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ORIGINAL ARTICLE

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Expression of Bcl-2 in human epithelial tumor (HeLa) cells enhances clonogenic survival following exposure to 5-fluoro-2′-deoxyuridine or staurosporine, but not following exposure to etoposide or doxorubicin

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Abstract A reduced capacity for apoptosis induction is considered to play a significant role both in the development of malignancy and in tumor cell resistance to chemotherapeutic drugs. The Bcl-2 oncoprotein inhibits apoptosis induced by antitumor agents at a point downstream of drug-target interactions. Stable expression of Bcl-2 in the human epithelial tumor (HeLa) cell line results in inhibition of apoptosis following exposure to the topoisomerase II poison, etoposide. However, Bcl-2 is unable to enhance clonogenic survival as a result of alternate pathways to reproductive death induced by the drug. *Purpose*: The purpose of this study was to further investigate the role of Bcl-2 in human epithelial tumor cell drug resistance using 5-fluoro-2′-deoxyuridine, staurosporine, and doxorubicin, in addition to etoposide. *Methods*: The ability of Bcl-2 to enhance clonogenic cell survival was studied by colony-forming assays, while delay of cell death induction was assessed by trypan blue viability measurements. The proportion of apoptotic cells was measured by morphological criteria, as well as detection of apoptotic DNA fragmentation using the terminal deoxynucleotidyl transferase assay. *Results*: Despite profound inhibition to loss of plasma membrane integrity for all agents tested, Bcl-2 was only able to significantly increase clonogenic survival following exposure to 5-fluoro-2′-deoxyuridine and staurosporine, but not following exposure to etoposide or doxorubicin. Furthermore, the time-course of apoptosis induction following exposure of HeLa cells to equitoxic concentrations of staurosporine and etoposide was profoundly different. *Conclusions*: These results indicate that Bcl-2 enhances clonogenic survival of human epithelial tumor cells in an agent-specific fashion, which may be determined by the initial cytotoxic lesion induced by a particular drug.

Key words Bcl-2 · Apoptosis · Drug resistance · Clonogenic survival · Human tumor cells

Abbreviations *FUdR* 5-fluoro-2′-deoxyuridine · IC_{50} concentration of drug resulting in 50% cytotoxicity by colony-forming assay compared to solvent-treated controls · *Tdt* terminal deoxynucleotidyl transferase · *WT* wild-type

Introduction

The *bcl-2* oncogene, originally identified at the t(14:18) chromosomal translocation found in the majority of human follicular lymphomas [25], is the founder member of a multigene family whose products interact to control the cellular commitment to programmed cell death, apoptosis [26]. Various human "solid" tumors also express unusually high levels of Bcl-2, which is not associated with the t(14:18) translocation [20, 22, 26]. Overexpression of the Bcl-2 protein has been shown to inhibit apoptosis induced by almost all of the chemotherapeutic agents in current use, through events which occur downstream of the initial cytotoxic lesion [6, 20, 26]. The limited number of correlative studies between Bcl-2 expression and clinical prognosis suggest an overall trend towards Bcl-2 conferring a poor outcome [20, 26]. Certainly this appears to be true for lymphoma [20]. However, an increasing number of reports indicate that in human epithelial tumors, particularly those of the breast and lung, Bcl-2 overexpression is associated with smaller, well-differentiated tumors which exhibit more favorable responses to hormonal and cytotoxic therapy [7, 14, 19, 22].

In a previous report, we described the development of a model system to study the sole contribution of Bcl-2 to drug resistance of human epithelial tumor (HeLa) cells, which express a dysfunctional p53 [15]. Despite the

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demonstration that etoposide-induced apoptosis is inhibited in these Bcl-2-expressing cells, to an extent which is consistent with other reports in the literature, enhanced clonogenic survival was not observed [15]. This study has now been extended to include other agents with differing mechanisms of cytotoxicity: doxorubicin, FUdR and staurosporine. In a similar manner to the action of etoposide, the stabilization of covalent (''cleavable'') complexes between the nuclear enzyme DNA topoisomerase II and DNA appears to be the initial cytotoxic lesion induced by doxorubicin [3]. FUdR inhibits thymidylate synthase, reduces the size of intracellular nucleotide pools and induces ''thymidylate stress'' [4]. The result of the thymidylate stress is the introduction of strand breaks into nascent DNA, possibly because of misincorporation of deoxyuridine monophosphate, with ensuing cell death via apoptosis [6]. Staurosporine is a broad-range protein kinase inhibitor which appears to induce apoptosis as its primary mechanism of cytotoxicity [12]. Also, at nontoxic concentrations, staurosporine potentiates antitumor drug action, possibly through unscheduled activation of cell-cycle control proteins [17, 18]. All of these agents have been shown to induce apoptosis in human tumor cells [10]. Our data indicate that, while Bcl-2 inhibits apoptosis induced by all four agents, it is only able to enhance clonogenic survival in a drug-specific fashion. Therefore, the perception of Bcl-2 as a multidrug resistance protein in human epithelial tumor cells should take these observations into account.

Materials and methods

Reagents and cell lines

The sources of all reagents have been described in previous reports [15–17], except for FUdR (Sigma Chemical Co., St. Louis, Mo.), and doxorubicin (Adriamycin) which was obtained from the J. Graham Brown Cancer Center Pharmacy as a clinical preparation. Cell culture conditions and derivation of the stable Bcl-2-expressing HeLa clones, along with a control vector-transfected clone, have also been described previously [15].

Drug treatments and cytotoxicity assays

Etoposide was prepared as a 100 m*M* stock in DMSO and stored at −20 °C. Immediately prior to treating cells, the etoposide was diluted to 10 m*M* in a final concentration of 30% DMSO. FUdR was dissolved in 0.9% saline immediately prior to treatment of cells. Staurosporine was dissolved in methanol as a 2 m*M* stock and stored at −20 °C. Immediately prior to cell treatment, the staurosporine was diluted in methanol if necessary. Doxorubicin was reconstituted in sterile distilled water to give a stock solution of 5 m*M* which was stored in aliquots at −20 °C. Subsequent dilutions were in distilled water. During experiments, control cultures received equivalent solvent treatment.

For colony-forming assays, between 700 and 70 000 cells were seeded into 10-cm diameter tissue culture plates and allowed to adhere overnight. Cells were then exposed to various concentrations of each drug for 24 h, washed twice with sterile phosphate-buffered saline (calcium and magnesium free) at 37 °C, and incubated in drug-free medium for 10–14 days. Colonies were stained with 2% crystal violet in methanol. Survival was expressed as a percentage of the colony-forming efficiency of solvent-treated controls. IC_{50} concentrations were read from each survival curve.

Cell viability assays, cytological techniques, and biochemical quantification of apoptosis

The methodologies for determination of cell viability by trypan blue exclusion, preparation and Wright/Giemsa staining of cell cytospins, and determination of the proportions of morphologically apoptotic or multinucleated cells have been described in detail in our previous reports [15, 16]. The Tdt assay [8] was also used to confirm the proportion of apoptotic cells, and generally differed by less than 10% from morphological estimations.

Statistics

Unless stated otherwise, numerical data presented are the means of at least two experiments. Standard errors, unless shown, were less than 10% of the respective data point. Student's *t*-test was used to evaluate whether differences between data sets were statistically significant. Only *P*-values < 0.05 were considered significant.

Results

Cell Lines

HeLa transfectants have been established which constitutively overexpress Bcl-2 [15]. Testing two of these clones (B-5 and B-6) has revealed a significant delay in both the loss of viability and the onset of apoptosis following a 4-h etoposide exposure compared to WT and a control vector-transfected clone (S-1) [15]. Notably, these characteristics are insufficient to confer a survival advantage when tested using clonogenic assays, probably owing to the ability of etoposide to cause clonogenic death through gross chromosomal damage in the absence of apoptosis [16]. Because of these somewhat surprising results, we hypothesized that Bcl-2 may only be able to enhance clonogenic survival in an agent-specific fashion. The results of additional experiments to investigate cross-resistance patterns conferred by Bcl-2 are described below.

Bcl-2 enhances clonogenic survival in an agent-specific fashion

The survival of HeLa S-1 and B-5 clones was tested by clonogenic assay following 24-h exposures to the thymidylate synthase inhibitor, FUdR, the protein kinase inhibitor, staurosporine, and the topoisomerase II poisons, doxorubicin and etoposide. The data presented in Fig. 1 clearly demonstrate that Bcl-2 expression caused enhanced clonogenic survival following exposure to FUdR or staurosporine, but not following exposure to doxorubicin or etoposide. For FUdR, the increase in survival of B-5 cells reached statistical significance at concentrations of 500 n*M* and 1 μ*M* (Fig. 1A). No colonies were observed to grow when S-1 cells were exposed to 2.5 μ*M* FUdR over four separate experiments, even when 70 000 cells were seeded per plate.

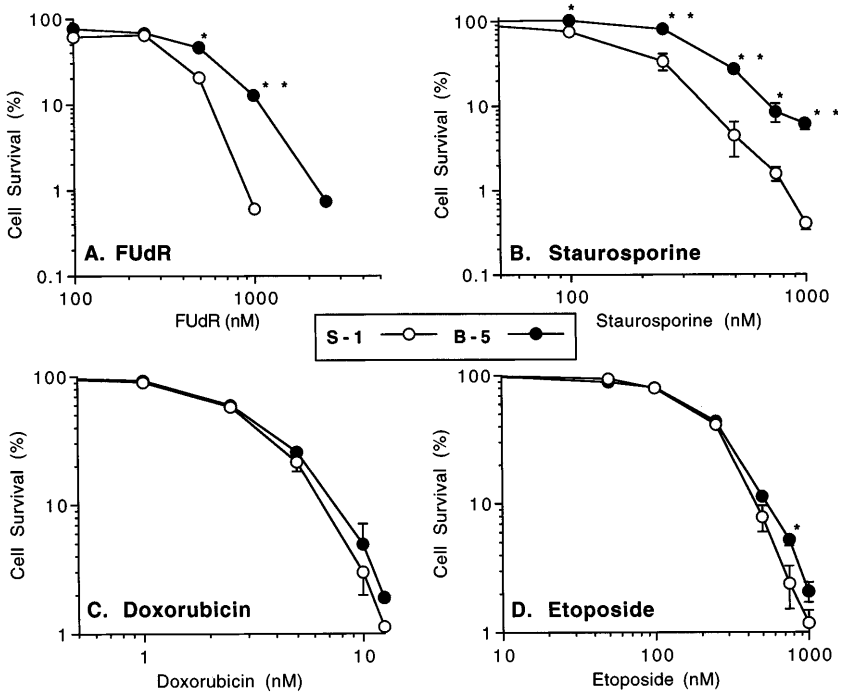

Fig. 1A–D Survival of HeLa S-1 (*open circles*) and B-5 (*filled circles*) cells following exposure to FUdR (**A**), staurosporine (**B**), doxorubicin (**C**) and etoposide (**D**). Survival was assessed by colony-forming assays following 24-h exposures to each drug. Each data point represents the mean ± SE of three or four separate experiments. SEs are shown where they are >10% of the respective survival value (*$P < 0.05$, **$P < 0.01$, S-1 vs B-5 cells)

Consequently, B-5 cells were calculated to exhibit at least a 135-fold increase in survival at this drug concentration.

The increase in B-5 cell survival compared to S-1 cells was significant at all staurosporine concentrations tested (Fig. 1B). At the three highest drug concentrations (500 n*M*, 750 n*M* and 1 μ*M*), the survival of B-5 cells ranged between 5- and 15-fold higher than that of S-1 cells. The results with FUdR and staurosporine contrasted with those obtained with doxorubicin and etoposide. The differences in survival between S-1 and B-5 cells did not reach statistical significance at any of the doxorubicin concentrations tested (Fig. 1C). The marginal increase in B-5 cell survival at doxorubicin concentrations of 5, 10 and 12.5 n*M* did not exceed 1.7-fold that of S-1 cells. Similarly for etoposide, a statistically significant increase in B-5 cell survival was not observed at five out of six drug concentrations tested. The increase in B-5 cell survival at 750 n*M* etoposide was 2.2-fold that of S-1 cells. At all other etoposide concentrations tested, the survival of B-5 cells did not exceed 1.7-fold that of S-1 cells. Therefore, while B-5 cells exhibited a slight tendency for higher survival than S-1 cells following exposure to doxorubicin or etoposide, the differences were not statistically significant.

Table 1 compares IC_{50} values read from individual survival curves of HeLa WT and transfected clones exposed to the four drugs. There were no statistically significant differences in IC_{50} values between all clones following exposure to doxorubicin or etoposide, as

Table 1 IC_{50} values (n*M*) of HeLa clones exposed to FUdR, staurosporine, doxorubicin or etoposide. Values are means ± SE. Survival was estimated by colony-forming assay after 24-h drug exposures (*Fold S-1* ratio of IC_{50} values, *nd* not determined). *P*-values are by Student's *t*-test

Drug	WT	S-1	B-5	B-6
Doxorubicin ($n=3$)	3.13 ± 0.21	2.85 ± 0.18	3.00 ± 0.13	2.33 ± 0.44
Fold S-1	1.1	1.0	1.1	0.82
P-value vs WT	–	0.64	0.63	0.18
P-value vs S-1	0.64	–	0.54	0.36
Etoposide ($n=4$)	189 ± 21	198 ± 21	214 ± 17	169 ± 16
Fold S-1	0.95	1.0	1.1	0.85
P-value vs WT	–	0.76	0.61	0.52
P-value vs S-1	0.76	–	0.57	0.30
FudR ($n=3$)	181[a]	204 ± 43	409 ± 58	404[a]
Fold S-1	nd	1.0	2.0	nd
P-value vs S-1	nd	–	0.036	nd
Staurosporine ($n=4$)	194 ± 22	184 ± 23	368 ± 21	355 ± 32
Fold S-1	1.1	1.0	2.0	1.9
P-value vs WT	–	0.76	<0.001	0.005
P-value vs S-1	0.76	–	<0.001	0.004

[a] Results of a single experiment

anticipated from the composite survival curves shown in Fig. 1. In contrast, the IC_{50} values for B-5 and B-6 cells exposed to FUdR or staurosporine were increased approximately twofold compared with WT and S-1 cells (Table 1). These differences in IC_{50} values reached a high degree of statistical significance. Moreover, these data demonstrate that a twofold difference in IC_{50} can translate into a >15-fold increase in cell survival at a single drug concentration (e.g. compare FUdR or staurosporine IC_{50} values between S-1 and B-5 cells in Table 1 and cell survival at 1 μ*M* of each agent in Fig. 1A,B).

Bcl-2 delays drug-induced loss of cell viability

Owing to the ability of Bcl-2 to enhance clonogenic survival in an agent-specific fashion in our HeLa clones, experiments were performed to determine the effects of Bcl-2 on loss of plasma membrane integrity. This technique has become the generally accepted method to estimate the effects of Bcl-2 on resistance to chemotherapeutic drugs [6, 20, 26]. In this set of experiments, S-1 cells were again compared to B-5 cells following a 24-h exposure to a single concentration of FUdR, staurosporine, doxorubicin or etoposide. Attempts were made to use a concentration of each drug that was relevant to the clonogenic data represented in Fig. 1, rather than supralethal drug concentrations. Fig. 2 confirms the results of our previous study with etoposide [15], but also illustrates that the loss of cell viability in B-5 cells was significantly delayed compared to S-1 cells, regardless of the agent tested.

The data presented in Fig. 2 indicate that the effects of Bcl-2 on loss of plasma membrane permeability following doxorubicin or etoposide exposure may be somewhat attenuated compared to the effects following exposure to FUdR and staurosporine. However, there still remains a striking disparity between inhibition of cell death and increase in clonogenic survival caused by Bcl-2 in HeLa cells exposed to topoisomerase II poisons (compare Figs. 1 and 2).

Throughout the course of these experiments, no significant variations in total cell number were detected between S-1 and B-5 cells which could explain the protective effects of Bcl-2 on loss of cell viability as an artifact of rapid cell lysis (and consequently loss) from the B-5 population (data not shown).

The time-courses of cell death caused by equitoxic concentrations of etoposide and staurosporine differ markedly

In order to begin to define the mechanisms by which Bcl-2 may enhance clonogenic survival following exposure to FUdR or staurosporine but not following exposure to doxorubicin or etoposide, experiments were performed to monitor cell death processes in S-1 cells exposed to equitoxic (by clonogenic assay) concentrations of staurosporine or etoposide. Exposure to 750 n*M* staurosporine or etoposide for 24 h allowed 2.1 ± 0.5% and 2.1 ± 0.4% cell survival, respectively ($n = 3$). Despite this similarity, the time-course of loss of cell viability was markedly different for each drug

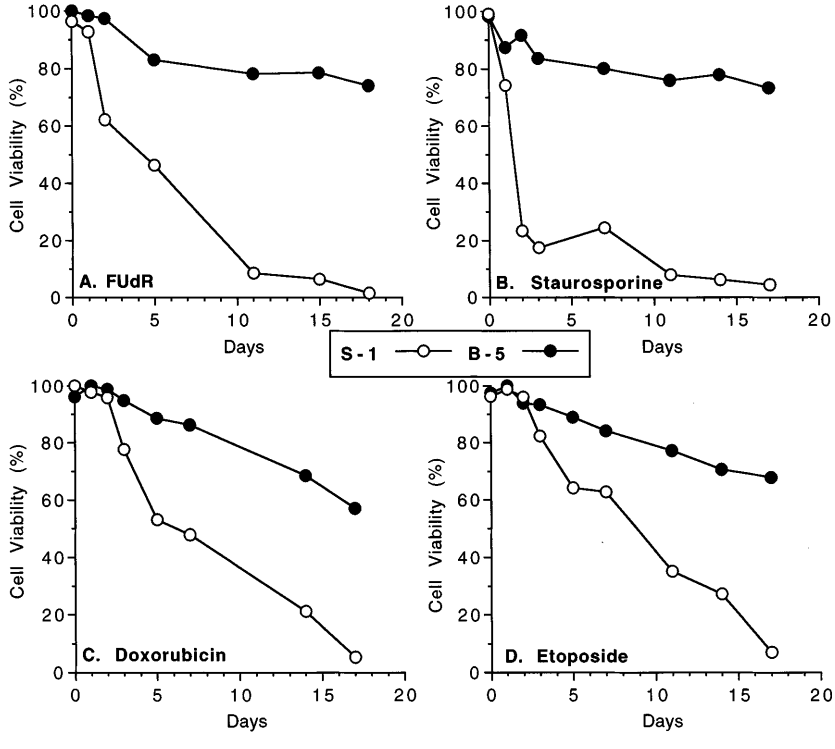

Fig. 2A–D Viability of HeLa S-1 (*open circles*) and B-5 (*filled circles*) cells following 24 h exposures to 1 μ*M* FUdR (**A**), 1 μ*M* staurosporine (**B**), 25 n*M* doxorubicin (**C**) and 1 μ*M* etoposide (**D**). Viability was assessed by the ability to exclude 0.2% trypan blue. Data presented are from a single experiment, which is representative of at least two separate experiments. *Days* on the abscissae denotes the number of days following initiation of the drug treatment

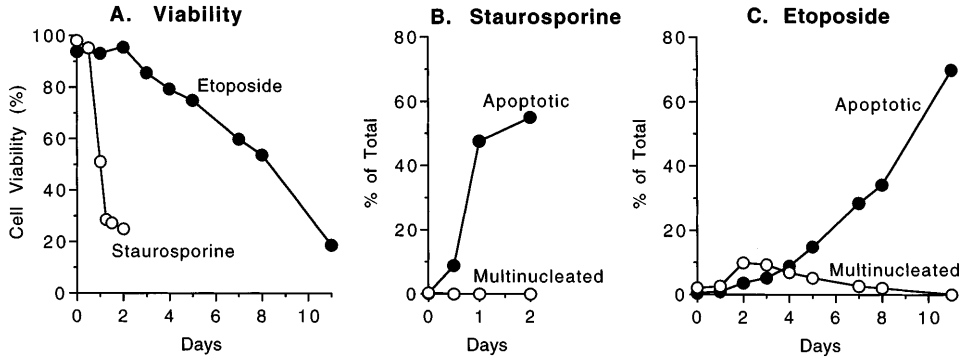

Fig. 3A–C Behavior of HeLa S-1 cells following exposure to equitoxic (by clonogenic assay) concentrations of staurosporine or etoposide. Cells were exposed to 750 n*M* of each drug for 24 h, following which viability was estimated by the ability to exclude trypan blue (**A**). The proportions of morphologically apoptotic or multinucleated cells were also estimated by examination of cell cytospins following exposure to staurosporine (**B**) or etoposide (**C**). Data presented are from a single experiment, which is representative of at least two in separate experiments. *Days* on the abscissae denotes the number of days following initiation of the drug treatment

(Fig. 3A). Staurosporine caused approximately a 50% reduction in cell viability within 24 h of administration, whereas S-1 cells required around 8 days following initiation of the 24-h etoposide exposure to achieve a similar reduction in plasma membrane integrity. The difference in loss of viability was also reflected in the respective time-courses of the proportion of morphologically apoptotic cells (Fig. 3B,C): approximately 50% of staurosporine-treated cells were apoptotic within 24 h of drug exposure, whereas between 8 and 11 days were required for the same effect following the initiation of etoposide treatment. These results were confirmed using the Tdt assay which detects apoptotic DNA fragmentation, and comparable differences were observed between staurosporine and etoposide in the induction of apoptosis (data not shown).

The appearance of apoptotic cells in the S-1 population exposed to etoposide was preceded by the detection of large multinucleated cells (Fig. 3C) [15]. Multinucleated cells were not detected following staurosporine exposure (Fig. 3B).

Discussion

The Bcl-2 oncoprotein has been shown to inhibit apoptosis induced by almost all of the chemotherapeutic agents in current use [6, 20, 26]. Because of this, it is generally assumed that Bcl-2 overexpression in human tumor cells is associated with a multidrug resistance phenotype. While our studies confirm that Bcl-2 does indeed inhibit the induction of apoptosis and delay the loss of membrane integrity in cells exposed to agents with differing mechanisms of action (this study; [15]) we have now demonstrated that Bcl-2 is only able to enhance clonogenic survival in HeLa cells in an agent-specific fashion.

We have proposed previously that etoposide-induced multinucleation is sufficient to cause reproductive death in HeLa cells [15]: inhibition of apoptosis by Bcl-2 resulted in the accumulation of multinucleated cells which exhibited no increased capacity for clonogenic survival compared to non-Bcl-2-expressing cells. These cells eventually lose plasma membrane integrity without exhibiting the morphological or DNA degradative characteristics of apoptosis (MJ Elliott and RB Lock, unpublished observations). The data presented above indicate that, in HeLa cells, the impact of apoptosis on reproductive survival is strikingly different for dissimilar agents, and may depend upon the nature of the initial cytotoxic insult imposed by a particular drug. Inhibition of apoptosis by Bcl-2 allowed increased clonogenic survival following exposure to FUdR or staurosporine, but not to doxorubicin or etoposide. While the degree of resistance to staurosporine and FUdR appears modest in terms of IC_{50} values, the survival protection afforded by Bcl-2 at equivalent drug concentrations may be >15-fold (compare S-1 and B-5 cell survival at 1 μ*M* FUdR and staurosporine, Fig. 1) and is therefore likely to be of clinical significance.

The most plausible explanation for the limitation imposed upon Bcl-2 to enhance clonogenic survival in an agent-specific fashion in our model system is that the extensive DNA damage caused by topoisomerase II poisons results in reproductive death owing to loss or mutation of essential genetic material [2, 21, 24]. In this instance, apoptosis functions in a secondary role to eliminate a reproductively incompetent cell, which results in the extended time-course of apoptosis induction observed in etoposide-treated cells (Fig. 3). In contrast, it would appear that apoptosis plays a primary role in cell death induced by staurosporine (protein kinase inhibition) and FUdR (thymidylate stress), for the simple reason that inhibition of apoptosis by Bcl-2 expression significantly enhances clonogenic cell survival. Moreover, induction of apoptosis by staurosporine was extremely rapid compared to that caused by etoposide, which suggests differential signaling pathways towards apoptosis caused by each agent. However, the resistance to staurosporine and FUdR is not absolute considering the high level of enforced Bcl-2 expression in these

transfectants [15], and additional pathways to cell death appear to be invoked by these agents.

Our observations add to the growing body of evidence that indicates that affecting the rate of onset of drug-induced apoptosis by specific alterations in Bcl-2 expression or p53 functional status does not necessarily translate into enhanced survival in cells of epithelial or mesenchymal origin [9, 15, 27]. The molecular mechanism underlying a cell's commitment to death in the absence of apoptosis is not restricted to the specific lesions induced by topoisomerase II poisons [27], and may have to be determined empirically for each antitumor agent. However, the data presented above appear to be the first demonstration that Bcl-2 exhibits drug-specific effects on clonogenic survival in the same model system.

HeLa cells express a dysfunctional p53 response to DNA damage, because of the human papillomavirus E6 protein [11]. Recent studies indicate an inverse relationship between Bcl-2 and p53 (presumably mutant) expression in certain human solid malignancies [1, 7], which may represent an important aspect of tumor developmental biology. Clearly, additional in vitro and in vivo studies are required of the effects of Bcl-2 on chemotherapeutic drug action, in which the cellular context of Bcl-2 expression is taken into account.

A concern raised in the performance of these studies is that, by plating out relatively low numbers of cells for colony formation, additional stresses are imposed upon cells which may somehow negate the protective effects of Bcl-2 that are observed when assessing the viability of pooled cell populations. Indeed, some three-dimensional model systems have been shown to behave quite differently from cells grown as a monolayer in conferring chemotherapeutic drug resistance [13]. This concern is negated somewhat by the demonstration above that enhanced clonogenic survival conferred by Bcl-2 is agent-specific, but in addition requires the testing of our HeLa-transfected clones grown as xenografts in immune-deprived mice. These experiments are currently underway.

Our results may also contribute to understanding the apparent conundrum in breast cancer, which may extend to other tumors of epithelial origin, in which Bcl-2 overexpression is associated with well-differentiated, estrogen receptor-positive, p53-negative (and therefore presumably WT) tumors which exhibit more favorable responses to hormonal and cytotoxic therapy [7, 14, 23]. Doxorubicin is probably the most active single chemotherapeutic agent used in the treatment of breast cancer, although 5-fluorouracil is also included in combination [5]. Our data indicate that Bcl-2 overexpression would not be predicted to increase clonogenic survival of human epithelial tumor cells exposed to doxorubicin.

Finally, while our studies do not question the importance of apoptosis in physiological cell death and in tumor cell responses to chemotherapeutic drugs, they do serve to highlight the importance of alternative cell death processes which must be considered for individual drugs. Currently, we are testing the generality of these observations in other human tumor model systems both in vitro and in vivo.

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