A Hypoxia-Regulated Adeno-Associated Virus Vector for Cancer-Specific Gene Therapy[1]

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Abstract

The presence of hypoxic cells in human brain tumors is an important factor leading to resistance to radiation therapy. However, this physiological difference between normal tissues and tumors also provides the potential for designing cancer-specific gene therapy. We compared the increase of gene expression under anoxia (<0.01% oxygen) produced by 3, 6, and 9 copies of hypoxia-responsive elements (HRE) from the erythropoietin gene (*Epo*), which are activated through the transcriptional complex hypoxia-inducible factor 1 (HIF-1). Under anoxic conditions, nine copies of HRE (9XHRE) yielded 27- to 37-fold of increased gene expression in U-251 MG and U-87 MG human brain tumor cell lines. Under the less hypoxic conditions of 0.3% and 1% oxygen, gene activation by 9XHRE increased expression 11- to 18-fold in these cell lines. To generate a recombinant adeno-associated virus (rAAV) in which the transgene can be regulated by hypoxia, we inserted the DNA fragment containing 9XHRE and the *LacZ* reporter gene into an AAV vector. Under anoxic conditions, this vector produced 79- to 110-fold increase in gene expression. We believe this hypoxia-regulated rAAV vector will provide a useful delivery vehicle for cancer-specific gene therapy. *Neoplasia* (2001) **3,** 255–263.

Keywords: hypoxia, brain tumor cells, hypoxia-responsive element, adeno-associated virus, gene therapy.

Introduction

Solid tumors are heterogeneous and are composed of physiologically distinct subpopulations of cells. One important feature of human solid tumors is the presence of a hypoxic microenvironment [1]. Pioneering studies by Thomlinson and Gray [2] demonstrated that hypoxic cells arose in tumors whenever tumor growth produces cells that are >150 μm from a blood vessel. Clinical studies using an oxygen electrode to measure the oxygen levels in patients' brain tumor tissues have found a significant number of hypoxic cells in every tumor examined [3]. Hypoxia in solid tumors confers resistance to standard radiotherapy and chemotherapy [4,5]. Furthermore, oxygen-deprived tumor cells are predisposed to a more malignant phenotype, as characterized by increases in local invasion, metastatic spread, and genetic instability [6,7]. Inhibition of cellular proliferation by hypoxia also may contribute to a resistant phenotype, because current cancer treatment modalities are primarily effective against rapidly dividing cells. In brief, hypoxia within tumors most likely contributes to a poor therapeutic outcome, regardless of which cancer treatment is used [7].

In addition to conferring resistance to cancer therapy, recent clinical and experimental studies have also suggested that the cellular response to hypoxia can result in dramatic alterations in the expression of a variety of genes, many of which may lead to more aggressive phenotypes. For example, hypoxia in solid tumors mediates selection of cells with diminished apoptotic potential and with genetic alterations such as loss of the p53 tumor suppressor gene or overexpression of the apoptotic inhibitor protein Bcl-2 [8].

Studies of molecular responses to hypoxia have identified the major mediator for cellular hypoxic responses as a transcriptional activator hypoxia-inducible factor 1 (HIF-1) [9]. HIF-1 is a heterodimeric basic helix–loop–helix (bHLH) protein consisting of two subunits, HIF-1α and HIF-1β. HIF-1α is the unique, O_2-regulated subunit that determines HIF-1 activity [10]. Under hypoxic conditions, HIF-1 binds to the hypoxia-responsive element (HRE) in the enhancer region of its target genes and turns on gene transcription. HIF-1 responsive genes include glycolytic and gluconeogenic enzymes in energy metabolism, vascular endothelial growth factor (VEGF), transcription factors, glucose transporters, tyrosine hydroxylase and erythropoietin (Epo), a hormone that regulates erythropoiesis in accordance with the oxygen-carrying capacity of the blood. The core consensus sequence for HRE has been identified as (A/G)CGT(G/C) [11].

Several studies have demonstrated overexpression of HIF-1α in cancer cells and tissues. For example, increased

Abbreviations: HRE, hypoxia-responsive element; HIF-1, hypoxia-inducible factor 1; rAAV, recombinant adeno-associated virus; bHLH, basic helix–loop–helix; Epo, erythropoietin; CMEM, complete growth medium; MEM, Eagle's minimum essential medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; SDS, sodium dodecylsulfate; β-gal, β-galactosidase; MOI, multiplicity of infection; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactosidase; VEGF, vascular endothelial growth factor

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expression of HIF-1α protein has been detected in rat and human prostate cancer cell lines [12]. Recently, immunohistochemical analysis of HIF-1α has revealed increased expression in a variety of tumor types compared with the respective normal tissues, including brain, colon, breast, gastric, lung, skin, ovarian, pancreatic, prostate, and kidney [13,14]. These results suggest that HIF-1α overexpression is associated with tumor progression and invasion, possibly by increased cell growth and metastatic potential.

Although the presence of hypoxic cells in human brain tumors is an important factor leading to resistance to cancer treatments, this physiological difference between normal tissues and tumors also provides the potential for designing cancer-specific gene therapy protocols [4]. Over the last 10 years a number of viral vectors including adeno-associated virus (AAV) have been developed to deliver transgenes that are potentially useful for treating central nervous system malignancies [15]. However, a fundamental problem for cancer gene therapy is the lack of tumor specificity; proteins that are therapeutic in malignant cells also may be harmful to surrounding normal tissue. One way to circumvent this problem is to use transcriptionally targeted vectors that can restrict the expression of the therapeutic proteins primarily to cancer cells [16].

We have previously demonstrated the feasibility of this approach by employing a trimer of a minimal (31 bp) HRE from the human Epo gene to regulate the expression of the pro-apoptotic BAX gene [17]. In the present study, we sought to improve the ratio of hypoxic induction of gene expression. We compared the induction of gene expression from constructs containing an SV40 minimal promoter and 3X, 6X, or 9XHRE. Our results indicate that 6XHRE produced a similar increase in gene expression under anoxic conditions as observed earlier for 3XHRE [17]. However, 9XHRE increased the amount of gene expression markedly in both cell lines, as compared with the lower HRE copy numbers. We subsequently characterized induction of gene expression by 9XHRE under intermediate oxygen concentrations. In stable clones transfected with a construct containing 9XHRE and the suicide gene BAX, gene expression was increased under hypoxic conditions. Finally, we report development of a novel recombinant adeno-associated virus (rAAV) in which the expression of a transgene is under the control of HREs and occurs only under hypoxic conditions.

Materials and Methods

Plasmid Construction

Based on the published HRE sequence from the 3′ enhancer region of the Epo gene [18], we designed pairs of oligonucleotides that contain three tandem repeats of the HRE (G CCC *TAC GTG CT*G TCT CAC ACA GCG CCT GTC) [17]. In addition, 6X and 9XHREs were generated by tandem ligation of the 3XHRE oligonucleotide pairs. These oligonucleotides were inserted into the multiple cloning sites of the mammalian expression vector pβgal-promoter (Clontech, Palo Alto, CA), which contains an enhancerless SV40 promoter situated upstream of the *LacZ* gene. The resulting constructs were named pH3LacZ, pH6LacZ, and pH9LacZ, respectively (Figure 1). To construct pH9BAX, the *LacZ* gene in pH9LacZ was deleted using *Hin*dIII and *Nde*I enzymes and replaced with a murine pro-apoptotic BAX cDNA fragment flanked by *Hin*dIII and *Nde*I sites. To construct the plasmid pH9LacZ-AAV, the DNA fragment containing 9XHRE and the *LacZ* gene flanked by restriction enzyme sites *Sma*I and *Sal*I were inserted into pSSV9 (Figure 1). The plasmid pCMVLacZ-AAV was similarly

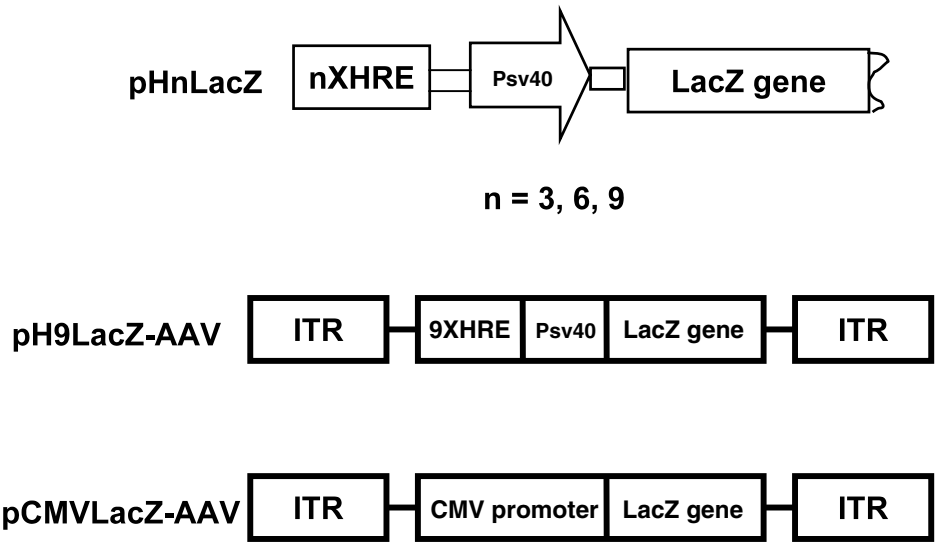

Figure 1. *Structural map of plasmids pH3LacZ, pH6LacZ, pH9LacZ, and rAAV vectors. In pHnLacZ plasmids, the reporter gene LacZ was under the transcriptional control of different copy numbers of HRE and an SV40 minimal promoter. In pH9LacZ-AAV, the LacZ with nine copies of HRE and the SV40 minimal promoter was inserted between the two inverted repeat regions (ITR). The control vector pCMVLacZ-AAV contains a CMV promoter upstream of the LacZ gene.*

constructed by inserting the CMV promoter and *LacZ* gene fragment into pSSV9 (Figure 1) [19].

Cell Culture

Human glioblastoma cell lines U-251 MG and U-87 MG and human embryonic kidney cell line 293 were maintained in complete growth medium (CMEM), which consists of Eagle's minimum essential medium (MEM) supplemented with 1% nonessential amino acids and 10% fetal bovine serum (FBS). Cultures were incubated in an humidified atmosphere containing 5% CO_2 at 37°C.

Induction of Hypoxia in Tumor Cells

Cells were seeded into glass petri dishes, allowed to attach overnight and then placed into gastight aluminum gassing jigs. The jigs were subjected to five rounds of evacuation and flushing with either 95% air and 5% CO_2 (oxic conditions) or 95% N_2 and 5% CO_2 (anoxic conditions) on a shaking platform at room temperature. We produced other hypoxic mixtures by using known mixtures of 95% O_2/5% CO_2 and 95% N_2/5% CO_2, which were ordered from the manufacturer (Nellcor Puritan Bennett, Pleasanton, CA). After the last round of gas exchange, the jigs were placed in a 37°C incubator and incubated for 16 hours.

Transient Transfection

Cells (4×10^5) were plated into 6-cm glass petri dishes and grown for 16 to 18 hours until cell growth reached log phase and cell density was ~50% confluent. Multiple sets of dishes in either duplicate or triplicate were prepared for each plasmid sample. Transfections were performed using 6 μl of lipofectamine (2 mg/ml, Gibco BRL Life Technologies, Gaithersburg, MD), 5 μg of plasmid containing *LacZ* and various copy number of HREs, and 0.5 μg of DNA containing the luciferase reporter gene (to correct for transfection efficiency) in 2 ml of serum-free CMEM for 5.5 hours. Then, the transfection solution in each dish was replaced with 4 ml of fresh CMEM and incubated at 37°C for 24 hours. One set of dishes was grown under normal oxic conditions and the other sets were subjected to different hypoxic conditions for 16 hours. Cells were then harvested and assayed for β-galactosidase (β-gal) activity by the enzymatic method described below.

Stable Transfection

Cells were stably transfected with pH9BAX using lipofectamine reagent. For U-251 MG, 2×10^5 cells/well were transfected with 5 μg of DNA and 7.5 μl of lipofectamine. Thirty-six hours after addition of the DNA and lipofectamine, growth medium was changed and cells were incubated in growth medium containing 600 μg/ml G418 (Gibco BRL Life Technologies) 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. Stably transfected clones were maintained in medium containing 600 μg/ml G418.

rAAV Production

rAAV was produced by using the three-plasmid cotransfection system as described previously [20]. Briefly, human embryonic kidney 293 cells were grown in MEM containing 10% FBS in 15-cm plastic petri dishes to 70% confluence. To generate AAVH9LacZ virus, the cells were cotransfected with 17 μg of plasmid pH9LacZ-AAV per dish along with 17 μg of plasmid pHLP19 and 17 μg of plasmid pLadeno5 per dish. pHLP19 has AAV *rep* and *cap* genes, which provide the *trans* functions of rAAV. pLadeno5 has the adenoviral VA, E2A and E4 regions that mediate rAAV replication. To generate AAVCMVLacZ virus, plasmid pCMVLacZ-AAV was used in place of pH9LacZ-AAV in the above cotransfection process. The medium was changed after 16 hours to CMEM. After an additional 24 hours, the cells were collected and lysed by three freeze–thaw cycles. Viral supernatants were generated by centrifugation at 10,000$\times g$ for 5 minutes and further purified by CsCl-gradient ultracentrifugation; the titer for each rAAV was determined by dot blot assay. This assay provides a titer of total number of particles per unit volume. The supernatant containing rAAV was stored in aliquots at −80°C and thawed for use immediately before each experiment.

rAAV Infection

U-251 MG cells were grown to 70% confluence in 6-cm glass petri dishes. For infection, the growth medium was replaced with rAAV-containing medium and dishes were incubated for 48 hours at 37°C in 5% CO_2. At the end of the incubation period, cells were subjected to β-gal expression analysis using either an enzymatic assay or an X-gal *in situ* staining assay.

Western Immunoblotting

Total cellular protein was extracted from cell lines by lysing cells in 0.5 ml of phosphate-buffered saline (PBS) containing 0.5% deoxycholate, 1% Nonidet P-40 and 0.1% sodium dodecylsulfate (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 method [21] (Bio-Rad, Hercules, CA) 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 before loading. Equal amounts of total protein were separated on an SDS–polyacrylamide gel for 1.5 hours at 150 V using a Bio-Rad minigel apparatus (Bio-Rad). The protein gel was transferred to a polyvinylidene difluoride membrane (Amersham, Buckinghamshire, UK) 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 using Ponceau-S (Sigma, St. Louis, MO). 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 either the anti-human HIF-1α antibody (Novus Biologicals, Littleton, CO) or the anti-murine BAX antibody (Genzyme, Cambridge, MA) 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 a peroxidase-labeled secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 hour at room temperature. After three washes in PBS plus 0.05% Tween 20, the membrane was incubated for 1 minute in enhanced chemiluminescence buffer (Amersham) and proteins were visualized on an X-ray film using exposure times varying from 30 seconds to 5 minutes.

β-gal and Luciferase Assays

Transfected cells were washed with PBS, removed from the glass surface using a rubber policeman and transferred into Eppendorf tubes. Cells were lysed using the "freeze–thaw" method, centrifuged, and the supernatants were collected. The supernatants were assayed for β-gal activity using a chemiluminescent method according to the manufacturer's directions (Clontech). Briefly, an aliquot of supernatant was incubated with substrate at room temperature for 1 hour and the light intensity was measured using a Model 20e luminometer (Turner Designs, Sunnyvale, CA). The cotransfected luciferase activity of the extract was determined using a kit from Promega (Madison, WI). An aliquot of the supernatant was incubated with the luciferase substrate luciferin, and the light intensity emitted by the sample was measured using the luminometer. β-Gal activity was normalized to the cotransfected luciferase activity to standardize the efficiency of transient transfections. In some transfections, β-gal activity was normalized to cellular protein level, which was measured using the Bradford method [21].

X-gal In Situ Staining

Cells were fixed in 0.2% glutaraldehyde and 2% paraformaldehyde for 5 minutes, and washed twice with PBS. The cells were immersed in a staining solution containing 100 mM sodium phosphate (pH 7.3), 1.3 mM $MgCl_2$, 3 mM $K_3Fe(CN)_6$, 3 mM $K_4Fe(CN)_6$, and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal, 1 mg/ml) and incubated at 37°C for 18 hours. The stained cells were washed twice with PBS and examined under a light microscope.

Results

Induction of HIF-1α Expression under Anoxic Conditions in Glioblastoma Cell Lines

To determine whether HIF-1α expression was induced under anoxic conditions, we analyzed HIF-1α levels under both oxic and anoxic conditions in two glioblastoma cell lines using Western blots. After exposure to either oxic or anoxic conditions for 16 hours, cells were harvested and total protein was extracted from these cells. Equal amounts (25 μg) of protein were analyzed by 7% SDS–polyacrylamide gel and immunoblotting was performed using a monoclonal anti-human HIF-1α antibody. U-87 MG cells showed minimal expression of HIF-1α under oxia and a strong signal under anoxia; by densitometry measurement there was about a 15-fold increase in gene expression under anoxia (Figure 2). In U-251 MG cells, HIF-1α showed strong expression under oxia, and anoxia exposure increased expression only mildly ($\sim$1.4-fold) (Figure 2).

Effect of HRE Copy Number on Anoxia-Induced Gene Expression

More than a dozen hypoxia-regulated genes have been identified as containing HREs in their flanking regions [9]. In our study, we have used an HRE derived from the human Epo 3′ flanking region in its natural context. To investigate the influence of HRE copy number on the induction ratio of hypoxic to oxic heterologous gene expression in these cells, 3X, 6X, and 9XHRE were inserted adjacent to an SV40 minimal promoter in a mammalian expression vector that contained the *LacZ* gene (Figure 1). We have previously compared pH3LacZ gene expression under both oxic and anoxic conditions in U-251 MG and U-87 MG cells [17]. In the present paper, we first asked whether increasing HRE copy number from 3 to 6 or 9 would affect gene expression under oxic conditions. We transiently transfected these plasmids into U-87 MG and U-251 MG human glioblastoma cells using the lipofectamine-mediated method. To correct for variation in the efficiencies of transfection, we included in the DNA/liposome complex a control plasmid containing the luciferase reporter gene. Forty hours after transfection, we collected protein extracts from transfected cells and assayed them for β-gal activity, which was normalized by the cotransfected luciferase activity. As shown in Table 1, for U-251 MG cells, increasing HRE copy number above 3 resulted in very similar basal gene expression under oxic conditions. For U-87 MG cells, basal gene expression

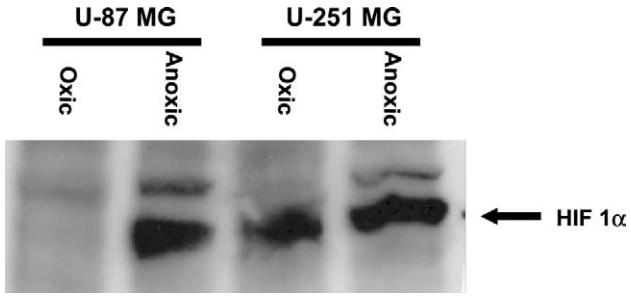

Figure 2. *Cell type–specific induction of HIF-1α under anoxic conditions. After exposure to either oxic or anoxic conditions for 16 hours, U-87 MG and U-251 MG cells were harvested and lysed. Equal amounts (25 μg) of proteins were analyzed by 7% SDS-PAGE and immunoblotting was performed using a monoclonal anti-human HIF-1α antibody (Novus Biologicals). U-87 MG cells showed minimal expression of HIF-1α under oxia and significantly increased HIF-1α under anoxia. In contrast, in U-251 MG cells HIF-1α showed considerable expression under oxic conditions and increased only slightly under anoxic conditions.*

Table 1. Gene Expression in Oxic Cells Exposed to Gene Constructs Containing *LacZ* under the Control of 3X, 6X, and 9XHREs.

Plasmid	Normalized β-gal Activity (relative light units/μg)*	
	U-251 MG	U-87 MG
pH3LacZ	23.3±7.0	28.7±5.1
pH6LacZ	29.9±2.0	22.3±3.7
pH9LacZ	28.8±2.8	45.2±8.5

*Numbers with errors in the table are the means and standard deviations of three independent samples.

remained similar for 3X and 6XHRE, however, for 9XHRE the average basal gene expression was about two-fold higher than for either 3X or 6XHRE (Table 1).

This increase in basal gene expression under oxic conditions, however, was very small compared to that observed under anoxic conditions. To measure this, we transiently transfected the plasmids into U-251 MG and U-87 MG cells. Twenty-four hours after transfection, cells were made anoxic for 16 hours. At the end of the anoxic period, we collected protein extracts from transfected cells and assayed them for β-gal activity. The β-gal activity in cells that were maintained under oxic conditions served as a control. In U-251 MG cells, 3XHRE increased gene expression about four-fold under anoxia (Figure 3), consistent with our previous findings [17]. Expanding HRE copy number to 6

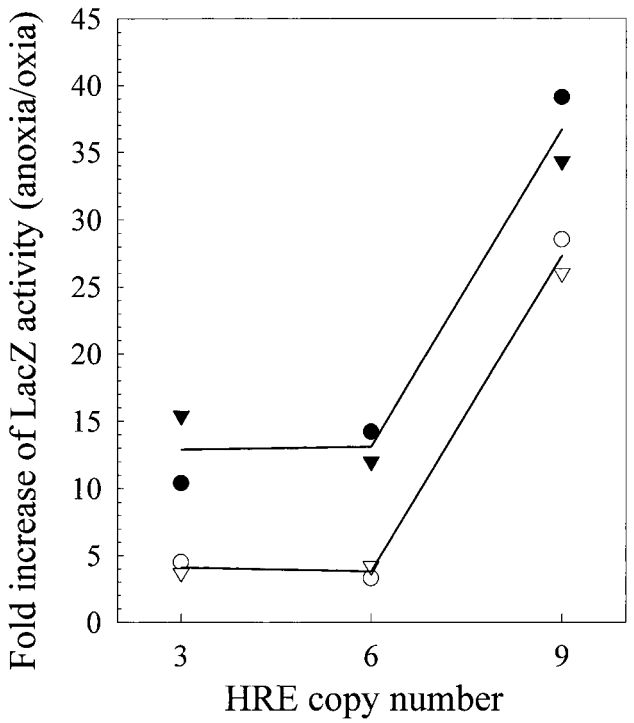

Figure 3. *Anoxia-induced LacZ expression depends on HRE copy number. U-87 MG and U-251 MG cells were transiently transfected with plasmids containing the LacZ reporter gene and 3X, 6X, and 9XHRE. Transfected cells were incubated under either anoxic or oxic conditions for 16 hours before analysis for LacZ activity. The relative level of LacZ expression in oxic cells was designated as 1.0. The actual values obtained from two independent experiments are shown for U-87 MG cells (−●−, −▼−) and U-251 MG cells (−○−, −▽−).*

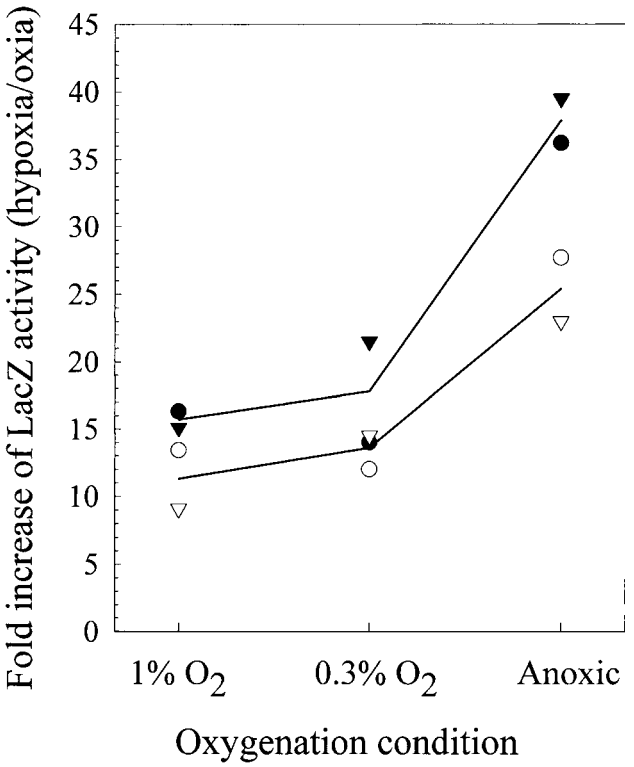

Figure 4. *Differential regulation of LacZ expression in pH9LacZ under intermediate oxygen concentrations. U-87 MG and U-251 MG cells were transiently transfected with the plasmid containing LacZ reporter gene and 9XHRE. Transfected cells were incubated under different oxygen concentrations for 16 hours before analysis for LacZ activity. The relative level of LacZ expression in oxic cells was designated as 1.0. The actual values obtained from two independent experiments are shown for U-87 MG cells (−●−, −▼−) and U-251 MG cells (−○−, −▽−).*

did not further increase gene expression compared with 3XHRE. However, when HRE copy number was increased to 9, we observed that gene expression increased to ~27-fold. We observed similar patterns of induced gene expression in U-87 MG cells; both 3X and 6XHRE produced about a 13-fold increase in gene expression, whereas 9XHRE further increased gene expression to ~37-fold (Figure 3).

Effect of 9XHRE on Gene Expression under Intermediate Oxygen Concentrations

Because many hypoxic tumor cells exist under intermediate oxygen levels [22], a successful hypoxia-targeted gene

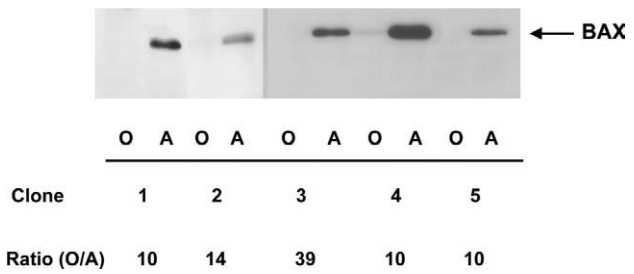

Figure 5. *Western blot showing BAX expression under Oxic (O) and Anoxic (A) conditions in pH9BAX−stably transfected U-251 MG clones. The intensity of the BAX protein was quantified using densitometry; the fold increase in BAX expression under anoxic conditions ranged from 10 to 39.*

Table 2. Infection of U-251 MG Cells by the recombinant AAV, AAVH9LacZ*.

Experiment	Normalized β-gal Activity† (relative light units/μg)	Fold of Increase (Anoxic/Oxic)
Oxic		
1	56.0 ± 2.7	
2	76.7 ± 15.5	
Anoxic		
1	6140 ± 786	110
2	6055 ± 548	79

*Multiplicity of infection (MOI) = 25.
†Numbers with errors in the table are the means and standard deviations of four independent samples.

therapy will need to be functional under a range of hypoxic conditions. Therefore, we determined whether 9XHRE could increase gene expression under two intermediate levels of hypoxia. For these studies, we transiently transfected pH9LacZ into both U-87 MG and U-251 MG cells. Transfected cells were then incubated under 1% oxygen, 0.3% oxygen or anoxic conditions. After a 16-hour incubation period, cells were harvested and β-gal activities were assayed. Compared to oxic conditions, gene expression in U-251 MG cells increased 11- and 14-fold under 1% and 0.3% oxygen, respectively, whereas the fold increase under anoxia was ~25 (Figure 4). Similarly, gene expression in U-87 MG cells increased 16- and 18-fold under 1% and 0.3% oxygen, respectively (Figure 4), whereas the fold increase under anoxia was ~38.

Evaluation of an HRE Vector that Expressed BAX Under Hypoxia

We demonstrated previously that overexpression of the pro-apoptotic BAX gene can kill anoxic tumor cells [17], indicating BAX might be a potentially useful suicide gene in hypoxia-targeted gene therapy. To determine whether the 9XHRE vector system could be employed to specifically express a suicide gene under hypoxic conditions, we generated pH9BAX. We then transfected this plasmid into U-251 MG cells and selected for stably transfected clones. Five clones were analyzed for BAX expression under both oxic and anoxic conditions using the Western blot method. As shown in Figure 5, under oxic conditions there was no detectable BAX expression in all five clones, indicating the 9XHRE regulatory system displayed minimal promoter leakage. When these clones were incubated under anoxic conditions for 16 hours, however, BAX expression was significantly upregulated in all clones. Densitometric scanning of the Western blots indicated that BAX expression increased from 10- to 39-fold, compared with the minimal expression measured under oxic conditions.

Hypoxic Induction in Glioblastoma Cells Infected with AAVH9LacZ Virus

To examine the magnitude of hypoxic induction in cells infected with the AAVH9LacZ virus, exponentially growing U-251 MG cells were infected with the recombinant virus. One day after infection, we incubated cells under anoxic or oxic conditions for 16 hours and then measured the levels of

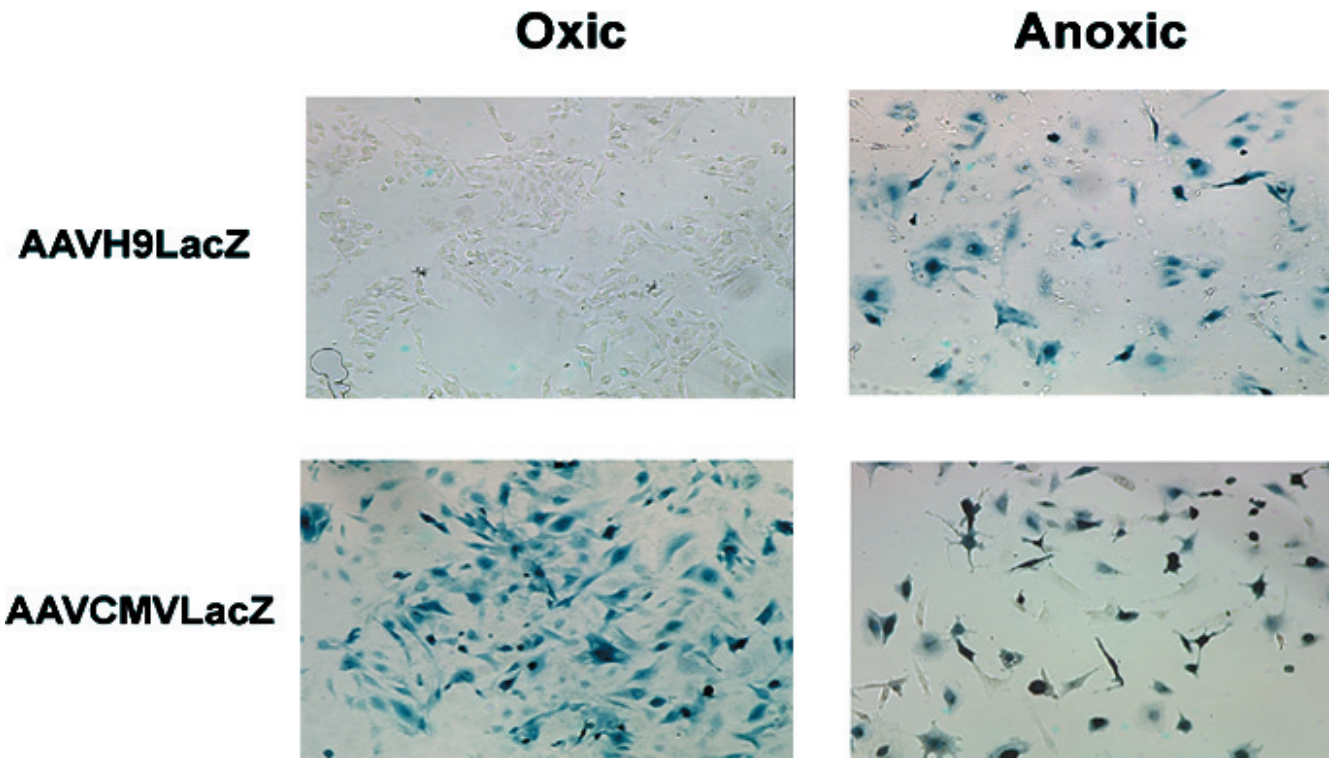

Figure 6. *β-gal expression in U-251 MG cells. U-251 MG cells (3×10^5) were infected with either AAVH9LacZ or AAVCMVLacZ at an MOI of 25 and incubated under either oxic or anoxic condition for 16 hours. At the end of incubation, cells were assayed for β-gal expression by the X-gal in situ staining method as described in the Materials and Methods section.*

β-gal quantitatively using a chemiluminescent assay. Significant increases of β-gal were detected in the anoxic cells compared with the oxic cells. As shown in Table 2, anoxic incubation gave 79- to 110-fold increase in gene expression compared with oxic conditions in two independent experiments. In addition, we found that the absolute level of gene expression from 9XHRE/Psv40 promoter was about 70% of that from the strong constitutive CMV promoter under anoxic conditions. Infected U-251 MG cells were also stained for β-gal expression using the X-gal *in situ* staining method. As shown in Figure 6, β-gal expression was minimal when cells were incubated under oxic conditions. However, anoxic incubation led to a significant increase in β-gal expression. As a control, β-gal expression from AAVCMVLacZ virus showed strong expression under both oxic and anoxic conditions.

Discussion

Hypoxia is a well-recognized feature of human brain tumors and is an important factor in tumor angiogenesis, invasion, and malignant growth [13]. A recent clinical study by Rampling and colleagues [3] made multiple measurements of pO_2 at various locations in primary and metastatic brain tumors. The mean pO_2 in tumors ranged from 0.01% to 3.2%, and the percentage of measurements that were <0.3% in each of these tumors ranged from 9.5 to 68.5%. Solid tumors cannot grow beyond a volume of several cubic millimeters in the absence of vascularization, and there is an inverse relation between tumor hypoxia and patient survival [9]. Overall, both basic and clinical studies have pointed out that tumor hypoxia is a significant problem for tumor resistance to radiotherapy and chemotherapy [4].

Our goal is to develop a gene therapy strategy to specifically kill hypoxic tumor cells. In combination with radiation therapy, which can efficiently kill well-oxygenated cells, this gene therapy approach should improve the therapeutic outcome for brain tumor patients.

HIF-1 is the master regulator of hypoxia-inducible gene expression and increased expression of HIF-1α protein has been detected in cancer cells. In this study, we first measured the expression of HIF-1α in two human glioblastoma cell lines. We found that HIF-1α protein in U-87 MG cells was minimal under oxic conditions but increased significantly when cells were incubated for 16 hours under anoxic conditions. In contrast, HIF-1α protein in U-251 MG cells was detected under both oxic and anoxic conditions, with only a slight increase in response to anoxic exposure. However, the absolute levels of gene expression under oxic conditions as measured by β-gal activities were essentially equivalent in both cell lines. We postulate that U-251 MG cells may have developed mechanisms to suppress activity of HIF-1 and render it nonfunctional under oxic conditions. It will be interesting to compare the expression levels of HIF-1 target genes such as VEGF between these two cell lines. These two cell lines are also heterogeneous with respect to other biologic properties. For example, these cells exhibit different sensitivities to anoxia; exposure of U-87 MG cells for 16 hours kills ~50% of the cells, whereas very few U-251 MG cells are killed under these conditions (Ruan and colleagues, unpublished data). Furthermore, it is known that U-87 MG cells carry a wild-type p53 gene, whereas p53 in U-251 MG cells is mutated [23]. Loss of wild-type p53 function in tumor cells has been implicated in enhancing HIF-1α levels in response to hypoxia [24]. Our data support the notion that the mutated p53 gene in U-251 MG cells might result in increased expression of HIF-1α even under oxic conditions.

Several studies by others indicate that the hypoxic environment can be used to activate heterologous gene expression driven by HRE. For example, Dachs and colleagues [25] showed that an HRE from the mouse phosphoglycerate kinase-1 gene could be used to control expression of marker and the therapeutic genes *in vitro* and within a solid tumor *in vivo*. In another study, Shibata and colleagues [26] have developed a hypoxia-responsive vector containing 5XHRE derived from the human VEGF gene combined with a CMV minimal promoter. Cells transfected with the vector exhibited over 500-fold increased transgene expression under hypoxia, similar to the level of the intact CMV promoter.

The amount of induced gene expression required to achieve selective tumor cell killing under hypoxia needs to be empirically determined for any given experimental system. Too low a ratio will not kill enough tumor cells, whereas too high a ratio may cause an adverse effect on surrounding normal cells. Therefore, in this study we investigated how increased gene expression under hypoxic conditions depends upon the HRE copy number. We chose the 31-bp human Epo HRE sequence for this study, and we investigated the hypoxia-induction ratio in plasmids containing 3X, 6X, and 9XHRE. Our results indicate that 9XHRE constructs produced the highest level of gene expression in both human glioblastoma cell lines studied. It remains to be determined whether increasing HRE copy number beyond 9 will further increase the hypoxia-induction ratio.

In our model systems, HRE copy number is an important component in the regulation of hypoxia-induced gene expression. Our data also indicate that the SV40 minimal promoter is a suitable basal promoter to be used in combination with HRE enhancers, because increased gene expression occurred under hypoxic conditions without any important increase in gene expression under oxic conditions (i.e., leakage). Boast and colleagues [27] also found that the SV40 minimal promoter and 3XHRE from the polyglycerate kinase gene gave a 146-fold induction of gene expression under hypoxia without increased basal level expression.

Because we wish to develop a therapy that kills tumor cells under hypoxic conditions, we have tested hypoxic induction of the suicide gene BAX. BAX is a member of the Bcl-2 protein family and overexpression of BAX causes apoptosis in both cell cultures and animals [28]. We have recently analyzed BAX expression in U-251 MG clones stably transfected with the construct containing 3XHRE and BAX [29]. Our results suggested that 3XHRE may be

insufficient to increase BAX expression under hypoxia in these clones. Because gene induction under hypoxia in the context of 9XHRE is significantly higher than that in 3XHRE, we decided to generate stable U-251 MG clones transfected with pH9BAX and examine hypoxic induction of BAX expression. There was minimal or undetectable BAX expression under oxic conditions in all clones, indicating no promoter leakage. Under anoxic conditions, we observed strong induction of BAX expression, ranging from 10- to 39-fold. Although we do not yet know how much increase in BAX gene expression is needed to kill hypoxic cells, studies on other therapeutic genes, such as cytosine deaminase and HSV-thymidine kinase genes, have found that only an approximately two- to seven-fold increase in gene expression was sufficient for hypoxic cell killing [25,30]. The magnitude of BAX expression needed to kill hypoxic cells in each of our model systems will have to be determined empirically.

Because it is unlikely that all tumor cells *in vivo* can be transfected with the suicide gene, the bystander effect will need to play a prominent role in successful gene therapy [31]. The development of the stably transfected pH9BAX clones will allow us to study any bystander effect that may be elicited by BAX. Ideally, the bystander effect will occur primarily in tumor cells and have limited effect on adjacent normal cells. It will be necessary to determine what the optimal level of BAX induction is to achieve such a beneficial bystander effect.

To exploit hypoxia-targeted gene therapy using an HRE-regulated system, it will be important to use an effective delivery system. Recently, an adenoviral vector, in which the transgene was under transcriptional control of HRE, produced inducible gene expression in response to hypoxia in a range of cell lines [32]. However, there are problems associated with adenovirus that could hamper its potential application for gene therapy in humans. These include the host immune reaction and inflammatory response elicited by viral genes and the reported toxicity of adenovirus to human tissues such as liver [33]. We decided to use an AAV vector system, because in the rAAV vector, both viral genes are removed and replaced with the desired therapeutic gene. Therefore, the risk of immune reaction is minimized because the vector cannot code for its own gene products [34]. Also, AAV is efficient in delivering its gene to both dividing and nondividing cells. This capability is important for the type of gene therapy that we propose, because most hypoxic cells are expected to be quiescent.

Finally, it will be critical that the AAV vector be able to reach tumor cells and it has to be minimally toxic to the surrounding normal brain tissue. The broad tropism of AAV makes targeting gene expression particularly important, and the goal for a targeted gene delivery system is to achieve low basal activity with high inducible expression levels. For example, hepatocarcinoma-specific expression of a therapeutic gene in rAAV has been demonstrated by employing the α-fetoprotein enhancer and albumin promoter; α-fetoprotein is expressed in most hepatocarcinomas with no detectable levels in normal adult liver tissue [35]. These tumor-specific parameters were also achieved in our rAAV, AAVH9LacZ, through the regulation of hypoxic conditions. When we inserted the DNA fragment containing 9XHRE and *LacZ* into the AAV vector, the resulting rAAV could be specifically regulated by hypoxia. *LacZ* expression was 79- and 110-fold higher under anoxic conditions compared to oxic conditions in U-251 MG human glioblastoma cells. X-gal *in situ* staining experiments also confirmed increased gene expression in AAVH9LacZ-infected U-251 MG cells under anoxic conditions. Furthermore, the activity of the 9XHRE/Psv40 promoter is comparable with that of CMV promoter under anoxic conditions, indicating this promoter can lead to strong expression of the target gene. Similar HRE-regulated systems have been described previously. When an expression cassette containing 3XHRE derived from the human phosphoglycerate kinase gene linked with the SV40 minimal promoter was configured into an adenoviral vector, a range of cell types displayed low basal transgene expression and highly inducible level under hypoxia [32].

In summary, we have developed a novel rAAV in which transgene expression was specifically regulated by hypoxia, resulting in strong induction under hypoxic conditions. We believe this hypoxia-regulated rAAV vector will provide a useful delivery vehicle for tumor-targeted gene therapy, and we plan to test this hypothesis *in vivo*.

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