CEM-C1 cells, along with GC-resistant CEM-R6 and the GC-sensitive CEM-C7H2 cells as controls, were subjected to whole-cell radioligand binding assays (Fig. 2). Corresponding Scatchard analyses revealed about 18,000 GC binding sites/cell in CEM-C7H2 but only about 7,000 binding sites/cell in CEM-C1 cells (Fig. 3). The GR-defective CEM-R6 line expressed no ORs detectable in this assay, as was expected from previous studies (27). To obtain information about the transactivation properties of the OR in CEM-C1 cells, we performed transient transfection assays using reporter plasmids expressing bacterial CAT under the control of a GC-inducible MMTV promoter. Treatment of transfected CEM-C7H2 cells resulted in an ~60-fold increase of CAT activity versus ~10-fold induction in CEM-C1 (Fig. 4). No induction of CAT activity was obtained by transient transfection of the receptor-deficient cell line CEM-R6. The lower induction of CAT activity in CEM-C1 cells correlated well with GR expression values obtained by the above radioligand binding assays. Taken together, these data suggest that CEM-C1 cells either express lower levels of functional GR or a GR whose transactivation and/or ligand binding capacity is significantly reduced, possibly by a mutation in the GR gene.

Molecular Analyses of the GR Gene. To analyze the possibility of mutations within the coding region of the GR, we first investigated whether CEM-C1 cells carry the ligand-binding mutation L753F (leucin to phenylalanin), observed previously in a heterozygous state in CEM-C7H2 cells (24), and in homo- or hemizygous states in some GC-resistant CEM derivatives (25). Dot blot analyses of PCR-amplified GR DNA revealed that CEM-C1 cells were heterozygous for...
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The L753F mutation (Fig. 5). To determine whether the other chromosome carried a mutation in the GR gene, we PCR-amplified, cloned and sequenced the mRNA corresponding to the L753 wild-type allele. Sequence analysis of its entire coding region revealed no aberrations from the normal GR sequence. Thus, CEM-C1 cells, like GC-sensitive CEM-C7H2 cells, express one GR allele carrying the L753F mutation and another one that is devoid of any coding region mutation.

Phenotype Reversion by Overexpression of Exogenous GR. On the basis of the above data, we speculated that the GC resistance in CEM-C1 cells might be due to insufficient quantitative expression of the functional GR allele. To test this hypothesis, we established several stably transfected CEM-C1 derivatives expressing rat GR that is known to function in human cells (21) and can easily be distinguished from the endogenous (i.e., human) GR. Initial experiments were performed with constructs expressing the OR under control of the CMV promoter/enhancer (clones BF10 and BF11). However, because we subsequently noted that the CMV promoter might be partially transcriptionally repressed by the GR, we established additional stably transfected cell lines using the chicken β-actin expression vector pRCf3-ratGR for GR expression (clones 4B2 and 404). As shown in Fig. 6 (left panel), expression of the exogenous rat GR at the mRNA level and ligand binding correlated well. Only BF10 expressed high levels of ratGR mRNA but did not bind corresponding high levels of GC. This might be due to reduction in transgene expression over time since radioligand binding and apoptosis assays were performed with cells that had been kept in culture several weeks longer than those used for RNA preparation. The highest GR expression level (as measured by GC binding) was found in clone 4G4, followed by clones 4B2 and BF10. Clone BF11, in contrast, did not differ significantly from the parental CEM-C1 line in this respect. Upon treatment with dexamethasone for 3 days, the GR-overexpressing cell lines 4G4, 4B2, and BF10 underwent apoptosis, with percentages of apoptotic cells roughly corresponding to the level of GR expression and approaching levels seen in CEM-C7H2 (Fig. 6, right panel). In contrast, CEM-C1 and clone BF11, which did not express more GR than the parental CEM-C1 line, showed no significant increase of hypodiploidic nuclei in flow cytometric apoptosis assessment upon GC treatment.

DISCUSSION

GC resistance is a major problem in the treatment of acute lymphatic leukemia, yet very little is known about the underlying mechanisms. We and others use the acute leukemic cell line CCRF-CEM and its derivatives as a human model for GC-induced apoptosis and resistance toward this phenomenon. Previous studies have shown that GC-resistant CEM derivatives, like other human and mouse GC-resistant leukemia lines, carry loss-of-function mutations in both GR alleles, explaining their GC-resistant phenotype (24–27). The CEM-C1 subline, in contrast, has been described to express biochemically functional GR and was thought to owe its GC resistance to mutations downstream of the GR (29).

In this study, we present a detailed molecular analysis of the GR in CEM-C1 cells that challenges the above concept. We first confirmed and extended previous evidence for the presence of functional GRs in CEM-C1 cells. Like others, we observed specific GC binding with an affinity typical for the GR. Moreover, the functionality of the GR was supported by transient transfection assays with a GC-responsive reporter construct that showed GC-dependent reporter regulation in CEM-C1 cells consistent with previous findings showing GC induction of glutamine synthase activity (a paradigm for an endogenous GC-responsive gene) in such cells (29). Finally, our molecular analyses revealed that CEM-C1 expressed a mutation-free GR allele. The other GR allele carried a point mutation (L753F). However, this mutation is present in the highly GC-sensitive CEM-C7 line as well and, hence, cannot by itself explain GC resistance in CEM-C1. Although our investigations supported the previously reported presence of functional GR in CEM-C1 (29), they differed from that report in a quantitative aspect. Thompson’s laboratory found comparable induction of glutamine synthase by dexamethasone in CEM-C1 and CEM-C7, and only a small reduction in GR expression in CEM-C1 as compared to CEM-C7 cells, whereas we found significantly lower levels of GR in CEM-C1 than in GC-sensitive CEM-C7H2. To test whether reduced GR expression might account for GC resistance, we increased the GR level by stably overexpressing exogenous GR in CEM-C1 sublines. This treatment restored GC sensitivity, suggesting that GC resistance in CEM-C1 results from insufficient expression of functional GRs and is not the consequence of a defect distal of the GR.

How might the different conclusions drawn from our work and previous studies (29, 34) suggesting a post-receptor defect be explained? The latter conclusion was mainly based on the observations that the CEM-C1 GR was functional and biochemically indistinguishable from that in CEM-C7 cells (29), and that somatic hybrids between CEM-C1 and the GR-negative CEM-C7 derivative ICR27 were GC sensitive, proving the functionality of the CEM-C1 GR with respect to apoptosis induction if expressed in a different cellular context (34). The slightly reduced GR level in CEM-C1 as compared to GC-sensitive CEM-C7 was not considered significant. Our analyses confirmed the functionality of the CEM-C1 GR; however, we noted a more pronounced difference in GC binding between the GC-sensitive and -resistant CEM sublines (perhaps due to small fluctuations in GR

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Fig. 4. Representative MMTV-CAT assay of GC-sensitive CEM-C7H2 (●), GC-resistant, receptorless CEM-R6 (□), and GC-resistant CEM-C1 cells (●) transiently transfected with an MMTV-CAT reporter construct and incubated for 24 h in the absence or presence of 0.1 μM dexamethasone. Columns, means of induction over background of CAT activity by dexamethasone from an experiment performed in duplicate. Similar results were obtained in two additional experiments.

Fig. 5. Dot blot analysis of PCR-amplified genomic DNA corresponding to the region surrounding codon 753 of the GR gene from CEM-C1 cells and a normal control person hybridized to radioactively labeled oligonucleotides recognizing the wild-type sequence (wt, top row) or the L753F mutated sequence (L753F, bottom row), respectively.

S. Geley, unpublished data.
expression of ORs per cell required for OC sensitivity is, however, not precisely known and may differ from one leukemia cell type to another due to differences in expression of co-activators, accessory molecules, and other factors. In view of the clinical significance of these phenomena, further studies on the molecular basis of OR down-regulation and potential enhancement of OR expression by pharmacological agents seem warranted.

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REFERENCES


Fig. 6. A, whole-cell radioligand binding and Northern blot analyses of GC-sensitive CEM-C7H2, GC-resistant CEM-C1, and CEM-C1 sublines stably transfected with constructs constitutively expressing rat ORs (BF10, BF11, 4B2, and 4G4). Columns, means (bars, SD) of specifically bound radioactivity from an assay performed in triplicate. For each line, expression of exogenous (rat) OR is shown on the mRNA level in the Northern blot shown beneath. The endogenous (human) OR migrates at a different position in the gel and cannot be seen in this blot. The amount of mRNA per lane is reflected in the signal obtained with the housekeeping gene probe α-tubulin. B, flow cytometric apoptosis analysis of 0.1 μM dexamethasone-treated GC-sensitive CEM-C7H2, GC-resistant CEM-C1, and CEM-C1 sublines stably transfected with constructs constitutively expressing rat OR (BF10, BF11, 4B2, and 4G4). The percentage of apoptotic nuclei (% gated) is indicated in the top left corner of each diagram.
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