Hypoxia-inducible expression of BAX: application in tumor-targeted gene therapy

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Object. The presence of hypoxic cells in human brain tumors contributes to the resistance of these tumors to radiation therapy. However, because normal tissues are not hypoxic, the presence of hypoxic cells also provides the potential for designing cancer-specific gene therapy. Suicide genes can be expressed specifically in hypoxic conditions by hypoxia-responsive elements (HREs), which are activated through the transcriptional complex hypoxia-inducible factor-1 (HIF-1).

Methods. The authors have transfected the murine *BAX–green fluorescent protein (GFP)* fusion gene under the regulation of three copies of HRE into U-87 MG and U-251 MG cells and selected stably transfected clones. Even though BAX was expressed under both oxic and anoxic conditions in these clones, cell survival assays demonstrated increased cell killing under anoxic as compared with oxic conditions. Cells obtained from most of these clones did not grow in vivo, or the tumors exhibited highly variable growth rates. However, cells obtained from the U-251 MG clone A produced tumors that grew as well as tumors derived from parental cells, and examination of the tumor sections under fluorescent microscopy revealed GFP expression in localized regions. Western blot analyses confirmed an increased BAX expression in these tumors. Analysis of the results suggests that HRE-regulated BAX can be a promising tool to target hypoxic brain tumor cells. However, there are measurable levels of BAX-GFP expression in this three-copy HRE-mediated expression system under oxia, suggesting promoter leakage. In addition, most clones did not show significant induction of BAX-GFP under anoxia. Therefore, the parameters of this HRE-mediated expression system, including HRE copy number and the basal promoter, need to be optimized to produce preferential and predictable gene expression in hypoxic cells.

KEY WORDS • BAX • hypoxia • gene therapy • glioblastoma multiforme • suicide gene

Therapy for malignant gliomas currently relies on a multimodality approach involving surgery, radiation therapy, and chemotherapy. However, the median survival time of patients with the most malignant glioma, GBM, is increased by only a few months when current treatments are applied.² After surgery, radiation therapy is the most effective treatment for patients with malignant gliomas,³⁹ and the efficacy of the treatment increases with increasing radiation dose.⁴⁰ The dose-limiting tissue is the normal brain that surrounds the tumor, and the pivotal issue in the radiation therapy of gliomas is clearly a matter of therapeutic ratio—doses of radiation that are tolerable to the patient are insufficient to control the tumor. Clearly, there is a need to devise strategies that markedly improve the therapeutic ratio, and this will require that we exploit differences between normal and malignant tissues.

One important abnormal characteristic found in most solid tumors, including human brain tumors, is the pres-

ence of regions with reduced oxygen concentrations.^{18,22} From a radiobiological perspective, these hypoxic cells are the most important subpopulation because they are much more resistant than oxic cells to radiation-induced damage.¹³ Historically, the radiobiologically important hypoxic cells have been thought to be those that arise in tumors when tumor growth produces cells that are further than 150 μm from a blood vessel.³⁴ Such chronic hypoxia is produced by an insufficient blood supply that occurs partly because tumor cells grow faster than the endothelial cells that make up the blood vessels and partly because the newly formed vascular supply is disorganized.³⁶ Acute hypoxia can also develop due to intermittent blood flow, a phenomenon that is known to occur in a variety of disease states including tumors.^{4,7,37} Hypoxia affects the proliferation of cancer cells and their responsiveness to radiotherapy, because anoxic cells ($\text{PO}_2 \leq 0.5$ mm Hg) require approximately three times the radiation dose needed to kill

Abbreviations used in this paper: cDNA = complementary DNA; CMEM = Complete Eagle's minimum essential medium; Epo = erythropoietin; GBM = glioblastoma multiforme; GFP = green fluorescent protein; HIF-1 = hypoxia-inducible factor-1; HRE = hypoxia-responsive element; PBS = phosphate-buffered saline; SDS = sodium dodecylsulfate.

oxic cells ($PO_2 \geq 20$ mm Hg).¹³ Cells at intermediate oxygen levels (0.5 mm Hg $< PO_2 < 20$ mm Hg) display intermediate sensitivities to radiation and are also important contributors to the overall efficacy of radiation therapy.⁴⁴

The investigators of several studies have shown that the regulation of gene expression by oxygen is an important feature of many biological processes, and hypoxia is a powerful modulator of gene expression.^{23,28,29} Therefore, it should be possible to target hypoxic cells by developing a gene therapy strategy that uses plasmids containing suicide genes that are selectively expressed under hypoxic conditions. One example of hypoxia-regulated gene expression is provided by *Epo*, a hormone that regulates erythropoiesis in accordance with the oxygen-carrying capacity of the blood. The *Epo* gene contains an HRE consensus sequence in the 3' enhancer region.⁵ Hypoxia-inducible factor-1 is a transcription factor that accumulates in hypoxic cells, binds to HREs, and "turns on" the promoter regulating *Epo*.³⁰ Previously, we transfected plasmids containing three copies of HRE derived from the human *Epo* gene into human brain tumor cells and tested their ability to induce *LacZ* gene expression under oxic and anoxic conditions.²⁴ Gene expression under anoxic conditions increased approximately 12-fold for U-87 MG cells and approximately fourfold for U-251 MG cells, indicating HRE is functional in both brain tumor cell lines.

In the present study, we used HREs to regulate expression of the *BAX* suicide gene in response to hypoxia. A member of the Bcl-2 family of proteins, *BAX* can initiate apoptotic cell death in cell culture and in animals.^{14,21,46} We demonstrated previously that cells transiently transfected with *BAX* under HRE regulation are preferentially killed through apoptosis in anoxic conditions.²⁴ In this present study, we transfected a vector containing a *BAX-GFP* fusion gene under the regulation of three HREs into two human GBM cell lines, and we isolated clones that were stably transfected with this plasmid. The GFP marker protein allows us to determine quickly the level and location of *BAX* expression in cells and in animal xenografts. The *BAX-GFP* fusion protein has been successfully used by other investigators, who found that the *BAX-GFP* fusion protein can produce cell killing as effectively as the *BAX* protein.⁴² Stably transfected clones that were made from these cell lines exhibit increased cell-killing ability under anoxic conditions. We implanted cells from *BAX*-containing clones into the flanks of athymic mice, and one of these clones produced tumors that grew as well as parental wild-type tumors. We detected gene expression in tissues removed from these tumors by observing GFP via fluorescence microscopy and by measuring *BAX* expression using Western blot analysis. The results of our studies indicate that *BAX* can be stably transfected into GBM cells. Such cells will be important for conducting future in vitro and in vivo bystander effect studies.

MATERIALS AND METHODS

Formation of DNA Constructs Containing HREs

The expression vector pH3BG was used for all experiments (Fig. 1). In this vector, the cDNA for marker protein GFP was fused at the C-terminus of the murine *BAX*

Fig. 1. Structural map of plasmid pH3BG. The expression of the fusion gene *BAX-GFP* was under regulation of three copies of HRE and the SV40 minimal promoter. The neomycin-resistant cassette (NeoR) was driven by the SV40 early promoter/enhancer.

cDNA.⁴³ The fusion gene was placed under the transcriptional control of SV40 minimal promoter and three copies of HREs derived from the human *Epo* gene. In the control construct pH3GFP, GFP cDNA alone was under the control of SV40 minimal promoter and three copies of HREs. Both constructs contain a neomycin-resistant cassette under transcriptional control of the SV40 early promoter/enhancer, allowing stably transfected cells to be selected using G418.

Maintenance of Cell Cultures

Two human GBM cell lines, U-251 MG and U-87 MG, were maintained in CMEM, which consists of Eagle's minimum essential medium supplemented with nonessential amino acids, glutamine, and 10% fetal calf serum. Cultures were incubated in a humidified atmosphere containing 95% air and 5% CO_2 at 37°C.

Production of Stably Transfected Cells

Cells were stably transfected with pH3BG or pH3GFP by using Lipofectamine reagent. For U-87 MG, cells (2×10^5 /well) were plated into six-well dishes and transfected with 3.5 μ g of DNA and 5 μ l of Lipofectamine. For U-251 MG, cells (2×10^5 /well) were transfected with 5 μ g of DNA and 7.5 μ l of Lipofectamine. Thirty-six hours following transfection, growth medium was changed and cells were incubated in growth medium containing 600 mg/ml G418 to select for stably transfected clones. Growth medium was replaced with fresh medium containing G418 every 2 to 3 days to maintain the concentration of active G418. After 10 to 12 days of G418 selection, individual surviving clones were isolated and expanded in six-well dishes. Detectable levels of GFP expression were demonstrated in all clones under oxic conditions when examined using the fluorescence microscope. The G418-resistant clones were subjected to another round of selection, in which clones that expressed the highest levels of GFP (estimated using fluorescence microscopy) under anoxic conditions were selected for further analysis. Stably transfected clones were maintained in medium containing 600 μ g/ml G418.

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Induction of Hypoxia in Tumor Cells

Cells were seeded into glass petri dishes, allowed to attach overnight, and then placed into gas-tight aluminum gassing jigs. The jigs were subjected to five rounds of evacuation and flushing with either 95% air and 5% CO₂ (oxic conditions) or 95% N₂ and 5% CO₂ (anoxic conditions) on a shaking platform at room temperature. After the last round of gas exchange, the jigs were placed in a 37°C incubator and incubated for 16 hours.

Measurement of Cell Survival by Using a Colony-Forming Efficiency Assay

On the day before assay, SF-126 cells received a 40-Gy dose of irradiation and were plated in six-well dishes at a density of 5×10^4 cells/well to serve as a feeder layer. On the assay day, test cells were trypsinized with 0.05% trypsin, counted, and plated into wells containing the irradiated feeder layer; dishes were incubated for 14 days. The resulting colonies were stained with methylene blue (0.66% solution in 95% ethanol), and colonies containing at least 50 cells were counted. Plating efficiency was calculated as the ratio of the number of colonies formed to the number of cells seeded. The survival fraction was calculated as the plating efficiency of cells that were exposed to anoxic conditions divided by that of cells that were kept under oxic.¹²

Western Protein Immunoblotting

Total cellular protein was extracted from cell lines or tumor tissues by lysing cells in 0.5 ml of PBS containing 0.5% deoxycholate, 1% Nonidet P-40, and 0.1% SDS at pH 7.4. To reduce sample viscosity, DNA was sheared by passage through an 18-gauge needle. Samples were boiled for 3 minutes, immediately cooled on ice, and spun for 5 minutes at 10,000 rpm in a microcentrifuge to remove insoluble material. Supernatants were assayed for protein concentration using the Bradford method³ and then stored at -70°C. Before electrophoresis, sample buffer containing bromophenol blue was added to reach a final concentration of 10% glycerol, 2% SDS, and 50 mM Tris-Cl, pH 6.8. Protein samples were boiled for 3 minutes and cooled prior to loading. Equal amounts of total protein were separated on a 13% SDS-polyacrylamide gel for 1.5 hours at 150 V by using a mini-gel apparatus. The protein gel was transferred to a polyvinylidene difluoride membrane for 1 hour at 100 V in transfer buffer containing 25 mM Tris-HCl, 40 mM glycine, and 20% methanol at pH 8.3. The membrane was stained for total protein by using Ponceau-S. The membrane was blocked with PBS containing 5% skim milk powder and 0.05% Tween 20 at pH 7.4. After incubation for 1 hour at room temperature, fresh blocking solution containing the anti-murine BAX antibody was added, and the membrane was incubated for 1 hour. The membrane was washed three times in PBS containing 0.05% Tween 20 at room temperature and followed by incubation with peroxidase-labeled secondary antibody for 1 hour at room temperature. After three washes in PBS combined with 0.05% Tween 20, the membrane was incubated for 1 minute in enhanced chemiluminescence buffer, and proteins were visualized on an x-ray film by using exposure times varying from 30 seconds to 5 minutes.

Implantation of Subcutaneous Tumors in Athymic Mice

Cells in culture were trypsinized, washed with CMEM to remove the enzyme, and harvested by centrifugation. The cell pellets were resuspended in CMEM, and 5×10^6 cells in 0.1 ml suspensions were injected subcutaneously into the flanks of 5-week-old female athymic mice according to procedures previously described.²⁰ The animals were examined daily until tumors became palpable. Thereafter, the length and width of the tumors were measured with calipers; these measurements were converted into volumes by multiplying the length $\times$ width² $\times$ 0.5. Each animal's weight was measured at least once a week. Protocols for all animal experiments were approved by the UCSF Committee on Animal Research.

Detection of GFP Expression by Using Fluorescence Microscopy

Cells were grown in petri dishes containing a coverslip, washed with PBS, and then fixed with 4% paraformaldehyde for 10 minutes. After fixation, cells were washed twice with PBS, stained for nuclei by using 10 µg/ml propidium iodide and immediately examined under fluorescence microscopy. The animals were killed by cervical dislocation, and the tumors were removed. Tissues were placed in embedding medium for frozen tissue and immediately snap-frozen in liquid nitrogen. Frozen tissues were cut into 8-µm sections, which were placed on slides, stained, and examined as described previously.

Sources of Supplies and Equipment

We obtained the Lipofectamine reagent and the G418 from Gibco BRL (Gaithersburg, MD). The mini-gel apparatus is manufactured by Bio-Rad (Hercules, CA). The polyvinylidene difluoride membrane and the chemiluminescence buffer were purchased from Amersham Corp. (Buckinghamshire, UK). We acquired the Ponceau-S from Sigma Co. (St. Louis, MO); the anti-murine BAX antibody from Genzyme Co. (Cambridge, MA); the athymic mice from Simonsen (Gilroy, CA); and the embedding medium for frozen tissue from Sakura Finetek USA Inc. (Torrance, CA). Dr. Mark Israel (Brain Tumor Research Center, UCSF) kindly provided the murine BAX cDNA. The cell lines were generously provided by the UCSF Neurosurgery Tissue Bank (San Francisco, CA).

RESULTS

Generation of Glioblastoma Clones With Stably Transfected BAX-GFP

After cells were transfected with pH3BG, 10 clones from U-251 MG cells and six clones from U-87 MG were selected by examining GFP autofluorescence under anoxic conditions. We also generated control clones of U-251 MG and U-87 MG cells that were stably transfected with pH3GFP. We conducted BAX confirmation studies by using Western blot methodology, and BAX was detected in nine of 10 U-251 MG clones and in three of six U-87 MG clones.

Two clones derived from each cell line were selected for additional study. The cells were incubated under either oxic or anoxic conditions for 16 hours before Western blot

Fig. 2. Western blot analysis indicating expression of BAX-GFP fusion protein in stably transfected U-87 MG and U-251 MG clones. After a 16-hour exposure to either oxic (O) or anoxic (A) conditions, BAX-GFP stably transfected U-87 MG and U-251 MG clones were harvested and lysed. Equal amounts (25 µg) of protein were analyzed by SDS–polyacrylamide gel, and immunoblotting was performed using an antimurine BAX antibody. The U-87 MG and U-251 MG cells that were transfected with pH3GFP were used as controls.

analysis. A single band of transfected BAX-GFP protein was observed in stably transfected clones, and no background band was detected in pH3GFP-transfected control cells (Fig. 2). As compared with the oxic control, exposure to anoxia induced BAX expression approximately twofold in U-251 MG clone A cells. However, there was either no induction or only modest induction of BAX under anoxia in the other three clones (Fig. 2). These Western blots were reprobed using GFP antibody. Within each cell line, we have observed similar levels of GFP expression in both control cells (GFP alone) and BAX-GFP transfected cells (data not shown).

Increased Cell Killing in BAX-GFP Stably Transfected Cells Under Anoxic Conditions

We also subjected the selected clones to oxic or anoxic conditions for 16 hours, after which they were collected and assayed for cell survival. Control U-87 MG cells were inherently sensitive to anoxia; approximately 50% of the cells survived after a 16-hour exposure (Table 1). However, expression of BAX under anoxia in U-87 MG clones produced significantly more cell killing, resulting in only approximately 10 to 20% cell survival. Under oxic condi-

tions, BAX-GFP-transfected U-87 MG cells survived as well as control cells. Table 2 shows the survival data for the U-251 MG control cells and selected clones. Compared with U-87 MG cells, control U-251 MG cells were more resistant to anoxia; approximately 90% of cells survived the 16-hour exposure. In BAX-GFP-transfected U-251 MG clones, cell survival decreased to 40 to 50% under anoxia. In contrast, both control and BAX-GFP-transfected U-251 MG cells were shown to have similar plating efficiencies under oxic conditions.

Implantation of BAX-GFP Stably Transfected Cells into the Flanks of Athymic Mice

To test the effect of BAX on killing hypoxic cells in vivo, we inoculated the four clones subcutaneously into the right flanks of adult female athymic mice. Each clone was injected into five mice. Three clones (U-251 MG Clone B and U-87 MG Clones C and D) did not grow as tumors or were shown to have highly variable growth rates among individual mice as compared with that seen in parental tumors. However, in all five mice injected with U-251 MG clone A cells tumors grew, and the tumor growth among the five mice was quite uniform (Fig. 3). The growth rate is comparable with that of control U-251 MG cells.²¹

BAX Expression in Flank Tumors

To determine whether BAX was expressed in tumors that developed from U-251 MG clone A cells, three tumors were removed from animals and protein lysates were prepared. Western blot analysis was performed using anti-BAX antibody. Strong expression of BAX-GFP protein was seen in all three clone A tumors (Fig. 4), whereas in a tumor derived from control U-251 MG cells no expression of the fusion protein was shown. To explore the distribution pattern of HRE-mediated BAX-GFP expression in tumors, tissues were embedded and cryosectioned, and GFP expression was examined by fluorescence microscopy. The GFP-BAX fusion protein was detected in the tumor tissue, and the intensity of GFP-BAX expression was variable among different regions (Fig. 5A). Compared with propidium iodine staining of the same slide (Fig. 5B), BAX-GFP expression was localized in distinct regions within the tumor, probably reflecting the heterogeneity in hypoxia that occurs within the tumors.

TABLE 1 Cell killing produced by stably transfected BAX under anoxic conditions in U-87 MG cells			
Clone	Oxygen Condition	Plating Efficiency*	Survival Fraction (95% CI)
control	oxic	0.52 ± 0.02	1
	anoxic	0.26 ± 0.02	0.5 (0.48, 0.53)
clone C	oxic	0.47 ± 0.02	1
	anoxic	0.09 ± 0.01	0.19 (0.14, 0.24)
clone D	oxic	0.64 ± 0.02	1
	anoxic	0.08 ± 0.01	0.12 (0.06, 0.18)

* Values are presented as the means ± standard errors assuming a Poisson distribution. Abbreviation: CI = confidence interval.

TABLE 2 Cell killing produced by stably transfected BAX under anoxic conditions in U-251 MG cells			
Clone	Oxygen Condition	Plating Efficiency*	Survival Fraction (95% CI)
control	oxic	0.36 ± 0.06	1
	anoxic	0.32 ± 0.09	0.89 (0.84, 0.96)
clone A	oxic	0.37 ± 0.02	1
	anoxic	0.19 ± 0.01	0.51 (0.51, 0.52)
clone B	oxic	0.31 ± 0.03	1
	anoxic	0.13 ± 0.02	0.42 (0.41, 0.44)

* Values are presented as the means ± standard errors assuming a Poisson distribution. Abbreviation: CI = confidence interval.

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Fig. 3. Graph demonstrating the growth of tumors derived from BAX-GFP stably transfected U-251 MG clone A. Athymic mice were injected subcutaneously with 5×10^6 cells to generate flank tumors. The tumor size was monitored by measuring 2 diameters at right angles with calipers and converted to volume by the formula $\text{length} \times \text{width}^2 \times 0.5$. The animals were killed when their tumor sizes reached approximately 2000 mm³.

DISCUSSION

Solid tumors are heterogeneous and typically composed of physiologically distinct subpopulations. Over 40 years ago, the pioneering work conducted by Thomlinson and Gray³⁴ demonstrated that the sensitivity of tumor cells to radiation-induced damage depends on the presence of oxygen in the tumor's microenvironment. Since their discovery, the authors of many subsequent studies have confirmed the presence of radioresistant hypoxic cells in tumors, including brain tumors.^{18,22} Radiation therapy can effectively kill oxic tumor cells; however, hypoxic cells pose an obstacle in attempts to cure brain tumors with radiation. Clearly, we need to devise new therapeutic strategies to target selectively this radioresistant cellular subpopulation in tumors.

During the past decade, a number of gene therapy vectors have been developed to deliver transgenes that have potential therapeutic application in treating malignancies of the central nervous system.⁴² However, the principal impediments to current gene therapy approaches are the lack of efficacy and/or selectivity. This is particularly relevant to suicide gene therapy for brain tumors because it requires in vivo delivery of vector, and it demands that the suicide gene product be limited as much as possible to malignant tissues.³²

Fig. 4. Western blot analysis indicating expression of BAX in mouse flank tumors derived from BAX-GFP stably transfected U-251 MG clone A. All three tumors derived from a BAX-GFP stably transfected clone showed strong expression of BAX (lanes 1, 2, and 3). In contrast, wild-type U-251 MG tumor (lane 4) showed no BAX expression.

Fig. 5. Photomicrographs showing GFP expression in tumor tissues derived from BAX-GFP stably transfected U-251 MG clone A examined under fluorescence microscopy. (A) autofluorescence from GFP in tumor tissue; (B) nuclei staining of the same tissue sample by propidium iodide. Original magnification $\times 63$ for both photomicrographs.

In this study, we exploit the targeted gene therapy approach by using a transcriptional regulatory element under the control of oxygen level. A major advantage of this gene therapy lies in its potential for producing cytotoxicity selectively in hypoxic tumor cells. When used in conjunction with radiation therapy, which preferentially kills oxic cells, the result should be to reduce the overall tumor burden, without causing additional damage in the well-oxygenated normal brain tissues. Therefore, this treatment combination may result in improved therapeutic effects in these patients.

Studies conducted to evaluate the molecular mechanisms of hypoxia-regulated gene expression provide evidence that hypoxia induces gene expression via the cis-acting DNA sequence HRE, which interacts with the transcriptional activator HIF-1.⁵ Genes that are regulated by oxygen include glycolytic and gluconeogenic enzymes in energy metabolism, vascular growth factors, transcription factors, glucose transporters, tyrosine hydroxylase, and *Epo*.²⁹ The HIF-1 is a heterodimeric basic helix-loop-helix-PAS domain transcription factor containing α and β subunits, and HIF-1 is unique among mammalian transcription factors with respect to demonstrated specificity and sensitivity of induction by hypoxia.⁴¹ Expression of the HIF-1 α subunit, which is unique to HIF-1, is precisely regulated by cellular O₂ concentration such that levels of HIF-1 α protein and HIF-1 DNA-binding activity increase exponentially as O₂ concentration decreases.¹⁷ Gene knockout studies indicate that HIF-1 α is essential for embryonic development, solid tumor formation, and tumor vascularization.^{6,16,25} Overexpression of HIF-1 α has recently been identified in multiple types of human cancers and their metastases, including brain, lung, prostate, breast, pancreatic, and colon cancers.^{47,48} In contrast to the specificity of HIF-1 α , the HIF-1 β subunit, which was identified previously as the aryl hydrocarbon receptor nuclear translocator,¹⁵ heterodimerizes with several other mammalian basic helix-loop-helix-PAS proteins including the aryl hydrocarbon receptor.^{9,33,35}

Other investigators have placed reporter genes and suicide genes under the control of HREs. In a study conduct-

ed by Dachs, et al.,⁸ the authors tested the ability of hypoxia to control selectively gene expression and increase cell killing. They placed a CD2 marker gene or a gene that produces the enzyme cytosine deaminase under the control of HRE derived from murine phosphoglycerate kinase gene, and they showed that CD2 was induced in vitro only when oxygen concentrations were less than 2%. These investigators also demonstrated that activation of cytosine deaminase under hypoxic conditions caused significant cell killing when the prodrug 5-fluorocytosine was present. Recently, Shibata, et al.,³¹ have used multiple copies of HRE derived from the human *vascular endothelial growth factor* gene to achieve hypoxia-specific expression of therapeutic genes. When cells were transfected with the *Escherichia coli nitroreductase* gene (which can activate the anti-cancer prodrug CB1954) under the regulation of HRE, marked growth inhibition was achieved under hypoxic conditions.

Since the Bcl-2 family was discovered, identification of its many members has progressed rapidly. These members can be divided into pro- and antiapoptotic subfamilies. These factors interact with each other and regulate the important cellular processes of apoptosis involved in tissue development, homeostasis, and tumor cell response to radio- and chemotherapy.^{19,45} Our work has focused on BAX as a potential suicide gene;²⁴ BAX is a member of the proapoptotic subfamily. The function of BAX protein is associated with loss of the mitochondrial membrane potential and with events typical of apoptosis, including cytosolic accumulation of cytochrome c, caspase activation, cleavage of poly (ADP-ribose)-polymerase, and DNA fragmentation.^{1,10,11} Overexpression of BAX has been shown to sensitize human breast cancer cells to radiation-induced apoptosis.²⁷ In addition, overexpression of BAX in human glioma cells results in increased sensitivity to apoptosis-inducing agents.³⁸

In this current study, we generated *BAX-GFP* stably transfected clones in two human GBM cell lines and investigated the cell-killing effect of BAX-GFP under hypoxia in these clones. We found that when the *BAX-GFP*-transfected clones were exposed to anoxia for 16 hours, they demonstrated an increased cell-killing ability, indicating that BAX-GFP overexpression can effectively initiate apoptosis and lead to cell death.

We observed expression of the transfected *BAX-GFP* fusion gene under oxic conditions in the stable clones, suggesting that the HRE-mediated expression system is "leaky." In addition, although BAX expression was increased after 16 hours of exposure to anoxia in U-251 MG clone A cells, it was not increased under anoxia in the other stable clones. Lack of increased BAX expression in the two U-87 MG clones under anoxic conditions may be due to the strong proapoptotic activity of BAX, in which even a small increase in BAX leads to cell death. This hypothesis is consistent with recent studies reported by Vogelbaum, et al.,³⁸ who showed that glioma cells with wild-type *p53* such as U-87 MG cells were extremely sensitive to increased BAX expression, and in our early studies we demonstrated that overexpression of BAX in U-87 MG leads to apoptosis.²⁴ However, the lack of BAX induction under anoxia in U-251 MG clone B cells cannot be explained by the aforementioned mechanism, because

high levels of BAX expression were observed in glioma cells containing mutant *p53* such as U-251 MG.³⁷ We think another mechanism may account for increased cell death under anoxia in U-251 MG clone B. It has been shown that hypoxia can reduce cellular adenosine 5'-triphosphate levels, resulting in the translocation of BAX from cytosol to mitochondria,²⁶ where the apoptosis pathway is initiated. In U-251 MG clone B cells, anoxic conditions can promote translocation of BAX from cytosol to mitochondria, thereby increasing cell killing. This could decrease the number of BAX-containing cells in the population.

To determine whether HRE-mediated expression of BAX in human tumor xenografts could be regulated by the tumor's hypoxic microenvironment, we implanted BAX stably transfected clones into mice as flank tumors. Although three out of four clones did not grow as tumor xenografts, tumors derived from U-251 MG clone A grew as well as those derived from untransfected control cells. Moreover, BAX was expressed in these tumors, indicating the expression plasmid pH3BG was functional in this in vivo model. Finally, examination of GFP expression revealed that BAX expression was restricted to distinct regions of the tumor tissues, consistent with the localized presence of hypoxia in these tumors.

CONCLUSIONS

These findings indicate that HRE-regulated BAX can be stably transfected into GBM cells. Overexpression of BAX in human GBM cells can result in increased cell killing under anoxic conditions. When a *BAX-GFP* stably transfected clone was implanted as flank tumors in athymic mice, BAX expression was detected in distinct regions of tumor tissues, suggesting inducible expression of under hypoxic conditions. However, our current hypoxia-regulated expression system contains some degree of promoter leakage under oxic conditions, and the majority of clones did not show significant induction of BAX-GFP expression under anoxia. Therefore, the parameters of this HRE-mediated expression system, including HRE copy number and the basal promoter, need to be optimized to produce preferential and predictable gene expression in hypoxic cells.

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