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Transfection of C6 glioma cells with the bax **gene and increased sensitivity to treatment with cytosine arabinoside**

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Genes known to be involved in the regulation of apoptosis include members of the **bcl-2** gene family, such as inhibitors of apoptosis (**bcl-2** and **bcl-xl)** and promotors of apoptosis (**bax**). The authors investigated a potential approach for the treatment of malignant gliomas by using a gene transfection technique to manipulate the level of an intracellular protein involved in the control of apoptosis.

The authors transfected the murine **bax** gene, which had been cloned into a mammalian expression vector, into the C6 rat glioma cell line. Overexpression of the **bax** gene resulted in a decreased growth rate (average doubling time of 32.96 hours compared with 22.49 hours for untransfected C6, and 23.11 hours for clones transfected with pcDNA3 only), which may be caused, in part, by an increased rate of spontaneous apoptosis (0.77 ± 0.15% compared with 0.42 ± 0.08% for the vector-only transfected C6 cell line; p = 0.038, two-tailed Student's t-test). Treatment with 1 µM of cytosine arabinoside (ara-C) resulted in significantly more cells undergoing apoptosis in the cell line overexpressing **bax** than in the vector-only control cell line (23.57 ± 2.6% compared with 5.3 ± 0.7% terminal deoxynucleotidyl transferase-mediated biotinylated-deoxyuridine triphosphate nick-end labeling technique-positive cells; p = 0.007). Furthermore, measurements of growth curves obtained immediately after treatment with 0.5 µM ara-C demonstrated a prolonged growth arrest of at least 6 days in the cell line overexpressing **bax**.

These results can be used collectively to argue that overexpression of **bax** results in increased sensitivity of C6 cells to ara-C and that increasing **bax** expression may be a useful strategy, in general, for increasing the sensitivity of gliomas to antineoplastic treatments.

Key Words * apoptosis * chemotherapy * brain tumor * bcl-2 **gene family * gene therapy * cell cycle * rat**

The prognosis for patients with malignant gliomas remains dismal, a fact that has sustained interest in the development of new therapeutic strategies. Multiple chemotherapeutic agents have been directed at interrupting processes vital for cell cycle progression and/or cellular growth and proliferation, but recently interest in the ability of these agents to induce programmed cell death (apoptosis) in tumors has increased.[18] However, gliomas are resistant to most chemotherapeutic agents. An increased understanding of the genes involved in the regulation of apoptosis has led to the hypothesis that dysregulation of apoptosis underlies tumor development, progression, and chemoresistance[8,19,36] and

that therapies directed at altering the levels of expression of these genes may mediate and/or potentiate the effects of commonly used chemotherapeutic agents.[4,10,19,38]

The **bcl-2** gene family is composed of a group of related genes that either promote or prevent apoptosis.[5,25,34] Members of the family include genes such as **bcl-2**, which is antiapoptotic, and **bax**, which is proapoptotic. Another **bcl-2** gene family member, **bcl-x**, produces two transcripts, **bcl-x_L** (long), which is antiapoptotic, and **bcl-x_S** (short), which is proapoptotic. These various proteins share significant homology and form either hetero- or homodimers. Heterodimerization of BCL-2 and BAX inhibits apoptosis, whereas homodimerization of BAX promotes apoptosis. The ratio of expression of pro- and antiapoptotic genes likely determines whether a cell lives or dies after an insult.[24] Overexpression of **bcl-2** or **bcl-x** protects cells from apoptosis after a number of different insults,[8,10] whereas overexpression of **bax** renders cells more sensitive to apoptosis-inducing stimuli.[4,32,38] Similarly, loss of **bcl-2** or **bcl-x** can result in excessive apoptosis during development, whereas loss of **bax** can prevent apoptosis.[13,15,22]

The molecular characterization of gliomas has revealed a number of abnormalities, such as **p53** mutations, amplification of the epidermal growth factor receptor, and overexpression of **ras**.[2,35,37] However, there are no data supporting the role of mutations or dysregulation of **bcl-2** gene family members in the pathogenesis or progression of these tumors. A recent study of human gliomas noted an association between wild-type **p53** and **bcl-2** overexpression, whereas in most cases gliomas with mutant **p53** did not show **bcl-2** overexpression.[1] The only study directed at screening for **bax** gene mutations was negative.[9] However, gliomas are sensitive to manipulations of the levels of expression of **bcl-2**; several investigators have shown that production of **bcl-2** overexpression in glioma cell lines protects them from different types of apoptosis-inducing stimuli.[17,23,39] The effect of **bax** overexpression in gliomas has not yet been characterized.

Gene transfection strategies directed at treating gliomas have included overexpression of **p53** or **s-Myc**, tumor necrosis factor alpha, $p21^{WAF1/CIP1}$, interleukin-1ß converting enzyme, antisense glial fibrillary acidic protein complementary DNA, and viral thymidine kinase.[3,11,16,20,27,29,40] In general, these manipulations have slowed tumor growth, and in one study, adenovirus-mediated transfection of **p53** caused spontaneous apoptosis in three of six tumor lines examined.[11] Initial reports of experiments with **bax** overexpression in breast and ovarian carcinomas have demonstrated dramatic increases in sensitivity to chemotherapeutic agents.[4,32,38]

The rat C6 glioma cell line, like most malignant gliomas encountered clinically, is resistant to DNA-damaging treatments. The C6 glioma cells typically do not undergo apoptosis after even high doses of cytosine arabinoside (ara-C) or ionizing radiation. Thus, C6 is a reasonable glioma cell line in which to test the hypothesis that overexpression of **bax** alone is sufficient to increase sensitivity to an apoptosis-inducing stimulus. In this report, we describe the effect of **bax** overexpression in C6 cells. Our findings have led us to suggest that manipulation of the relative levels of **bcl-2** family members may be a useful strategy for increasing the sensitivity of gliomas to conventional chemotherapeutic agents.

MATERIALS AND METHODS

*Generation of a Stable Cell Line Overexpressing Murine **bax***

The murine **bax** gene was cloned into the pcDNA3 mammalian expression vector and transfected into the C6 rat glioma cell line with a cationic lipid reagent as recommended by the manufacturer. Murine

bax codes for a protein that has more than 98% homology to rat BAX. Transfected cells were grown and selected in the presence of G418 (500 mg/ml) for 10 to 14 days prior to the isolation of individual clones, and 12 clones were expanded. Clones overexpressing BAX were identified by Western blot analysis. Simultaneously, we created lines of C6 cells transfected with the pcDNA3 vector only (C6.pcDNA3). We tested several C6.pcDNA3 clones that have growth characteristics similar to untransfected C6 and selected one for use as a control line (C6.pcDNA3.10) in the experiments described in this report. The C6.pcDNA3.10 line demonstrated the same growth rate and resistance to chemotherapy as the parent C6 cell line. The C6 cells were maintained in standard medium consisting of Dulbecco's modified Eagles medium with 15% newborn bovine serum, 0.6% neomycin, 2 mM L-glutamine, and penicillin/streptomycin (100 µg/ml). Clones were maintained in the same medium plus G418 (500 mg/ml). All experiments were performed in G418-free medium.

Assessment of Proliferation

We used a modified version of a previously described colorimetric assay to measure growth curves.[31] Cells were plated into 96-well microplates at a density of 1000 cells per well in 200 µl of medium. Individual plates were used for each time point, with three to five wells plated for each cell type and treatment condition. After treatment, the cells in each plate were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at intervals ranging from 0 to 48 hours. After fixation, plates were washed with PBS, stained with 1% crystal violet for 4 hours, washed with distilled water, and allowed to air dry. The crystal violet was solubilized with 200 µl of 1% sodium dodecyl sulfate (SDS) per well. Optical density (OD) was determined with the aid of a microplate reader with absorbance read at 570 nm. The ODs were averaged for each cell type and time point. We verified the accuracy of this assay independently and determined its accurate working range by comparing assessments of cell number made using the colorimetric assay with those made using traditional cell counts.

Determination of Doubling Time

Data obtained from the proliferation assay were fitted with a logarithmic regression ($y = b*m^x$, where y = OD reading and x = time). The coefficients b and m were determined by using a commercially available curve-fitting program. The doubling time was calculated by the following formula: Doubling Time = ln(2)/ln(m).

Quantitative Assessment of Apoptosis

We used two techniques to assess evidence of apoptosis. The presence of chromatin condensation was determined in cells plated on 35-mm plastic petri dishes modified for ultraviolet (UV) illumination; a glass coverslip was glued over a 15-mm diameter hole made in the center of the dish. Cells were fixed in 4% paraformaldehyde, washed with PBS, and stained with **bis**-benzamide, a fluorescent DNA-binding dye. The cells were viewed at 345/460-nm wavelengths with a fluorescent microscope equipped with a UV filter, and the number of cells showing chromatin condensation were counted. Cells that demonstrated several clumps of densely coalesced nuclei were counted as apoptotic, and nuclear fragments that were not associated with cytoplasm, when viewed under phase-contrast light microscopy, were not counted.[33] Three random X 200 fields were counted in this manner. Phase-contrast microscopy was used to determine the total cell count for each field examined, and the percentage of cells demonstrating chromatin condensation was calculated.

Cells were also stained by using terminal deoxynucleotidyl transferase-mediated

biotinylated-deoxyuridine triphosphate nick-end labeling (TUNEL). This assay reveals nuclei undergoing DNA fragmentation in a manner specific for apoptosis. Cells were washed with PBS and fixed with 4% paraformaldehyde in PBS for 1 hour, after which they were washed with PBS and blocked for 15 minutes in proteinase K. Prelabeling and labeling were performed with a commercially available TUNEL kit, which was used according to the manufacturer's instructions. FluoroLink Cy3-Streptavidin was added at a dilution of 1:1000 and the samples were incubated in the dark for 20 minutes, washed with PBS, and viewed with the aid of a UV light-equipped microscope.

Western Protein Immunoblotting

Cells that were in the growth phase were washed twice in PBS, scraped, and pelleted. The cell pellet was lysed in RIPA buffer (0.15 M NaCl; 0.01 M Tris, pH 7.5; 1% Triton X-100; 1% Nadeoxycholate; 1% SDS; 0.001% ethylenediamine tetraacetic acid) containing the protease inhibitors aprotinin (1:100) and leupeptin (1:100) and incubated on ice for 20 minutes. The cells were centrifuged again and the supernatant was transferred to a fresh microcentrifuge tube. The total protein concentration of each protein lysate was determined by using a modified Bradford reaction. Equal amounts of protein (50 µg) from each lysate were denatured in 2 X Laemmli buffer and loaded on a 15% SDS-polyacrylamide gel for SDS-polyacrylamide gel electrophoresis (PAGE) with appropriate protein standards loaded in one well. After electrophoresis, the proteins were transferred onto Immobilon-P membranes. Each membrane was blocked in nonfat milk (5%) in Tris-buffered saline (20 mM Tris, pH 7.5; 150 mM NaCl) with 0.05% Tween, and blotted with a primary antibody against BAX (#651, 1:500 dilution) followed by an anti-rabbit immunoglobulin G horseradish peroxidase-labeled secondary antibody (1:5000). The reaction was developed by means of a chemiluminescence reagent and exposed on autoradiography film. Bands were digitized and integrated densities were determined by means of commercially available software. The anti-BAX antibody used does not cross react with BCL-2 or BCL-X (S Korsmeyer, personal communication, 1996)**.**

Fluorescence Analysis

A monolayer of cells was fixed in cold 70% ethanol for at least 2 hours. After PBS washes, the cells were treated with RNase A (100 µg/ml in PBS) for 30 minutes at room temperature. Cells were stained with propidium iodide at a final concentration of 25 µg/ml for 1 hour. Cell cycle analysis was conducted on a fluorescence-activated cell sorter (FACS) 440 flow cytometer interfaced to a data acquisition/analysis system. The resulting DNA histograms were analyzed for their cell phase distribution with the aid of commercially available software.

Sources of Supplies and Equipment

The pcDNA3 mammalian expression vector was purchased from Invitrogen, Carlsbad, CA, and the cationic lipid reagent (Lipofectamine) and standard medium from Life Technologies, Grand Island, NY. The curve-fitting program (Microsoft Excel) was acquired from Microsoft Corp., Redmond, WA. The **bis**-benzamide (Hoechst 33258) molecular probe was purchased from Hoechst Pharmaceuticals, Eugene, OR. The TUNEL kit was obtained from Trevigen, Gaithersburg, MD, and the FluoroLink Cy3-Streptavidin, protein standards, and ECL chemiluminescence reagent were purchased from Amersham International, Buckinghamshire, England.

The UV light-equipped microscope was purchased from Nikon, Tokyo, Japan. The test kit for the Bradford reaction was purchased from Biorad, Inc., Hercules, CA. The Immobilon-P membranes were

acquired from Millipore, Bedford, MA. The secondary antibody and autoradiography film were obtained from Caltag, Burlingame, CA, and Eastman Kodak, Rochester, NY, respectively. The FACS 440 flow cytometer was purchased from Becton Dickinson, Mountain View, CA, the CICERO data acquisition/analysis system from Cytomation, Fort Collins, CO, and the MCYCLEAV distribution analysis software from Phoenix Flow Systems, San Diego, CA. Unless otherwise noted, all reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

RESULTS

Generation of Clones With Stable Overexpression of BAX

We transfected C6 cells with the full-length murine **bax** gene cloned into the pcDNA3 vector or with the pcDNA3 vector alone. Lysates from 12 independent clones derived from the transfection with the vector containing the **bax** gene were analyzed by SDS-PAGE/Western blotting with an antibody against BAX. A total of five clones demonstrated **bax** overexpression. Figure 1A shows an example of a Western blot test used to screen for BAX expression; two clones (C6.Bax.4 and C6.Bax.7) showed relatively high levels of expression. In Fig. 1B, BAX expression in one of these clones (C6.Bax.7) is compared with that demonstrated by the parent C6 cell line and with a cell line transfected with the vector only (C6.pcDNA3.10). Tubulin expression was used to verify that equal amounts of protein were loaded in each lane. Bands were digitized, analyzed by means of the NIH Image software, and a BAX/tubulin ratio was calculated for each lane (C6:0.30; C6.pcDNA3.10:0.35; C6.Bax.7:0.65). Hence, BAX was expressed at levels more than twofold greater in the C6.Bax.7 cell line than in the parent C6 line. This blot is representative of several blots performed with separate lysates; in all cases BAX expression was at least twofold greater in the transfected cells.

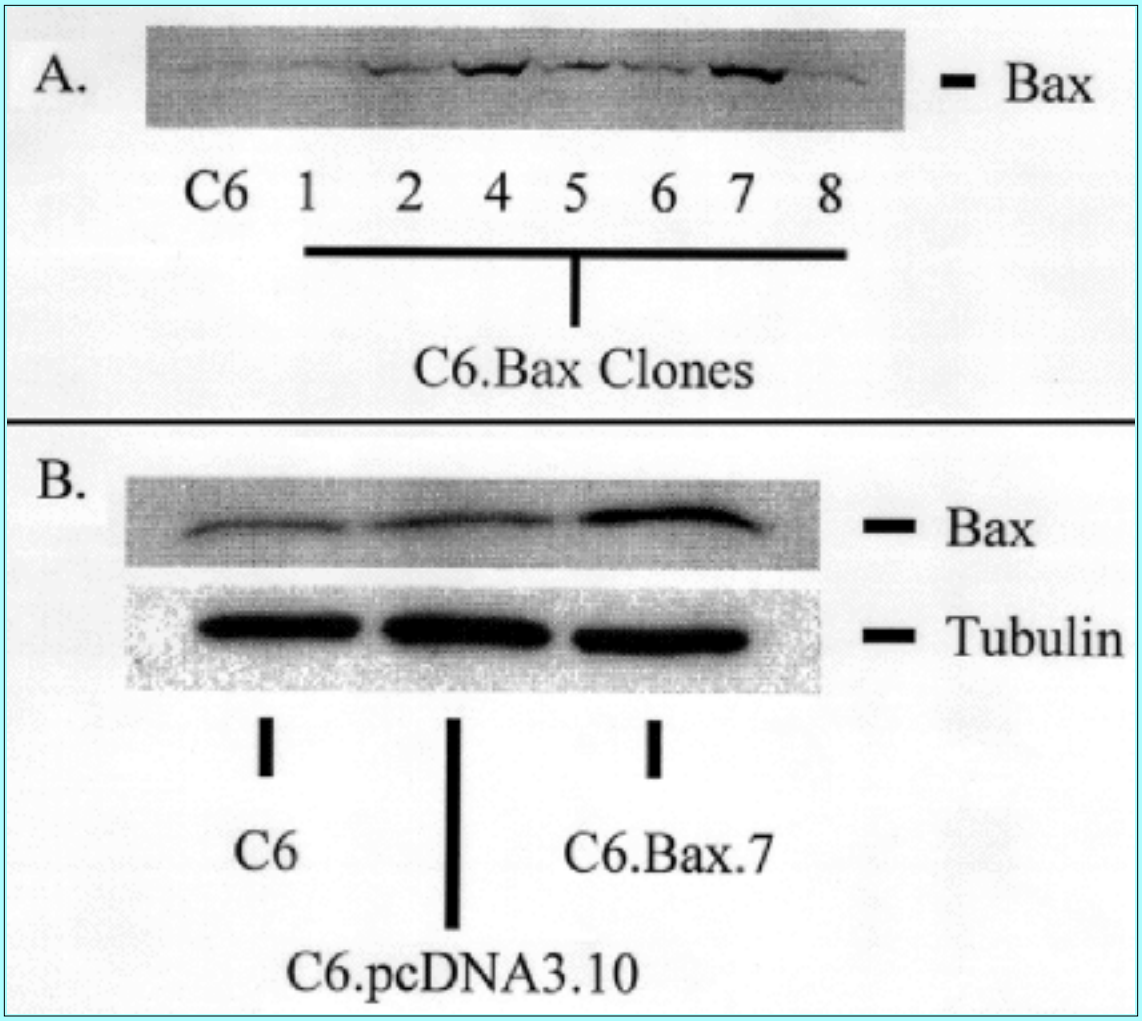

Fig. 1. Western blot gels showing BAX expression. A: Example of a blot used to screen BAX expression in transfected clones. Each lane was loaded with 50 µg of protein. The highest levels of BAX expression were seen in clones C6.Bax.4 and C6.Bax.7. B: Quantitative analysis of BAX expression by untransfected C6 and C6 cell lines transfected with vector only (C6.pCDNA3.10), or with the vector containing the murine **bax** gene (C6.Bax.7). Equivalent amounts of protein (50 µg) were loaded in each lane and the blots were probed for BAX and tubulin. Note the similar expression of tubulin in each cell line and overexpression of **bax** in C6.Bax.7.

Overexpression of** bax **and Growth Rate of C6 Glioma Cells

The growth rates of two clones overexpressing BAX (C6.Bax.4 and C6.Bax.7), four vector-only control lines, and the parent C6 line were assessed by means of the microplate growth assays (Fig. 2). Doubling time was determined by fitting a logarithmic function to each growth curve (Table 1). The doubling time for the C6 cell line was 22.49 hours (r = 0.96) and for the vector-only control lines it ranged between 22.46 and 24.94 hours (23.31 ± 1.11 hours [mean ± standard deviation {SD}], r = 0.95-0.99). The doubling time for two of the cell lines overexpressing **bax** was 32.51 (r = 0.93 [C6.Bax.4]) and 33.42 hours (r = 0.98 [C6.Bax.7]). Hence, both lines overexpressing the **bax** gene had slower growth rates than the parent C6 and the vector-only control lines, which had similar growth rates.

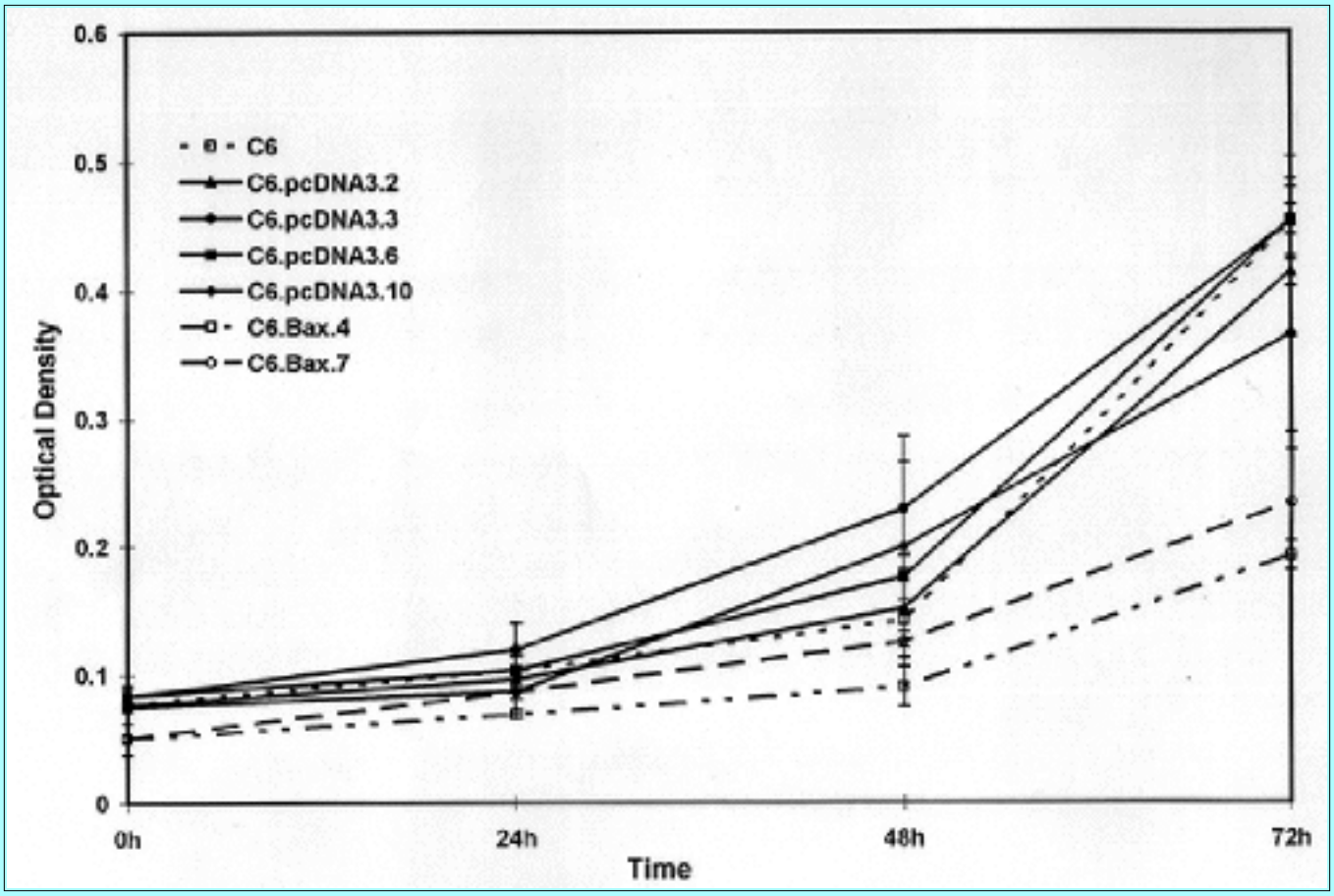

Fig. 2. Graph showing growth curves of C6 cells, C6 clones transfected with vector only or with vector containing the **bax** gene. Individual points were determined by means of a colorimetric assay. Each point represents the mean ± SD of five wells for the C6 and vector-only clones and the mean ± SD of three wells for the clones transfected with **bax**. Zero hour is the first time point for cell measurement, usually 24 hours after plating. Cells were examined in exponential growth phase. By 72 hours of growth, a distinct difference in growth rates was observed between the clones transfected with **bax** and those transfected with vector only or untransfected.

TABLE 1
DOUBLING TIMES OF SELECTED CLONES OF C6 GLIOMA CELLS

Clone	Doubling Time (hrs)	No. of Wells*
C6 (parent)	22.49	5
C6.pcDNA3.2	22.74	5
C6.pcDNA3.3	24.94	5
C6.pcDNA3.7	22.46	5
C6.pcDNA3.10	23.08	5
C6.Bax.4	32.51	3
C6.Bax.7	33.42	3

* Number of microplate wells tested for each time point.

Cell cycle analysis of the two lines revealed no significant differences in the percentage of cells in the G_1, S, or G_2/M phases (Table 2). Examination of untreated C6.Bax.7 and C6.pcDNA3.10 cells stained using the TUNEL protocol revealed nearly twice as many TUNEL-positive C6.Bax.7 cells as C6.pcDNA3.10 cells (0.77 ± 0.15% compared with 0.42 ± 0.08%, respectively; $p = 0.038$, two-tailed Student's t-test). The similar cell cycle profiles of these two lines together with the increased rate of

spontaneous apoptosis indicate that the difference in doubling time may be caused in part by a higher rate of spontaneous apoptosis in the **bax** transfected cell line.

TABLE 2
CELL CYCLE ANALYSIS OF VECTOR-ONLY AND **BAX**-TRANSFECTED C6 CELLS

Phase (%)	C6.pcDNA3.10	C6.Bax.7
G_1	54.6	55.3
S	12.3	13.1
G_2/M	33.1	31.6

Sensitivity to Treatment With Ara-C

We treated C6.Bax.7 and C6.pcDNA.10 cells with ara-A for 72 hours at doses ranging from 0.1 to 5 µM and then assayed them for the occurrence of apoptosis with TUNEL staining or by applying morphological criteria after staining with **bis**-benzamide. Treatment of C6.pcDNA3.10 and C6.Bax.7 cells with 5 µM ara-C resulted in near-total cell loss in both clones, whereas treatment with 0.1 µM ara-C did not induce apoptosis in either clone. Treatment with 0.5 or 1 µM ara-C caused a large percentage of C6.Bax.7 cells to undergo apoptosis. In contrast, the same doses rarely caused apoptosis in C6.pcDNA3.10 cells (Fig. 3). The percentage of cells undergoing apoptosis after treatment with 0.5 or 1 µM ara-C was determined by calculating the fraction of TUNEL-positive cells in each low-power field examined (Fig. 4). These results demonstrate that overexpression of **bax** results in a more than fourfold increase in apoptosis (23.57 ± 2.6% compared with 5.3 ± 0.7% [mean ± SD], p = 0.007) after treatment with 1 µM ara-C.

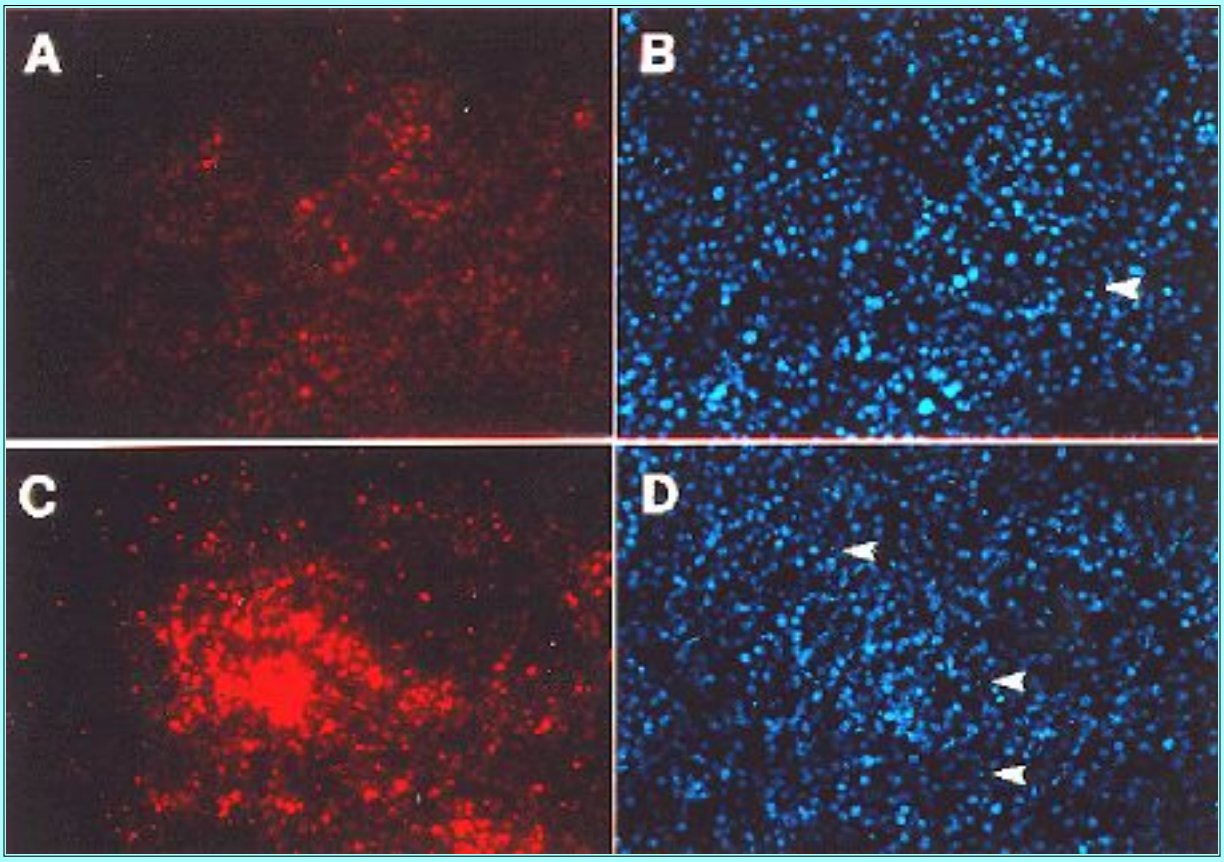

Fig. 3. Photomicrographs showing induction of apoptosis in C6 cells transfected with vector only (A and B) or with vector containing the **bax** gene (C and D). Cells were treated with

0.5 µM ara-C for 72 hours. They were stained using the TUNEL protocol (A and C), which reveals only nuclei of cells undergoing apoptosis, or with **bis**-benzamide (B and D), which stains all nuclei but allows for identification of apoptotic nuclei based on morphological criteria. Cells were viewed at X 200 magnification with the aid of a microscope equipped with fluorescent optics. Treatment of C6.pCDNA3.10 with ara-C produces only occasional TUNEL-positive cells (A) or chromatin condensation with **bis**-benzamide (B). Staining reveals most nuclei to be intact; in only the occasional cell is there fragmentation typical of chromatin condensation. C: In contrast, many TUNEL-positive C6.Bax.7 cells are seen after treatment with ara-C. D: The same cells as in (C) stained with **bis**-benzamide showing multiple nuclear fragments consistent with the frequent occurrence of chromatin condensation. **Arrowheads** point to examples of chromatin condensation revealed by staining with **bis**-benzamide (B and D).

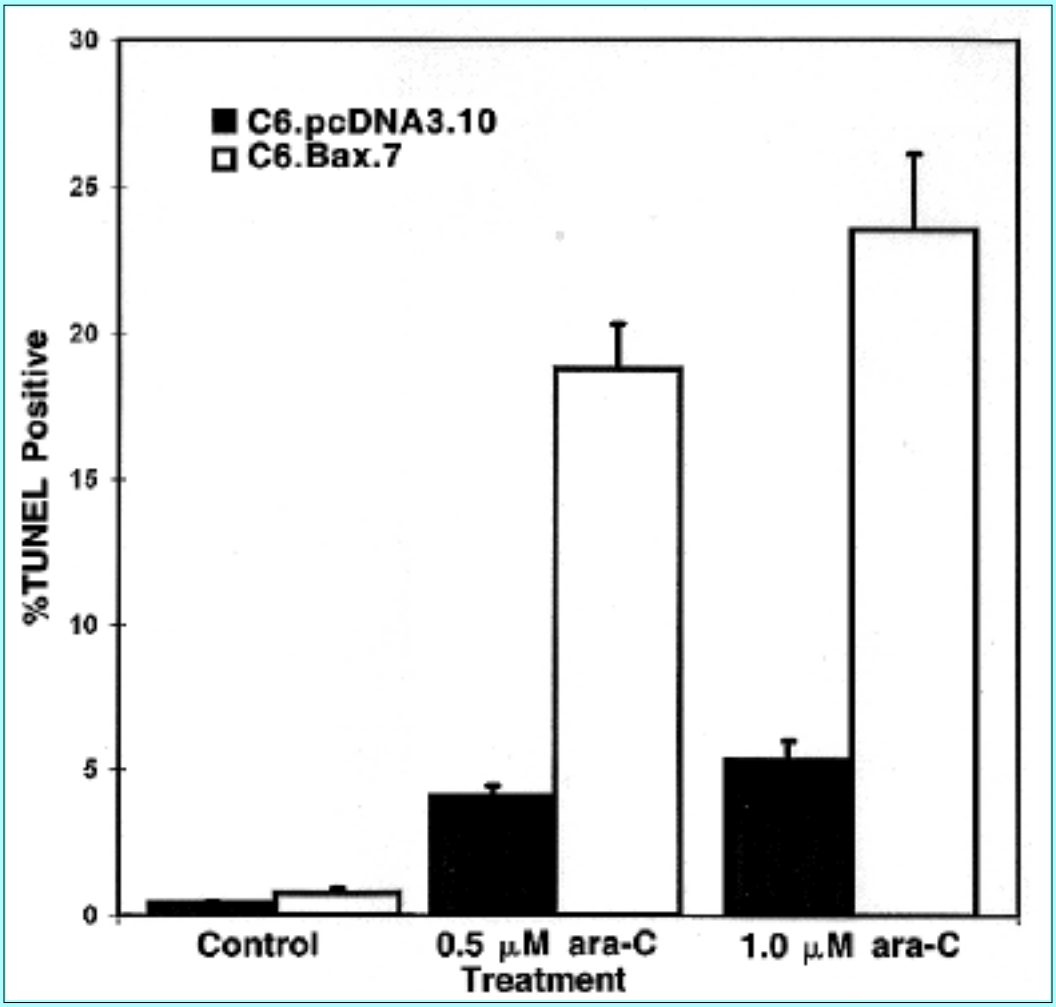

Fig. 4. Bar graph showing quantitative assessment of the prevalence of TUNEL-positive cells following treatment with 0.5 µM or 1 µM ara-C. The percentage of TUNEL-positive cells was determined for three separate high-power fields, and an average ± SD was determined. A significantly increased percentage of TUNEL-positive cells was seen in the C6.Bax.7 cells compared with the C6.pCDNA3.10 line at both doses ($p < 0.008$, two-tailed Student's t-test). A small but statistically significant increase in the percentage of TUNEL-positive cells was also seen in the untreated (control) C6.Bax.7 cells ($p = 0.038$, two-tailed Student's t-test).

Impairment of Growth in Glioma Cells Overexpressing BAX

To examine further the effects of ara-C on the proliferation of glioma cells overexpressing BAX, we measured growth curves after 72 hours of treatment with ara-C. Cells were plated, allowed to attach for 24 hours and treated with 0.5 µM ara-C. After 72 hours, the cells were washed with fresh medium to remove the ara-C. The cells were allowed to grow for 1 week, during which time proliferation was measured by means of the microplate assay. Hence, proliferation was assessed prior to treatment (which was used to normalize the assay for each clone), immediately following treatment, and at 48-hour intervals thereafter. Figure 5 shows that the C6.pCDNA3.10 cells continued to grow during treatment with and after washout of ara-C. In contrast, the C6.Bax.7 cells had a prolonged impairment of growth lasting more than 6 days after washout of ara-C. We assayed for the presence of apoptosis in these cells at 72 hours and 6 days after washout of ara-C, but saw only rare TUNEL-positive cells. The FACS analysis of these cells revealed a highly disturbed cell cycle pattern from which no clear picture of G_1 or G_2 arrest could be determined.

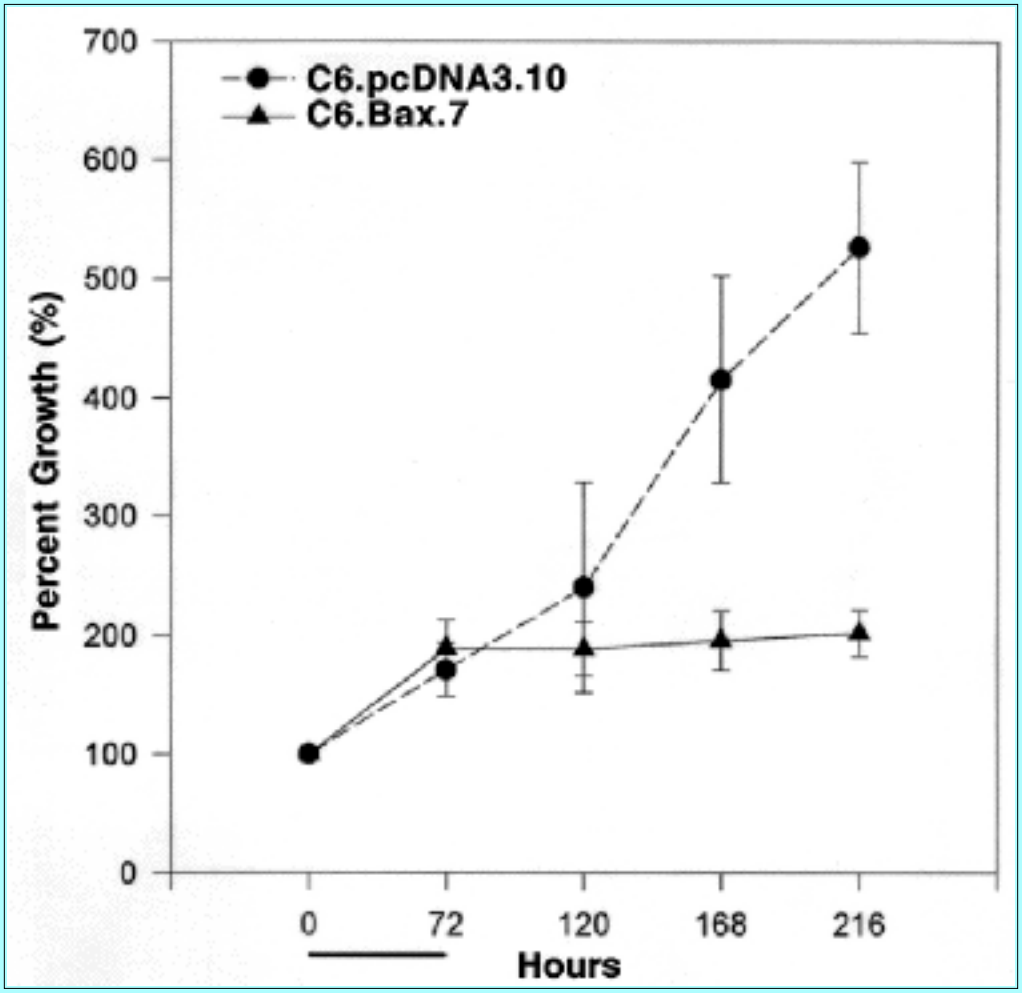

Fig. 5. Graph showing growth curve assay of C6.pcDNA3.10 and C6.Bax.7 cells after treatment with 0.5 µM ara-C. Each time point represents an average ± SD of three wells and is normalized to a point determined at the initiation of treatment (0 hour). Cells were treated with ara-C for 72 hours (bar under x axis), growth was assayed, and the drug was washed out and replaced with normal medium. Growth was determined at 48 hour intervals thereafter. Growth of the C6.Bax.7 cells was severely impaired over the 6 days after washout of the drug, despite regular replacement with fresh medium.

DISCUSSION

Our study shows that overexpression of **bax** in a rat glioma cell line results in significantly increased sensitivity to treatment with ara-C, which is a DNA-intercalating agent that has been used frequently in clinical chemotherapy protocols. Previous work in our laboratory has shown that untransfected C6 cells do not undergo apoptosis after treatment with even high doses of ara-C. Other investigators have noted that **bax** overexpression in ovarian[32] and breast[4,30,38] carcinomas has resulted in a similar increase in sensitivity to a variety of apoptosis-inducing stimuli. Interestingly, chemotherapy-induced apoptosis after **bax** overexpression does not necessarily require the presence of functional p53,[32] which makes **bax** overexpression an attractive therapeutic modality because up to 50% of malignant gliomas have mutations of **p53**.[2,28,35,37]

The slowing of the growth rate and slight increase in the rate of spontaneous apoptosis of the tumor lines overexpressing **bax** are in contrast to a previous report that **bax** overexpression without any subsequent treatment was insufficient to cause an increase in the rate of spontaneous tumor cell death.[32] However, that conclusion was based on an examination of trypan blue exclusion data only and did not involve direct determination of tumor cell growth rates. In another study, **bax**-transfected cells implanted into SCID mice without any additional treatment showed a reduced rate of tumor formation.[4] Our finding of a small but statistically significant increase in the rate of spontaneous apoptosis in untreated glioma cells overexpressing **bax** (Fig. 4) may be sufficient to explain the difference in growth rates over a long period of time. However, our experimental paradigm did not allow us to examine directly whether **bax** overexpression alone is sufficient to cause apoptosis; we do not think that the small increase in the rate of spontaneous apoptosis we observed is adequate to contradict previous data showing that **bax** overexpression does not cause apoptosis by itself.[38] Furthermore, we do not believe that this small decrease in the growth rate will have significant therapeutic potential by itself. However, our data support the hypothesis that **bax** acts to sensitize cells to apoptosis-inducing stimuli.

Treatment of glioma cells overexpressing **bax** resulted in a profound disruption of growth that lasted for many days. We did not observe a large increase in spontaneous apoptosis during this time interval, which was one possible hypothesis to account for the lack of growth after treatment with ara-C had ended. Our attempts to characterize the cell cycle by means of FACS analysis during this time interval revealed a highly disturbed pattern that could not be easily defined because statistically significant peaks were absent. Initial reports characterizing the effects of members of the **bcl-2** gene family on the cell cycle indicate that overexpression of **bcl-2** tends to slow the cell cycle[6,26] and overexpression of **bax** tends to speed it up or reverse the cell cycle-slowing effect of **bcl-2**.[6,7] A more detailed analysis of the effect of protracted treatment with ara-C on glioma cells overexpressing **bax** is in progress.

Many new antitumoral therapies have focused on mechanisms of inducing apoptosis. Strategies have included increasing the expression of functional p53, overexpression of tumor necrosis factor alpha, overexpression of $p21^{WAF1/CIP1}$, and overexpression of interleukin-1ß converting enzyme.[3,11,16,20,40] Protein kinase C inhibitors also cause apoptosis in gliomas.[12,14] The importance of apoptosis in mediating cell death from a potential antitumoral therapy in gliomas was noted by Weller, et al.,[39] who showed that overexpression of **bcl-2** prevented Fas/APO-1 antibody-mediated apoptosis. Also, manipulation of **p53** expression may serve to increase **bax** expression because p53 is a transcriptional activator of **bax**.[21] Hence, activation of specific signal transduction cascades or DNA transcriptional elements may result in altered levels of expression of members of the **bcl-2** gene family, which may enhance sensitivity to apoptosis-inducing stimuli, which

include chemotherapeutic agents and ionizing radiation. However, the association between the level of expression of members of the **bcl-2** gene family and response to therapy or outcome in gliomas remains to be determined.

CONCLUSIONS

In summary, overexpression of **bax** in the rat glioma cell line C6 results in slowing of the rate of growth and a dramatic increase in sensitivity to ara-C. These findings demonstrate that increasing the level of BAX in C6 glioma cells results in an increased sensitivity to apoptosis-inducing agents and indicate that direct manipulation of the level of expression of members of the **bcl-2** gene family may be a useful strategy for the treatment of primary brain tumors.

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References

1. Alderson LM, Castleberg RL, Harsh GR IV, et al: Human gliomas with wild-type p53 express bcl-2. **Cancer Res 55:**999-1001, 1995

2. Anker L, Ohgaki H, Ludeke BI, et al: p53 protein accumulation and gene mutations in human glioma cell lines. **Int J Cancer 55:**982-987, 1993

3. Asai A, Miyagi Y, Sugiyama A, et al: Negative effects of wild-type p53 and s-Myc on cellular growth and tumorigenicity of glioma cells. **J Neurooncol 19:**259-268, 1994

4. Bargou RC, Wagener C, Bommert K, et al: Overexpression of the death-promoting gene bax-alpha which is downregulated in breast cancer restores sensitivity to different apoptotic stimuli and reduces tumor growth in SCID mice. **J Clin Invest 97:**2651-2659, 1996

5. Boise LH, González-Gárcia M, Postema CE, et al: bcl-x, a bcl-2 related gene that functions as a dominant regulator of apoptotic cell death. **Cell 74:**597-608, 1993

6. Borner C: Diminished cell proliferation associated with the death-protective activity of Bcl-2. **J Biol Chem 271:**12695-12698, 1996

7. Brady HJM, Gil-Gómez G, Kirberg J, et al: Bax alpha perturbs T cell development and affects cell cycle entry of T cells. **EMBO J 15:**6991-7001, 1996

8. Chiou SK, Rao L, White E: bcl-2 blocks p53-dependent apoptosis. **Mol Cell Biol 14:**2556-2563, 1994

9. Chou D, Miyashita T, Mohrenweiser HW, et al: The BAX gene maps to the glioma candidate region at 19q13.3, but is not altered in human gliomas. **Cancer Genet Cytogenet 88:**136-140, 1996

10. Dole M, Nuñez G, Merchant AK, et al: bcl-2 protein inhibits chemotherapy-induced apoptosis in neuroblastoma. **Cancer Res 54:**3253-3259, 1994

11. Gomez-Manzano C, Fueyo J, Kyritsis AP, et al: Adenovirus-mediated transfer of the p53 gene

produces rapid and generalized death of human glioma cells via apoptosis. **Cancer Res 56:**694-699, 1996

12. Hamilton HB, Hinton DR, Law RE, et al: Inhibition of cellular growth and induction of apoptosis in pituitary adenoma cell lines by the protein kinase C inhibitor hypericin: potential therapeutic application. **J Neurosurg 85:**329-334, 1996

13. Hockenbery DM: bcl-2 in cancer, development and apoptosis. **J Cell Sci 18 (Suppl):**51-55, 1994

14. Ikemoto H, Tani E, Matsumoto T, et al: Apoptosis of human glioma cells in response to calphostin C, a specific protein kinase C inhibitor. **J Neurosurg 83:**1008-1016, 1995

15. Knudson CM, Tung KSK, Tourtellotte WG, et al: Bax-deficient mice with lymphoid hyperplasia and male germ cell death. **Science 270:**96-99, 1995

16. Kondo S, Barna BP, Kondo Y, et al: WAF1/CIP1 increases the susceptibility of p53 non-functional malignant glioma cells to cisplatin-induced apoptosis. **Oncogene 13:**1279-1285, 1996

17. Kondo S, Barnett GH, Hara H, et al: MDM2 protein confers the resistance of a human glioblastoma cell line to cisplatin-induced apoptosis. **Oncogene 10:**2001-2006, 1995

18. Lotem J, Sachs L: Regulation by bcl-2, c-myc, and p53 of susceptibility to induction of apoptosis by heat shock and cancer chemotherapy compounds in differentiation-competent and -defective myeloid leukemic cells. **Cell Growth Diff 4:**41-47, 1993

19. Lowe SW, Ruley HE, Jacks T, et al: p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. **Cell 74:**957-967, 1993

20. Merzak A, Raynal S, Rogers JP, et al: Human wild type p53 inhibits cell proliferation and elicits dramatic morphological changes in human glioma cell lines **in vitro**. **J Neurol Sci 127:**125-133, 1994

21. Miyashita T, Reed JC: Tumor suppressor p53 is a direct transcriptional activator of the human **bax** gene. **Cell 80:**293-299, 1995

22. Motoyama N, Wang F, Roth KA, et al: Massive cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice. **Science 267:**1506-1510, 1995

23. Murai N, Ueba T, Takahashi JA, et al: Apoptosis of human glioma cells in vitro and in vivo induced by a neutralizing antibody against human basic fibroblast growth factor. **J Neurosurg 85:**1072-1077, 1996

24. Oltvai ZN, Korsmeyer SJ: Checkpoints of dueling dimers foil death wishes. **Cell 79:**189-192, 1994

25. Oltvai ZN, Milliman CL, Korsmeyer SJ: bcl-2 heterodimerizes in vivo with a conserved homolog, bax, that accelerates programmed cell death. **Cell 74:**609-619, 1993

26. O'Reilly LA, Huang DCS, Strasser A: The cell death inhibitor Bcl-2 and its homologues influence control of cell cycle entry. **EMBO J 15:**6979-6990, 1996

27. Ram Z, Culver KW, Walbridge S, et al: In situ retroviral-mediated gene transfer for the treatment of brain tumors in rats. **Cancer Res 53:**83-88, 1994

28. Rubio MP, von Deimling A, Yandell DW, et al: Accumulation of wild type p53 protein in human astrocytomas. **Cancer Res 53:**3465-3467, 1993

29. Rutka JT, Hubbard SL, Fukuyama K, et al: Effects of antisense glial fibrillary acidic protein complementary DNA on the growth, invasion, and adhesion of human astrocytoma cells. **Cancer Res 54:**3267-3272, 1995

30. Sakakura C, Sweeney EA, Shirahama T, et al: Overexpression of bax sensitizes human breast cancer MCF-7 cells to radiation-induced apoptosis. **Int J Cancer 67:**101-115, 1996

31. Scragg MA, Ferreira LR: Evaluation of different staining procedures for the quantification of fibroblasts cultured in 96-well plates. **Anal Biochem 198:**80-85, 1991

32. Strobel T, Swanson L, Korsmeyer S, et al: BAX enhances paclitaxel-induced apoptosis through a p53-independent pathway. **Proc Natl Acad Sci USA 93:**14094-14099, 1996

33. Tornusciolo DR, Schmidt RE, Roth KA: Simultaneous detection of TDT-mediated dUTP-biotin nick end-labeling (TUNEL)-postive cells and multiple immunohistochemical markers in single tissue sections. **Biotechniques 19:**800-805, 1995

34. Tsujimoto Y, Finger LR, Yunis J, et al: Cloning of the chromosome breakpoint of neoplastic B cells with the t(14,18) chromosome translocation. **Science 226:**1097-1099, 1984

35. Van Meir EG, Kikuchi T, Tada M, et al: Analysis of the **p53** gene and its expression in human glioblastoma cells. **Cancer Res 54:**649-652, 1994

36. Vaux DL, Cory S, Adams JM: "**bcl**-2 gene promotes haemopoietic cell survival and cooperates with **c-myc** to immortalize pre-B cells. **Nature 335:**440-442, 1988

37. von Deimling A, Louis DN, Wiestler OD: Molecular pathways in the formation of gliomas. **Glia 15:**328-338, 1995

38. Wagener C, Bargou RC, Daniel PT, et al: Induction of the death-promoting gene **bax-a** sensitizes cultured breast-cancer cells to drug-induced apoptosis. **Int J Cancer 67:**138-141, 1996

39. Weller M, Malipiero U, Aguzzi A, et al: Protooncogene bcl-2 gene transfer abrogates Fas/APO-1 antibody-mediated apoptosis of human malignant glioma cells and confers resistance to chemotherapeutic drugs and therapeutic irradiation. **J Clin Invest 95:**2633-2643, 1995

40. Yu JS, Sena-Estaves M, Paulus W, et al: Retroviral delivery and tetracycline-dependent expression of IL-1beta-converting enzyme (ICE) in a rat glioma model provides controlled induction of apoptotic death in tumor cells. **Cancer Res 56:**5423-5427, 1996

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