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Research paper

Bcl-2 expression correlates with apoptosis induction but not loss of clonogenic survival in small cell lung cancer cell lines treated with etoposide

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The influence of *bcl*-2 oncogene expression on etoposide-induced apoptosis and clonogenic survival was investigated in five small cell lung cancer (SCLC) cell lines, three of which were *bcl*-2-expressing and two of which were non-*bcl*-2-expressing. The *bcl*-2-expressing lines displayed a lower apoptosis propensity than the non-*bcl*-2-expressing lines. When *bcl*-2-expressing cells were incubated in cystine/methionine-free (CMF) medium, etoposide-induced apoptosis was restored to levels comparable to those seen in non-*bcl*-2-expressing lines. However, the endpoint of clonogenic survival after drug treatment did not display any consistent pattern that correlated with *bcl*-2 status. In addition, treatment of the two *bcl*-2-expressing cell lines with etoposide in CMF medium did not modify their clonogenic survival curves compared to treatment in regular medium. These results are consistent with the idea that *bcl*-2 expression modulates etoposide-induced apoptosis but not clonogenic survival.

Key words: Apoptosis, *bcl*-2, etoposide, small cell lung cancer, thiols.

Introduction

Etoposide (VP-16) is an important drug in the treatment of solid tumors, especially in small cell lung cancer (SCLC).[1] Although it is effective in the clinic, the presence of drug-resistant tumor cells often obviates cure of this disease.[2] It is now recognized that the failure of the drug-treated cells to undergo apoptosis is one possible mechanism to explain such resistance.[3] Apoptosis, a mode of cell death during normal development,[4] is also a major mechanism by which chemotherapeutic agents kill cells.[3,5] Understanding how apoptosis is regulated at the molecular and biochemical levels may provide insight into means for overcoming blocks to this mode of cell death and restore tumor cell chemosensitivity.

Several genes are known to be involved in the regulation of apoptotic pathways.[6] However, two appear to be especially important for apoptosis after radiation or chemotherapy: the p53 tumor suppressor gene and the *bcl*-2 oncogene (and its associated family members). *bcl*-2 protein is localized to the membrane components of mitochondria, the endoplasmic reticulum and the nuclear envelope.[7,8] Its overexpression blocks apoptosis caused by many different agents, including etoposide.[9,10] The possibility that *bcl*-2 is a drug-resistance protein is the subject of intensive research because abnormal expression of this oncoprotein has been detected in a variety of human cancers, including SCLC.[11,12]

How *bcl*-2 controls apoptosis is not understood but many theories have been proposed.[13] One is that *bcl*-2 may act as an antioxidant or stimulate other cellular antioxidants to block a putative redox-mediated step in the cascade of events required for apoptosis.[14] Other reports link cellular thiols, specifically glutathione (GSH), with the ability of *bcl*-2 to block apoptosis and provide evidence that the basal concentration of GSH is higher in *bcl*-2-expressing cell lines.[15,16] Recently, our laboratory has demonstrated that one of *bcl*-2's functions may involve a redistribution of GSH to the nucleus and that high nuclear GSH levels may block apoptosis.[17]

In this report, we studied the effects of *bcl*-2 overexpression on etoposide-induced apoptosis in SCLC cell lines. The *bcl*-2-expressing cell lines were more resistant to the apoptosis-inducing effects of etoposide than non-*bcl*-2-expressing cell lines. Moreover, we show that etoposide-induced apoptosis was restored after incubation in cystine/methionine-free

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(CMF) medium. However, expression of *bcl*-2 did not correlate with clonogenic survival of SCLC cells exposed to etoposide.

Materials and methods

Cell culture

Methods for the culture and characterization of the cell lines used were as previously described.[18] Five SCLC cell lines were used for this study: three classic SCLC (H510, H69 and H889) and two variant SCLC (H82 and N417) lines were obtained from the American Type Culture Collection (Rockville, MD). The lines were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 μg/ml penicillin and 10 μg/ml streptomycin (Gibco/BRL, Gaithersburg, MD).

Drug treatment

Etoposide (Bristol, Princeton, NJ) was freshly diluted into complete medium before each experiment. The cells were treated at 40 μM for 6 h in a 37°C incubator. The drug-containing medium was then removed, fresh complete medium was added to the cultures and they were returned to a 37°C incubator for the appropriate time.

Apoptosis assay

Apoptosis was detected using the Apoptosis Detection System (Promega, Madison, WI) which involves labeling the 3′ OH ends of fragmented DNA through the use of terminal deoxynucleotidyl transferase (TUNEL). Briefly, after etoposide treatment, cells were cytocentrifuged onto glass slides (500 r.p.m. for 5 min), air dried and fixed in 4% formalin for 10 min at room temperature. The cells were then stained following the procedure specified by the manufacturer and imaged using a fluorescent microscope. Apoptotic cells (TUNEL-positive cells) were expressed as a percentage of total cells (TUNEL-positive cells + TUNEL-negative cells).

Clonogenic survival

Clonogenic cell survival was assessed using a modification of the protocol by Dynlacht *et al.*[19] Cells at a concentration of 10^6/ml in 37°C medium were treated with etoposide at different concentrations (10, 20 and 40 μM). After 6 h, the drug-containing medium was removed and replaced with fresh medium. Serial dilutions of 10- and 100-fold were made, and 200 μl of each cell suspension was transferred into each of eight wells of the first row of a 96-well plate using a multichannel pipettor. After the suspension in the first row was mixed, 100 μl was transferred into the adjacent well, which had previously been filled with 100 μl of medium. This was repeated for every well, resulting in a serial dilution of cells by a factor of 2 for each set of wells. The plates were returned to a 37°C incubator and after 24 h an additional 100 μl of medium was added into each well. The plates were returned to the incubator and the cells were allowed to grow for 7 days.

Cell survival experiments in which the effects of CMF medium were tested were performed as above except that the cells were treated with etoposide in CMF medium. Experiments testing the effects of CMF medium after drug treatment were also performed as above except that each well of the 96-well plates was filled with CMF medium and after 24 h an additional 100 μl of medium containing twice the normal cystine and methionine concentrations was added to each well.

After 7 days, 25 μl of a 500 μl/ml solution of MTT (Sigma, St Louis, MO) was inoculated into each well of the 96-well plates and the plates were incubated overnight at 37°C. The next day, each plate was scored for wells with positive MTT staining. The relative clonogenic survival following a given etoposide treatment was determined by comparing the cell dilution at which one-half of the wells were positive with the dilution in the control plate at which one-half of the wells were positive.

GSH assay

The method that was used to determine the GSH levels in SCLC cell lines was developed by Hissin and Hilf.[20] This method involves the use of *o*-phthalaldehyde (OPT) as a fluorescent reagent for GSH. Briefly, cells were washed once in PBS and then resuspended at 3×10^6/ml in cold lysis buffer consisting of 5% trichloroacetic acid, 1 mM EDTA and 0.1 M HCl (1:1:1, v/v/v). Following lysis, non-soluble material was spun out at 2500*g* for 20 min at 4°C. A sample (0.2 ml) of the supernatant was mixed with 3.6 ml of 0.1 M phosphate/5 mM EDTA buffer (pH 8.0) and 0.2 ml of OPT stock (0.1 mg/ml in methanol) was added. Fluorescence was read at 420 nm with excitation set at 350 nm. A standard curve determined using

known quantitites of GSH was used to convert fluorescence readings to GSH concentrations.

Western blot analysis for *bcl*-2 expression

bcl-2 protein expression in the SCLC cell lines was quantified by Western blot analysis on total cell lysates made from 5×10^6 cells. Briefly, lysates were subjected to gel electrophoresis through a 12% polyacrylamide gel. Following transfer to a nitrocellulose membrane (Amersham, Amersham, UK) at 100 V for 1 h, the membrane was blocked in 5% dried milk in PBS-T (0.1% Tween-20 in PBS) for at least 15 min. The membrane was then probed with 1:1000 primary antibody (Dako, Carpinteria, CA) for 2 h at room temperature followed by appropriate secondary antibody conjugated to horseradish peroxidase at a concentration of 1:1000. Four 15 min washes were performed in 5% dried milk in PBS-T prior to incubation with the secondary antibody. Chemiluminescence was detected using Enhance (ECL; Amersham). Quantification was performed by scanning laser densitometry (Molecular Dynamics, Sunnyvale, CA).

Results

Our study involved comparing five different SCLC cell lines, three classic and two variant lines. Western blot analysis of cell lysates (Figure 1) demonstrated that three cell lines expressed *bcl*-2 (H510, H889 and H69) and two did not (H82 and N417). These results are in agreement with previous reports for H69, H82 and H510.[11] We compared etoposide-induced apoptosis in these lines. As can be seen (Figure 2), the *bcl*-2-expressing cells were more resistant to etoposide-induced apoptosis than the non-*bcl*-2-expressing cells.

Previous work from our laboratory using murine lymphoma cell lines demonstrated that *bcl*-2-expressing cells have higher levels of GSH than their non-*bcl*-2-expressing counterparts and that apoptosis propensity could be restored to *bcl*-2-expressing cells by lowering GSH levels by incubation in CMF medium.[16] To extend this analysis to human tumor cell systems, we examined the ability of CMF medium to restore etoposide-induced apoptosis in the SCLC cell lines. The results (Figure 3A) indicate that incubation in CMF medium sensitized the *bcl*-2-expressing cell lines to etoposide induced apoptosis, but a similar effect was not observed with the non-*bcl*-2-expressing cells (Figure 3B). Little additional sensitization was achieved by incubating in CMF medium longer than during the drug exposure (data not shown). Moreover, in separate experiments, we treated the cells with etoposide in regular medium followed by incubation in CMF medium after the drug exposure for up to 24 h. In those cases, we observed no sensitization of the *bcl*-2-expressing cells (data not shown).

Previous reports have indicated that *bcl*-2-expressing cells have higher intracellular levels of GSH.[15,16] Therefore, we measured the GSH levels in the SCLC cell lines. The results showed that the level of GSH was higher in *bcl*-2-expressing cell lines than in the non-*bcl*-2-expressing cell lines (Table 1). We also determined the GSH levels after incubation in CMF medium. These experiments revealed that GSH levels decreased with time in CMF medium for four

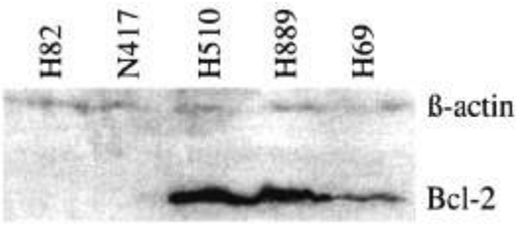

Figure 1. Expression of *bcl*-2 protein in SCLC cell lines as determined by Western blot analysis.

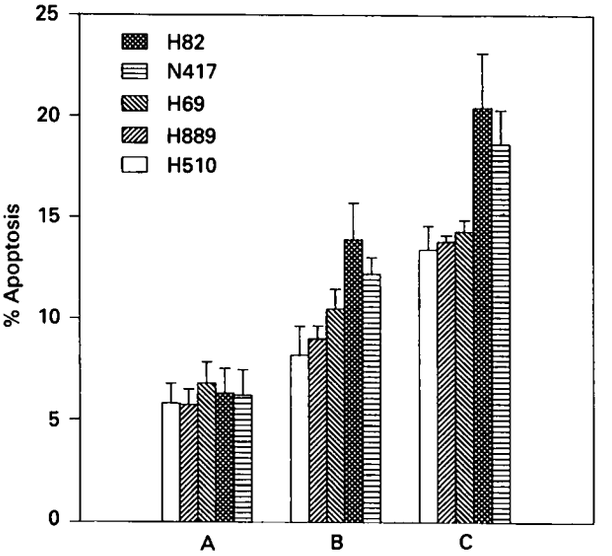

Figure 2. Quantification of apoptosis in SCLC cell lines exposed to etoposide. (A) Non-treated cells. (B) Cells treated with etoposide for 6 h, apoptosis assessed by TUNEL 18 h after drug treatment. (C) Cells treated with etoposide for 6 h, apoptosis assessed by TUNEL 24 h after treatment. Data presented are the means of four separate experiments. Error bars represent SDs.

cell lines (H510, H69, H82 and N417) but not for H889 (data not shown). In two of the *bcl*-2-expressing cell lines, H510 and H69, the levels decreased below the levels expressed by H82 and N417 growing in regular medium within 4 h of incubation in CMF medium and reached levels of 50% of control by 6 h (data not shown).

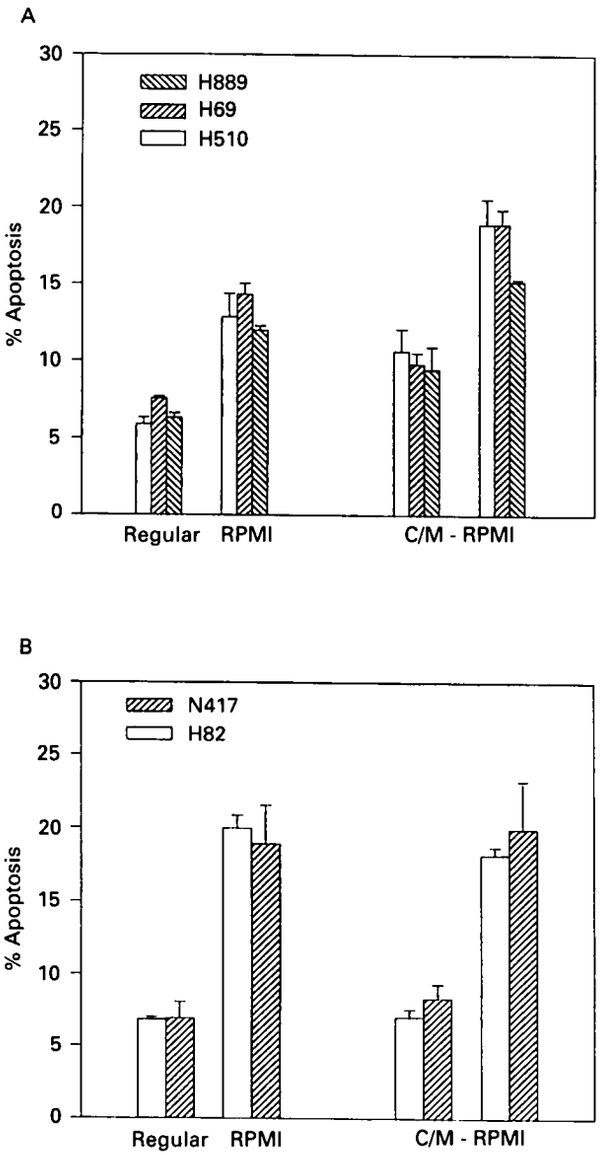

Figure 3. The influence of incubation in CMF medium on etoposide-induced apoptosis assessed by TUNEL. The cells were treated with etoposide for 6 h in regular RPMI medium or CMF medium. After treatment, cells were incubated for 24 h at 37°C in regular medium. (A) *bcl*-2-expressing cell lines (H510, H889, and H69). (B) Non-*bcl*-2-expressing cell lines (H82 and N417). For each treatment condition, the set of bars to the left represents the untreated controls and the set of bars to the right represents the etoposide-treated cells.

Whereas the results described above indicated that *bcl*-2 expression affected the apoptosis propensity of SCLC lines, it was also of interest to test whether *bcl*-2 could affect the clonogenic cell survival of these lines when treated with etoposide. All five SCLC lines were treated with graded concentrations of etoposide and plated for colony forming ability. The results are shown in Figure 4. Based on the apoptosis results, it was anticipated that the non-*bcl*-2-expressing lines would be more sensitive than the *bcl*-2-expressing lines; however, the survival curve analysis (Figure 4) indicated that only one of the non-*bcl*-2-expressing lines, H82, was more sensitive to etoposide. All of the other lines had similar sensitivity and were more resistant than H82. We also wanted to test if the increase in susceptibility to etoposide-induced apoptosis observed for *bcl*-2-expressing cells in CMF

Table 1.

Cell lines	GSH (nmol/10^6 cells)[a]
H82	6.4 ± 0.72[b]
N417	6.45 ± 0.58
H510	10.64 ± 0.4
H889	8.5 ± 0.94
H69	8.28 ± 0.51

[a]GSH was assayed using OPT.
[b]Data presented are the means and SDs of three separate experiments.

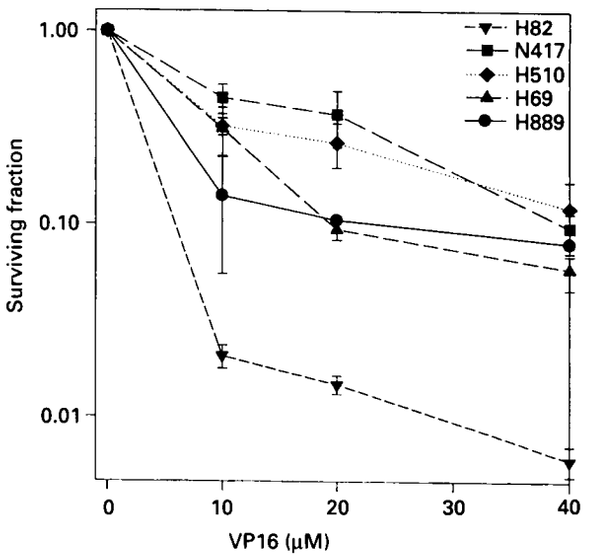

Figure 4. Clonogenic survival curves for SCLC cell lines. Cells were exposed to various concentrations of etoposide for 6 h. Error bars are shown where larger than the symbol plotted and depict SDs of three determinations.

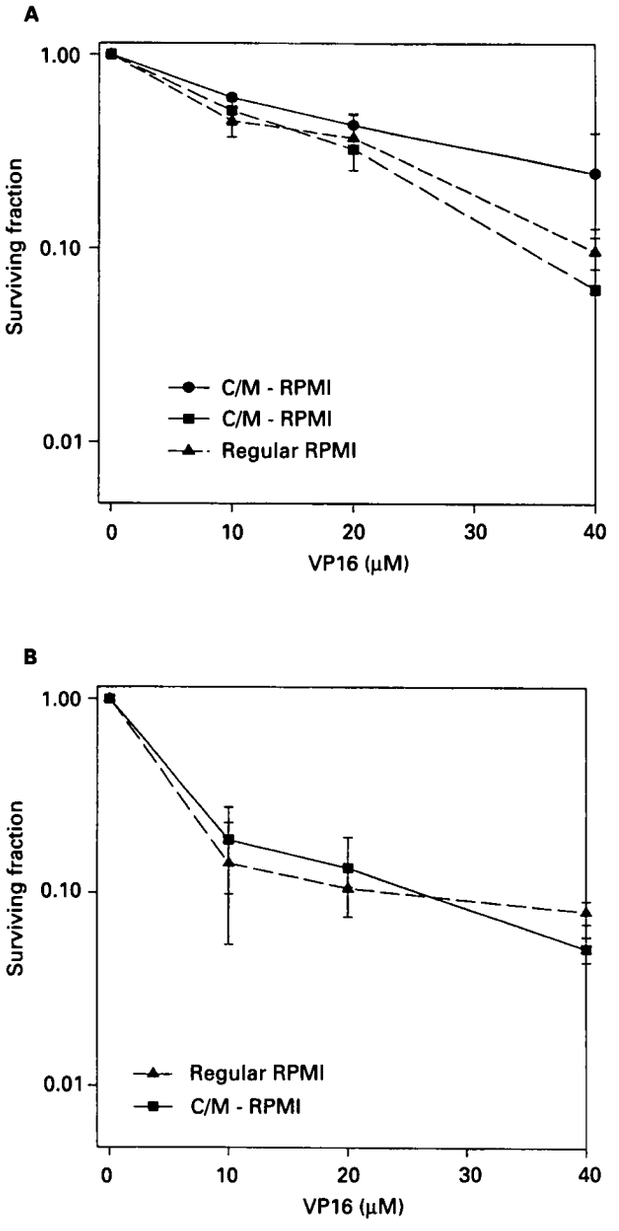

Figure 5. The influence of incubation in CMF medium on clonogenic survival for two *bcl*-2-expressing cell lines. (A) H510. Cells were incubated in CMF medium and etoposide for 6 h (black circles) or treated with etoposide for 6 h followed by incubation in CMF medium for 18 h (black squares). Cells in regular medium are represented by black triangles. (B) H889. Cells were incubated in CMF medium during the 6 h etoposide treatment and an additional 24 h after drug exposure (black squares). Cells in regular medium are represented by black triangles. Error bars are shown where larger than the symbol plotted and depict SDs of three determinations.

medium correlated with restored sensitivity as determined on the basis of a clonogenic assay. Therefore, H510 and H889 cells were treated with etoposide combined with CMF medium under different conditions (during drug treatment or after drug treatment). The survival curve analysis did not show any enhanced cytotoxic effect of etoposide when combined with CMF medium (Figure 5).

Discussion

This study has demonstrated that overexpression of *bcl*-2 among SCLC cell lines suppresses apoptosis but has no consistent effect on loss of clonogenic survival following treatment with etoposide. The results demonstrating the ability of *bcl*-2 expression to suppress etoposide-induced apoptosis agree with several similar reports in the literature.[21-23] However, as has been aptly pointed out,[24] most studies on apoptosis do not include assessment of clonogenic survival, an omission that has impeded understanding of the relationships between various markers for cell death and the cytotoxicity of anticancer drugs. Our finding that apoptosis does not correlate with clonogenic cell death is, however, similar to two other reports concerning *bcl*-2 expression in human tumor cells of epithelial origin.[24,25]

In the first of these reports, Yin and Schimke[24] examined the response of HeLa cells, in which *bcl*-2 expression was under the control of a tetracycline-repressible promoter, to aphidicolin. Aphidicolin induced apoptosis that was blocked when *bcl*-2 expression was allowed, but *bcl*-2 expression did not enhance clonogenic survival following aphidicolin treatment. They concluded that, although *bcl*-2 was able to block or delay the appearance of apoptotic features, aphidicolin-treated cells were committed to apoptosis upstream of *bcl*-2 activity. In the second study, Lock and Stribinskiene[25] tested HeLa cells transfected with *bcl*-2 expression vectors for etoposide-induced apoptosis. *bcl*-2 inhibited the appearance of cells with apoptotic features following exposure to etoposide but did not influence clonogenic survival. They concluded that, even though *bcl*-2 blocked or delayed cell death by apoptosis, the etoposide-treated cells were destined to become incapable of further cell divisions due to other modes of cell death produced by etoposide, namely mitotic or reproductive cell death. Although our data presented here are consistent with both of these theories, we favor the latter because we have previously hypothesized a similar theory to explain the failure of human cells derived from solid

tumors to undergo apoptosis following exposure to another DNA-damaging agent, ionizing radiation.[26]

On the other hand, the relationship between apoptosis and loss of clonogenic survival appears to be intact in cells of lymphoid origin. Numerous studies have demonstrated that *bcl*-2-expressing lymphoid cells are resistant to etoposide, radiation and other agents when assayed for apoptosis, and that this resistance translates to enhanced clonogenic survival.[16,23,27,28] Thus, apoptosis may be the primary mode of cell death contributing to loss of clonogenic survival in lymphoid cells, whereas in non-lymphoid cells the primary mode responsible for loss of clonogenic survival may be mitotic death.[26] Based on this theory, suppressing apoptosis in non-lymphoid cells through *bcl*-2 expression would not necessarily lead to enhanced clonogenic survival.

A previous study from our laboratory showed that overexpression of *bcl*-2 inhibits radiation-induced apoptosis in murine lymphoma cells and that depleting intracellular thiols, specifically GSH, through the use of CMF medium could reverse this resistance.[16] In the present study, we attempted to sensitize *bcl*-2-expressing SCLC cells to etoposide using an identical strategy. Although, we successfully restored etoposide-induced apoptosis in the *bcl*-2-expressing lines to levels similar to those achieved in the non-*bcl*-2-expressing lines, the CMF medium did not enhance loss of clonogenic survival. This inability is consistent with the theory presented above regarding differences in the role of apoptosis in lymphoid and non-lymphoid cells. However, the enhancement of apoptosis in these cells by CMF medium confirms our earlier conclusions regarding the relationship between *bcl*-2 expression and cellular thiols,[16] including additional endpoints besides apoptosis; the data in Table 1 confirm the observations reported by us[16] and others[15] that *bcl*-2-expressing cells have higher intracellular levels of GSH.

Moreover, in a recent report, we have demonstrated in several different types of cells that *bcl*-2-expression causes a redistribution of GSH to the nucleus where it apparently blocks caspase activity associated with apoptosis.[17] Preliminary assessment suggests a similar phenomenon occurs in these *bcl*-2-expressing SCLC cell lines (unpublished results). Therefore, in spite of its inability to modify clonogenic survival in some cells, *bcl*-2 still exerts its biochemical functions in those situations. *bcl*-2 may, through this biochemical activity, modify other cellular functions, e.g. growth rate,[29,30] that are important in tumor response. Further, in analogy to two recent studies,[31,32] *bcl*-2's ability to suppress apoptosis may affect tumor response *in vivo* even in the absence of an effect on clonogenic survival determined on the same tumor cells *in vitro*. These reports support continued efforts to understand the biochemical mechanism responsible for the inhibition of apoptosis by *bcl*-2 and the further development of strategies for reversing this resistance to enhance tumor response to drug therapies.

Acknowledgments

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