Cancer Chemother Pharmacol (1999) 44: 367–371

ORIGINAL ARTICLE

Michael D. Story · Nena Mirkovic
Nancy Hunter · Raymond E. Meyn

Bcl-2 expression correlates with apoptosis induction but not tumor growth delay in transplantable murine lymphomas treated with different chemotherapy drugs

Received: 22 September 1998 / Accepted: 9 February 1999

Abstract *Purpose*: Previously, we have reported that the bcl-2-expressing murine lymphoma cell line LY-ar is resistant to chemotherapy-induced apoptosis when compared to the non-bcl-2-expressing LY-as cell line. The intent of the present study was to determine whether this relationship extends to lymphomas produced from these cell lines in syngeneic mice, after treatment with the same chemotherapy agents. *Methods*: LY-ar and LY-as tumors were grown in the hind legs of syngeneic mice. They were subsequently exposed to graded doses of cisplatin (CP), etoposide (VP-16), Adriamycin (ADR), cytarabine (ara-C), cyclophosphamide (CY), or camptothecin (CAM). Apoptotic bodies were scored in histological sections of tumors that had been stained with hematoxylin and eosin. Tumor growth delay was determined on tumors that were treated when they were 8 mm in diameter. Thereafter, tumor diameter was measured daily with a vernier caliper until they had grown to a maximum of 16 mm in diameter. *Results*: When transplanted into host animals, tumors derived from these two cell lines and treated in vivo with CP, VP-16, ADR, ara-C, CY, and CAM displayed apoptotic propensities similar to those seen in the same cell lines when treated in vitro. Generally, for all the drugs tested, apoptotic indices in LY-as tumors were significantly higher than in LY-ar tumors. However, tumor growth delay measurements could not be predicted with any accuracy from the apoptotic indices. For some drugs LY-ar tumors were more sensitive than LY-as tumors (CP, Vp-16, ADR, ara-C), yet LY-ar tumors were more resistant to CY. *Conclusions*: Despite considerable interest in using apoptotic indices as predictors of treatment outcome, the data presented here suggest that these relationships are very complex. This may be especially true for chemotherapy agents for which effects in vivo are complicated by pharmacokinetics, host effects, and tumor cell heterogeneity.

Key words Apoptosis · Tumor growth delay · Cisplatin · Adriamycin · Etoposide · Cyclophosphamide · Camptothecin · Cytarabine

M.D. Story · N. Mirkovic[1] · N. Hunter · R.E. Meyn (✉)
Department of Experimental Radiation Oncology,
The University of Texas, M.D. Anderson Cancer Center,
1515 Holcombe Blvd., Houston, TX 77030, USA
e-mail: rmeyn@mdanderson.org
Tel. +1-713-792-3424; Fax +1-713-794-5369

[1] *Present address*:
Department of Radiation Oncology,
Loyola University Medical Center Maywood,
IL 60153, USA

Introduction

Correlations between the expression of certain oncogenes or tumor suppressor genes in clinical specimens with patient prognosis have yielded mixed results. For example, in the case of bcl-2, a gene that controls apoptosis, expression of its protein has been associated with an unfavorable prognosis in neuroblastoma [2], prostate cancer [7], and lymphoproliferative disorders [1, 4]. On the other hand, other studies have suggested that bcl-2 positivity may predict a favorable prognosis in tumors of the breast [5], thyroid [18] and lung [9]. Most of these investigations did not assess the type of treatment that patients received as independent variables, i.e. surgery alone vs. surgery plus chemotherapy or radiotherapy. This might ultimately be important because bcl-2 has been shown to inhibit radiation- and chemotherapy agent-induced apoptosis in cells treated in vitro [13, 17]. Thus, bcl-2 expression may be a major determinant of response to cancer therapy [12].

Results from such in vitro studies may not directly translate to the in vivo situation. Experiments conducted with model tumor systems in vivo have yielded evidence that is contradictory to that seen in vitro. For instance, tumor cells in which p21, the downstream target of p53, has been knocked out are more sensitive to radiation-induced apoptotic death but do not display a corresponding change in in vitro clonogenic survival

[11, 19]. Yet when these same p21-deficient cells are grown as tumor xenografts they are significantly more sensitive to ionizing radiation as measured by tumor growth delay [20].

In a previous study we have shown that the upregulation of bcl-2 and other factors in a murine lymphoma cell line, LY-ar, reduces chemotherapy agent-induced apoptosis when compared to the non-bcl-2-expressing LY-as cell line and that resistance is manifested at the level of in vitro clonogenic survival. Whether the development of a chemoresistant phenotype by LY-ar cells in vitro would also result in a resistant phenotype in vivo is unknown and is certainly open to question given the conflicting reports in the literature. To address this question, transplantable tumors, derived from LY-ar and LY-as cells, were grown in syngeneic mice. Apoptotic propensity and tumor growth delay were measured in these tumors and compared after treatment of the host with cisplatin (CP), etoposide (VP-16), Adriamycin (ADR), cyclophosphamide (CY), cytarabine (ara-C), and camptothecin (CAM).

Materials and methods

Mice and tumor development

Inbred C3Hf/kam mice were maintained in a specific pathogen-free colony at a density of five per cage. Mice of either sex were used at the age of 11–13 weeks for these experiments. Single-cell suspensions of $1–2 \times 10^6$ LY-ar or LY-as cells were inoculated into the subcutis of the right thigh to generate solitary tumors. When the tumors reached a size of 6–8 mm in diameter, the chemotherapy agents were diluted and administered interperitoneally in a solution of sterile water, with the exceptions of VP-16, which was diluted in a normal saline solution, and CAM, which was administered as a suspension in sesame oil. Tumor volume was determined daily using a vernier caliper to obtain three mutually orthogonal diameters. Some mice were sacrificed at 0, 8, 12, and in some cases 24 h after drug administration in order to process the tumor tissue for the scoring of apoptosis. Following sacrifice of the mice in a closed CO_2 chamber, the tumors were excised and placed in neutral-buffered 10% formalin. The tissue was processed and embedded in paraffin blocks. Sections of 2–4 μm thickness were mounted on slides and stained with hematoxylin and eosin (H & E).

Assay for apoptotic bodies

Coded slides were scored blindly by N.M. for apoptotic bodies by microscopic examination of the stained tumor sections at a magnification of 400× as described previously [14]. Five fields of non-necrotic areas were selected, and 100 nuclei in each field were scored as apoptotic, necrotic or normal. Nuclei were considered apoptotic if they were shrunken and darkly stained.

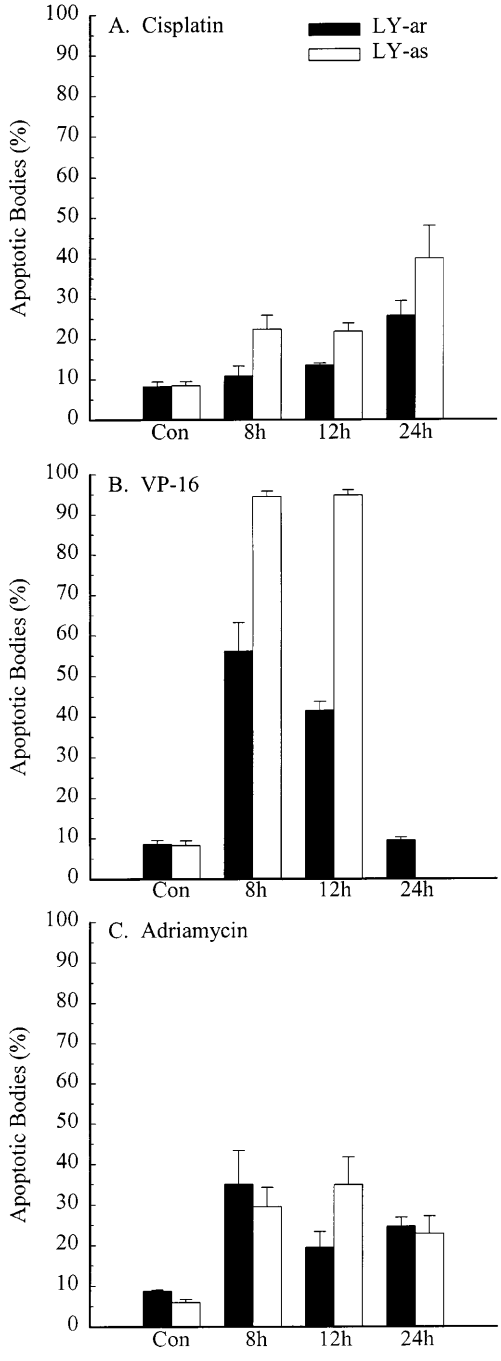

Fig. 1A–C Apoptotic indices measured in LY-ar and LY-as tumors grown in the hind legs of mice and treated by (**A**) CP, (**B**) VP-16, and (**C**) ADR. Apoptotic bodies were counted 0, 8, 12 and 24 h after treatment. Error bars reflect the standard deviation of apoptotic bodies measured in the three to five animals used for each data point

Results

As shown in Fig. 1, the ratios of apoptotic bodies in LY-ar and LY-as tumors after treatment with three of the drugs (CP, VP-16, ADR) was similar to and could be predicted from those reported previously for in vitro exposures [15, 16]. With the possible exception of ADR, where a difference was only noted 12 h after treatment, the apoptosis levels in LY-ar tumors were less than those in LY-as tumors (Fig. 1). After treatment with CP and VP-16, the LY-as tumors had between 1.5- and 2-fold higher levels of apoptosis at all times examined except for 24 h. At 24 h after VP-16 exposure, apoptosis could not be measured in LY-as tumors because of the loss of apoptotic cells due to secondary necrosis and clearance

from the tumor site. That trend was displayed by LY-ar tumors as well, as evidenced by the decline in apoptotic bodies as represented in Fig. 1.

This analysis was extended to three additional agents, CAM, ara-C and CY. The apoptotic indices for LY-as and LY-ar tumors treated with these agents are presented in Fig. 2. As for the other agents, apoptosis in the LY-as tumors was generally higher than in LY-ar tumors. This was especially true in the case of CY, where LY-as tumors had three- to eightfold higher apoptosis levels than did LY-ar tumors.

The differences in chemotherapy agent-induced apoptosis between the LY-as and LY-ar tumors did not, however, translate into the predicted differences in tumor growth delay for each drug examined. Indeed, it appeared that, for many of the agents, the LY-ar tumors were more sensitive than the LY-as tumors when assessed on the basis of tumor growth delay (Figs. 3 and 4). The times for the treated tumors to grow from 8 mm to 12 mm, relative to the untreated controls, were cal-

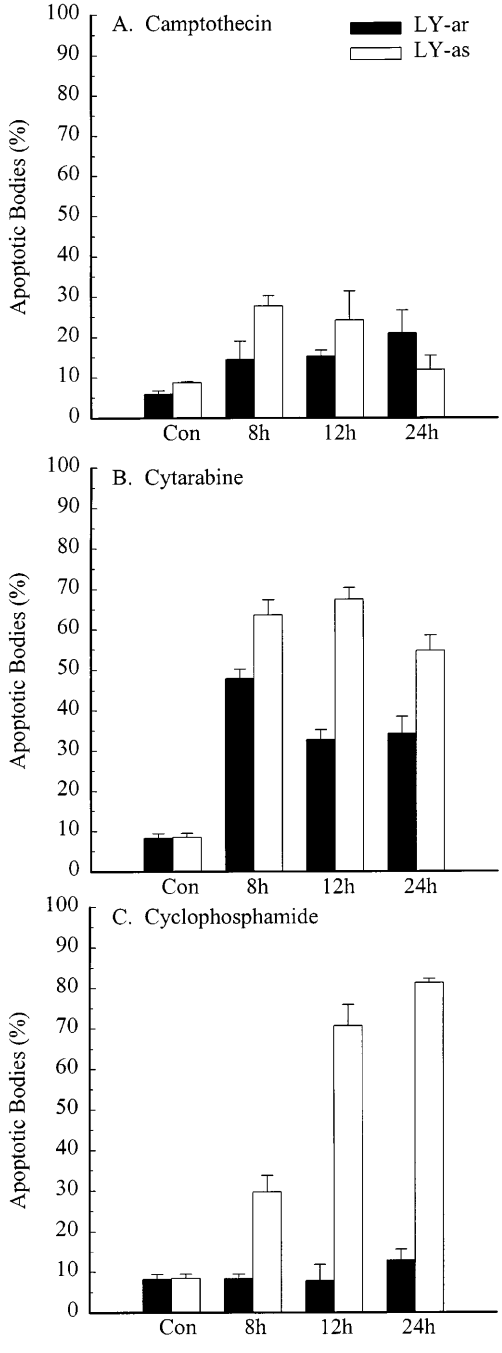

Fig. 2A–C Apoptotic indices measured in LY-ar and LY-as tumors grown in the hind legs of mice and treated by (**A**) CAM, (**B**) ara-C, and (**C**) CY. Apoptotic bodies were counted 0, 8, 12 and 24 h after treatment. Error bars reflect the standard deviation of apoptotic bodies measured in the three to five animals used for each data point

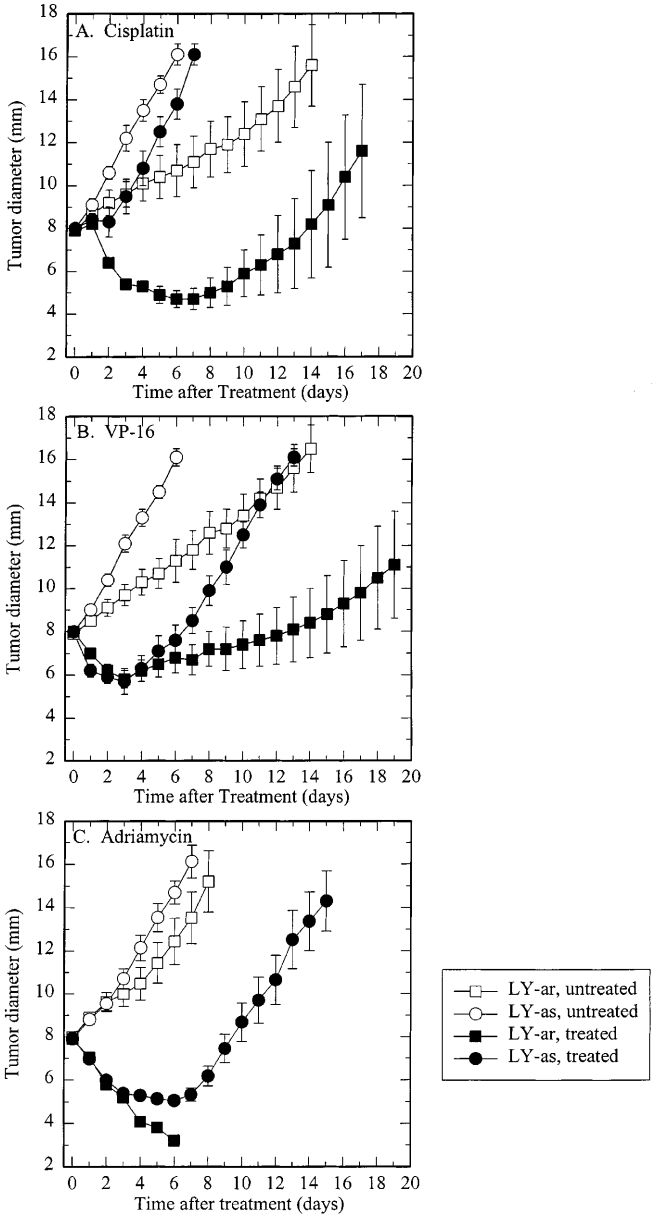

Fig. 3A–C Tumor growth delay in LY-ar and LY-as tumors after treatment with (**A**) CP, (**B**) VP-16, and (**C**) ADR. Error bars reflect the standard deviation of tumor sizes measured in the three to five animals used for each data point

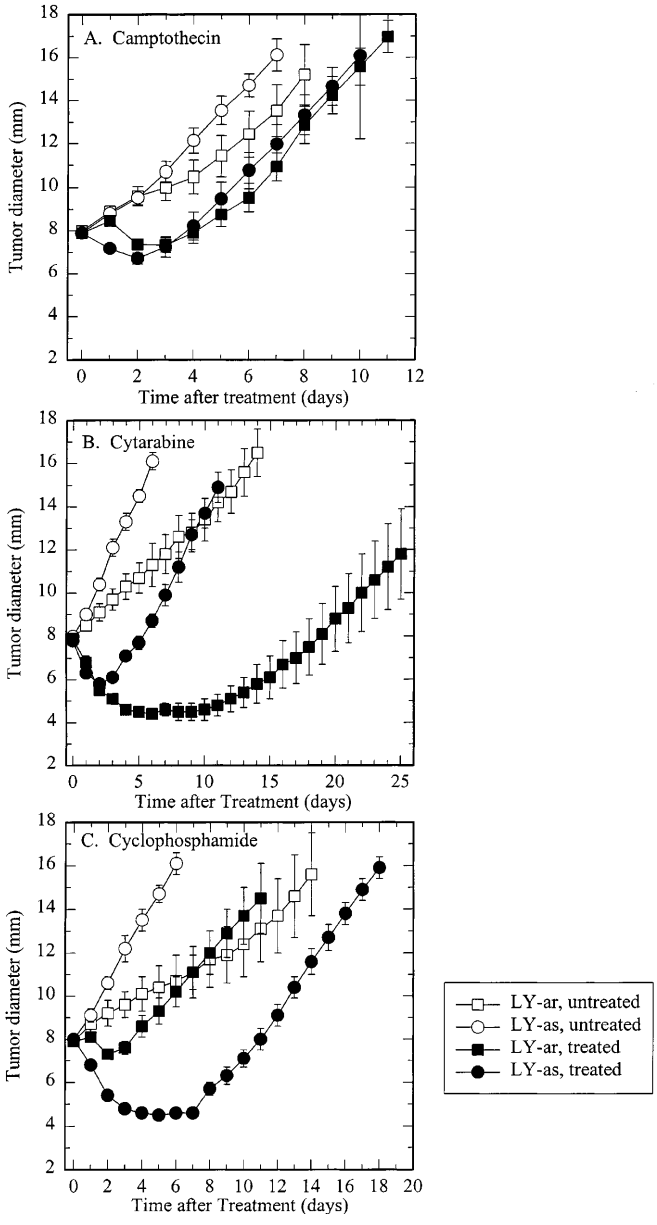

Fig. 4A–C Tumor growth delay in LY-ar and LY-as tumors after treatment with (**A**) CAM, (**B**) ara-C, and (**C**) CY. Error bars reflect the standard deviation of tumor sizes measured in the three to five animals used for each data point

culated for each growth curve shown in Figs. 3 and 4, and these values are given in Table 1. As can be seen from these results, for CP, ADR, VP-16, and ara-C, the LY-ar tumors had larger growth delays than the LY-as tumors. However, for the LY-ar tumors, the response to CY was less than that for the LY-as tumors while for CAM the tumor growth delay was within experimental error. The difference was especially dramatic for CY, with which the growth delay in LY-ar tumors was on the order of only one-tenth that of LY-as tumors. In addition, untreated LY-ar tumors grew at about half the rate of the LY-as tumors: the times to grow from 8 mm to 12 mm were 3.8 ± 1.4 days for LY-as tumors and 8.1 ± 0.9 days for the LY-ar tumors.

Table 1 Tumor growth delay in days for LY-as and LY-ar tumors after treatment with CP, VP-16, ADR, ara-C, CY, and CAM. Delay measurements were calculated as the additional time required for tumors to grow from 8 mm to 12 mm in diameter as compared to untreated tumors

Drug	Dose (mg/kg)	Tumor growth delay (days)	
		LY-as	LY-ar
Adriamycin	16	9	> 16
Etoposide	40	6.7	13
Cisplatin	8	1.7	8.4
Camptothecin	4	3	1.9
Cytarabine	600	5.5	17.6
Cyclophosphamide	100	11.7	0
Cyclophosphamide	200	26.6	2.2

Discussion

We have previously reported that, as for radiation exposures [16], LY-ar cells are resistant to apoptosis and display increased clonogenic survival following exposure to CP, VP-16, and ADR in vitro when compared to LY-as cells [15]. Because one might expect that LY-ar tumors would be resistant when treated with these same agents in vivo, we examined apoptosis and tumor growth delay in LY-ar and LY-as tumors implanted in syngeneic mice to see if such generalities would hold. The result was mixed. The apoptotic propensity of the transplanted tumors did correlate well with our previous in vitro data, but the results of the tumor growth delay experiments bore little relationship to those in the in vitro clonogenic survival assays [15]. In fact, for four of the six agents, LY-ar tumors had greater growth delay than LY-as tumors exposed to those drugs. Tumor growth delay was, therefore, not predictable from in vitro determinations of either apoptosis or clonogenic survival or even from the in vivo apoptosis results.

The use of tumor growth delay as an endpoint in this study may be confounded by the different growth rates of the two model tumors. LY-as tumors grew at approximately twice the rate of LY-ar tumors. However, the fact still remains that all of the ADR-treated LY-ar tumors, and one of the LY-ar tumors with CP showed complete regression. None of the LY-as tumors at any dose or treatment completely regressed and, in any event, there is no reason to assume that slower-growing tumors are more sensitive to chemotherapy.

Tumor microenvironment may also play a role in the alteration of chemosusceptibility. That role is probably associated with drug metabolism or transport, as the growth delay of these tumors induced by ionizing radiation, which delivers dose to a tumor instantaneously and uniformly, was predictable from the in vitro data [16]. The role of tumor microenvironment is probably apoptosis-independent, as the in vitro and in vivo

apoptosis ratios between cell or tumor types were similar. This result is interesting in that Wouters et al. have reported an increase in resistance to radiation by HCT116 tumor cells that are p21-deleted, via an apoptosis-independent mechanism acting through the loss of p21 function when grown as solid tumors but not as cell cultures [20]. They speculated that p21, in addition to its role in controlling cell cycle progression, regulates a pathway that is responsible for the acquired resistance of cells grown as solid tumors.

Bcl-2 may also have biochemical activities independent of its ability to inhibit apoptosis. For example, in several cell systems, bcl-2 expression appears to slow tumor cell growth [8, 10], similar to our observation reported here. Moreover, bcl-2 has recently been shown to suppress tumor angiogenesis [6]. Thus, it is possible that bcl-2 affects the metabolism of certain drugs by as yet unknown mechanisms and these mechanisms affect tumor growth delay more than apoptosis inhibition. Such mechanisms would be expected to affect the response to different drugs differentially, and it is therefore noteworthy that two of the agents examined, CY and CAM, yielded the expected result, i.e. LY-ar tumors displayed less growth delay than did the LY-as tumors. That the relationships between bcl-2 expression, apoptosis induction, and biological assessments of tumor cell response might be dependent on the chemotherapy agent in question has also been suggested by other investigators [3].

In summary, the results presented here coupled with the in vitro results presented elsewhere demonstrate that whereas bcl-2 expression inhibits chemotherapy agent-induced apoptosis, the response of tumors treated in vivo is not necessarily predictable from their bcl-2 status or apoptosis propensity. Moreover, the relationships between these parameters are highly dependent on the cancer treatment agent in question. Understanding these relationships in more detail may ultimately help explain the role of apoptosis in treatment response and enable additional markers to be identified to predict prognosis following therapy.

Acknowledgements This work was supported in part by NIH grants CA69003 (R.E.M.), CA62209 (M.D.S.), and the Institutional Core Grant CA16672 from the National Cancer Institute. All animals used in this study were cared for according to the "Principles of Animal Care", NIH publication No. 85-23, as revised in 1985.

References

1. Campos L, Rouault JP, Sabido O, Oriol P, Roubi N, Vasselon C, Archimbaud E, Magaud JP, Gnyotat D (1993) High expression of bcl-2 protein in acute myeloid leukemia cells is associated with poor prognosis to chemotherapy. Blood 18: 3091
2. Castle VP, Heidelberger KP, Bromberg J, Ou X, Dole M, Nunez G (1993) Expression of apoptosis-suppressing protein Bcl-2 in neuroblastoma is associated with unfavorable histology and N-myc amplification. Am J Pathol 143: 1543
3. Elliott MJ, Stribinskiene L, Lock RB (1998) 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. Cancer Chemother Pharmacol 41: 457
4. Gascoyne RD, Adomat SA, Krajewski S, Krajewska M, Horsman DE, Tolcher AW, O'Reilly SE, Hoskins P, Coldman AJ, Reed JC, Connors JM (1997) Prognostic significance of Bcl-2 protein expression and Bcl-2 gene rearrangement in diffuse aggressive non-Hodgkin's lymphoma. Blood 90: 244
5. Joensuu H, Pylkkäen L, Toikkanen S (1994) Bcl-2 protein expression and long-term survival in breast cancer. Am J Pathol 145: 1191
6. Koukourakis MI, Giatromanolaki A, O'Byrne KJ, Whitehouse RM, Talbot DC, Gatter KC, Harris AL (1997) Potential role of bcl-2 as a suppressor of tumour angiogenesis in non-small-cell lung cancer. Int J Cancer 74: 565
7. McDonnell TJ, Troncoso P, Brisbay SM, Logothetis C, Chung LWK, Hsieh JT, Tu SM, Campbell ML (1992) Expression of the protooncogene bcl-2 in the prostate and its association with emergence of androgen-independent prostate cancer. Cancer Res 52: 6940
8. O'Reilly LA, Harris AW, Tarlinton DM, Corcoran LM, Strasser A (1997) Expression of a bcl-2 transgene reduces proliferation and slows turnover of developing B lymphocytes in vivo. J Immunol 159: 2301
9. Pezzella F, Turley H, Kuzu I, Tungekar MF, Dunnill MS, Pierce CB, Harris A, Gatter K, Mason DY (1993) Bcl-2 protein in non-small-cell lung carcinoma. N Engl J Med 329: 690
10. Pietenpol JA, Papadopoulos N, Markowitz S, Villson JKV, Kinzler KW, Vogelstein B (1994) Paradoxical inhibition of solid tumor cell growth by bcl-2. Cancer Res 54: 3714
11. Polyak K, Waldman T, He TC, Kinzler KW, Vogelstein B (1996) Genetic determinants of p53-induced apoptosis and growth arrest. Genes Dev 10: 1945
12. Reed JC (1995) BCL-2: prevention of apoptosis as a mechanism of drug resistance. Hematol Oncol Clin North Am 9: 451
13. Sentman CL, Shutter JR, Hockenberry D, Kanagawa K, Korsmeyer SJ (1991) *Bcl*-2 inhibits multiple forms of apoptosis but not negative selection in thymocytes. Cell 67: 879
14. Stephens LC, Ang KK, Schultheiss TE, Milas L, Meyn RE (1991) Apoptosis in irradiated murine tumors. Radiat Res 127: 308
15. Story MD, Meyn RE (1999) Modulation of apoptosis and enhancement of chemosensitivity by decreasing cellular thiols in a mouse B-cell lymphoma cell line that overexpresses bcl-2. Cancer Chemother Pharmacol 44: 362
16. Story MD, Voehringer DW, Malone CG, Hobbs ML, Meyn RE (1994) Radiation-induced apoptosis in sensitive and resistant cells isolated from a mouse lymphoma. Int J Radiat Biol 66: 659
17. Strasser A, Harris AW, Cory S (1991) Bcl-2 transgene inhibits T cell death and perturbs thymic self-censorship. Cell 67: 889
18. Viale G, Roncalli M, Grimelius L, Graziani D, Wilander E, Johansson H, Bergholm U, Coggi G (1995) Prognostic value of Bcl-2 immunoreactivity in medullary thyroid carcinoma. Hum Pathol 26: 945
19. Waldman T, Lengauer C, Kinzler KW, Vogelstein B (1996) Uncoupling of S phase and mitosis induced by anticancer agents in cells lacking p21. Nature 381: 713
20. Wouters BG, Giaccia AJ, Denko NC, Brown JM (1997) Loss of p21Waf1/Cip1 sensitizes tumors to radiation by an apoptosis-independent mechanism. Cancer Res 57: 4703