Inhibition of Glutathione Synthesis Reverses Bcl-2-mediated Cisplatin Resistance

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ABSTRACT

Cisplatin is a potent cytotoxic agent that functions as a bivalent electrophile, forming both interstrand and intrastrand DNA cross-links. Cisplatin-mediated DNA damage results in cell cycle arrest and initiation of apoptotic cell death. Increased cellular glutathione concentrations have been closely correlated with cisplatin resistance but do not reduce the extent of cisplatin-DNA adduct formation. One hypothesis to explain the ability of glutathione to inhibit cisplatin cytotoxicity is that glutathione, through its antioxidant function, plays a role in apoptotic regulatory pathways. We tested this hypothesis using MCF-7 breast cancer cells transfected with the apoptotic inhibitor Bcl-2. Bcl-2 overexpression in MCF-7 cells was associated with a nearly 3-fold increase in cellular glutathione levels and with increased resistance to cell death after cisplatin exposure. Treatment of MCF-7 lines with buthionine sulfoximine, an inhibitor of glutathione synthesis, normalized glutathione levels in Bcl-2 and control transfectants and completely abrogated Bcl-2-mediated cisplatin resistance without affecting Bcl-2 expression. Bcl-2 overexpression and up-regulation of glutathione were not associated with a change in either cisplatin-DNA adduct formation or repair over time. These results suggest that Bcl-2-mediated cisplatin resistance in MCF-7 cells is dependent on up-regulation of glutathione production, which contributes to cell survival by mechanisms independent of cisplatin inactivation or inhibition of DNA adduct formation. A similar dependence on glutathione for Bcl-2-mediated inhibition of cisplatin toxicity was confirmed in a second cell line, the lymphocytic precursor FL5.12. Taken together, these data suggest that apoptotic signaling after genotoxic exposure can be inhibited by the antioxidant activity of glutathione. Inhibition of glutathione synthesis or modulation of glutathione stores in tumors that overexpress Bcl-2 may comprise a novel anticancer strategy.

INTRODUCTION

Cisplatin is one of the most commonly used chemotherapeutic agents, with demonstrated activity against a diverse spectrum of malignancies (1). The cisplatin molecule consists of a single platinum atom, coordinated with two amide groups and two reactive chlorides. Intracellularly, the relatively low intracellular chloride concentration drives replacement of one or both of the chloride moieties by H2O, resulting in positively charged species that react with nucleophilic sites to cross-link DNA, RNA, and protein (2). At pharmacologically relevant doses, the levels of binding to RNA and protein are low, and cisplatin cytotoxicity appears to correlate with DNA adduct formation (3–5). Intrstrand purine-purine cross-links are the most common DNA lesion induced by cisplatin, although interstrand cross-links can also occur and may be an important trigger of subsequent cell death (6–8).

Cellular responses to cisplatin exposure include inhibition of DNA replication, growth arrest in the G2 phase of the cell cycle, RNA transcriptional inhibition, and initiation of programmed cell death (9–11). The signaling pathway from cisplatin-induced DNA damage to irreversible apoptotic commitment has not been fully defined (6). Overexpression of Bcl-2 or related antiapoptotic Bcl-2 family members can lead to cisplatin resistance, suggesting that the mitochondrial events regulated by Bcl-2, including cytochrome c release and caspase activation, are important components of the DNA damage-initiated apoptotic pathway (12–16).

Multiple mechanisms of cisplatin resistance in tumor cells have been proposed, including decreased cellular uptake and increased cellular efflux of cisplatin (2). Cisplatin uptake appears to be dependent on both passive and active transport, although the mechanisms of active transport have not been elucidated (17). Cisplatin is not a substrate either for the P-glycoprotein drug efflux pump encoded by MDR-1 or for other related cell surface efflux pumps, but active export of cisplatin has been described (18–20). Increased DNA lesion repair through up-regulation of components of the nucleotide excision repair pathway may contribute to cisplatin resistance, as proposed for a number of resistant tumors demonstrating up-regulation of XPA or ERCC1 gene expression (21–23). Selection for cisplatin resistance in some models has been associated with development of mismatch repair deficiency (24–27). Failure to appropriately recognize cisplatin adducts in cells with defects in mismatch repair is hypothesized to lead to replication bypass of lesions, resulting in frequent incorporation of incorrect nucleotides and failure to trigger cell death.

Intracellular cisplatin inactivation by glutathione has also been proposed as a mechanism of cisplatin resistance (28, 29). Glutathione is the most abundant intracellular thiold, present at concentrations of 0.1–10 mM, and serves as a critical cellular antioxidant (30, 31). The reduction-oxidation (redox) state of a cell is largely determined by the balance between reactive oxygen species generated and endogenous expression of thiol buffers such as glutathione (32). Glutathione is required for the maintenance of protein sulfhydryl groups in a reduced state and for resistance to oxidative stress through detoxification of reactive oxygen species. Glutathione can similarly detoxify many exogenous toxins, including cisplatin, through the formation of glutathione adducts (28, 33). High relative expression of members of the glutathione S-transferase family, enzymes that catalyze coupling of glutathione to multiple reactive substrates, has been correlated with low response rate to cisplatin therapy (34–36).

Intracellular glutathione stores can be down-regulated by BSO through inhibition of γ-GCS, the rate-limiting enzyme for glutathione synthesis. Conversely, glutathione concentrations can be augmented by N-acetyl cysteine, which is converted intracellularly to the rate-limiting substrate for glutathione synthesis. Suppression of intracellular glutathione by addition of BSO to cancer lines in culture has been shown to increase cisplatin sensitivity; conversely, elevation of glutathione production by addition of N-acetyl cysteine leads to in-
creased cisplatin resistance (37). One explanation for this correlation is that increased glutathione concentrations enhance inactivation of cisplatin in the cytoplasm through the action of glutathione S-transferase, thereby decreasing cisplatin-mediated DNA damage. However, quantitative analysis of the extent of cisplatin-DNA adduct formation has failed to demonstrate a clear correlation between the level of DNA platination and cellular glutathione content, despite the correlation of glutathione content with cisplatin resistance (38). Taken together, these data suggest that total cellular glutathione content is an important determinant of cisplatin resistance but that glutathione-mediated cisplatin resistance may depend on factors other than cisplatin inactivation by glutathione-cisplatin adduct formation.

An alternative hypothesis for how increased glutathione leads to relative cisplatin resistance is that by serving as a redox buffer, glutathione may inhibit some of the mitochondrial alterations associated with apoptotic commitment. One of the early cellular alterations associated with apoptosis is an increase in the generation of reactive oxygen species by mitochondria (39). Up-regulation of glutathione or a shift in relative glutathione concentration within mitochondria could prevent cellular damage caused by oxygen free radicals through direct detoxification.

Bcl-2 is an apoptotic inhibitor that has been localized to the outer mitochondrial membrane, as well as other intracellular membranes (40). Bcl-2 overexpression has been shown to increase cisplatin resistance in a number of experimental models (12–16). Bcl-2 overexpression in the mitochondrial outer membrane has been shown to inhibit the characteristic increase in reactive oxygen species in cells exposed to a number of apoptotic triggers (39, 41). Bcl-2-deficient mice have several phenotypic alterations associated with chronic oxidative stress, suggesting that one function of Bcl-2 is the regulation of antioxidant pathways (39, 42).

Here we present data demonstrating that Bcl-2-mediated cisplatin resistance is associated with up-regulation of glutathione concentration, that glutathione synthesis is required for Bcl-2-mediated cisplatin resistance, and that the increased glutathione concentration in Bcl-2-overexpressing cells contributes to resistance by a mechanism independent of DNA platination. These data support the hypothesis that glutathione provides resistance to cisplatin primarily through inhibition of apoptotic commitment rather than through direct inhibition of cisplatin-mediated DNA damage.

**MATERIALS AND METHODS**

**Cell Lines and Culture Conditions.** MCF-7 breast cancer cells were grown at 37°C, 5% CO₂, in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 1.5 mg/ml NaHCO₃, and 100 units/ml penicillin/streptomycin. MCF-7 cells were stably transfected by electroporation (300 V, 960 μF) with the plasmid pSFFV-Neo (MCF7.Neo) or pSFFV-Bcl-2, the same vector containing the full-length human BCL2 cDNA (MCF7.Bcl-2), and selected in 1 μg/ml G418. Multiple clones of both MCF7.Neo and MCF7.Bcl-2 were isolated by limiting dilution and screened by Western blot for Bcl-2 expression. FL5.12 cells were stably transfected with the same constructs and maintained as described previously (43). BSO was used when indicated, at 0.5 mM.

**Western Blotting.** Protein extracts were prepared in radioimmunoprecipitation assay buffer and quantitated using a modified Bradford assay (Bio-Rad). Total glutathione quantitation was performed by enzymatic assay (44).

**Glutathione Determination.** Glutathione concentrations were measured as described previously (37). Briefly, cells grown in T-75 flasks to 80% confluence were harvested using trypsin, washed in cold PBS, and resuspended in 0.154 m NaCl. Protein extracts were prepared by multiple freeze/thaw cycles and sonication and normalized for total protein content using the modified Bradford assay (Bio-Rad). Total glutathione quantitation was performed by enzymatic assay (44).

**Cytotoxicity Assays.** Relative cisplatin cytotoxicity of the various cell lines was evaluated using a tetrazolium XTT colorimetric assay. All assays were performed in triplicate, and all experiments were performed multiple times. Cells were plated in 96-well dishes at a concentration of 3000 cells/well in 50 μl of medium. After overnight incubation, 50 μl of medium containing 1 mM BSO were added. The following day, 100 μl of medium containing cisplatin were added to achieve concentrations ranging from 0 to 240 μM in a total volume of 200 μl. After continued incubation for 2 or 4 days, 50 μl of medium containing 1 mg/ml XTT and 7.6 μM phenazine methosulfate were added to each well. After incubation for 1–4 h, optical absorbance at 450 nm was measured in a multplate reader. Absorbance measurements were expressed as a percentage relative to an untreated control.

**Platination Assays.** Quantitative assessment of cisplatin-DNA adduct formation was based on atomic absorption spectroscopy. Exponentially growing cells on triplicate dishes were exposed to 200 μM cisplatin for 1 h at 37°C. Cells were then harvested using trypsin and washed twice with ice-cold PBS. Cell pellets were incubated for 5 h at 37°C in lysis buffer [10 mM Tris-HCl (pH 8.0), 0.1 mM NaCl, 0.1 mM EDTA, 0.5% SDS, and 20 μg/ml RNase] and then incubated overnight at 50°C after the addition of 100 μg/ml proteinase K. Total genomic DNA was isolated by phenol/chloroform extraction and ethanol precipitation; resuspended in 500 μl of 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA; and quantitated by absorbance at A260 nm. Platinum concentration was assayed with a Perkin-Elmer model 1100 flameless atomic absorption spectrometer (Perkin-Elmer, Norwalk, CT) monitoring 265.9 nm. The temperature program used was as follows: ramp over 30 s to 90°C and hold for 30 s; ramp over 10 s to 110°C and hold for 10 s; ramp over 30 s to 300°C and hold for 30 s; ramp over 45 s to 1500°C and hold for 60 s; and atomize at 2700°C with no ramping. Argon gas flow was 800 ml/min during all heating steps except atomization, when it was interrupted. Platinum concentrations were determined by comparison with a standard curve performed on the same day as the assay (45). Standard curve concentrations were 4.9, 24.5, and 68.6 ng/ml. Quality control samples of 12.2 and 49 ng/ml were assayed in duplicate with each run.

**RNase Protection Assays.** Cell lines to be analyzed were grown to 80% confluence. Total cellular RNA was extracted using the Trizol reagent (Life Technologies, Inc., Gaithersburg, MD). RNA integrity was confirmed by agarose gel electrophoresis, and RNA yield was quantified spectrophotometrically. The 5'-GCS cDNA was provided by Dr. Andrew Godwin (Fox Chase Cancer Center, Philadelphia, PA), and the GS cDNA was provided by Dr. Philip Board (John Curtin School of Medical Research, Canberra, Australia). The G-CSF probe cloned in pSP72 was linearized with SfI, yielding a 312 bp antisense template and a 286-bp protected fragment, and the GS probe in pGEM7Zf was linearized with PstI, yielding a 238-bp antisense template and a 212-bp protected fragment. A γ-actin probe was included in all assays as an internal loading control. 32P-labeled riboprobes were generated for each template according to the manufacturer’s instructions (Promega, Madison, WI). After DNase treatment, the riboprobes were phenol extracted and ethanol precipitated. Labeled riboprobes were hybridized with either total RNA (30 μg) from samples or mRNA control overnight at 50°C, digested with 10 mg/ml RNase A, ethanol precipitated, and electrophoresed on a 6% polyacrylamide gel. After drying, the gel was exposed for autoradiography. All assays were done in duplicate, and mRNA expression was quantitated using a digital phosphorimager (ChemiImager 5500; Alpha Innotech Corp.).

**Statistical Analysis.** To characterize cell survival after different doses of cisplatin, a quadratic regression model was used, beginning with the lowest non-zero dose and applying a logarithmic transformation to the dose [i.e., survival proportion = α + β log dose + β² log (log dose)²]. If the proportion of cells surviving at a given dose was zero, the data from all higher doses were dropped. With this adaptation, these models fit the data well, with R² ranging from 0.80 to 0.98. LD₅₀ and 95% CIs for the control lines and Bcl-2 transfected lines were constructed using an inverse prediction method (46).

To examine the difference in the extent of DNA repair after exposure to
cisplatin between the control cell lines and Bcl-2 cell lines, linear regression models were used with the concentration of platinum bound to DNA regressed on the elapsed time after exposure to cisplatin (i.e., concentration = α + β₁ * time + β₂ * BCL2 + β₃ * time * BCL2, where BCL2 is an indicator variable equal to 1 for Bcl-2 cell lines and 0 for the Neo cell lines). Once model fitting was completed, the statistical significance of β₂ and β₃ was determined.

**RESULTS**

**Glutathione Concentration Is Increased by Bcl-2 in MCF-7 Cells.** MCF-7 breast cancer cells were transfected with an expression construct driving the human Bcl-2 cDNA (pSFFVNeo-Bcl-2) or with an empty vector control (pSFFVNeo). Two clonal lines from each transfection were selected for further analysis based on relative Bcl-2 expression on intracellular glutathione content, total cellular glutathione concentration in the control and Bcl-2 transfectants was determined (Fig. 2). The Bcl-2-overexpressing lines were found to have glutathione concentrations averaging 117 nmol/mg protein (SD = 39 nmol/mg protein). In contrast, control lines were found to have less than half the glutathione content of Bcl-2 stable transfectants, averaging 50 nmol/mg protein (SD = 16 nmol/mg protein).

**Bcl-2-mediated Protection from Cisplatin Toxicity Is Dependent on Glutathione.** To evaluate the capacity of Bcl-2 to inhibit cytotoxicity in response to cisplatin, this panel of clones was incubated in the presence of variable concentrations of cisplatin, and viability was monitored by XTT assay on day 4 after cisplatin exposure (Fig. 3A). Bcl-2 was found to markedly increase cisplatin resistance in MCF-7 cells. LD₅₀ for the control cells was 16.7 μM (95% CI, 14.6–19.3) compared with 56.8 μM (95% CI, 43.9–70.3) for the Bcl-2 overexpressing lines. These results were consistent with observations in other cell types that Bcl-2 overexpression can increase chemotherapeutic resistance.

To evaluate whether the relative increase in survival in Bcl-2 transfectants after cisplatin exposure was in part dependent on the up-regulated glutathione concentrations in these cells, XTT assays were performed in parallel in the presence of BSO. BSO depleted glutathione stores in both the Neo transfectants and Bcl-2 transfectants to a similar basal level (Fig. 2) but did not affect cell viability in the absence of cisplatin. Glutathione depletion reversed the cisplatin resistance of both Bcl-2 transfectants; the cisplatin cytotoxicity profiles were similar in all four cell lines after preincubation with 0.5 mM BSO (Fig. 3B), with no statistically significant difference in LD₅₀ [13.2 μM (95% CI, 10.2–17.2) for the control cells and 18.9 μM (95% CI, 15.0–22.8) for the Bcl-2-overexpressing lines].

These results suggested that Bcl-2 overexpression in MCF-7 resulted in increased resistance to cisplatin-mediated death and that this increased resistance was dependent on cellular glutathione production. An alternative explanation would be that BSO exposure had resulted...
in suppression of Bcl-2 expression in the Bcl-2 transfectants. However, immunoblotting in the presence or absence of BSO revealed no significant decrease in Bcl-2 expression in either of the cell lines (Fig. 1, compare Lanes 2–5 with Lanes 6–9). The ratio of Bcl-2 expression level in the presence of BSO versus that in the absence of BSO was estimated to be 0.97 ± 0.24 for Bcl-2-1 and 1.07 ± 0.03 for Bcl-2-2.

It was possible that the dependence on glutathione for Bcl-2-mediated cisplatin resistance was unique to MCF-7 cells. To evaluate this possibility, we analyzed cisplatin sensitivity in FL5.12 cells, a lymphoid precursor line, stably transfected with either control plasmid (FL5.Neo) or the same vector containing the Bcl-2 gene (FL5.Bcl-2). Cells were treated with varying concentrations of cisplatin in the presence or absence of 0.5 mM BSO, and viability was determined by propidium iodide exclusion at 48 h. Treatment with BSO was not toxic to cells in the absence of cisplatin. At low cisplatin concentrations, Bcl-2 was able to maintain partially increased resistance to cisplatin-mediated cell death even in the presence of BSO. However, exposure to 64 μM cisplatin demonstrated an effect consistent with that seen in MCF-7 cells: preincubation with BSO suppressed the ability of Bcl-2 to inhibit cisplatin toxicity (Fig. 4). These results indicate that maintenance of glutathione in both FL5.12 and MCF-7 cells contributes to the ability of Bcl-2 to inhibit cisplatin-mediated cell death.

Cisplatin Resistance in Bcl-2 Transfectants Is Not Associated with Inhibition of DNA Platination. Glutathione depletion in MCF-7 Bcl-2 transfectants led to loss of the increased apoptotic resistance despite continued overexpression of Bcl-2. This observation suggests that the increase in glutathione concentration conferred by Bcl-2 overexpression contributes to the cisplatin resistance of these cells. The increased glutathione concentration could inhibit cell death through effects on mitochondrial integrity and function, for example by suppressing the damaging effects of mitochondrial reactive oxygen species. Alternatively, a doubling of the cellular glutathione stores could enhance resistance by detoxifying cisplatin within the cell through the activity of glutathione S-transferase. If the latter hypothesis were correct, this would be reflected in a decrease in the extent of cisplatin-DNA adduct formation. Quantitative analyses of DNA platination were performed using atomic absorption spectroscopy after a 1-h incubation with cisplatin (Fig. 5). Despite the presence of elevated glutathione levels in the Bcl-2-transfected lines, there was no significant difference in the extent of platination between the Bcl-2 transfectant and control transfectant cell lines (P = 0.412).

Another possible contribution of glutathione to survival after cisplatin exposure is enhanced DNA damage repair through facilitation of platinum adduct removal from genomic DNA. To evaluate this possibility, cells were incubated in the presence of cisplatin for 1 h and then washed free of cisplatin. The rate of platinum adduct removal over 24 h was monitored by atomic absorption spectroscopy (Fig. 6). Using a linear model of the concentration of platinum bound to DNA, the removal rate of platinum-DNA adducts in MCF-7 cells was estimated to be 3.70 fmol/μg/h. There was no significant difference in the removal rate between Bcl-2 transfectants and control transfectants (P = 0.861).

Glutathione Up-Regulation in Bcl-2-overexpressing Cells Is Not Dependent on Up-Regulated Expression of Genes Encoding Glutathione Synthetic Enzymes. Glutathione up-regulation has been reported in multiple cell lines after Bcl-2 transfection, consistent with the effects reported here in MCF-7 cells (41, 47, 48). This up-regulation could be due to overexpression of key biosynthetic enzymes, increased activity of these enzymes, increased intracellular...
supply of glutathione precursors, or inhibition of glutathione degradation. Glutathione synthesis is dependent on two enzymes, γ-GCS and GS (30). In addition, extracellular glutathione can be partially metabolized for import and intracellular regeneration through a pathway dependent on the enzyme γ-GT (30). To begin to evaluate this mechanism for the up-regulation of glutathione in Bcl-2-overexpressing cells, expression of the genes encoding γ-GCS, GS, and γ-GT was quantitated by RNase A protection of total cellular RNA derived from each of the MCF-7 transfectant lines. No consistent differences between Neo and Bcl-2 transfected lines were noted in expression of any of these regulators of glutathione biosynthesis (Table 1, Fig. 7A). The γ-GCS enzyme controls a reaction that is typically rate-limiting for glutathione synthesis. Expression of this factor was further investigated by Western blotting of protein extracts from Neo- and Bcl-2-transfected MCF-7 cells. Consistent with the RNase protection data, γ-GCS protein levels were not elevated in the Bcl-2 transfectants relative to controls (Fig. 7B).

DISCUSSION

Introduction of Bcl-2 into MCF-7 cells is associated with up-regulated intracellular content of glutathione, a key regulator of cellular redox balance, and with increased resistance to cell death after cisplatin exposure. The increased cisplatin resistance conferred by Bcl-2 in these cells is dependent upon maintenance of cellular glutathione. The mechanism by which increased glutathione production in Bcl-2-transfected cells contributes to apoptotic resistance after cisplatin exposure cannot be explained by effects on either DNA adduct formation or DNA damage repair. Taken together, these observations suggest that the cellular glutathione level is regulated in part by Bcl-2 and that glutathione contributes to apoptotic resistance through maintenance of mitochondrial or cellular homeostasis.

Increased production of reactive oxygen species is observed in cells exposed to a diverse array of apoptotic triggers and precedes cellular commitment to programmed cell death. Bcl-2 expression in the outer mitochondrial membrane can decrease the concentration of reactive oxygen species in mitochondria after an apoptotic trigger and facilitates continued mitochondrial bioenergetic function (39, 49). One mechanism by which Bcl-2 may promote these effects is through up-regulation of glutathione, leading to rapid detoxification of reactive oxygen species and inhibition of free radical-mediated mitochondrial damage.

Several studies have implicated Bcl-2 in regulating antioxidant pathways. Initial defects noted in mice lacking Bcl-2 included the diencephalic damage.

Table 1 - Quantitative analysis of gene expression in MCF-7 lines by RNase protection

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Cell line</th>
<th>Expression level normalized to γ-actin</th>
<th>Average</th>
<th>SD</th>
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<tr>
<td>γ-GCS</td>
<td>Neo-1</td>
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<td>1.09</td>
<td>0.15</td>
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<td></td>
<td>Neo-2</td>
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<td></td>
<td>Bcl-2-1</td>
<td>1.07</td>
<td>1.04</td>
<td>0.04</td>
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<tr>
<td></td>
<td>Bcl-2-2</td>
<td>1.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GS</td>
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<td>0.50</td>
<td>0.23</td>
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<tr>
<td></td>
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<td></td>
<td>Bcl-2-2</td>
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Fig. 7. Expression of γ-GCS is not altered by Bcl-2 transfection. A. RNase protection assay. 32P-labeled probes for γ-actin and γ-GCS were mixed and hybridized with either purified tRNA or total cellular RNA from MCF-7 transfectants and subjected to RNase A digestion, and products were separated by PAGE. Lanes from left to right include a 32P-labeled 100-nucleotide ladder (Marker), probe mix hybridized to tRNA only (tRNA), and probe mix hybridized to RNA from each of the indicated MCF-7 transfectant lines. The final three lanes demonstrate free probe for γ-actin (Lane 1), γ-GCS (Lane 2), and the combination of the two probes used for RNase A protection (Mix). Quantitative measurement of γ-GCS expression level relative to that of γ-actin for each cell line is presented in Table 1. B. Western blot. Total cellular protein extracts were prepared from each of the four MCF-7 stable transfectant lines and normalized for total protein content. Shown is a Western blot probed with an anti-γ-GCS antibody. Relative expression is 1.00 ± 0.06 in Neo clones versus 0.93 ± 0.02 in Bcl-2 clones.

to H2O2 after UV light exposure, further indicate the involvement of Bcl-2 as a regulator of intracellular redox potential (50, 51).

Mitochondria, as the sites of aerobic respiration, are the principal generators of reactive oxygen species in the cell. Mitochondria are dependent upon glutathione to detoxify reactive oxygen species, preventing oxidative damage (30, 32). Despite this dependence, mitochondria are unable to synthesize glutathione. Glutathione stores within mitochondria are derived from active transport across the mitochondrial membrane against an electrochemical gradient (52). Mitochondrial glutathione concentrations are regulated and have been implicated in apoptotic regulation (53, 54). At baseline, the concentration of glutathione in mitochondria has been found to be similar to that of the cytoplasm. However, in cells exposed to oxidative stress, the concentration of mitochondrial glutathione is maintained at the expense of a decreasing cytoplasmic pool (55, 56). Depletion of the mitochondrial (but not cytoplasmic) glutathione pool is associated with markedly increased sensitivity to antimycin A, which blocks oxidative phosphorylation in complex III of the electron transport chain, leading to generation of reactive oxygen species (57). These observations suggest that mitochondrial glutathione stores are highly regulated by the cell and may affect the cellular sensitivity to apoptotic stimuli.

One mechanism by which glutathione may protect cells against apoptotic cell death may be through the preservation of critical protein sulfhydryl groups in a reduced state, preventing formation of covalent disulfide bridges. This function has been mimicked through the use of monovalent and bivalent thiol-reactive agents (58, 59). Cross-linking of thiol groups by the bifunctional thiol-reactive agent diamine leads to alterations in mitochondrial membrane complexes associated with apoptotic initiation and triggers programmed cell death in intact cells (58). In contrast, monovalent thiol-reactive compounds (which impede disulfide bridge formation) inhibit apoptosis in response to
diamine, glucocorticoids, and γ-radiation. Use of monovalent thioredoxin-reactive agents targeted to the mitochondrial matrix demonstrates that this protection appears to be dependent on stabilization of thiol residues within the mitochondrial matrix or inner membrane. Consistent with the hypothesis that Bcl-2 may prevent cell death in part through modulation of antioxidant pathways, Bcl-2 cannot prevent cell death initiated by the bivalent thioredoxin-reactive agent diamine (59). These data demonstrate an association between Bcl-2 expression and antioxidant function. Studies of apoptotic initiation under hypoxic conditions, where generation of reactive oxygen species would be expected to be suppressed, have indicated that Bcl-2 can protect cells from some apoptotic stimuli independent of inhibition of reactive oxygen species production (60, 61). Recent observations have challenged this assumption, demonstrating that in multiple cell types, physiological hypoxia results in a paradoxical increase in the production of reactive oxygen species (62, 63). Bcl-2 may be able to affect the apoptotic threshold of cells by multiple mechanisms, only some of which are dependent on antioxidant function.

In addition to its expression on the mitochondrial outer membrane, Bcl-2 is expressed in the outer nuclear membrane and endoplasmic reticulum (40). Bcl-2 overexpression in lymphocytes has been associated with up-regulated glutathione concentration and with an increase in the ratio of nuclear to cytoplasmic glutathione (64). This suggests that Bcl-2 expression in critical intracellular membranes may regulate distribution of glutathione to organelles, affecting their local redox potential and antioxidant capacity. The regulation of glutathione production and intracellular distribution may be an important mechanism by which Bcl-2 family members determine apoptotic sensitivity of a particular intracellular compartment, and alterations in levels within either mitochondria or nuclei could prove to be relevant. It would be of interest to evaluate relative glutathione concentrations in the mitochondrial matrix of control and Bcl-2-overexpressing cells and compare the platinum adduct load of mitochondrial and nuclear DNA after cisplatin exposure in control and Bcl-2-overexpressing cells.

Finally, although glutathione is the most prevalent redox buffer within cells, other cellular buffers, including the flavoprotein thioredoxin, may also play critical roles. Thioredoxin expression is up-regulated in several human tumors and has been implicated in both cancer cell growth and apoptotic resistance (32, 65, 66). It has not been determined whether Bcl-2 or other apoptotic regulators can influence the levels of thioredoxin or whether such modulation may also contribute to chemotherapeutic resistance in human tumor cells. The data presented here suggest that further investigation of the ability of Bcl-2 family members to alter cellular redox balance as a mechanism of chemoresistance is warranted.

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REFERENCES


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