Int. J. Radiation Oncology Biol. Phys., Vol. 55, No. 4, pp. 1027–1036, 2003

0360-3016/03/$–see front matter

ELSEVIER

doi:10.1016/S0360-3016(02)04507-8

BIOLOGY CONTRIBUTION

INHIBITION OF HYPOXIA INDUCIBLE FACTOR 1α CAUSES OXYGEN-INDEPENDENT CYTOTOXICITY AND INDUCES p53 INDEPENDENT APOPTOSIS IN GLIOBLASTOMA CELLS

SIMON DAI, M.S.,* MARY LIN HUANG, M.D.,* CHANG Y. HSU, M.D., PH.D.,† AND K. S. CLIFFORD CHAO, M.D.*

Departments of *Radiation Oncology and †Neurology, Washington University School of Medicine, St. Louis, MO

Purpose: Hypoxia, which activates the hypoxia inducible factor-1 alpha (HIF-1α) pathway, is a common feature in malignant gliomas and has been linked with tumor cell survival and therapy resistance. In this study, we examined the effect of antisense inhibition of HIF-1α on the survival, apoptosis and responses to chemotherapy in U-87 malignant glioma cells.

Methods and Materials: Hypoxia (1% oxygen) was achieved in a tri-gas incubator with intermittent N_2 gas flushing or in a gas tight-module sealed with 94% N_2, 1% O_2 and balance CO_2. HIF-1α inhibition was achieved with antisense phosphorothioate oligodeoxynucleotide (AS-HIF ODN), delivered using cytofectin GSV3815. HIF-1α expression level was monitored by a hypoxia-responsive luciferase reporter assay and verified by northern blot and immunoblot analyses. Cell viability was quantified by a colorimetric microtiter plate MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay. Apoptotic cell death was detected with a colorimetric caspase-3 assay, as well as using terminal transferase-catalyzed *in situ* end labeling (TUNEL) staining.

Results: Antisense HIF-1α phosphorothioate oligodeoxynucleotide (AS-HIF ODN) treatment suppressed HIF-1α expression by up to 80% under both normoxic and hypoxic conditions as measured by a hypoxia-responsive reporter assay and confirmed by northern and western blot analyses. Antisense knockdown of HIF-1α resulted in significant reduction in U-87 cells survival and an acceleration of apoptosis, which did not involve p53 transactivation. Pretreatment of cells with Z-Val-Ala-Asp (-OCH_3)-fluoromethylketone (Z-VAD), a broad-spectrum caspase inhibitor largely eliminated this effect of AS-HIF. Caspase-3 specific activity was markedly induced 3 days after AS-HIF treatment when increased cell death was also noted. Transient overexpression of HIF-1α in U-87 cells neutralized apoptosis-inducing effect of AS-HIF. AS-HIF treatment did not affect viability of primary astrocytes and was selectively more toxic to U-87 glioma cells than normal human fibroblasts. The HIF-1α antisense treatment exerted an oxygen-independent, and additive but not synergistic effect to the cytotoxicity of cisplatin, etoposide, and vincristine.

Conclusions: These results together indicate that suppression of HIF-1α-expression may be a promising strategy that is selective for reducing the survival and facilitating chemotherapeutic efficacy of malignant glioma.

Hypoxia inducible factor-1, Antisense, Apoptosis, Chemotherapy, Malignant glioma.

INTRODUCTION

Malignant gliomas are highly proliferative and infiltrating, and may contain regions of necrosis and hypervascularization, a feature suggestive of hypoxia. Surgery, radiation, and chemotherapy have been unsuccessful in treating malignant gliomas due to chemoresistance and frequent reoccurrence. Dismal overall prognosis remains for patients with malignant glioma (1). A new approach to target hypoxic tumor cells with intensity-modulated radiation therapy has been in active development to overcome tumor resistance to radiation (2). Understanding the molecular mechanism underlying hypoxia-mediated tumor resistance to chemotherapy or radiation may improve the efficacy of current therapies.

Adaptation to hypoxia selects tumor cells for malignant phenotype, which is characterized by angiogenesis, invasion, metastasis and resistance to chemo- and radiotherapy. Hypoxia inducible factor-1 alpha (HIF-1α), the inducible

Reprint requests to: K. S. Clifford Chao, M.D., Department of Radiation Oncology, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd.-97, Houston, TX 77030-4009. Tel: 713-792-4573; Fax: 713-745-6994; E-mail: cchao@mdanderson.org

Supported by grants from Washington University School of Medicine-Pharmacia Biomedical Research Support Program to K.S.C. and C.Y.H. Chang Hsu is a recipient of a Lowell B. Miller Memorial Research Grant (National Brain Tumor Foundation).

Acknowledgments—The authors thank Yali Du and Dr. Jan Xu for excellent technical assistance.

Received Feb 20, 2002, and in revised form Oct 9, 2002. Accepted for publication Oct 16, 2002.

subunit of the heterodimeric transcription factor HIF-1, plays key roles during the pathophysiologic adaptation of tumor cells (3). HIF-1α messenger ribonucleic acid (mRNA) is constitutively expressed but its protein is quickly degraded under normoxic condition (4, 5). Accumulation of HIF-1α protein increases instantly and exponentially in response to physiologic hypoxia through the inactivation of an oxygen-dependent HIF-1α prolyl hydroxylase (4–9). Genes that are transcriptionally activated by HIF-1 play important roles in glucose metabolism, cell proliferation, angiogenesis, cell survival, and apoptosis. Overexpression of HIF-1α and its downstream genes such as glucose transporter type 1 (Glut-1) and vascular endothelial growth factor (VEGF) have been identified as markers of treatment failure and poor prognosis in many cancers including malignant glioma, head and neck, cervical, breast, and prostate cancers (3). In glioblastoma multiforme (World Health Organization grade IV malignant glioma), HIF-1α overexpression was associated with poor survival (10). Enhanced HIF-1α expression coincides with accelerated trophoblast growth and proliferation during human early embryo development (11), and absence of HIF-1α expression in mouse embryonic stem (ES) cells retards solid tumor formation (12). In the present study, using antisense oligodeoxynucleotide (ODN) specifically targeting the expression of HIF-1α mRNA, we were able to establish that disruption of HIF-1α expression selectively reduces survival and facilitates apoptosis in malignant glioma cells. Unlike other conventional measures, the tumoricidal effect of HIF-1α inhibition is independent of tumor oxygenation.

METHODS AND MATERIALS

Cell culture and hypoxia treatment

The human glioma U-87 MG, human embryo fibroblast MRC-5 cell lines were obtained from the American Type Culture Collection (Manassas, VA). Human glioma cell lines LN-18, LN-229 and LNZ-308 were kindly provided by Dr. de Tribolet of Center Hospitalier Universitaire Vaudois (Lausanne, Switzerland). Rat primary astrocytes were prepared as follows: 1–2-day-old Sprague-Dawley rat brain cortex was loosely homogenized with 25 mL Dulbecco's modified Eagle's medium (DMEM) culture medium containing 10% fetal bovine serum, filtered with 80-μ nylon mesh and centrifuged at 1,000 rpm for 10 min. The pellets were washed twice with the culture medium. The cells were plated into 75-mm flasks (1.5 brains/flask) and incubated for 7–10 days until confluence. Cells were cultured in DMEM (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum, 100 unit/mL penicillin and 100 μg/mL streptomycin at 37°C, in an atmosphere of 5% CO_2 and 21% O_2 (normoxia). For hypoxia experiments, cells were exposed either in a Tri-gas hypoxia incubator (Forma Scientific, Marietta, OH) maintained with 1% O_2, 5% CO_2 and 94% N_2 under intermittent flushing with nitrogen, or flushed inside a gas-tight humidified module three times with a tri-gas mixture of 1% O_2 5% CO_2 and 94% N_2 and then sealed and kept in a humidified incubator at 37°C.

HIF-1α antisense and drug treatment

The antisense HIF-1α phosphorothioate ODN (AS-HIF) and sense control (S-HIF) sequences were described previously by others (11). We, however, predicted the folding of HIF-1α mRNA using RNADraw program and verified that the AS-HIF target sequence on HIF-1α mRNA was indeed in an accessible loop structure. Unlabeled and 5-carboxyfluorescein (FAM dye)-labeled phosphorothioate ODNs were custom-synthesized and reverse-phase high-pressure liquid chromatography purified at IDT Inc. (Coralville, Iowa). Subconfluent U-87 glioma cells were incubated in serum containing medium with indicated concentration of antisense or sense ODNs premixed with cytofectin GSV3815 reagent (Glen Research, Sterling, VA) in Opti-MEM-I (Invitrogen, Grand Island, NY) at final cytofectin concentration of 2.5 μg/mL as recommended by the manufacturer. In some experiments, repeated ODN treatment was given every 24 h for 3 days. We included sense ODN and vehicle only controls in all experiments. Cisplatin, etoposide and vincristine (Sigma) were prepared in stock concentrations of 1 mM, 1 mM and 1 μM in sterile water, dimethyl sulfoxide, and sterile water, respectively. Drugs were mixed into culture medium at the time of application. The concentration ranges tested for cisplatin at 1–50 mM, etoposide at 0.5–25 mM and vincristine 0.5–25 nM, covered most clinically relevant concentrations for these drugs.

Transient transfections and reporter luciferase assay

HIF-1α expression vector pcDNA3.1-HIF1α was constructed by inserting the 3.4 kb fragment of HIF-1α complementary deoxyribonucleic acid (cDNA) (a gift from Dr. G. Semenza) that contains the complete coding sequence between Kpn I and BamH I of the multiple cloning site (MCS) of pcDNA3.1(+) vector (Invitrogen). The pGL2 (Promega) –based HIF-1α reporter plasmid pVEGF-Luc was a gift from Dr. A. Damert of Max Planck Institute. The p53 responsive plasmid, pBP100Luc, a luciferase reporter construct also based on pGL2, containing p53-binding sites ($\sim$100 bp) adopted from intron 1 of mdm2 gene, was a gift from Dr. N. Horikoshi. The control Renilla luciferase pRL-TK plasmid was from Promega. A 10:1 mixture of pVEGF-Luc and pRL-TK, 10:1 mixture of pBP100Luc and pRL-TK, or 10:10:1 mixture of pcDNA3.1-HIF1a, pVEGF-Luc and pRL-TK, was co-transfected into subconfluent U-87 cells seeded in 6-well or 12-well culture plates, by SuperFect reagent (Qiagen) at a concentration of 5 μg/mL in the presence of 10% serum for 2 h followed by replacement with fresh medium. Luciferase expression was quantified with Dual Luciferase Assay System (Promega) using a TD-2 Luminometer (Turner Designs, Sunnyvale, CA).

Northern blot analysis

Total cellular RNA isolated using Trizol reagent (Invitrogen) was electrophoresed (10 μg/lane) in 0.9% agarose gels

containing 2.2 M formaldehyde and blotted onto positively charged GeneScreen Plus nylon membrane (DupontNEN, Boston, MA) by downward capillary transfer and fixed with UV crosslinking. cDNA probes for HIF-1α (Gift of Dr. G. Semenza), HIF-2α (Gift of Dr. S. McKnight), Glut-1 (IMAGE clone# 540862), VEGF (IMAGE clone# 850729) and β-actin (ClonTech, Palo Alto, CA) were labeled with α-[^{32}P]-deoxycytidine triphosphate (DupontNEN) using Prime-a-Gene Random Priming kit (Promega). Filters were prehybridized for 1 h in aqueous hybridization buffer followed by hybridization for 12 h at 68°C. Filters were then washed at 68°C once in 0.5× saline sodium citrate (SSC), 0.1% sodium dodecyl sulfate (SDS) for 10 min, and then twice in 0.1× SSC, 0.1% SDS for 5 min. Images were captured from exposed Storage Phosphor Screen with a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA). For reprobing, filters were stripped of bound radioactivity in 10 mM Tris-Cl, pH 7.4, 0.2% SDS at 75°C for 2 h.

Assay for cell viability

In vitro cell viability was determined by a colorimetric MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay. Cells (1×10^4) were plated in triplicate in 24-well culture plate and allowed to attach overnight before treatments were applied. After treatment, the number of viable cells was determined by measuring absorbance at 490 nm using an Elx800 universal microplate reader (BioTek, Burlington, VT) following bio-reduction by intracellular dehydrogenases of the tetrazolium compound MTS (Promega, Madison, Wisconsin) in the presence of the electron-coupling reagent phenazine methosulfate.

Terminal transferase-catalyzed in situ end labeling (TUNEL) assay

A modified TUNEL assay was performed using Apoptag kit (Intergen, Purchase, NY) according to manufacturer's instructions. Cells were cultured and treated directly on chamber slides. After fixing with 10% formalin in phosphate-buffered saline (PBS), pH 7.4 for 10 min at room temperature, cells were permeabilized with ethanol:acetic acid 2:1 for 5 min. Terminal deoxynucleotidyl transferase labeling of fluorescence-labeled nucleotides was performed at 37°C in a humidified chamber for 1 h.

Caspase-3 activity assay

Caspase-3 activity was determined with a colorimetric CaspACE Assay System (Promega) following the manufacturer's instructions. Briefly, treated cells were incubated with or without Z-VAD (Biomol, Plymouth Meeting, PA), a broad-spectrum caspase inhibitor, for 24 h before cell lysate was prepared. The cell lysate was incubated with 50 μM Ac-DEVD-p-nitroanilide, a caspase-3 substrate, for 4 h at 37°C. The amounts of released p-nitroanilide were measured in a micro titer plate reader at 405 nm. Protein concentrations in cell lysate were determined with a BioRad protein assay reagent on a microplate reader using bovine serum albumin as the standard. Specific caspase activities were expressed as fmol pNA released/h/μg protein.

Immunoblot analysis

Nuclear proteins were prepared for the detection of HIF-1α. Cells were washed twice with PBS, resuspended in buffer A (10 mM HEPES-KOH [pH 7.8], 10 mM KCl, 0.1 mM ethylenediaminetetraacetic acid [EDTA] [pH 8.0], and 0.1% Nonidet P-40 [NP-40], 1 mM dithiothreitol [DTT], 1 mM orthovanadate, 2 mM phenylmethylsulfonyl fluoride [PMSF]), and mixed by vortexing. Cell nuclei were recovered by centrifugation at 500 × *g* for 5 min. The pellet was resuspended in buffer C (50 mM HEPES-KOH [pH 7.8], 420 mM KCl, 0.1 mM EDTA [pH 8.0], 5 mM $MgCl_2$, and 20% glycerol, 1 mM DTT, 1 mM orthovanadate, 2 mM PMSF, 1 μg/mL leupeptin, 1 μg/mL aprotinin and 1 μg/mL pepstatin) and gently mixed at 4°C for 30 min. After centrifugation at 14,000 × *g* at 4°C for 30 min, the supernatant containing the nuclear protein fraction was recovered and immediately frozen on dry ice and stored at –80°C. Protein concentrations were determined as described above. Fifty microgram per lane nuclear proteins were resolved by 12% and 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), respectively, transferred to nitrocellulose, and blocked with 5% nonfat milk for 1 h. The filters were then incubated with 1:200 diluted mouse anti-HIF-1α monoclonal antibody (BD Transduction Laboratories, Lexington, KY), 1:500 rabbit anti-p21 or 1:500 mouse anti-Bcl-2 monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), followed by washing and incubation with peroxidase-conjugated anti-mouse or anti-rabbit IgG. The blots were then visualized using an enhanced chemiluminiscence kit (Amersham, Uppsala, Sweden).

RESULTS

FAM dye-labeled AS-HIF phosphorothioate ODN in solution failed to enter U-87 cells as expected, even at high concentrations. Packaged with cytofectin GSV 3815, a cationic lipid formulated with the fusogenic lipid dioleoylphosphatidyliethanolamine, the labeled ODN was readily taken up by U-87 cells. At 0.017 μM, approximately 35% of cells showed intense nuclear labeling after 24 h. At least 95% of cells were labeled at a concentrations of 0.25 μM or above. The fluorescence appeared mainly in the nuclei, with other compartments and organelles weakly labeled. The effective dose of cytofectin at 2.5 μg/mL was not toxic to U-87 cells, as analyzed by visual inspection and repeated MTS cell viability assays (data not shown).

Inhibition of HIF-1α transactivation was measured as drop in the relative light unit (RLU) in U-87 cells cotransfected with a hypoxia-responsive firefly luciferase reporter and a constitutive low-expression renilla luciferase reporter plasmids. No inhibition of HIF reporter activity was seen with S-HIF treatment at concentrations up to 1 μM. At 0.05 μM, the AS-HIF treatment achieved 75% and 76% reporter activity inhibition under normoxia and hypoxia

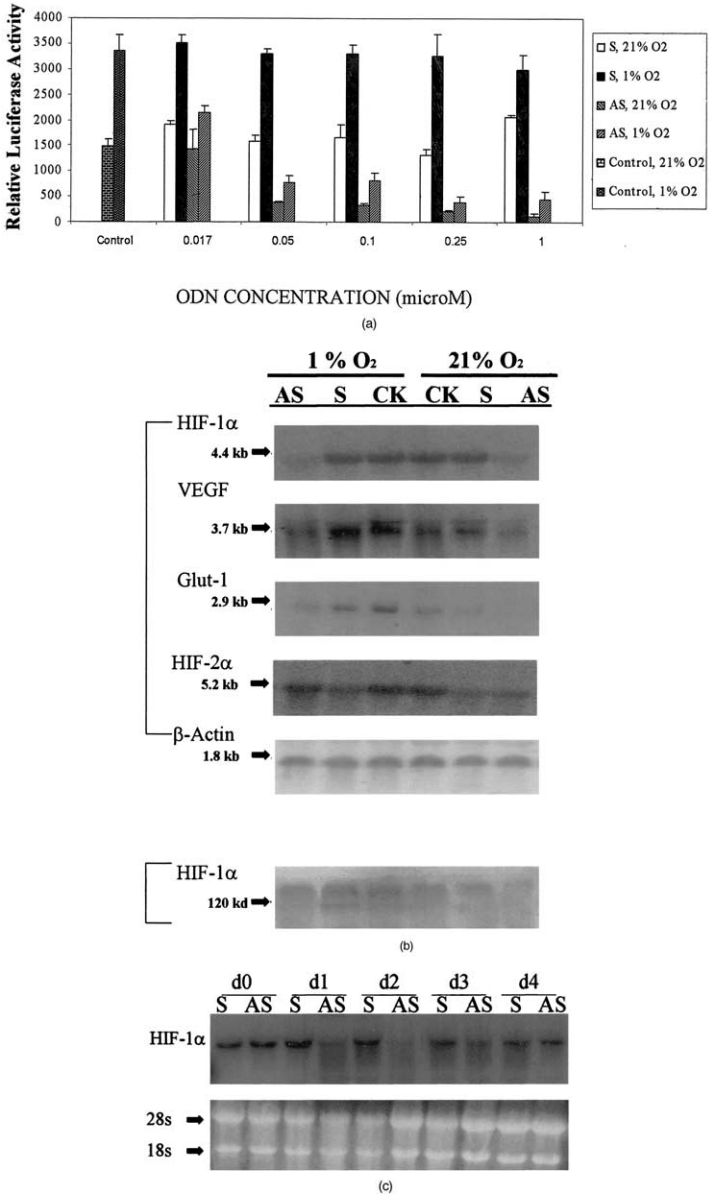

Fig. 1. Antisense inhibition of HIF-1α expression and transactivation. (a) 2.5×10^5 U-87 cells/well were seeded onto 6-well culture plates, allowed to attach overnight, and then transiently transfected with HIF-1α reporter pVEGF-Luc and control vector pRL-TK. 24 h later, cells were treated with AS-HIF or S-HIF ODN at 0.017, 0.05, 0.10, 0.25 or 1.0 μM, or vehicle alone. Hypoxia started 4 h after the initiation of ODN treatment and last for 16 h. Relative luciferase activity is quantified as the ratio of amount of firefly luciferase light to that of renilla luciferase. Plotted data represent the mean of three independent experiments. (b) Subconfluent U-87 cells in T-75 culture flasks were treated with 0.25 μM AS-HIF (*AS*), S-HIF (*S*), or vehicle (*CK*) for 4 h followed by 16-h exposure to hypoxia or normoxia. Top: Ten microgram total RNAs were electrophoresed on formaldehyde agarose gel and analyzed for HIF-1α, Glut-1, VEGF, HIF-2α or β-Actin mRNA by northern blots as described in "Methods and Materials." Lower: 50 μg/lane nuclear proteins resolved on SDS-PAGE were immunoblotted with anti-HIF-1α monoclonal antibody as described in "Methods and Materials." (c) 50% confluent U-87 cells were treated with 0.25 μM AS-HIF or S-HIF ODN. Cells were harvested at 0, 24, 48, 72 and 96 h after ODN treatment and total RNAs were extracted analyzed for HIF-1α mRNA. Lower panel shows ethidium bromide stain of total RNAs on nylon blot.

(1% O_2), respectively. Significant inhibition of HIF reporter activity was achieved with AS-HIF concentrations ranging from 0.05 μM to up to 1 μM. The degree of inhibition was comparable between normoxic and hypoxic conditions at these concentrations (Fig. 1A).

The primary antisense action for phosphorothioate ODNs is believed to be through ribonuclease H–mediated mRNA cleavage (13). Next, we determined if AS-HIF-1α indeed caused targeted HIF-1α mRNA depletion in Northern blot analyses. HIF-1α mRNA level was largely depleted by 0.25 μM AS-HIF within 24 h under hypoxic as well as normoxic conditions. Because the VEGF promoter used in the luciferase reporter assay contains both HIF-1 and HIF-2 binding sites, we also determined whether the AS-HIF-1α also interfered with HIH-2α expression. As shown in Fig. 1B, HIF-2α mRNA level was unaffected by the same HIF-1α-

specific antisense treatment. In a time course study, HIF-1α mRNA level did not recover until 4 days after the antisense treatment (Fig. 1C). The antisense inhibition of HIF-1α transactivation was further supported by results derived from western blot for HIF-1α as well as northern blot for VEGF and Glut-1. AS-HIF suppressed HIF-1α protein levels under both hypoxia and normoxia. Noticeably, unphosphorylated HIF-1α (lower molecular weight band) content was absent in AS-HIF treated hypoxic cells. We notice that in AS-HIF treated cells, VEGF and Glut-1 mRNA level was still induced by hypoxia but to a lesser extent, consistent with the fact that VEGF and Glut-1 genes respond to the transcription promotion of both HIF-1 and HIF-2 (Fig. 1B).

AS-HIF treatment negatively affected U-87 glioma cell viability. Within 4 days of the antisense treatment, survival was significantly reduced at concentration above 0.05 μM, as measured by MTS cell viability assay. At 0.25 μM, there were approximately 60% and 75% reduction in the number of viable cells under hypoxic and normoxic conditions respectively. No significant reduction in cell viability was seen in sense ODN (S-HIF) treated groups as compared to vehicle-treated or untreated controls. There was a clear dose-dependent effect of AS-HIF on cell survival between 0.017 μM and 1 μM (Fig. 2A). The AS-HIF effect on cell death was apparent 2–3 days after treatment. Significant decrease in cytoskeleton and increase in cell surface blebbing were observed in cells treated with 0.25 μ 77 AS-HIF for 48 h (data not shown).

The constitutive expression of HIF-1α mRNA in normal and cancer cells (4) raised the concern that the AS-HIF effect on cell viability may be indiscriminate between normal and cancer cells. We therefore tested the growth inhibitory effect of AS-HIF on normal human fibroblasts MRC-5 as well as rat primary astrocytes. The rat HIF-1α coding sequence has been published (Genbank #U22373) and AS-HIF target sequence in rats is the same as in humans. HIF-1α mRNA was abundantly expressed in rat primary astrocytes and was also detected in MRC-5 fibroblasts. A single dose of 0.25 μM AS-HIF treatment for 24 h effectively knocked down HIF-1α mRNA level in both cells (Fig. 2B). Comparing with S-HIF and vehicle treatment for three consecutive days, survival of rat normal astrocytes was not affected by AS-HIF. In fact, we observed slight increase in cell number in AS-HIF treated rat astrocytes. The reason for this increase is unknown. The difference in cytotoxicity between U-87 glioma cells and MRC-5 normal fibroblasts appeared to be dose-dependent. MRC-5 cell viability was unaffected by AS-HIF at 0.05 μM, whereas only 30% U-87 cells survived the 3-day treatment. At concentration of 0.25 μM or below, S-HIF control had minimal effect on survival of MRC-5 cells. Toxicity of S-HIF increased at concentrations at 1 μM or higher, which is not unusual for nonspecific effect of phosphorothioate ODN (Fig. 2C).

We then investigated whether AS-HIF induced cell death involved caspase activation. AS-HIF activation of caspase-3 in U-87 cells was confirmed by a specific caspase-3 colorimetric assay using Ac-DEVD-p-nitroanilide as substrate. At 48 and 96 h caspase-3 activity was 5- to 6-fold higher in AS-HIF than S-HIF and vehicle-treated cells (Fig. 2D). Z-VAD virtually blocked AS-HIF activation of caspase-3 (Fig. 2D). An increase in DNA fragmentation accompanied AS-HIF activation of caspase-3. Approximately 16% AS-HIF treated cells stained positive for DNA strand breaks based on TUNEL stain at 72 h post treatment as compared to less than 2% in S-HIF and vehicle treated cells (Fig. 2E). Z-VAD treatment also reduced AS-HIF-induced U-87 cell death by 80% (Fig. 2F). Because S-HIF and vehicle had little effects in activating caspase-3 (Fig. 2D), Z-VAD was not expected to have much impact on cell survival in cells treated with S-HIF or vehicle as shown in Fig. 2F.

U-87 glioma cells express wild-type p53 (14). We explored the involvement of p53 in the AS-HIF-mediated caspase-dependent apoptosis by analyzing p53 transactivation with a p53-responsive luciferase reporter pBP100 as well as the expression of p53 target genes, namely p21 and Bcl-2 using western blot. The p53 reporter assay was validated by a 3.5- and 3.1-fold induction of relative luciferase activity in U-87 cells exposed to 5 μM camptothecin for 24 h under normoxia and hypoxia, respectively. Hypoxia (24 h at 1% O_2) did not induce p53 response, neither did 24 h AS- or S-HIF treatments at concentrations of 0.05 μM and 0.25 μM (Fig. 3). We also compared the effects of AS-HIF on cell survival in four glioma cell lines, namely U-87, LN-18, LN-229 and LN-Z308. U-87 possesses a wild type p53. LN-18 and LN-229 express mutant p53s whereas LN-Z308 is a deletion/translocation heterozygote (14). Percent survival after 0.25 μM AS-HIF treatment for 3 days were 45%, 61%, and 55% for U-87, LN-229 and LN-Z308, respectively. Overall, we found no correlation between p53 status and the AS-HIF effect on survival of glioma cells.

To study the role of HIF-1α in promoting glioma cell survival, we transiently transfected U-87 cells with a pcDNA3.1-HIF1α expression vector. Transfected cells showed 4- and 2.6-fold higher HIF reporter response under normoxia and hypoxia, respectively, as compared to pcDNA3.1 empty vector-transfected cells (Fig. 4A). Hypoxia protects glioma cells against the cytotoxicity of chemotherapeutic agents. In U-87 cells transfected with control vector pcDNA3.1, cisplatin, etoposide, and vincristine were effective in reducing U-87 cell population by approximately 50% at 5 μM, 0.5 μM and 0.5 nM, respectively under normoxic condition. However, a 4-h precondition at 1% O_2 before adding these chemotherapeutic agents followed by a 48-h incubation under 1% O_2, the concentrations required to achieve the similar level of cell population reduction were increased to approximately 25 μM, 10 μM, and 5 nM, respectively. Overexpressing HIF-1α protected U-87 cells against the effect of chemotherapy drugs. U-87 cells over expressing HIF-1α were found to be resistant to the killing effect of cisplatin, etoposide and vincristine to similar extent as control vector-transfected cells under hypoxia (Fig. 4B).

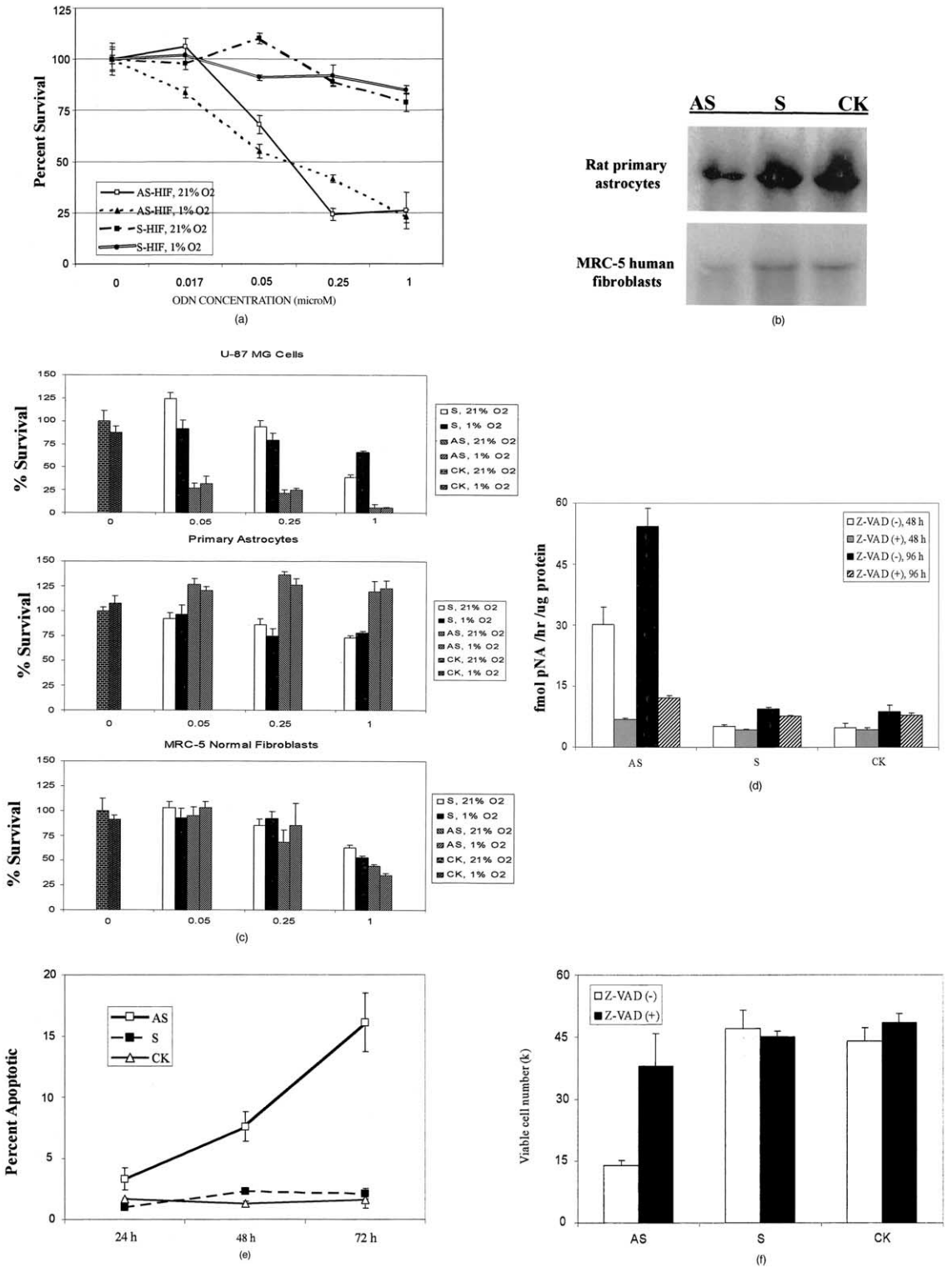

Fig. 2. AS-HIF suppresses U-87 glioma cell survival and induces caspase-dependent apoptosis. (a) 1×10^4 cells/well were seeded on 24-well culture plates and allowed to attach and adapt to either normoxic or hypoxic culture conditions overnight. Normoxic and hypoxic cells were then treated with 0.017, 0.05, 0.25 and 1 μM of AS-HIF or S-HIF, or vehicle alone. Viable cell numbers were determined 4 days after receiving ODN treatment by MTS assay as described in "Methods and Materials." Data are plotted as percent survival using vehicle control cells as 100%. (b) Subconfluent rat primary astrocytes or MRC-5 human fibroblasts were treated with 0.25 μM AS-HIF, S-HIF, or vehicle for 24 h under normoxia. Total RNAs were extracted and 10 μg total RNA/lane were subjected to northern blot analysis for HIF-1α mRNA as described in "Methods and Materials." (c) 1×10^4 cells/well of U-87 glioma cells, MRC-5 fibroblasts or rat primary astrocytes were seeded on 24-well culture plates and allowed to attach and adapt to hypoxic or normoxic culture condition overnight. Cells were then treated with AS-HIF or S-HIF ODN at given concentrations, or vehicle alone daily

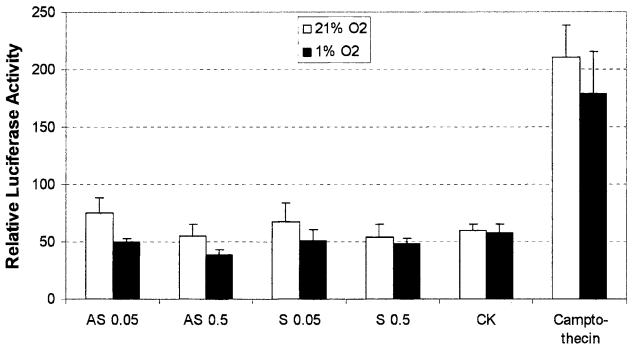

Fig. 3. AS-HIF treatment does not lead to p53 upregulation. 2×10^5 U-87 cell/well seeded on 6-well culture plates were co-transfected with 10:1 mixture of pBP100-Luc and pRL-TK. Twenty-four hours later, cells were exposed to the indicated concentrations of AS- or S-HIF, vehicle alone, or vehicle with 5 μM camptothecin. Hypoxia started at 4 h after the addition of ODNs or drug and lasted for 24 h. Relative luciferase activity is quantified as the ratio of amount of firefly luciferase light to that of renilla luciferase. Plotted data represent the mean of three independent experiments.

The implication of HIF-1α overexpression in tumor malignancy and resistance to therapies led us to hypothesize that HIF-1α may be directly involved in hypoxic protection against cytotoxic chemotherapy and depleting HIF-1α might overcome hypoxia-induced chemoresistance. To test this hypothesis, we pretreated U-87 cells with various concentrations of AS-HIF, S-HIF at concentrations of 0.05 μM, 0.10 μM, 0.25 μM, or vehicle for 24 h then subjected cells to 1% O_2 hypoxia for 4 h before applying various concentrations of cisplatin, etoposide and vincristine. In all AS-HIF concentrations, we found that when normalized sense control, the effects of HIF-1α antisense and chemotherapy drugs on tumor cell survival were additive in nature and no synergistic interaction could be established. Figure 5 shows the results of hypoxic U-87 cells treated with 0.25 μM AS-HIF plus 5 μM cisplatin, 5 μM etoposide, or 0.5 nM vincristine. Similar trend was observed at various chemotherapy dose levels (data not shown). Interestingly, the tumoricidal effect of AS-HIF was not attenuated by hypoxia, either given alone or in combination with the three drugs. Pretreatment with 0.25 μM AS-HIF combined with 5 μM cisplatin, 5 μM etoposide, or 0.5 nM vincristine achieved significant suppression of hypoxic tumor cell number by 78%, 82% and 67%, respectively.

DISCUSSION

AS-HIF effect is oxygen independent

In this study, we demonstrated an effective antisense strategy that successfully knocks down HIF-1α expression under both normoxic and hypoxic conditions in U-87 glioma cells. HIF-1α protein is constitutively expressed but quickly degraded beyond detection in most cells under normal condition (6). The reason for the existence of such seemingly wasteful process is not understood. HIF-1α and HIF pathway related genes are highly conserved in vertebrates from *Caenorhabditis elegans* to humans (9, 15). Permanent loss of HIF-1 expression as seen in HIF-1α knockout mouse is lethal (12), and antisense knockdown of HIF-1α mRNA expression reduces proliferation of PC12 cells (16). Nevertheless, loss of ability to induce HIF-1α under hypoxia is tolerated in some cells such as Hepa1c4 cells (17). Beyond an extremely rapid response mechanism to hypoxic stress, maintaining basal level HIF-1α expression may be essential to cellular function, especially so for rapidly growing tumor cells. We showed that significant inhibition of HIF reporter activity was achieved with AS-HIF concentrations ranging from 0.05 μM to up to 1 μM. U-87 cells treated with 0.05 μM of AS-HIF showed reduction in luciferase reporter activity by 75 and 76% under normoxia and hypoxia (1% O_2), respectively. Thus, the antisense strategy to knock down HIF employed in the present study is independent of oxygen tension. It should be noted, however, that the ability of hypoxia to induce HIF function was still partially preserved even with HIF antisense knockdown. It is therefore plausible that the suppression of basal HIF-1α expression by AS-HIF likely accounts for its oxygen-independent effects. The VEGF promoter used in the luciferase reporter assay contains both HIF-1 and HIF-2 binding sites. However, the AS-HIF-1α used in the present study was specific for HIH-1α and did not alter HIH-2α expression suggesting the specificity of this antisense ODN.

AS-HIF caused glioma cell death and induced apoptosis

Results from the present study indicate that the inhibition of HIF-1α transactivation compromised cell viability and facilitate apoptosis through a caspase-dependent, but p53-independent mechanism in U-87 glioma cells. After 0.25 μM AS-HIF treatment for 4 days, viable cell number de-

for three consecutive days. Viable cell numbers were measured by MTS assay on the fourth day, 24 h after last treatment. (d) 1×10^4 U-87 cells/well seeded and treated as above on 24-well culture plates. 50 μM general caspase inhibitor Z-VAD was added at the beginning of ODN treatment and was added again every 24 h. Caspase-3 activities were determined 3 days after the treatment. Experiments were conducted under normoxia. (e) Subconfluent U-87 cells were treated with 0.25 μM AS-HIF or S-HIF ODN, or vehicle for 24 h and were then trypsinized. 1×10^6 cells/well were seeded onto 6-well culture plates in sets of two, one of which were added 50 mM Z-VAD at seeding and added again 24 h late. Cell extracts were prepared at 24 and 48 h after seeding. Caspase-3 activity, in fmol pNA/h/mg protein, was determined by measuring the release of free p-nitroanilide from Ac-DEVD-p-NA, as described in "Methods and Materials." Experiments were conducted under normoxia. (f) Confluent U-87 cells cultured on 2-well glass chamber slides were treated with 0.25 mM AS-HIF or S-HIF ODN or vehicle alone. At 24, 48, and 72 h post treatment, slides were harvested for detection of DNA breakage by TUNEL assay as described in "Methods and Materials." Negative controls without biotinylated nucleotides were run for all samples. At least 500 cells were counted under microscope for each sample. Shown results are representative of at least three independent experiments. Experiments were conducted under normoxia.

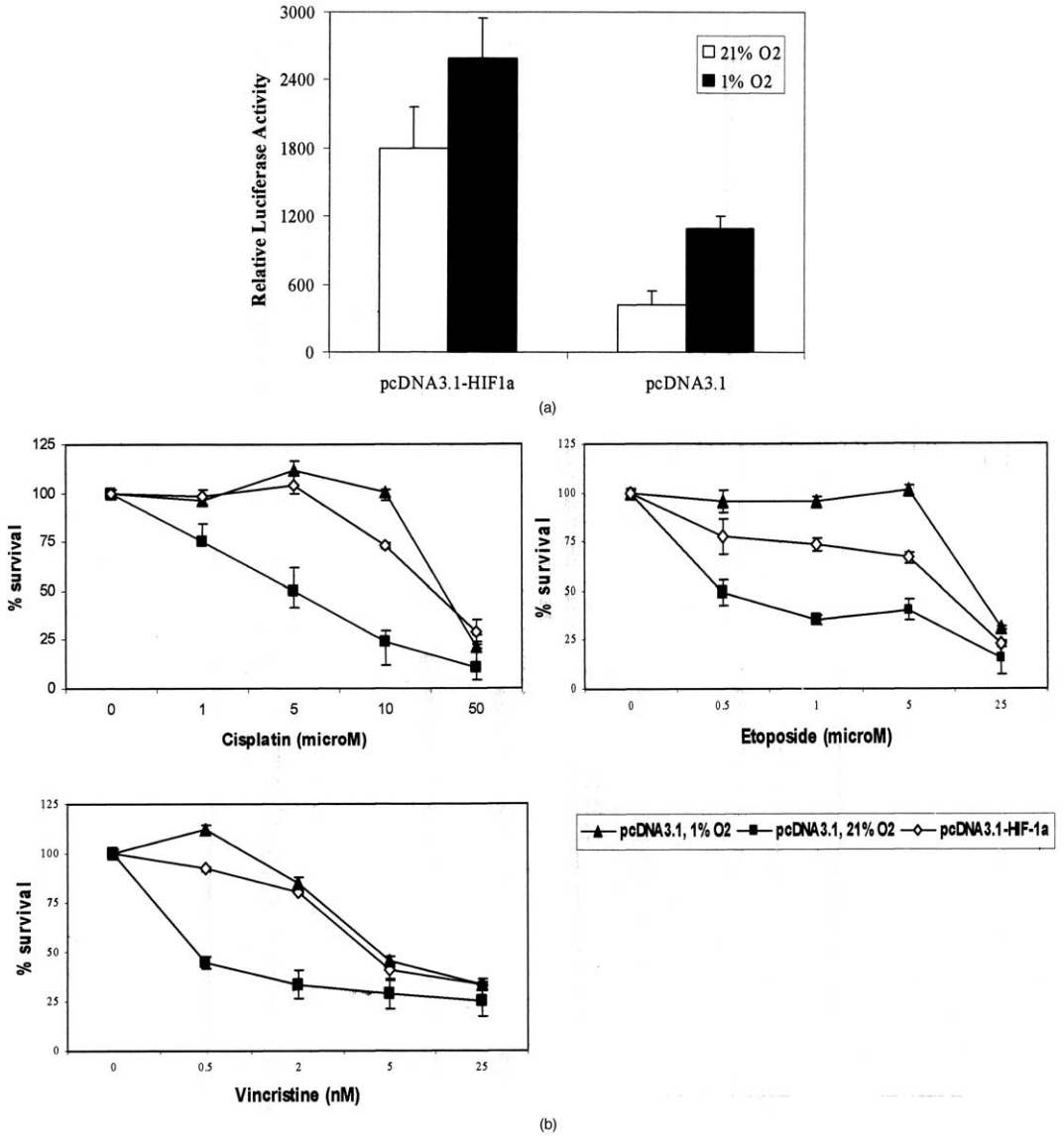

Fig. 4. Overexpression of HIF-1α confers chemo-resistance to U-87 cells. (a) 2.5×10^5 U-87 cell/well seeded on 6-well culture plates were co-transfected with 10:1:10 mixture of pVEGF-Luc, pRL-TK, and either pcDNA3.1-HIF-1α or pcDNA3.1 vector without insert. 24 h later, cells were exposed to hypoxia or normoxia for 16 h before being harvested for luciferase assay as described in "Methods and Materials." (b) 1×10^4 U-87 cells/well were seeded on 24-well culture plates and transiently transfected with pcDNA3.1-HIF-1α or pcDNA3.1 empty vector. Twenty hours later, transfected cells were conditioned to either normoxia (21% O_2) or hypoxia (1% O_2) for a period of 4 h before being treated with various concentrations of cisplatin, etoposide and vincristine for 48 h under correspondent oxygen conditions.. Cell survival was measured MTS assay. Survival of empty vector pcDNA3.1-transfected cells receiving no drug a was set as 100%.

creased 60% and 75% under hypoxic and normoxic conditions respectively. A dose-dependent effect of AS-HIF on cell survival was established between 0.017 μM and 1 μM. U-87 cells express functional wild-type p53 but normally resist apoptosis. Upregulation of wild-type p53 is not adequate to induce apoptosis phenotype (14, 18). The accumulation of HIF-1α protein is known to stabilize wild-type p53 presumably through direct interaction of unphosphorylated HIF-1α with wild-type p53 and possibly also HDM2 (19). The absence of unphosphorylated HIF-1α in AS-HIF treated U-87 cells, and hence likely interruption of HIF-1α interaction with p53 and HDM2 may partially explain the fact that p53 function and expression of p53 target genes did not change by HIF-1α depletion. A p53-independent apoptotic pathway, which triggers mitochondrial caspase activation events, may be responsible for glioma cell death induced by AS-HIF. Interestingly, we showed the same antisense strategy did not induce cell death in astrocytes in primary cultures. The mechanism by which AS-HIF selectively induces apoptosis in U-87 cells but not astrocytes is not clear. By activating glycolytic metabolism and angiogenic growth, HIF-1α has an important function in cell

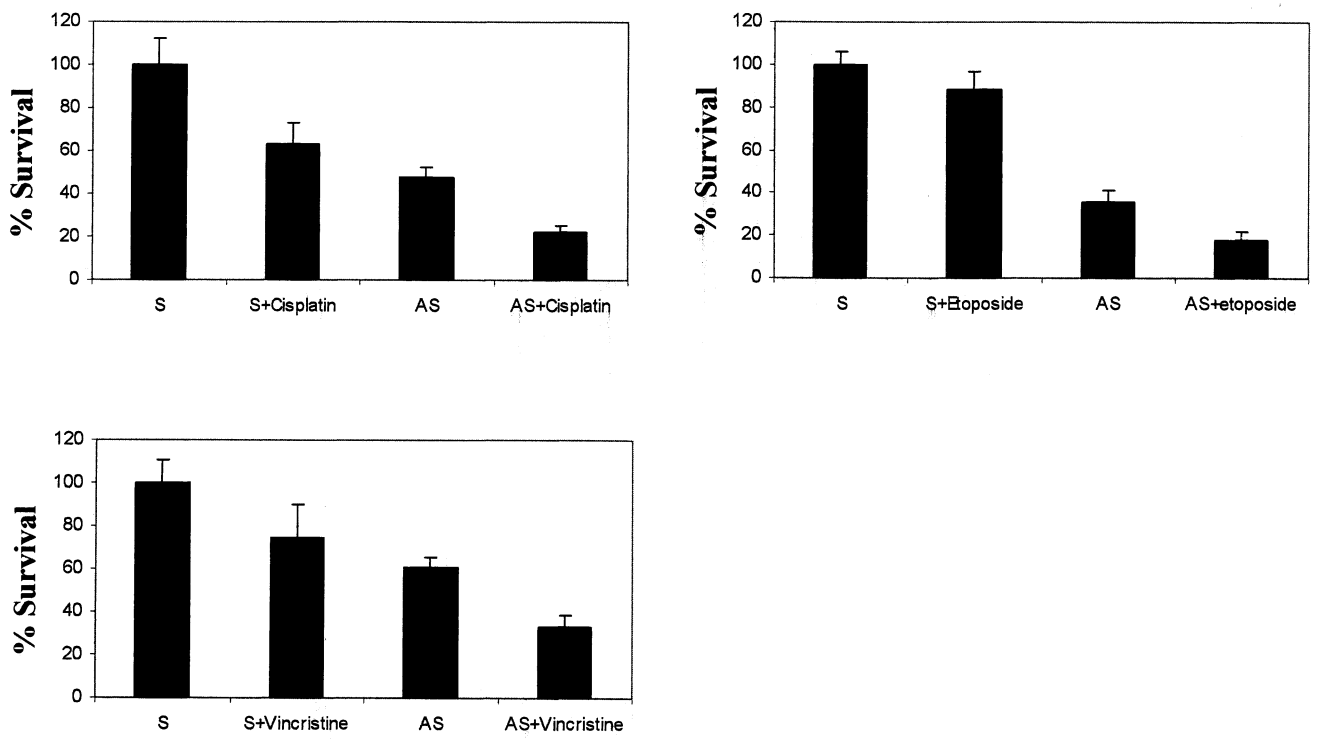

Fig. 5. Effect of HIF-1α inhibition on U-87 glioma cell response to chemotherapy drugs cisplatin, etoposide and vincristine. Subconfluent U-87 cells in T-75 flasks were treated with 0.25 μM AS-HIF or S-HIF ODN, or vehicle for 24 h. Viable cells were then detached with trypsin-EDTA, counted and seeded onto 24-well plate at 1×10^4 cells/well and allowed to adapt to hypoxic (1% O_2)condition for 16 h before addition of 5 μM cisplatin, 5 μM etoposide, or 0.5 nM vincristine. After 48-h incubation with drugs under hypoxia, viable cell numbers were measured by MTS assay. Plotted results are representative of three independent experiments.

survival. However, HIF-1α is also involved directly and indirectly in activating proapoptotic proteins (19, 20). It is possible that neoplastic cells may be more dependent upon HIF-1α because of a higher metabolic demand and a unique cell survival mechanism involving genes downstream of HIF-1α. Results presented in this study support the contention that HIF-1α is a significant mechanism for survival in U-87 cells but not in non-neoplastic astrocytes. A transient suppression of HIF-1α expression in U-87 glioma cells may shift the balance of apoptotic and pro-apoptotic forces in favor of cell death.

AS-HIF exerts additive cytotoxic effect with chemotherapeutic agents on glioma cells

Overexpression of HIF-1α is common in human glioma and is speculated to be one of the causes of glioma resistance to chemotherapy and radiation. Thus, inhibition of HIF-1α may sensitize resistant glioma cells to the cytotoxic effect of radiation and chemotherapeutic agents. We found that when normalized to sense control, the effects of HIF-1α antisense and chemotherapy drugs on tumor cell survival were additive in nature and that suppressing HIF-1α did not exert synergistic sensitization of hypoxic U-87 cells to the cytotoxic effect of chemotherapeutic agents. Cell survival was reduced by 5 μM cisplatin, 5 μM etoposide, or 0.5 nM vincristine by 72%, 65% and 53% with 0.25 μM AS-HIF, and by 37%, 12%, and 25% without AS-HIF, respectively. Furthermore, we found that the antisense-mediated additive effect was not altered by oxygen tension, suggestive of separate mechanisms of cell killing are in operation for AS-HIF and chemotherapeutic agents respectively. Despite numerous reports that HIF-1α expression in tumors serves as negative prognostic indicator, a causal effect of HIF-1α in chemotherapy or radiation resistance, to our knowledge, has not been reported. Tumor hypoxia is a heterogeneous event and the consequence of it can be multidimensional. Through HIF-dependent gene transcription pathways, severe hypoxia triggers cytoprotective mechanisms but can also contribute to the activation of proapoptotic genes such as Nip3, a member of the Bcl-2 protein family (15). Complete understanding of the molecular events under hypoxia will be needed to dissect the delicate roles played by HIF-1α in hypoxic tumor resistance. New line of evidence is emerging that HIF-independent pathway contributes significantly to the immunity of cancer cells to apoptotic stimuli. The upregulation of inhibitor of apoptosis protein-2 (IAP-2) by severe hypoxia is HIF-independent and does not involve reoxygenation/reperfusion injury (21). Taken together, we suggest that the mechanism for hypoxic tumor cell resistance may be complex and involve HIF-dependent as well as -independent pathway(s).

HIF-1 is a potential cancer treatment target

The molecular mechanisms underlying tumor adaptation to hypoxia and angiogenic growth are currently unfolding. Deliberate tumor oxygen deprivation results in starvation and programmed cell death. However, it is well known that sublethal level of hypoxia stimulates heterogeneous neovascularization and selects for more malignant phenotypes, which eventually contribute to the progression and metastasis of tumor. The limitation of antiangiogenesis therapy for cancer is increasingly being recognized. HIF-1α, given its pivotal role in tumor hypoxia, apoptosis and angiogenesis regulation, fits the profile of a more elaborate target for cancer molecular therapy (22). It has been shown that gene knockdown using antisense HIF-1α retroviral vector reduces tumor vessel density and enhances cancer immunotherapy (23) and that disruption of HIF-1α co-activational pathway and subsequent attenuation of HIF-dependent gene expression lead to diminution of tumor growth (24). Still, there are few choices that are currently available for direct and specific inhibition of HIF-1α. Recent discovery of the cellular oxygen sensor –HIF prolyl hydroxylase (7–9), the key negative regulator of HIF-1α, is likely to aid the study of HIF-1α inhibition. Targeting HIF-1α in human glioma maybe advantageous in a number of aspects. First, oxygen independent induction of apoptosis by HIF-1α inhibition can potentially overcome therapy problems arising from local hypoxia. Second, HIF-1α is believed to play a major role in hypoxic cancer cell invasion and metastasis (25). Clinically, the reversal of invasive and infiltrating growth nature of malignant glioma maybe more desirable over a tumoricidal approach. Third, sustained inhibition of neovascularization may be achieved through the modulation of HIF-1α expression rather than direct antagonism of angiogenic stimuli.

In summary, we demonstrated that antisense specific knockdown of HIF-1α is accompanied by reduced viability and increased apoptosis in glioma cells *in vitro* under both normoxic and hypoxic conditions. The effect of AS-HIF is additive to that of chemotherapy drugs. An antisense strategy directed at HIF-1α may be a useful adjunct to the treatment of malignant glioma in which HIF expression is robust.

REFERENCES

1. Maher E, Furnari F, Bachoo R, *et al.* Malignant glioma: Genetics and biology of a grave matter. *Genes Dev* 2001;15: 1311–1333.
2. Chao KSC, Bosch WR, Mutic S, *et al.* A novel approach to overcome hypoxic tumor resistance—ATSM-guided intensity modulated radiation therapy. *Int J Radiat Oncol Biol Phys* 2001;49:1171–1182.
3. Semenza G. HIF-1: Using two hands to flip the angiogenic switch. *Cancer Metastasis Rev* 2000;19:59–65.
4. Wiener C, Booth G, Semenza G. In vivo expression of mRNAs encoding hypoxia-inducible factor 1. *Biochem Biophys Res Commun* 1996;225:485–488.
5. Jiang B, Semenza G, Bauer C, *et al.* Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O_2 tension. *Am J Physiol* 1996;271:C1172–1180.
6. Jewell U, Kvietikova I, Scheid A, *et al.* Induction of HIF-1alpha in response to hypoxia is instantaneous. *FASEB J* 2001;15:1312–1314.
7. Ivan M, Kondo K, Yang H, *et al.* HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O_2 sensing. *Science* 2001;292:464–468.
8. Jaakkola P, Mole D, Tian Y, *et al.* Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. *Science* 2001;292:468–472.
9. Epstein A, Gleadle J, McNeill L, *et al.* C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell* 2001;107:43–54.
10. Zagzag D, Zhong H, Scalzitti J, *et al.* Expression of hypoxia-inducible factor 1alpha in brain tumors: Association with angiogenesis, invasion, and progression. *Cancer* 2000;88:2606–2618.
11. Caniggia I, Mostachfi H, Winter J, *et al.* Hypoxia-inducible factor-1 mediates the biological effects of oxygen on human trophoblast differentiation through TGFbeta(3). *J Clin Invest* 2000;105:577–587.
12. Ryan H, Lo J, Johnson R. HIF-1 alpha is required for solid tumor formation and embryonic vascularization. *EMBO J* 1998;17:3005–3015.
13. Baker B, Monia B. Novel mechanisms for antisense-mediated regulation of gene expression. *Biochim Biophys Acta* 1999; 1489:3–18.
14. Weller M, Rieger J, Grimmel C, *et al.* Predicting chemoresistance in human malignant glioma cells: The role of molecular genetic analyses. *Int J Cancer* 1998;79:640–644.
15. Suzuki H, Tomida A, Tsuruo T. Dephosphorylated hypoxia-inducible factor 1alpha as a mediator of p53-dependent apoptosis during hypoxia. *Oncogene* 2001;20:5779–5788.
16. Sowter H, Ratcliffe P, Watson P, *et al.* HIF-1-dependent regulation of hypoxic induction of the cell death factors BNIP3, and NIX in human tumors. *Cancer Res* 2001;61: 6669–6673.
17. Jiang H, Guo R, Powell-Coffman J. The Caenorhabditis elegans hif-1 gene encodes a bHLH-PAS protein that is required for adaptation to hypoxia. *Proc Natl Acad Sci USA* 2001;98: 7916–7921.
18. Drutel G, Kathmann M, Heron A, *et al.* Two splice variants of the hypoxia-inducible factor HIF-1alpha as potential dimerization partners of ARNT2 in neurons. *Eur J Neurosci* 2000; 12:3701–3708.
19. Carmeliet P, Dor Y, Herbert J, *et al.* Role of HIF-1 alpha in hypoxia-mediated apoptosis, cell proliferation, and tumour angiogenesis. *Nature* 1998;394:485–490.
20. Cerrato J, Yung W, Liu T. Introduction of mutant p53 into a wild-type p53-expressing glioma cell line confers sensitivity to Ad-p53-induced apoptosis. *Neuro-oncol* 2001;3:113–122.
21. Bruick R. Expression of the gene encoding the proapoptotic Nip3 protein is induced under hypoxia. *Proc Natl Acad Sci* 2000;97:9082–9087.
22. Dong Z, Venkatachalam M, Wang J, *et al.* Up-regulation of apoptosis inhibitory protein IAP-2 by hypoxia: Hif-1-independent mechanisms. *J Biol Chem* 2001;276:18702–18709.
23. Kung A, Wang S, Klco J, *et al.* Suppression of tumor growth through disruption of hypoxia-inducible transcription. *Nat Med* 2000;6:1335–1340.
24. Sun X, Kanwar J, Leung E, *et al.* Gene transfer of antisense hypoxia inducible factor-1 alpha enhances the therapeutic efficacy of cancer immunotherapy. *Gene Ther* 2001;8:638–645.
25. Zhong H, DeMarzo A, Laughner E, *et al.* Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. *Cancer Res* 1999;59:5830–5835.