BCL-2 Expression and Mitochondrial Activity in Leukemic Cells With Different Sensitivity to Glucocorticoid-Induced Apoptosis

By Lou A. Smets, Joop Van den Berg, Dennis Acton, Bert Top, Henny Van Rooij, and Manon Verwijs-Janssen

The present study investigates the relationship between mitochondrial activity and the expression of the BCL-2 gene in a panel of six human and murine leukemia/lymphoma cell lines. The cell lines all contained normal glucocorticoid receptors but differed widely in sensitivity to dexamethasone, ranging from very sensitive S49 lymphoma to completely resistant HL-60 acute leukemia cells. In this panel, 10- to 15-fold differences in basal adenosine triphosphate (ATP) content and adenosine diphosphate (ADP)/ATP ratio were correlated with up to fivefold differences in bcl-2 protein (in human cells) and approximately 25-fold difference in bcl-2 mRNA content (all cell lines). Moreover, ATP content and BCL-2 gene expression were inversely correlated with glucocorticoid sensitivity and cell cycle length. In resistant cell lines, sensitivity to dexamethasone was restored by the mitochondrial inhibitors rotenone and meta-iodobenzylguanidine. This sensitization was not accompanied by detectable reductions in bcl-2 mRNA or protein content, suggesting that the inhibitors were capable of overriding BCL-2-mediated inhibition of apoptosis. Increased mitochondrial activity and (overexpressed) BCL-2 appeared closely related properties of glucocorticoid-resistant cells, sharing common cellular targets in hormone-induced apoptosis.

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MATERIALS AND METHODS

Cell lines and cytological assays. Cell lines HL-60, L1210 (subline 56.3), and S49 and culture conditions were as described in previous reports.16,19 Cell line JANEL was established by infection of normal human B cells with Epstein-Barr virus. The human non-Hodgkin's follicular lymphoma cell line DoHH2,20 carrying translocation t(14;18), was kindly donated by H. Kluin-Nelemans (Leiden University, The Netherlands). Human T-cell leukemia CEM-C7 line was obtained by courtesy of T. Schmidt (University of Iowa). Dex- methasone (DEX) was added from 1,000-fold ethanol concentrates to a final concentration of 10^{-7} mol/L throughout and lysis was scored microscopically. DNA per cell recordings were made by flow cytometry on ethanol-fixed and ethidium bromide stained cells as described previously.21

Metabolic studies. DNA and protein synthesis was assessed from the incorporation of ^3H-thymidine and ^14C-leucine, respectively, into acid-precipitable cell material and related to cellular protein according to routine procedures. Adenosine triphosphate (ATP) and adenosine diphosphate (ADP) content were determined in cold 0.5 N perchloric acid extracts and the relative ADP content was expressed by the molar ratio ADP/ATP \times 100\%.

^3H-dexamethasone binding. Specific ^3H-dexamethasone binding

THE TRANSCRIPTIONALLY deregulated BCL-2 gene increases the life-span of lymphoid cells1,2 and confers resistance to various inducers of programmed cell death (apoptosis).3,6 According to recent reports the bcl-2 protein complexes with death-accelerating homologs7 and functions in antioxidant pathways in preventing apoptosis.8 The precise biochemical activities of bcl-2 protein and its homologs are not known, however. Because of its localization to mitochondrial membranes,9 protection by BCL-2 has been previously associated with mitochondrial functions.4 An exclusive role of bcl-2 protein in mitochondria has been questioned, however, by the observation that in several cells the pattern of immunofluorescent staining is consistent with its localization to the endoplasmatic reticulum (ER) and the nuclear envelope as well.10,11 In fact, neither mitochondrial localization nor an integral membrane position are absolute requirements for bcl-2 function according to Hockenberry et al.8 Mitochondrial involvement has been specifically challenged by the observation that fibroblasts lacking mitochondrial DNA and thus deficient in oxidative phosphorylation, remain susceptible to protection by the transfected human BCL-2 gene.11

On the other hand, cells deficient in mitochondrial DNA still contain all nuclear encoded mitochondrial enzymes and retain important mitochondrial functions that include succinate dehydrogenase activity12 and the generation of an electrochemical gradient.13 There is circumstantial evidence for a critical role of mitochondrial activity in the susceptibility of leukemic cells to glucocorticoid (GC)-mediated lysis. Structural14 and functional15 damage to mitochondria has been observed early in GC-induced lysis of lymphoid cells, although a causal sequence has not been established. However, several leukemic cell lines, including fully resistant variants, can be sensitized to dexamethasone by inhibitors of mitochondrial respiration.16-18 The observation that leukemic cells are protected from GC-induced apoptosis by a relative abundance of bcl-2 protein3,6 but sensitized by respiration inhibitors adds to a notion of functional relationships between bcl-2 protein and mitochondrial activity in the lysis of leukemic cells.

To investigate the possibility relationship between BCL-2 and mitochondria, we have compared mitochondrial activity and BCL-2 expression in a panel of GC receptor-positive murine and human leukemic cell lines, selected for widely different susceptibility to the growth inhibitory and lytic effects of dexamethasone. In this report, we explain that the expression levels of the BCL-2 gene and cellular energy status are highly correlated phenomena and that both parameters are inversely related to sensitivity for GC-induced apoptosis and cell cycle length.
sites were measured in a whole-cell assay as described elsewhere. Briefly, cells were incubated with 2 pCi of 3H-dexamethasone (S × 10^3 mol/L) with or without a 1,000-fold excess of radioinert steroid to assess nonspecific binding. After 20 minutes postincubation in agonist-free growth medium the amount of cell-associated radioactivity was determined.

Chemicals, antibodies, and buffers. Anti-receptor antibody GR49.1 was kindly donated by Dr H.M. Westphal (Marburg, Germany); monoclonal MAI-510 (clone BuGR2) was purchased from Affinity BioReagents (Neshanic Station, NJ). Anti-bcl-2 monoclonal antibody (clone 124) was a kind gift of Drs Mason and Pezella (Manchester, UK).

Affinity Bioreagents (Neshanic Station, NJ). Anti bcl-2 monoclonal antibody (GAM-HRP) IgG was from Pierce (Rockford, IL), fluorescein isothiocyanate (FITC) coupled sheep-anti-mouse IgG was from Sigma (St Louis, MO), nitrocellulose filters were from Bio-Rad (Richmond, VA) and PVDF immobilon membranes were from Millipore (Bedford, MA). Enhanced chemiluminescence (ECL) detection kit for Western blotting, carnation (Los Angeles, CA) nonfat dry milk (5% (wt/vol)) and 0.1% (vol/vol) Tween-20 in PBS; blocking buffer no. 2: 0.15% (vol/vol) bovine serum albumin (BSA), 0.5 mmol/L EDTA, 0.5% (vol/vol) Tween-20 in PBS; SSC (20X): 88.2 g sodiumcitrate, 175.3 g NaCl, pH 7; lysis buffer: 250 mmol/L sucrose, 20 mmol/L KH2PO4, pH 7, 1 mmol/L EDTA, CaCl2 and MgCl2, both at 0.15 mmol/L.

**Western blotting.** The intracellular distribution of glucocorticoid receptors was determined by Western blotting. After disruption of the cells in lysis buffer, cytosolic and nuclear fractions were prepared by centrifugation at 14,000g and cell equivalent amount of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (7.5%) and transferred to nitrocellulose filters. The filters were blocked with buffer no. 1 and probed with anti-receptor antibody GR49.1 or BuGR2 (1:5,000 in blocking buffer no. 1) using GAM-HRP (1:1000 in blocking buffer no. 1) as secondary antibody. The immunoreactive proteins were visualized by the ECL technique according to the instructions of the manufacturer.

Relative levels of bcl-2 protein were determined in samples of 10^6 cells boiled in sample buffer, separated by SDS-PAGE (15%) and transferred to Immobilon-P, PVDF membranes. The membranes were incubated in blocking buffer no. 2 and probed with anti bcl-2 antibody (1:20 in blocking buffer no. 2). Detection was performed by GAM-HRP (1:3,000 in blocking buffer no. 2) as primary antibody. The immunoreactive proteins were visualized by the ECL technique according to the instructions of the manufacturer.

**Cell Line Description**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Description</th>
<th>Cell Cycle Time Sites Sensitivity to Dexamethasone</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL-60</td>
<td>Human myeloid leukemia</td>
<td>48 18,000 -</td>
</tr>
<tr>
<td>DoHH2</td>
<td>Human NH lymphoma</td>
<td>36 15,000 ±</td>
</tr>
<tr>
<td>Janel</td>
<td>Human B-cell leukemia</td>
<td>36 ND</td>
</tr>
<tr>
<td>CEM-C7</td>
<td>Human T-cell leukemia</td>
<td>18 20,000 +</td>
</tr>
<tr>
<td>L1210</td>
<td>Mouse leukemia</td>
<td>12 9,000+++</td>
</tr>
<tr>
<td>S49</td>
<td>Mouse lymphoma</td>
<td>10-12 20,000 +++</td>
</tr>
</tbody>
</table>

Cell cycle time was estimated from the doubling time during exponential growth. Specific 3H-DEX binding sites were determined as described in Materials and Methods. Sensitivity to 10^-7 mol/L of dexamethasone was rated as strong growth inhibition and massive cell lysis after 24 hours (+++), growth inhibition with marginal cell lysis after 48 hours (+) or no effect (−).

Abbreviation: ND, not done.

**RESULTS**

**Characterization of cell lines.** The presence of GC-binding sites, ranging from 9,000 in L1210 to 20,000 sites/cell in S49 and CEM-C7, was confirmed in conventional whole-cell binding assays. Nuclear translocation of DEX-ligated cytosolic receptors was also confirmed by immunoblotting of the GC receptor protein in cytosolic and nuclear fractions before and after 2 hours of incubation with the steroid. In spite of the presence of normal GC receptors and of the capability of nuclear translocation, the cell lines differed widely in susceptibility to the growth inhibitory and lytic effects of 10^-7 mol/L DEX. (Table 1). In S49 cells, 80% was lysed within 24 hours, whereas lysis of L1210 cells was observed only after 2 days. In CEM-C7 cells inhibition of growth started after 72 hours and was accompanied by the appearance of 20% to 40% lysed cells after 92 hours.

In S49 cells, 80% was lysed within 24 hours, whereas lysis of L1210 cells was observed only after 2 days. In CEM-C7 cells inhibition of growth started after 72 hours and was accompanied by the appearance of 20% to 40% lysed cells after 92 hours. DoHH2 and JANEL cells responded to DEX by a transient inhibition of growth during the first 24 hours, accompanied by a marginal degree of cell death of 10% to 20%. Subsequently, these cells resumed normal growth in the continuous presence of DEX. HL-60 cells were completely insensitive to DEX for 72 hours as reported previously. Overall, the cell lines with shorter cell cycle time were better responders to the steroid, but there was no relation with the number of specific GC binding sites.

**Mitochondrial activity.** The cell lines differed considerably in basal ATP content and energy status as reflected by

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Fig 1. ATP levels (■) and relative ADP content (□) in cell lines with (from left to right) increasing sensitivity to dexamethasone (cf, Table 1). ADP content is expressed as percentage of molar ATP content and is defined as the reciprocal of cellular energy charge. Values are mean ± SEM of duplicates of six to eight independent experiments.

ADP/ATP ratio (Fig 1). ATP levels were expressed relative to protein content to compensate for differences among the various cell lines. The observed values were well above (HL-60) or below (S49; L1210) the average ATP content reported for various leukemic and non-leukemic cell lines of ≈4 to 6 nmol/10⁶ cells,¹² corresponding with ≈20 mg/g protein. Likewise, the relative ADP content ranged significantly below and above the average value of ≈10% in actively growing cells in vitro. Overall, sensitivity to dexamethasone was inversely correlated with ATP content and proportional with decreasing energy status, i.e., increasing ADP/ATP ratio.

BCL-2 expression. The bcl-2/actin ratio was highest in HL-60 cells, exceeding by two-fold that of DoHH2 cells. EBV-transformed JANEL cells contained lower levels of bcl-2 protein but these were still higher than in CEM-C7 cells (Fig 2). The bcl-2/actin ratio of reference human leukocytes was 0.05, indicating the relative abundance of the protein in the human cell lines. As expected, mouse L1210 cells were negative for the human-specific antibody.

Because an antiserum directed against murine bcl-2 protein was not available, BCL-2 gene expression in all cell lines was assayed on Northern blots using a mouse cDNA probe. This probe detects a 7.5- and 2.4-kb transcript as previously observed by Negrini et al. Because of the absence of cross-hybridization with the 28S rRNA, an additional 5-kb message is also detected. The 7.5-kb bcl-2 mRNA levels in L1210 and S49 cells were compared by densitometry, and normalized against the 2-kb actin mRNA signal. The results (Fig 3C) indicated at least three-fold higher levels in L1210 cells. Mouse L1210 cells were compared with human DoHH2 cells, using the mouse cDNA probe. At comparable total mRNA loading levels (Fig 3A), expression of the major 7.5-kb transcript in DoHH2 cells was about eight-fold higher than in L1210 cells (Fig 3B). Considering the differences in the nucleotide sequences between mouse and human BCL2, this eight-fold higher level may be an underestimate. However, no obvious differences in mRNA levels were observed between DoHH2 and the other human cell lines HL-60, JANEL, and CEM-C7 (data not shown).

Reversal of GC-resistance by mitochondrial inhibitors. To test if increased ATP levels were instrumental in GC resistance, the cells were assayed for dexamethasone-mediated lysis in the presence of the inhibitors rotenone or metiodobenzylguanidine (MIBG). Inhibition of mitochondrial respiration with these complex I inhibitors or with doxycycline allows for a compensatory increase in glycolytic flux, maintaining ATP at lower but vital levels. MIBG caused a dose-dependent cytolytic response to dexamethasone in refractory DoHH2 cells. This became already apparent as early as 12 hours after combined treatment (Fig 4). Rotenone in the non-toxic concentration range of 0.5 to 2.0×10⁻⁶ mol/L also potentiated lysis (Fig 5). An excess of 10⁻⁶ mol/L of the GC-antagonist RU486 completely blocked rotenone-induced sensitization to dexamethasone, indicating that lysis was initiated by the hormone and not caused by toxic side effects of the inhibitor. The effects of rotenone and MIBG in JANEL cells were similar to those observed in DoHH2 cells. Sensitization of CEM-C7 and L1210 cells was as re-
Fig 3. Bcl-2 mRNA expression in leukemic cell lines. (A) Total RNA loading control of B. (B) Northern blot of bcl-2 mRNA levels in mouse L1210 cells (lane 1), control human DoHH2 cells (lane 2), and DoHH2 cells incubated for 21 hours with 10⁻⁶ mol/L rotenone (lane 3). (C) Northern blot of bcl-2 and actin mRNA levels in L1210 (lane 1) and S49 (lane 2) cells.

ported previously⁷ but observations in S49 cells were inconclusive because of toxicity of both inhibitors in the concentrations used, probably because of critically low basal ATP levels. However, HL-60 cells remained completely refractory to DEX in the presence of rotenone. With MIBG a weak and delayed (ie, after 4 days) response to the steroid was induced as reported earlier.¹⁶ ATP levels were reduced in cells grown with rotenone (10⁻⁶ mol/L) or MIBG (10 µg/mL). After 21 hours, the reductions varied between 70% (CEM-C7, L1210) and 40% (HL-60) and were all accompanied by a proportional decrease in energy charge. In HL-60 cells, refractory to sensitization by the inhibitors, the ATP levels (37 mg/g protein) and the relative ADP content (6%) of treated cells remained well above (ATP) or below (ADP/ATP) the basal values in the sensitive L1210 and S49 cells (cf, Fig 1).

Effects on BCL-2 expression and cell proliferation. The effect of mitochondrial inhibitors was studied in more detail in DoHH2 cells. There was no reduction in the level of bcl-2 protein during incubation in rotenone for 4 or 16 hours (Fig 6). In Northern blots, the major 7.5-kb messenger signal in DoHH2 was not detectably altered by incubation with MIBG or rotenone for 21 hours (Fig 3A,B). Immunocytochemistry with confocal laser scanning microscopy of bcl-2 protein distribution showed predominant perinuclear localization with the typical patchy staining as described for several other cells.⁸ At this level of resolution, no gross effects of the inhibitors alone or in combination with dexamethasone on bcl-2 content nor its intracellular distribution could be observed (data not shown). Consistent with the finding on bcl-2 protein levels (Fig 6), the incorporation of ³H-leucine was not significantly affected during 21 hours of incubation with the inhibitors. The number of specific ³H-DEX binding sites was only marginally reduced from 15,000 to 11,000 sites per cell, probably because of cell cycle arrest.¹⁶ Cell multiplication in 24 hours was reduced from 1.8 in controls to 1.2 in cells grown the presence of rotenone or MIBG. Flow-cytometric analysis of DNA per cell content indicated that this inhibition of growth was accompanied by an increase in the fraction of cells in G₀ phase from 0.35 to 0.75. The biochemical and cell kinetic findings in DoHH2 cells were similar to the reported effects of doxycycline¹⁸ and MIBG¹⁶,¹⁷ in leukemic cells, showing that inhibition of mitochondrial respiration with simultaneous glycolytic compen-
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DISCUSSION

The protective effect of the overexpressed BCL-2 gene against several apoptotic stimuli has been well documented, but the biochemical mechanisms involved are still a matter of intensive research. In view of its preferential association with mitochondrial membranes, protection from apoptosis by bcl-2 has been previously associated with mitochondrial activity. In view of intensive research. In view of its preferential association with mitochondrial membranes, protection from apoptosis by bcl-2 may act in the control of intracellular Ca^{2+} repartitioning and functions in antioxidant pathways.

The present observations in a panel of leukemic cell lines revealed a correlation between BCL-2 expression (Figs 2 and 3) and mitochondrial activity as reflected by ATP levels and energy status (Fig 1). Moreover, BCL-2 expression level and ATP content were inversely related to the susceptibility to dexamethasone and the rate of cell proliferation (Table 1). Variation in sensitivity to dexamethasone was not associated with the amount of ^{3}H-DEX binding sites nor with the nuclear translocation capability of the liganded GC-receptor. The notion that increased mitochondrial activity can interrupt the lytic signal, once initiated by binding of GC’s to their cognate receptors, finds additional support in the present and previous observations that inhibitors of mitochondrial respiration can potentiate GC-action and even induce sensitivity to steroid-mediated lysis in resistant cell lines. A comparison of the basal ATP levels (Fig 1) and the sensitizing effect of inhibitors suggests that a level of about 20 mg ATP/g protein (4 nmol/10^{6} cells) or the corresponding ADP/ATP ratio of about 0.20 are critical discriminators between sensitivity and insensitivity to GCs, irrespective of leukemic cell lineage. These observations agree with similar alterations in ATP levels and relative ADP content in Molt-4 leukemia cells sensitized to DEX by doxycycline. In this view, failing sensitization of HL-60 cells by rotenone and MIBG can be ascribed to the inability of the drugs to lower high basal ATP levels below this critical level. Conversely, the sensitivity of more rapidly growing cell lines (CEM-C7, L1210, S49) is plausibly explained by an intrinsically lower energy status as a consequence of enhanced energy requirements for rapid protein and nucleic acid synthesis.

Unlike in the human cell lines, the relative bcl-2 protein levels in the murine L1210 and S49 cells could only be estimated from mRNA levels. Although comparison of mRNA levels can only show a trend in bcl-2 protein content, there was a marked, ie, 25-fold difference in BCL-2 gene expression between fully resistant HL-60 and more sensitive S49 cells detected with a mouse cDNA probe. Because of sequence differences between human and murine messengers, this difference is probably an underestimate. Despite this, the relationship between BCL-2 gene expression on the one hand and the correlated variations in ATP content, cell cycle time and DEX sensitivity (Fig 1; Table 1) on the other, is not an obvious one. However, abrogation of GC-resistance by the inhibitors in DoHH2 cells without detectable effects on bcl-2 protein (Fig 6) or mRNA (Fig 3) content may exclude a trivial explanation that a low energy status on its own reduces BCL-2 expression. Therefore, overexpression of BCL-2 and elevated energy status may each afford protection by totally different mechanisms and the observed correlations could be coincidental, ie, the result of co-selection for two unrelated mechanisms of GC-resistance.

On the other hand, there are some grounds for the hypothesis that mitochondrial activity and bcl-2 protein may cooperate in modulating GC sensitivity. In cultured hematopoietic cells and in the B-cell compartment of transgenic mice, overexpression of the bcl-2 protein does not stimulate cell proliferation per se, but promotes survival by preventing irreversible cell cycle exit leading to cell death. In fact, the inverse correlation in our cell panel between BCL-2 expression and cell cycle length suggests that the proto-oncogene can reduce the rate of cell proliferation, allowing the recovery of energy charge required for the acquisition of resistance to DEX. The notion that the BCL-2 gene can suppress proliferation finds support in the observations that elevated levels of bcl-2 protein are associated with slowly growing, indolent tumors and can afford protection against the lethal effects of excessive or inappropriate mitogenic signals.

Finally, the observation that mitochondrial inhibitors were capable of overriding BCL-2–mediated protection in DoHH2 cells could indicate a more direct relationship between bcl-2 protein and mitochondrial activity. Current explanations on bcl-2 action concentrate on two equally attractive hypotheses, namely the regulation of intracellular Ca^{2+} repartitioning and scavenging of reactive oxygen species. It is conceivable that any role of bcl-2 in antioxidant pathways will be dependent on the cellular redox state, and thus, sensitive to complex I inhibitors in cells with a functional respiratory chain. Likewise, mitochondria are directly and indirectly implicated in Ca^{2+} homeostasis. It is of note that incubation of hepatocytes with MIBG results in an increased size of the mitochondrial Ca^{2+} pool, a process that is typically blocked...
by bcl-2 overproduction in a hematopoietic cell line resistant to apoptosis induced by growth factor withdrawal.27

In spite of suggestive correlations, the present experiments cannot provide for direct evidence that bcl-2 protein level is a metabolic checkpoint of GC-sensitivity, acting through mitochondrial functions. However, it is obvious that the physiologic role of bcl-2 in the response of leukemic cells to GC treatment can be fully appreciated only in the context of cell cycle control and cellular energy status. Irrespective of the precise mechanisms involved, the apparent possibility to override resistance to GC hormones in bcl-2 expressing human leukemic/lymphoma cells may be of clinical relevance. Increased bcl-2 protein levels are found in a expanding spectrum of hematologic malignancies, often without involvement of translocation t(14;18).23,34 In tissue culture models, the proto-oncogene appears a potential arbitrator of response to GC hormones and various antileukemic drugs26 and BCL-2 expression has been recently associated with poor outcome of chemotherapy in acute myeloid leukemia.35 Accordingly, antagonism of bcl-2 action by pharmacologic interventions of the type described in this report would appear a feasible strategy in the chemotherapy of leukemia/lymphoma with high expression of bcl-2 protein.

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