Bcl-2 Overexpression Decreases BCNU Sensitivity of a Human Glioblastoma Line Through Enhancement of Catalase Activity

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Abstract   The aim of this study was to evaluate the role of *bcl*-2 in 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) sensitivity of the ADFS human glioblastoma cell line in vitro and in vivo. To this end, the ADFS line expressing a low level of the bcl-2 protein was transfected with a bcl-2 expression vector. We found that bcl-2 overexpressing clones were less sensitive to in vitro BCNU treatment than the control clone. Cell cycle analysis demonstrated that while BCNU induced a consistent block in S/G2-M phases of the cell cycle in the control clone, it did not affect the cell cycle phase distribution of the two *bcl*-2 transfectants. The different sensitivity to BCNU was unrelated to the ability of *bcl*-2 to inhibit apoptosis, while bcl-2 appeared to protect *bcl*-2 transfectants from BCNU toxicity through an increase of catalase activity. The ability of the catalase inhibitor, sodium azide, to increase the BCNU sensitivity of the *bcl*-2 transfectants to levels of the BCNU-treated control clone substantiated the role of the catalase activity. The effect of bcl-2 in reducing sensitivity to BCNU was also confirmed by in vivo experiments. Xenografts of *bcl*-2 overexpressing tumors were less sensitive to BCNU treatment than xenografts originating from control cells. J. Cell. Biochem. 83: 473–483, 2001. © 2001 Wiley-Liss, Inc.

Key words: BCNU; glioblastoma; bcl-2; catalase

Human glioblastomas are largely resistant to current strategies of surgery, chemotherapy, and radiotherapy. Thus, the study of molecular mechanisms that characterize the growth and the response to antineoplastic treatment, especially in terms of chemoresistance, is particu-

larly urgent. It is also important to develop new strategies to improve the therapeutic response in glioblastoma patients. Chemoresistance of brain tumors, primarily related to the blood-brain barrier [Elliot et al., 1996], is also related to elevated levels of enzymes, such as O^6-methylguanine-DNA-methyltransferase (MGMT), that can remove the alkyl group induced by DNA-alkylating agents. In particular, MGMT represents one of the major protective factors against the cytotoxicity produced by alkylating agents, and a positive correlation between cellular resistance and MGMT expression was observed [Brent et al., 1985; Pegg, 1990].

The levels of cellular antioxidant enzymes were also shown to influence the sensitivity of tumor cells to anticancer drugs. High intracellular content of glutathione (GSH) decreases the cytotoxic and DNA interstrand cross-linking

activity of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), and a direct correlation was found between resistance to BCNU and the catalase activity [Keizer et al., 1988; Ali-Osman et al., 1990; Zhong et al., 1999].

The role of the genes, associated with glioblastoma progression and apoptosis, in the sensitivity to antineoplastic treatment is controversial. Some important changes in gene expression, involved in the development of glioma and in the regulation of apoptosis, are not implicated in the response to chemotherapy [Trepel et al., 1998; Weller et al., 1998]. In addition, the expression of genes, such as bcl-2, was seen to confer resistance to some cytotoxic drugs and radiation without affecting the sensitivity to other agents [Weller et al., 1995; Roth et al., 1998]. The aim of this study was to gain information about the role of bcl-2 in the response of glioblastoma to BCNU treatment both in vitro and in vivo.

Bcl-2 protein is expressed in some human glioblastoma cell lines and correlates with malignant transformation of human glial tumors in vivo [Roth et al., 1998]. BCNU is one of the most effective single chemotherapeutic agents against human brain tumors, and is also extensively used in combination therapy [Chamberlain and Kormanik, 1998]. Even though only a few papers have evidenced the ability of bcl-2 to confer in vitro resistance to BCNU in cancer lines [Weller et al., 1995; Fels et al., 2000], the mechanism by which bcl-2 modulates resistance to BCNU is not well understood, and in vivo studies have not yet been reported.

The ADFS human glioblastoma line, showing a low level of bcl-2 expression, was transfected with a bcl-2 expression vector and the sensitivity to BCNU was evaluated. In this study we demonstrated that bcl-2 overexpression reduces sensitivity of the human glioblastoma line to BCNU both in vitro and in vivo, and we have evidence that the increase of the catalase activity is responsible for the bcl-2–induced BCNU resistance.

MATERIALS AND METHODS

Tumor Cell Line

The ADFS glioblastoma cell line, established and characterized in our laboratory from a biopsy obtained from the surgical department of our institute, was maintained in RPMI 1640 medium supplemented with 10% FCS, L-glutamine and antibiotics, in a humidified atmosphere with 5% CO_2 at 37°C.

Transfection

ADFS cells ($\sim 1 \times 10^6$ cells/250 µl medium) were transfected by electroporation (1,600 V, 25 µF, Gene Pulser, Bio-Rad, Milano, Italy) with the expression vector pSFFVneo **bcl-2** [Del Bufalo et al., 1997] carrying the bcl-2 gene and the gene for the resistance to Neomycine (neo, Gibco). As control, ADFS cells were transfected with the plasmid carrying the neo-resistance gene alone. Transfected clones were collected after 20 days of selection in neo-containing medium (800 µg/ml) and bcl-2 expression was tested by Western blot analysis as previously reported [Del Bufalo et al., 1997]. Two bcl-2 overexpressing clones (AB53 and AB59) and a control clone (AN2) were used for all the experiments.

Drugs Preparation and In Vitro Treatment

BCNU (Bristol-Myers Syracuse, NY, USA) supplied for clinical use was dissolved in 10% ethanol at a concentration of 25 mM. The drug was stored at −80°C and adjusted with culture media to the final concentrations at the time of treatment. Exponentially growing cells were exposed to BCNU for 24–72 h at doses ranging from 5 to 75 µM. Experiments with a catalase inhibitor, were performed using 0.5 mM sodium azide for 6 h before BCNU treatment (50 µM for 24 h). Cells were washed, assayed for cell viability (trypan blue exclusion test), and counted (Coulter Counter, Kontron Instruments, Milano, Italy). Aliquots of drug-treated and control cells were differentially processed according to the analyses to be performed.

Colony-Forming Assay, Cell Cycle Phases Distribution and Apoptosis

To evaluate the cell colony-forming ability, cell suspensions from different samples were seeded into 60-mm Petri dishes (Nunc, Mascia Brunelli, Milano, Italy) for 10 days. Colonies were stained with 2% methylene blue in 95% ethanol and counted (1 colony > 50 cells). The surviving fractions were calculated as the ratio of absolute survival of the treated sample/survival of untreated control sample. All experiments were repeated four times in triplicate employing freshly prepared drug solutions.

Cell percentages in the different phases of the cell cycle were estimated as previously described [Del Bufalo et al., 1996]. For bromodeoxyuridine (BrdU, Becton-Dickinson San Jose, CA) incorporation, cells were pulsed with BrdU at a final concentration of 10 μM for 40 min and then resuspended in medium without BrdU. After the appropriate intervals, cells were harvested, resuspended in Phosphate Buffer Solution (PBS), fixed with ice-cold 70% ethanol, and stored overnight at 4°C. After this fixation step, DNA was denatured with 2N HCl for 30 min at room temperature and stained with FITC-conjugated anti–BrdU prior to the addition of Propidium Iodide (PI) as described by Mazel et al. [1996].

Apoptosis was detected by cytofluorimetric analysis of permeabilized PI-stained cells and by TUNEL assay (terminal deoxynucleotide transferase [TdT]-mediated dUTP nick-end labeling) of cytospin preparations using a commercial kit (ApopDETEK in situ apoptosis detection kit, Enzo Diagnostic, New York) as previously reported [Leonetti et al., 1999].

Catalase Activity Assay

All the measures were performed on cell homogenates prepared in PBS by probe sonication. Catalase activity was measured spectrophotometrically at 240 nm by the disappearance of H_2O_2 as described by Luck [1963]. One unit of activity corresponds to 1 μmol H_2O_2 disappeared/min. Protein assay was measured as reported, using bovine serum albumine as standard [Del Bufalo et al., 1997].

In Vivo Experiments

Male CD-1 nude (nu/nu) mice, 6–8 weeks old and 22–24 g in body weight were purchased from Charles River Laboratories (Calco, Italy). All procedures involving animals and their care were previously described and were in accordance with institutional guidelines in compliance with national and international laws and policies [Leonetti et al., 1999]. Each experimental group included eight mice. Tumor cells in exponential growth phase were harvested from the culture, washed with medium, and resuspended in cold medium without FCS and injected intramuscularly (i.m.) into the hind leg of the mice at 5×10^6 viable cells/mice. Viability was evaluated by trypan blue exclusion test. Treatments started at Day 4 after injection of cells when a tumor mass of ~200 mg was evident

in all the mice. BCNU was dissolved as described above and its optimal dose of 30 mg/Kg (10% of lethal dose, LD_{10}, Marcantonio et al., 1997) was intravenously (i.v.) administered at 15 mg/kg/day for two consecutive days. Preliminary experiments demonstrated that the treatment with the fractionated dose was less toxic than the single treatment in terms of body weight loss and drug deaths. Three cycles at 7-day intervals were administered. Tumor weight was calculated from caliper measurements according to the formula: tumor weight (mg) = length (mm) × width^2 (mm)/2. Antitumor efficacy of BCNU was assessed by the following end points: i) relative tumor weight inhibition (TWI%) calculated as 1-(mean tumor weight of treated mice/mean tumor weight of untreated) × 100; ii) tumor growth delay calculated as T–C where T and C represent respectively, the median time for treated and control mice to reach the size of 1,000 mg; iii) relative increase of life span (ILS%) calculated as [median survival time (in days) of treated mice/median survival time (in days) of control mice] -1%.

Statistical Analysis

The Mann-Whitney U-test was used for statistical analysis. Differences were considered significant at P values < 0.01 (two sided).

RESULTS

Bcl-2 Overexpression Decreases BCNU Sensitivity of the ADFS Cell Line

To examine whether *bcl*-2 plays a role in the BCNU sensitivity of glioblastoma cells, we transfected the ADFS human glioblastoma cell line with a *bcl*-2 expression vector. As control we also transfected ADFS cells with the native vector carrying the neomycine resistant gene. Figure 1 shows the levels of bcl-2 protein in AN2 control clone, and two *bcl*-2 overexpressing clones (AB53 and AB59) which were chosen for all the in vitro and in vivo experiments. AB53 and AB59 transfectants express bcl-2 protein about 5-fold more than the AN2 clone, when normalized to HSP 72/73 protein amount. The ADFS parental cells and the AN2 clone had similar levels of bcl-2 protein expression (data not shown).

Parental cells, the control clone and two *bcl*-2 transfectants were exposed to increasing doses of BCNU. Figure 2 shows the survival curves of
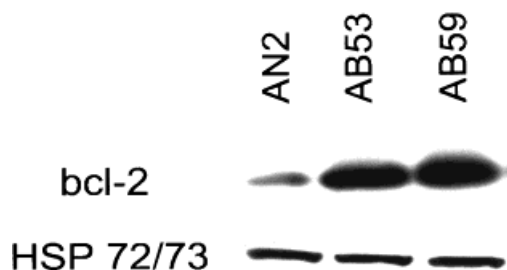
Fig. 1. Western blot analysis of the bcl-2 protein in the cell lysates of two *bcl*-2 transfectants (AB53 and AB59) and a control transfectant clone (AN2). The relative amounts of the transferred bcl-2 proteins were quantified and normalized to the correspondent HSP 72/73 protein amounts.

AN2, AB53, and AB59 cells exposed for 24 h to doses of BCNU ranging from 5 to 75 µM. The survival curve of the AN2 clone is characterized by a logarithmic decrease in the number of surviving cells as a function of increasing BCNU concentrations, implying that the cell population is sensitive to drug exposure. On the contrary, cell survival of the two *bcl*-2 transfectants does not decrease as a function of BCNU doses, indicating that a part of the cell population is relatively resistant to BCNU treatment. In fact, a similar value of cell survival is observed at the BCNU doses of 50 (about 75%) and 75 µM (about 65%). Survival curve of the ADFS line to BCNU is superimposable to that of the AN2 clone (data not shown).
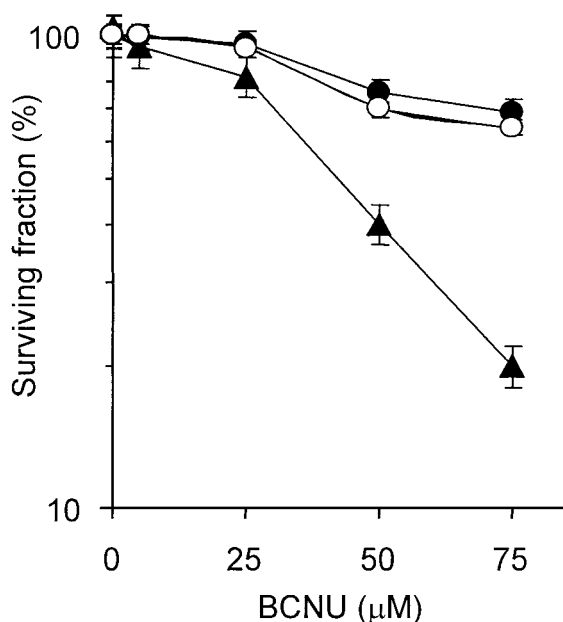

Fig. 2. Survival curves of AN2 cells (▲) and two *bcl*-2-transfectants AB53 (○) and AB59 (●) exposed for 24 h to doses of BCNU ranging from 5 to 75 µM. Values are means of four experiments (bars, S.D.).

Bcl-2 Overexpression Does not Affect Cell Proliferation

Since the effect of antineoplastic drugs depends strictly on the proliferative status of tumor cell lines [Tannock, 1978], we evaluated whether differences in sensitivity to BCNU between ADFS parental cells, the AN2 clone and *bcl*-2 transfectants could be related to differences in their proliferative activity. To this purpose, the cell proliferation after *bcl*-2 transfection was analyzed. No difference in doubling time (about 24 h), saturation density (about 4×10^5 cells/1.76 cm^2) and clonogenic ability (70−75%) were observed for all cell lines.

Since it has been demonstrated that *bcl*-2 expression can modulate cell cycle progression [Mazel et al., 1996; Roth et al., 2000], to confirm that *bcl*-2 does not affect cell proliferation of our model system, we followed the progression of S phase cells throughout the different phases of cell cycle. To this end, we used a 40-min pulse of BrdU and measured DNA content by cytofluorimetric analysis. Figure 3A shows the kinetics of progression of AN2 control and AB53 *bcl*-2 overexpressing clones through the stages of cell cycle after BrdU labeling. Figure 3B graphically depicts this effect. Both control and *bcl*-2 overexpressing clones labeled with BrdU to approximately the same extent during the pulse (about 50%, time 0 h). The progression of both types of cultures out of S phase, and into first G2/M and then G0/G1, is the same (time 4−12 h). In fact, about 35−40% of both control and *bcl*-2 overexpressing cells are labeled 4 h after the pulse of BrdU and only about 2% of both control cells and *bcl*-2 transfectant are labeled 12 h after the pulse of BrdU. Also the reappearence of BrdU positive cells into the S phase (beginning at 12 h for both types of lines) was overlapping. Sixteen hours after the pulse of BrdU about 5% of ADFS and AB53 labeled cells re-entered into the S phase. BrdU labeling of ADFS and AB59 cells is superimposable on that of AN2 and AB53 cells, respectively (data not shown).

BCNU Treatment Affects the Cell Cycle Distribution of AN2 Control Clone Differently to *bcl*-2 Transfectants

In an effort to elucidate the mechanism by which BCNU treatment causes different cytotoxicity on ADFS cells, the AN2 clone and *bcl*-2 transfectants, the ability of BCNU to induce cell
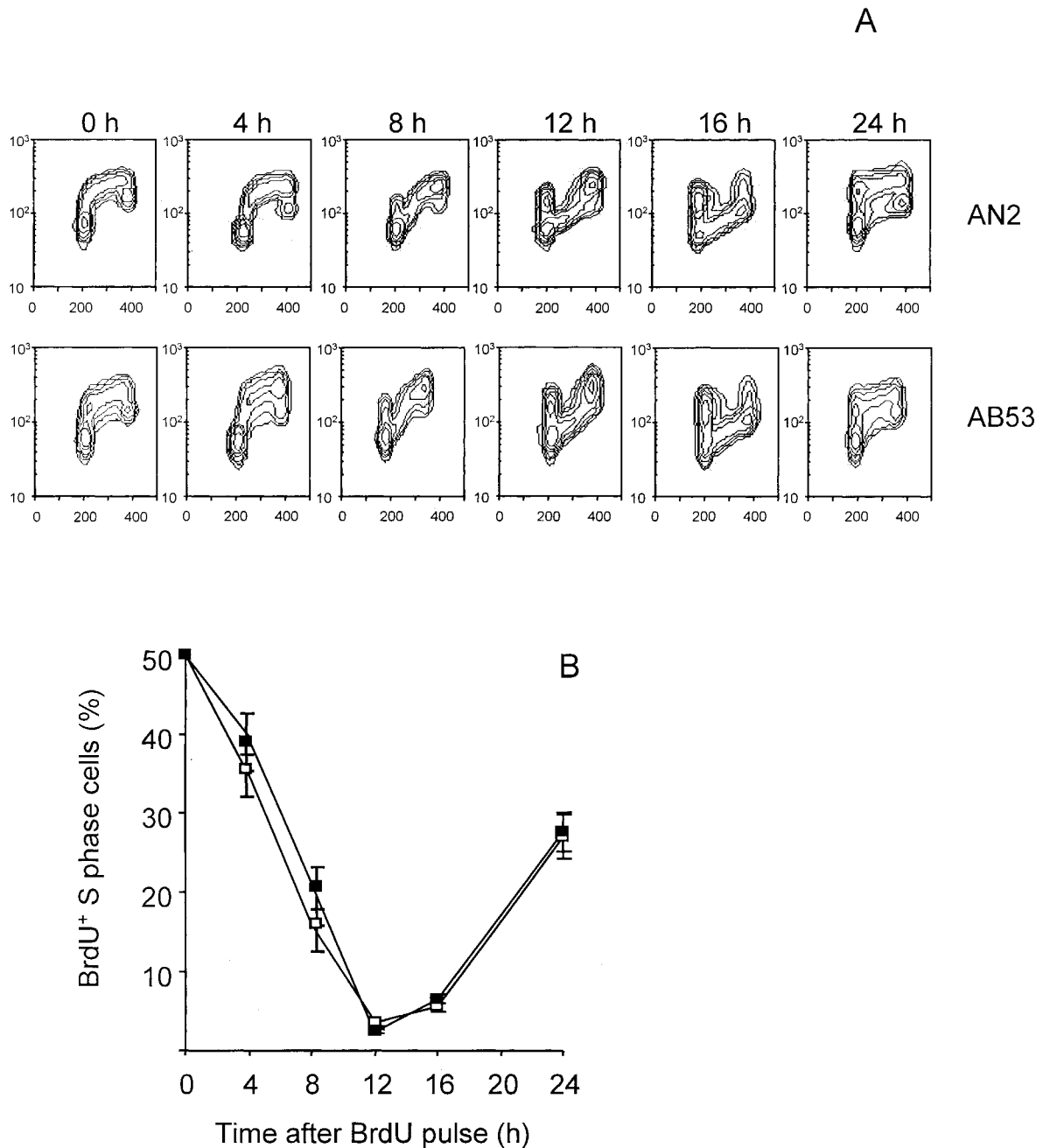
A

B

Fig. 3.   Kinetics of progression of AN2 cells and AB53 *bcl-2* transfectant through the stages of cell cycle. **A**: Cytofluotimetric analysis performed at the end of a 40 min-pulse with BrdU (0 h) and from 4 to 24 h after the end of the pulse. **B**: Graphic depiction of the kinetics of the progression of AN2 and AB53 cells through the S phase of the cell cycle. Values are means of three experiments (bars, S.D.).

cycle perturbation was analyzed by cytofluorimetric analysis. Cells were exposed to BCNU for 24 h at doses ranging from 5 to 75 μM, and cell cycle phase distribution analyzed at the end of treatment (Fig. 4). As expected BCNU induces accumulation of AN2 cells in the S and G2-M phases of the cell cycle that is already detectable at a dose of 25 μM, and more evident at the dose of 75 μM. This accumulation is concomitant with a decrease in the percentage of cells in the G1 phase. On the contrary, BCNU treatment does not determine cell cycle perturbations in the AB53 clone, despite the similar kinetic profile between the *bcl*-2 transfectant and the
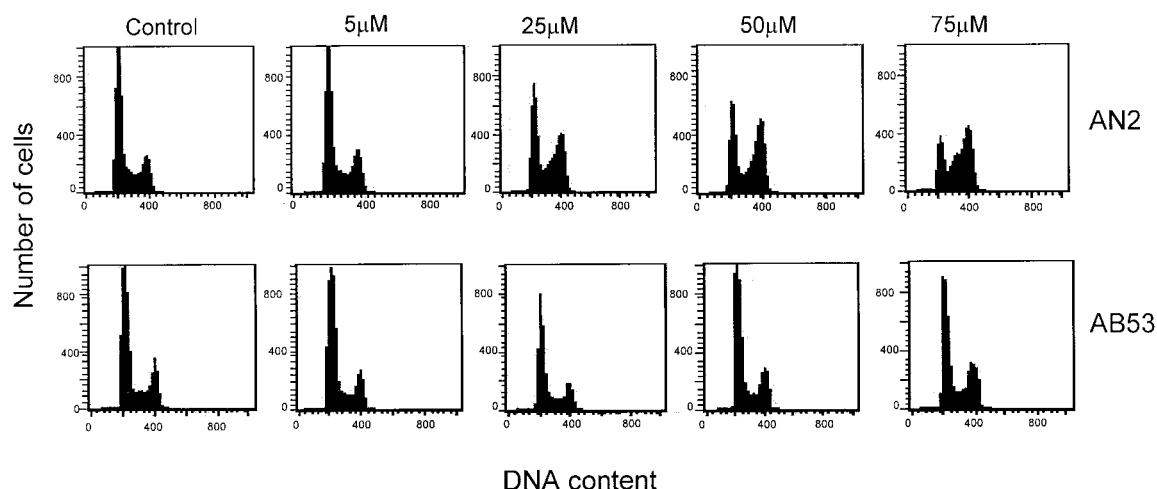
Fig. 4. Cell cycle phase distribution of AN2 cells and AB53 *bcl*-2 transfectant after BCNU treatment (from 5 to 75 μM for 24 h). Flow cytometric analysis after propidium iodide staining was performed at the end of treatment. Cell cycle distribution of untreated cells (Control) is also reported.

control clone. In particular, 90% of the AN2 cells are accumulated in the S and G2-M phases after 75 μM BCNU exposure, while no block was observed after BCNU treatment of AB53 cells. In fact, the percentage of AB53 cells in the S/G2-M phases is superimposable on that of untreated control cells. The cell cycle phase distribution after the BCNU treatment of ADFS and AB59 cells is similar to that observed in the AN2 and AB53 cells, respectively (data not shown).

The absence of a sub G1 peak, indicative of apoptosis, in the DNA histograms suggests that BCNU does not induce apoptosis in our model system even at the highest dose used (75 μM). Taking into account the crucial role of *bcl*-2 in the apoptotic program, and to confirm the hypothesis that differences in BCNU sensitivity observed after *bcl*-2 overexpression were not correlated to a different induction of the apoptotic program by BCNU, we evaluated the percentage of apoptotic cells after the BCNU treatment. A TUNEL assay of cytospin preparations was performed both at the end of treatment and on various days after the end of treatment. The dose of 50 μM BCNU was chosen because it reduces cell survival of parental cells about 60%, while it only slightly affects cell survival of the *bcl*-2 transfectants. Moreover, BCNU doses higher than 50 μM, which can potentially induce apoptosis, correspond to doses that cannot be used in clinical management because of their associated toxicity. Figure 5A shows the percentage of apoptotic

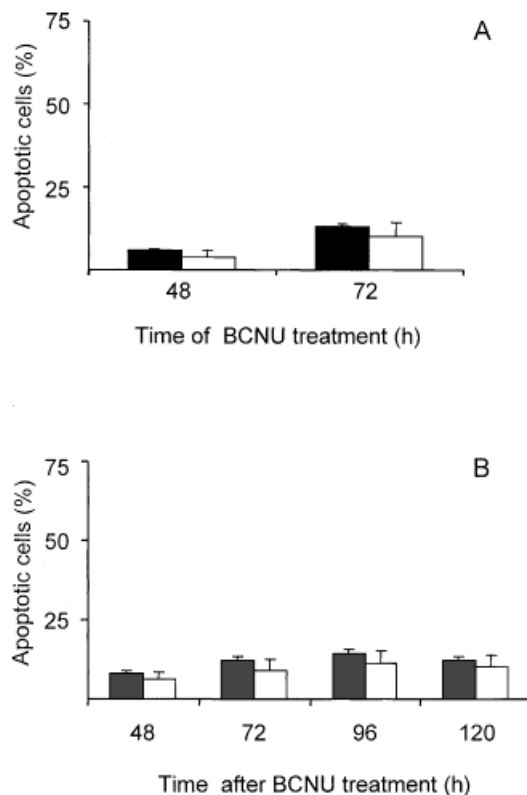**Fig. 5.** Percentage of apoptotic cells evaluated by TUNEL assay on the AN2 control cells (black columns) and the AB53 *bcl*-2 transfectant (white columns) treated with BCNU. **A**: Percentage of apoptotic cells evaluated after treatment with 50 μM BCNU for 48 and 72 h. **B**: Percentage of apoptotic cells evaluated from 48 to 120 h after the end of 50 μM BCNU treatment for 24 h. Values are means of four experiments (bars, S.D.).

cells evaluated after 48 and 72 h of continuous exposure to 50 μM BCNU. Figure 5B shows the percentage of apoptotic cells evaluated 48–120 h after the end of 50 μM BCNU administered for 24 h. No more than 15% of apoptotic cells were observed after BCNU exposure in all the lines and in all conditions used without a significant difference ($P > 0.05$) in the percentage of apoptotic cells between the control clone and the AB53 clone. The percentage of apoptotic cells after BCNU treatment of ADFS and AB59 cells was similar to that observed after BCNU exposure of AN2 and AB53 cells, respectively (data not shown).

Bcl-2 Overexpression Increases Catalase Activity

In order to evaluate whether *bcl*-2 modulates the classical determinants involved in BCNU resistance [Brent et al., 1985; Pegg, 1990], the expression of MGMT, the GSH content, and the activity of superoxide dismutase, glutathione peroxidase and catalase, were analyzed. We found that AN2 cells and *bcl*-2 transfectants are negative for MGMT protein expression. In additon, no differences in the activity of total glutathione peroxidase (about 30 U/mg protein), superoxide dismutase (about 125 U/mg protein), or in total glutathione content (about 60 nmol/mg protein), were found after *bcl*-2 overexpression. Oxidized glutathione was not detectable in any of the lines. On the contrary, higher levels of catalase activity were observed in *bcl*-2 transfectants when compared to the control clone (Fig. 6A). About 2-fold increase of catalase activity was observed after *bcl*-2 overexpression with values of about 25 for the control clone and about 55 U/mg protein for both *bcl*-2 transfectants. Catalase activity of the ADFS parental line is similar to that observed on the AN2 control clone (data not shown).

To evaluate the role of catalase activity on the BCNU-resistance induced by *bcl*-2, BCNU treatment (50 μM for 24 h) was performed after exposure to sodium azide (0.5 mM for 6 h), an inhibitor of catalase activity. At this dose and exposure time, the catalase activity was inhibited by 60–65% in both *bcl*-2 transfectants reaching values superimposable to those of parental cells. The response of parental cells, an AN2 clone and two *bcl*-2 transfectants in terms of clonogenic ability was analyzed
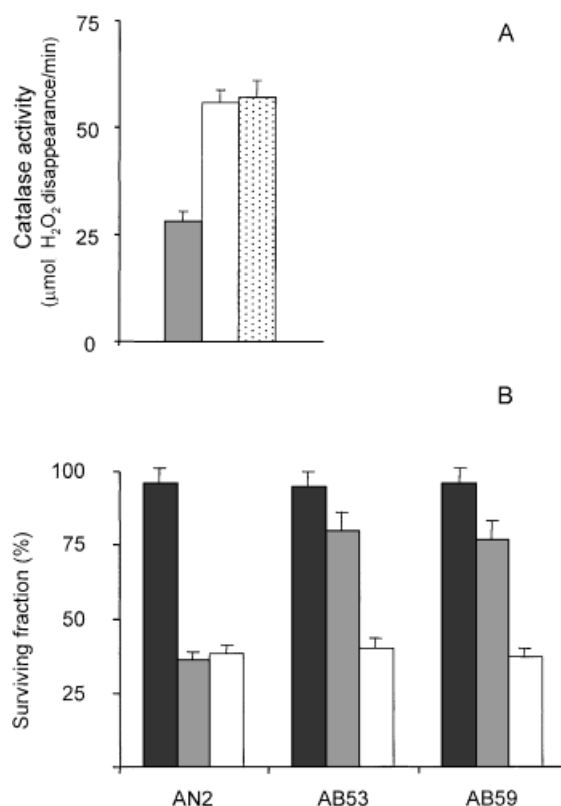

Fig. 6. **A**: Catalase activity in AN2 clone (gray column) and two *bcl*-2 transfectants AB53 (white column) and AB59 (dotted column). One unit of catalase activity corresponds to 1 μmol H_2O_2 disappearance/min. Values are the means of four experiments (bars, S.D.). **B**: Survival fraction of AN2 control cells and two *bcl*-2-transfectants (AB53 and AB59) exposed for 24 h at the dose of 50 μM BCNU alone (gray column) or preceded by 0.5 mM sodium azide treatment for 6 h (white column). Percentage of survival fraction relative to control is reported. The effect of sodium azide alone on the surviving fraction is also reported (black column).

(Fig. 6B). We found that treatment with a non-cytotoxic dose of sodium azide does not significantly affect the cell survival of AN2 cells treated with BCNU, the surviving fraction being 35% in the presence and 40% in the absence of the inhibitor. On the contrary, sodium azide reduces the BCNU sensitivity of *bcl*-2 transfectants to levels of the BCNU-treated control clone. In fact, while BCNU treatment determines a cell survival of about 75%, exposure to sodium azide followed by BCNU treatment reduces survival to about 40%, indicating that BCNU resistance in *bcl*-2 transfectants is due to the catalase activity. The effect of sodium azide on BCNU treated ADFS cells was similar to that on the AN2 clone (data not shown).

Bcl-2 Overexpression Decreases BCNU Sensitivity of ADFS Xenografts

To evaluate in vivo BCNU sensitivity, nude mice were i.m. implanted with AN2 control, AB53 and AB59 cells and treated with BCNU. A total dose of 90 mg/Kg BCNU was administered in mice bearing a tumor mass of about 200 mg. On the basis of preliminary experiments, the dose of BCNU was fractionated in three cycles with an interval time of 7 days. Each cycle was administered for two consecutive days injecting 15 mg/Kg starting the treatment at Day 4 after cells injection. This scheduling was well tolerated and did not produce any significant body weight reduction ($<10\%$) and toxic death. Table I shows the BCNU effect on the control clone and the *bcl*-2 transfectants. As appeared from the inconsistent tumor mass reduction (22 and 26%) and tumor growth delay (3 and 2 days) AB53 and AB59 clones were relatively unresponsive to BCNU treatment. Moreover, the lack of efficacy is confirmed by the survival of the mice that was superimposable to that of untreated mice. On the contrary, a partial response of AN2 tumors was observed at the end of BCNU treatment. In fact, about 50% of tumor inhibition is obtained. This effect was accompanied with a delay of 12 days in tumor regrowth and with a significant increase in animal survival (39%). These in vivo experiments clearly validate the different sensitivity observed in vitro. The response of the parental line is similar to that observed in the control clone (data not shown). Preliminary results obtained using an orthotopic implant confirm these results and demonstrated a reduced BCNU sensitivity of *bcl*-2 xenografts in terms of increase of life span.

Finally, to ascertain whether bcl-2 protein expression was maintained at a higher level in *bcl*-2 transfectants than control xenografts, we evaluated *bcl*-2 expression by immunohistochemistry. The staining intensity is remarkably higher in the AB53 clone compared to the AN2 control clone, demonstrating that the over-expression of the bcl-2 protein was maintained during tumor growth. The expression of bcl-2 on parental and AB59 xenografts was similar to that observed on AN2 and AB53 xenografts, respectively (data not shown).

DISCUSSION

In the present study we investigated whether *bcl*-2 is involved in the in vitro and in vivo sensitivity to BCNU of the ADFS glioblastoma line. To this end the ADFS line was transfected with an expression vector carrying the *bcl*-2 gene. The parental line, two *bcl*-2 transfectants and a control clone were used for in vitro and in vivo studies.

The up-regulation of *bcl*-2 observed in the two *bcl*-2 transfectants determines a reduced BCNU sensitivity both in vitro and in vivo. The dose of 75 μM was able to decrease in vitro cell survival of the ADFS parental line by about 80% whereas this same dose reduces in vitro cell survival of *bcl*-2 transfectants by about 30%. These in vitro results are supported by the in vivo data, where a significant difference in the tumor weight inhibition was observed between the parental mice treated with BCNU (50%) and *bcl*-2 xenografts treated with BCNU (22–26%). Furthermore, in vivo BCNU treatment of parental tumors determined a significant increase in life span (39%), whereas no differences were observed between untreated and BCNU– treated *bcl*-2 xenografts. Our results are in agreement with those of Weller et al. [1995] demonstrating the ability of *bcl*-2 to confer in vitro resistance to BCNU in glioma cell lines.

TABLE I. Antitumor Activity of BCNU After i.v. Treatment of the AN2 Control Clone and Two *bcl*-2 Transfectants (AB53 and AB59)

Lines	Treatment days[a]	Dose (mg/kg)	TWI (%)[b]	T−C[c] (days)	Toxicity[d]	Increase in life span (%)
AN2	4, 5−11, 12−18, 19	15	50	12	0/8	39
AB53	4, 5−11, 12−18, 19	15	26	3	0/8	0
AB59	4, 5−11, 12−18, 19	15	22	2	0/8	0

[a]Staged tumors (mean weight 200±20 mg) were i.v. treated with BCNU on days reported in the table. The day of tumor cell injection was considered Day 0.
[b]Tumor weight inhibition vs. untreated control group determined at Day 20 after tumor implant (Day 1 after the end of treatments).
[c]Tumor growth delay (mean times in days for treated and control tumors to reach the size of 1,000 mg).
[d]Number of toxic deaths/total number of mice.

Furthermore, to the best of our knowledge this is the first evidence indicating a role of *bcl*-2 in in vivo sensitivity to BCNU.

In addition, our results demonstrate that the overexpression of *bcl*-2, an antiapoptotic protein, determines a reduction of BCNU sensitivity, which is not associated with apoptosis inhibition. We found no differences in the ability of BCNU to induce apoptosis in the ADFS parental cells and the *bcl*-2 transfectants either in vitro or in vivo. Even at the highest doses of BCNU used for in vitro experiments (75 μM), and even when the analysis of apoptotic cells was performed several days after BCNU treatment, no more than 15% of apoptotic cells were observed in any of the lines with no significant differences between the control cells and the *bcl*-2 transfectants. Further, hardly any apoptotic cells were observed after in vivo BCNU treatment of control and *bcl*-2 overexpressing xenografts (data not shown). Moreover, BCNU doses higher than those used in this study, which can potentially induce apoptosis, correspond to doses that are not feasible in clinical management for their associated toxicity. Therefore, in our model system, *bcl*-2 inhibits BCNU induced necrotic cell death suggesting, as reported by other authors, some common steps in the necrotic and apoptotic death signaling pathways [Shimizu et al., 1996a, 1996b]. Moreover, results from Lee et al. [2000] indicate that apoptosis occurs in a rat glioma cell line only at a very high concentration of BCNU (500 μM for 48 h) while treatment with a lower dose of BCNU (50 μM for 48 h) resulted in a percentage of apoptotic cells similar to that of the control cells.

We also demonstrated that, despite the same kinetic cell cycle distribution of the *bcl*-2 overexpressing clones and of the control clone, BCNU determines an accumulation in the S/G2-M phases exclusively in the control clone. Thus, we hypothesized that the BCNU reduced cytotoxic effect on *bcl*-2 transfectants could be due to alterations on classical determinants of BCNU resistance, such as the expression of MGMT, or the pattern of antioxidant defenses induced by *bcl*-2 enforced expression. We found that parental cells and *bcl*-2 transfectants are negative for MGMT protein expression and no differences in GSH content, and the activity of superoxide dismutase and glutathione peroxidase, were observed after *bcl*-2 overexpression. On the contrary, higher levels of catalase activity were observed in the *bcl*-2 transfectants when compared to the parental line, suggesting that this increased enzymatic activity could be related to BCNU sensitivity. Proof of the close relationship between the BCNU effect and catalase activity appears from the cell survival obtained when BCNU treatment of the two *bcl*-2 transfectants was preceded by exposure to the catalase inhibitor sodium azide. We suggest that BCNU may induce oxidative cell damage through free radical injuries and that *bcl*-2 may induce resistance to BCNU through enhancement of the antioxidant enzyme catalase [Hemnani and Parihar, 1998; Izumi et al., 2000; Wang et al., 2000]. Shift of the cellular oxidation-reduction potential to a reduced state by *bcl*-2 has also been found by other authors [Hockenbery et al., 1993; Ellerby et al., 1996; Papadopoulos et al., 1998] thus indicating that protection by *bcl*-2 in part reflects potentiation of the antioxidant defense. To the best of our knowledge this is the first evidence indicating *bcl*-2 ability to increase catalase activity in human tumors. In agreement with our data Fandrey's group demonstrated that basal *bcl*-2 expression was significantly higher in HL60 cells characterized by an elevated endogenous catalase activity compared to the HL60 parental cells that show low level of bcl-2 protein and catalase activity [Katschinski et al., 2000]. In addition, the role of catalase on resistance has recently been demonstrated [Tome et al., 2001]. Tome et al. [2001] found that catalase-overexpressing thymocytes are resistant to dexamethasone.

In conclusion, our data demonstrated that in vitro and in vivo BCNU sensitivity in a glioblastoma line depends on the *bcl*-2 expression. The effect of *bcl*-2 on BCNU sensitivity is not due to the effect of *bcl*-2 on cell proliferation or apoptosis but to the effect of *bcl*-2 on the catalase activity.

Taken together, these results, the role of *bcl*-2 on the metastatic and angiogenic potential [Del Bufalo et al., 1997; Biroccio et al., 2000] and recent clinical studies which demonstrate a clear antitumoral effect of a *bcl*-2 antisense oligonucleotide (G-31319, Genta) in malignant melanoma [Jansen et al., 1998], indicate that the use of *bcl*-2 antisense in combination with BCNU could be useful in glioma treatment. Preclinical studies devoted to evaluate the efficacy of this combination are necessary.

ACKNOWLEDGMENTS

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