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Laboratory Investigation

Inhibition of hypoxia inducible factor-1α (HIF-1α) decreases vascular endothelial growth factor (VEGF) secretion and tumor growth in malignant gliomas

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Key words: angiogenesis, brain tumor, glioblastoma multiforme, hypoxia inducible factor, siRNA, vascular endothelial growth factor

Summary

Introduction: Hypoxia inducible factor-1α (HIF-1α) regulates vascular endothelial growth factor (VEGF), the presumed principal mediator of angiogenesis in malignant gliomas, under normal physiologic conditions. We examined the effect of HIF-1α on VEGF secretion, tumor growth, and angiogenesis in malignant gliomas.

Methods: We examined 175 human gliomas for expression of HIF-1α and its downstream-regulated proteins. HIF-1α expression and VEGF secretion in glioma cell lines under normoxia and hypoxia were examined using ELISA and Western blot. Malignant glioma cell lines were transfected with dominant-negative HIF-1α (DN-HIF-1α) expression vector or siRNA constructs against the HIF-1α gene. Growth studies were conducted on cells with the highest VEGF/HIF-1α inhibition isolated from stable transfected cell lines. MIB-1-labeling index and microvascular density (MVD) measurements were performed on the *in vivo* tumors.

Results: HIF-1 expression correlates with malignant glioma phenotype and was not confined to perinecrotic, pseudopalisading cells. VEGF and HIF-1 expression was high in glioma cell lines even under normoxia, and increased after exposure to hypoxia or growth factor stimulation. Cells transfected with DN-HIF-1α or HIF-1α siRNA demonstrated decreased HIF-1α and VEGF secretion. *In vivo* but not *in vitro* growth decreased in response to VEGF and HIF-1 inhibition. HIF-1 siRNA studies showed decreased VEGF secretion and *in vitro* and *in vivo* growth of glioma cell lines. MVD was unchanged but MIB-1 proliferation index decreased for both types of HIF-1 inhibition.

Conclusions: VEGF and HIF-1α are elevated in malignant gliomas. HIF-1α inhibition results in VEGF secretion inhibition. HIF-1α expression affects glioma tumor growth, suggesting clinical applications for malignant glioma treatment.

Introduction

Among the features that distinguish higher-grade gliomas, such as glioblastoma multiforme (GBM), from low-grade gliomas are vascular endothelial cell proliferation and peritumoral edema [1,2]. Vascular endothelial growth factor (VEGF) is thought to be the major mediator of these properties and has been implicated in glioma growth and angiogenesis [3,4]. VEGF expression is regulated by cellular hypoxia mediated by hypoxia inducible factor-1α (HIF-1α) under normal physiological conditions [5] and possibly under pathological situations, such as tumorigenesis.

Overexpression of HIF-1α has been described in common human cancers and their metastases, including a limited number of human brain tumors [6]. The role of HIF-1α in solid tumor growth is still not clear, but previous work suggests that this transcription factor is necessary for growth and angiogenesis of these tumors [7–10]. Although very little is known of the role of HIF-1α in glioma growth and angiogenesis, it is logical that similar processes take place in these tumors. We hypothesize that inhibition of HIF-1α will reduce secretion of VEGF and inhibit glioblastoma tumor growth.

Materials and methods

Patient data and tumor histology

Specimens from 175 patients were obtained under a University of Utah Institutional Review Board – approved protocol (Table 1). Tumors were graded by criteria set forth in the WHO classification of brain tumors [1,2]. A smaller number of tumor specimens from these same patients were obtained at the time of operation and immediately snap frozen in liquid nitrogen and stored at −80 °C. Tissue was macroscopically free of adjacent nontumorous tissue. Most tumor tissue was routinely processed in our pathology department and unstained histological slides were obtained for immunohistochemical analysis.

Immunohistochemistry for HIF-1α and VEGF

Formalin-fixed, paraffin-embedded surgical specimens were sectioned at a thickness of 6 μm, warmed to 60 °C for 20 min, deparaffinized in xylene, and hydrated in graded series of ethanol/H_2O. HIF-1α immunohistochemistry was performed using the Catalyzed Signal Amplification System (DAKO, Carpinteria, CA) that is based on streptavidin–biotin–horseradish peroxidase complex formation. In brief, after deparaffination and rehydration, slides were treated with target retrieval solution (DAKO) at 97 °C for 45 min, and the manufacturer's instructions were followed. Cells were incubated overnight with primary antibody, H1α67 (Novus Biologicals, Inc., Littleton, CO), at a dilution of 1:1000. Nuclei were lightly counterstained with toluidine blue. Negative controls were performed using nonimmune serum instead of primary antibody. The immunohistochemical analysis of HIF-1α was scored as follows: 0, no staining; 1, nuclear-positive cells in less than 1% of population; 2, nuclear-positive cells in 1–10% and/or weak cytoplasm staining; 3, nuclear-positive cells in 10–50% and/or distinct cytoplasm staining; 4, nuclear-positive cells in more than 50% of population [11]. Scores of 0 and 1 were considered negative for the protein of interest, and scores of 2, 3, and 4 were considered positive.

For VEGF, CAIX, and Glut-1 immunohistochemistry, sections were deparaffinized with xylene and rehydrated in a graded series of alcohols/water. Antigen retrieval was performed by microwave-heating sections immersed in 0.01 mol/l citrate buffer (pH 6.0). Endogenous peroxidase was blocked by incubation in 0.3% hydrogen peroxide, and nonspecific binding was blocked with 10% normal goat or horse serum, depending on the primary antibody. The specimens were then incubated overnight with either anti-VEGF Ab-1 polyclonal antibody (1:50 dilution; Calbiochem, Cambridge, MA), anti-CAIX goat polyclonal antibody (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), or rabbit anti-Glut-1 (1:100 dilution, Santa Cruz Biotechnology). Secondary antibodies and avidin–biotin complex incubations were performed with the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). The final reaction was treatment of the sections in peroxidase substrate solution, 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Vector Laboratories). After counterstaining with toluidine blue, sections were dehydrated in graded alcohols and xylene, and coverslips were applied with coverslip adhesive. Negative controls were performed by replacing the primary antibody with nonimmune serum, with all other steps performed as above.

Table 1. Patients with Gliomas Studied by Immunohistochemistry

Classification	Malignant			Low grade	
Tumor type	GBM	AO	AA	Low grade	Oligodendroglioma
Patients	94 (54%)	8 (5%)	12 (7%)	34 (19%)	27 (15%)
		114 (66%)		61 (34%)	
Age					
Mean	60	38	46	35	47
(yrs)		57		39	
Range	24–81	29–46	30–71	18–78	23–73
(yrs)		24–81		18–78	
Sex					
Male	62	3	6	18	12
		71 (62%)		30 (49%)	
Female	32	5	6	16	15
		43 (38%)		31 (51%)	

Measurement of MIB-1 index and microvascular density

Both MIB-1 index and microvascular density (MVD) are measured by immunohistochemical methods as described above. Primary antibodies were Ki-67 (clone Mib-1, 1:100, Santa Cruz Biotechnology) for MIB-1 index and anti-Von Willebrand factor (1:200, Pharmingen). Three pictures of each slide were taken at 400× (10 ocular×40 objective) using an Olympus Microfire camera and its accompanying software. The pictures were taken at random locations but were determined to be representative of the tissue. The images were then transferred to Image-Pro Plus 5.0, a graphic analysis program.

For MIB-1 index, stained cells were highlighted and counted. The MIB-1 index was calculated as the number of MIB-1-stained cells divided by the total number of cells stained. This was repeated 3 times for each picture and averaged. The results from all three pictures for each slide were averaged for the final MIB-1 index.

The MVD was calculated based on a method described previously [12]. Slides were examined for the most vascular area at 40× (10 ocular×4 objective). Three pictures of this 'hotspot' were taken at 200× (10 ocular×20 objective) as described above. The factor VIII-stained cells were counted. Any positive cell that was definitely separate from other stained cells and not thought to be contiguous or branching from other vessels was counted. The results from all three pictures for each slide were averaged for the resulting MVD (in mm^2).

Establishment of primary cell lines, cell culture, and growth factor studies

Tumor cells were obtained from the operating room and established as primary cultures as described previously [13]. Briefly, tumor cells were aseptically digested with 1 mg/ml collagenase A (Boehringer Mannheim, Indianapolis, Indiana) and 100 μl of 1 mg/ml deoxyribonuclease I (Sigma, St. Louis, MO) in 10–20 ml of Eagle's minimal essential medium (MEM) (Sigma). After 24 h of enzymatic digestion, the softened tissue and growth media were mechanically dissociated and placed in 75-ml tissue culture flasks.

Malignant glioma cell lines U251, U87, U138, A172, and T38G were obtained from American Type Culture Collection. Cells were grown in Eagle's MEM at 37 °C in a humidified incubator under 5% CO_2/95% air. Growth medium was changed twice weekly.

The effects of growth factor stimuli on VEGF secretion and HIF-1α expression were assessed by growing cells with media containing epidermal growth factor (EGF) (0.5 to 50 ng/ml), basic fibroblast growth factor (bFGF) (0.5 to 50 ng/ml), or platelet-derived growth factor BB (PDGF-BB) (0.5 to 50 ng/ml) in triplicate wells. The medium and cell monolayers were harvested 24, 48, and 72 h after plating. Media were assayed for VEGF secretion as described below. Cellular protein was isolated and assayed by Western blot as described below.

Cobalt chloride potentiates HIF-1α expression in a number of cell lines [14] and was used as a positive control at a dose of 600 μM.

Hypoxia chamber cell growth studies

For hypoxia experiments, cells were plated on 100-mm tissue culture dishes (Becton Dickinson, Franklin Lake, NJ) until they reached 80% confluence. The culture medium was changed immediately before placing the dishes in a GasPak Plus anaerobic culture chamber containing hydrogen and a palladium catalyst (GasPak Plus Hydrogen–CO_2 generators, Becton Dickinson) to remove all traces of oxygen. Cells were treated for 4–24 h. Once the seal of the hypoxia chamber was broken, 1 ml of medium from each group was removed and placed on ice. This was stored at −80 °C until protein measurement and VEGF enzyme-linked immunosorbent assay (ELISA) were performed. The remaining medium was decanted and the plates were placed in an ice-water bath to minimize the degradation of HIF-1α and washed with ice-cold DPBS and PIB (6.25 mM NaF, 1.25 mM Na_2V0_3). Cells were scraped from the culture dishes and cellular protein was isolated as described below for Western blot analysis.

Protein isolation from tumors and cell lines

Tumor tissue was snap frozen in the operating room and stored in liquid nitrogen. Tumor samples were chopped and 0.3 g of tissue was homogenized with a Polytron homogenizer in 3 ml of digest buffer containing 10 mM HEPES (pH 7.6), 0.1 mM EGTA, 2 mM DTT, 0.4 mM PMSF, 1 mM $NaVO_3$, 1× protease inhibitor cocktail (Sigma), 100 mM NaF, 10 mM $Na_4P_2O_7$. The homogenate was centrifuged at 580 RCF for 5 min at 4 °C. The supernatant was transferred to a new tube with glycerol added for a final concentration of 5%, then vortexed and centrifuged at 15,000 RCF for 15 min. The pellet was resuspended in 300 μl of lysis buffer (400 mM NaCl, 20 mM HEPES at pH 7.5, 10 mM NaF, 10 mM PNPP, 1 mM $NaVO_3$, 0.1 mM EDTA, 10 μM Na_2MoO_4, 10 mM β-glycerophosphate, 20% glycerol, 1× protease inhibitor cocktail, 1 mM DTT) and shaken gently at 4 °C for 30 min, then centrifuged at 30,000 RCF at 4 °C for 30 min and stored at −70 °C until ready for Western analysis.

Isolation of nuclear protein from malignant glioma cell lines grown in 100-mm dishes was performed on cells treated in the hypoxia chamber as described above. After growth medium from these cells was decanted, the monolayer was washed twice with ice-cold DPBS and PIB. The plates were kept in ice-water bath while the cells were dislodged into the second rinse using a cell lifter and then transferred to a 15-ml centrifuge tube. The cells were pelleted at 165 RCF for 5 min at 4 °C, then resuspended in 600 μl of HB buffer (20 mM HEPES at pH 7.5, 5 mM NaF, 0.1 mM EDTA, 10 μM Na_2MoO_4) and held in ice for 15 min. Nuclei were isolated by adding Nonidet P-40 (0.5% final), vortexing for 10 s, and spinning at 165 RCF for 4 min. The nuclei were resuspended in lysis buffer and shaken for 30 min, then clarified by centrifuging at 30,000 RCF for 20 min. The nuclear extract was stored at −80 °C until use. All steps were done on ice or at 4 °C.

Western blot

Total protein concentration of either type of cellular extract described above was determined spectrophotometrically in 96-well plates using the DC Total Protein Assay (Bio-Rad Laboratories, Hercules, CA) and read using a Benchmark microplate reader (Bio-Rad Laboratories). Equal amounts of protein (25–50 μg) were resolved using a SDS-PAGE slab gel (4–12% continuous gradient) and transferred to PVDF Hybond-p membrane (Amersham Pharmacia, Piscataway, NJ). The membrane was blocked and probed in Blotto (50 mM Tris at pH 7.5, 0.9% NaCl, 5% nonfat dry milk, 0.1% Tween 20) using a mouse IgG anti-human HIF-1α monoclonal antibody (nb100–296: 1:1800, Novus Biologicals, Littleton, CO) and a mouse IgG anti-actin monoclonal antibody (MAB 1501; 1:5000) (Chemicon Int., Temecula, CA). The Western blots were visualized using ECL chemiluminescent reagents (Amersham Pharmacia) and X-OMAT film (Eastman Kodak, Rochester, NY). A positive control for HIF-1α is provided with the antibody (HIF-1α protein isolated from cobalt chloride-treated COS-7 cells).

Enzyme-linked immunosorbent assay for VEGF

Medium containing cell supernatant was harvested from experimental groups as described above. VEGF concentrations in the growth medium were measured using a commercially available ELISA (R and D Systems, Minneapolis, MN). This system uses ELISA plates precoated with an anti-VEGF murine monoclonal antibody. Samples to be tested as well as standards for VEGF were added to wells in triplicate. After the unbound substances were washed away, an enzyme-linked polyclonal antibody specific for VEGF was added to the wells. The plates were again washed, substrate solution was added, and the color was allowed to develop. Absorbance at 450 nm was measured and corrected using the 540-nm reading on a Benchmark microplate reader. Data analysis was performed using Microplate Manager III software (Bio-Rad Laboratories). A standard curve was generated by plotting absorbance versus VEGF standards concentration using linear and nonlinear regression. The standard curve was linear from 0.015 to 2 ng/ml. Concentration of VEGF in the samples was calculated by interpolation from the standard

curve. Total protein was determined for normalizing ELISA.

Reporter gene transfected U251 cells and luciferase report assay

We obtained a U251 cell line stably transfected with an hypoxia response element (HRE)-driven luciferase-producing reporter plasmid (kind gift of Dr. Giovanni Melillo). This cell line is stably transfected with a pGL2-TK-HRE plasmid that was generated by subcloning three copies of the HRE (5′-GTGACTA-CGTGCTGCCTAG-3′) from the inducible nitric oxide synthase promoter into the pGL-2-TK promoter vector [15]. The pGL3-control (Promega) contains the firefly luciferase coding sequence under the control of the SV40 promoter and enhancer sequences. This cell line allows us to measure HIF-1 activity within the cells under various conditions.

Luciferase reporter assays were performed in 96-well OptiPlates (Packard Instrument, Inc., Meriden, CT) using Bright Glo luciferase assay reagents (Promega, Inc, Madison, WI).

Dominant-negative and wild-type HIF-1α constructs

Dr. Greg Semenza kindly provided the pCEP-4 plasmids containing wild-type (WT) and dominant-negative (DN) HIF-1α constructs [16]. The DN construct lacks both the basic DNA-binding domain and the carboxy-terminal transactivation domain. The DN construct presumably works by competing with the endogenous HIF-1α for heterodimerization with endogenous HIF-1β. Heterodimers of the DN HIF-1α and HIF-1β are biologically inactive because of loss of DNA binding activity [17]. pCEP4 is an episomal mammalian expression vector that uses the cytomegalovirus (CMV) immediate early enhancer/promoter for high-level expression of the gene of interest.

The DN form of HIF-1α, pCEP4/HIF-1αDN, was constructed by replacing the sequences encoding the basic domain of HIF-1α with a double-stranded, 16-base-pair oligonucleotide and deleting the sequences encoding the carboxyl terminus of HIF-1α (Figure 1) [5,17]. The constructs of interest were sequenced and verified with the GenBank data submission for HIF-1α. This construct was subcloned into a pcDNA3.1 vector containing multiple c-myc encoding regions for epitope tagging our construct. The new construct with expression of c-myc epitope tag was sequenced to confirm frame and correct orientation of all components.

RNA interference (RNAi) design

Small interfering RNAs (siRNAs) for HIF-1α were designed by searching the coding sequence of HIF-1α for two adenines followed by nineteen nucleotides that had a GC content below 45% and did not contain more than 3 thymines or adenines in a row. These sequences were tested for possible homology to other human and mouse genes with BLAST (http://www.ncbi.nlm.nih.gov/BLAST). Four potential siRNA sequences were selected and siRNA was prepared using Ambion's (Austin, TX) Silencer siRNA construction kit (Table 2). Briefly, two DNA primers with the sense and antisense siRNA sequence and a short sequence complimentary to the T7 promoter were annealed and filled in with Klenow DNA polymerase in separate reactions. T7 polymerase was used for *in vitro* transcription of the sense and antisense templates, which were then annealed and treated with RNase to digest the GGG overhangs left by T7 polymerase. The end product was a 19-nucleotide, double-stranded RNA with a UU overhang on each end. A negative control was designed by randomizing the sequence of one of the siRNAs and checking for nonhomology to any human or mouse gene by BLAST.

Creation of short hairpin RNA (shRNA) stable transfections

To study stable HIF-1α suppression, the pSilencer 2.1 U6-hygro plasmid from Ambion was used to express a small hairpin RNA (shRNA) controlled via the U6 promoter. Two DNA oligonucleotides containing both the sense and antisense siRNA sequences separated by a short loop sequence and having *Bam*HI- and *Hin*dIII-compatible overhanging ends are annealed and ligated into the linearized pSilencer plasmid. The two siRNA sequences found to best suppress HIF-1α transiently were used, along with the negative control. U-251 cells were transfected with Fugene transfection reagent (Roche, Alameda, CA) and transfectants were selected with 300 μg/ml hygromycin in Dulbecco's Modified Eagle Medium.

Xenograft tumor model

Transfected glioma cell lines were subcutaneously implanted on the flanks of CD-1 (nu/nu genotype) athymic nude mice of the Balb/c strain. Approximately 1×10^6 cells were placed on ice-cold Matrigel. Matrigel is liquid at 4 °C but at higher temperatures quickly polymerizes into a three-dimensional matrix. This allows the

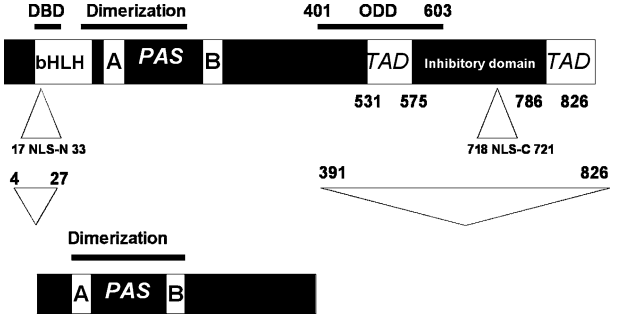


Figure 1. Schematic diagram of wild-type HIF-1α sequence demonstrating transactivation, oxygen degradation, and DNA-binding domains. The dominant-negative form of HIF-1α, HIF-1αDN, was constructed by replacing the sequences encoding the basic DNA-binding domain (DBD) of HIF-1α with a double-stranded, 16-base-pair oligonucleotide and deleting the sequences encoding the carboxyl terminus of HIF-1α. This construct acts presumably by competing with the endogenous HIF-1α for heterodimerization with endogenous HIF-1β. Heterodimers of the DN HIF-1α and HIF-1β are biologically inactive because of the loss of DNA-binding activity [17,18,19].

Table 2. Potential HIF-1 siRNA constructs used in this study

47-	CGUCGAAAAGAAAAGUCUCUU
495-	GCUUUUUUCUCAGAAUGAAUU
1094-	UCUUCAGAUAUGAAAAUGAUU
1589-	UUCAAGUUGGAAUUGGUAGUU
2042-	AAAUCUCAUCCAAGAAGCCUU
2450-	UUACUCAGAGCUUUGGAUCUU
5′-	CGUCGAAAAGAAAAGUCUCUU
3′-	UUGCAGCUUUUCUUUUCAGAG

growth of a number of different human tumors, including those from the central nervous system, in a xenograft model [20]. Our previous work demonstrated that Matrigel by itself does not form tumors and is not found histologically two weeks after implantation [20].

Methods of data analysis

Chi square analysis and Spearman rank-order correlation tests were performed to determine whether a relationship existed between HIF-1α and other hypoxia-responsive protein expression and tumor grade. Comparisons between cell lines of VEGF secretion and *in vitro* and *in vivo* tumor growth were made using Student's *t*-tests with a significance of $P < 0.05$.

Results

Patient demographics and tumor characteristics

Tumor specimens from 175 patients were obtained and graded (Table 1). One hundred fourteen (66%) of the tumors were considered malignant gliomas, including 99 (54%) GBM, 12 (7%) anaplastic astrocytomas (AAs), and 8 (5%) anaplastic oligodendrogliomas (AOs). Of the 94 glioblastoma tumors, 22 (22%) were recurrent tumors that had received prior radiotherapy. We did not include any duplicate tumors (primary resection and subsequent recurrence of the same tumor). Of 61 (34%) low-grade tumors included in this study, 34 (19%) were low-grade astrocytomas and 23 (15%) were oligodendrogliomas. Patients with malignant gliomas ranged from 24 to 81 years of age (mean 57), and 62% were male. Forty-nine percent of the low-grade tumor patients were male, with a mean age of 39 (range 18–78).

Expression of HIF-1α and its downstream-regulated proteins

Malignant gliomas (GBM, AA, and AO) demonstrated a higher frequency of immunohistochemical positivity for HIF than did lower-grade tumors (astrocytomas and oligodendrogliomas) ($P < 0.0001$) (Table 3). The same was true of proteins under the control of HIF-1, including VEGF ($P < 0.0001$) and Glut-1 ($P < 0.0001$) (Table 3). It is noted that although the findings are similar for CAIX, the difference between high-grade and low-grade tumors is less dramatic but is still highly statistically significant ($P = 0.003$). When astrocytomas are examined without the oligodendrogliomas, the difference is similar (Table 3). Interestingly, similar to other studies [21], if oligodendrogliomas are compared with AOs, no statistical difference is observed in expression of these proteins (Table 3). Twenty of the GBM tumors were recurrent and had received radiation prior to resection. No difference of expression of any of the proteins was observed between tumors from a first resection and tumors removed at recurrence that had received radiation therapy (data not shown). This is consistent with glioma cell line work with similar findings [22]. Interestingly, strong HIF-1α nuclear staining occurred throughout and was not confined to perinecrotic regions (Figure 2). This suggests that expression of HIF-1 (and other HIF-1-controlled proteins) is not simply a physiological response to hypoxia but may represent a pathological up-regulation of these proteins in higher-grade brain tumors.

Western blot analysis of tumors taken from human patients demonstrated that higher-grade tumors had higher expression of HIF-1α in both the cytoplasmic and nuclear cell isolates than did lower-grade tumors (data not shown). Interestingly, only the nuclear fraction for

Table 3. Immunohistochemistry for hypoxia-regulated molecules

All tumors			
Protein	All low-grade tumors	All malignant tumors	$\chi 2$
HIF-1	28/61 (46%)	92/114 (81%)	22.33, $P < 0.0001$
VEGF	25/61 (41%)	89/114 (78%)	20.58, $P < 0.0001$
CAIX	35/61 (57%)	92/114 (81%)	8.81, $P = 0.003$
Glut-1	25/61 (41%)	94/114 (82%)	27.57, $P < 0.0001$
Astrocytoma only			
Protein	Low-grade astrocytoma	Anaplastic astrocytoma glioblastoma	$\chi 2$
HIF-1	14/34 (41%)	82/102 (80%)	17.32, $P < 0.0001$
VEGF	11/34 (32%)	78/102 (76%)	20.14, $P < 0.0001$
CAIX	15/34 (44%)	83/102 (81%)	16.10, $P < 0.0001$
Glut-1	8/34 (23%)	83/102 (81%)	38.54, $P < 0.0001$
Oligodendrogliomas only			
Protein	Oligodendrogliomas	Anaplastic oligodendroglioma	$\chi 2$
HIF-1	16/27 (59%)	6/8 (75%)	0.3300, $P = 0.5657$
VEGF	16/27 (59%)	7/8 (87%)	0.5740, $P = 0.4487$
CAIX	22/27 (81%)	8/8 (100%)	0.3733, $P = 0.5412$
Glut-1	19/27 (70%)	7/8 (87%)	0.0116, $P = 0.9141$

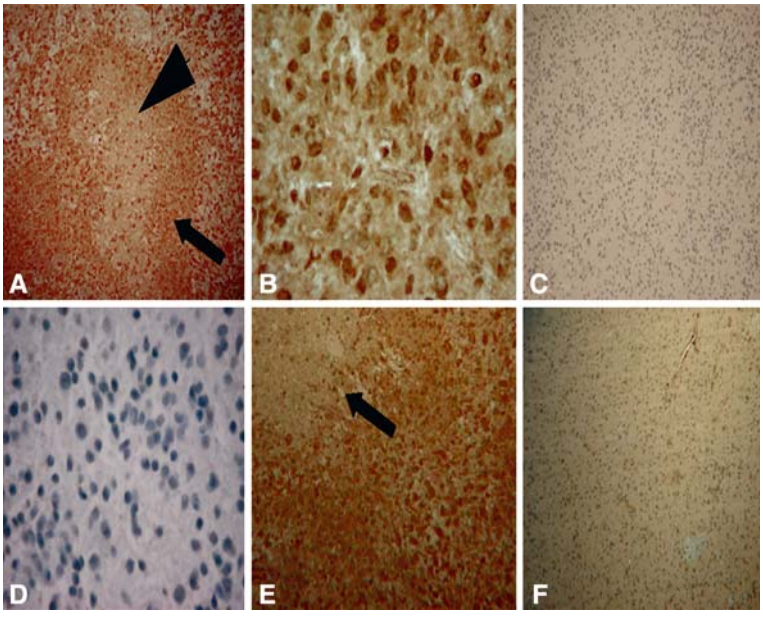


Figure 2. HIF-1α and VEGF immunohistochemistry. (a) GBM immunohistochemically stained for HIF-1α and toluidine blue as a counterstain, 10×. Sections demonstrate necrotic areas (arrowhead) with pseudopalisading cells (arrow) surrounding the necrotic areas. Strong HIF-1α nuclear staining is seen throughout, not confined to perinecrotic regions. (b) GBM demonstrating strong nuclear staining for HIF-1α throughout, 40×. (c) Low-grade astrocytoma negative for HIF-1α, 10×. (d) GBM, negative control (nonimmune serum substituted for antibody) for HIF-1α without nuclear staining, 40×. (e) GBM positive for VEGF, 10×. Section demonstrates necrotic area (arrow) and strong VEGF expression. (f) Low-grade astrocytoma negative for VEGF using same technique, 10×.

GBM demonstrated expression of HIF-1α. Examination of five different glioma cell lines (U251, U87, T98G, U138, A172) showed expression of HIF-1α even under normoxic conditions. Hypoxia significantly increased HIF-1 expression for all cell lines.

VEGF activity in intracranial tumors after stimulation by hypoxia and/or growth factors

We examined VEGF in primary glioma cell cultures and glioma cell lines under normoxic and hypoxic conditions. To correlate VEGF production to HIF-1α expression in glioma tumor cells, measurements were made of VEGF in media of the same cells used for the HIF-1α expression experiments. Media taken from culture dishes growing each cell line were examined using ELISA for VEGF protein. Baseline VEGF was relatively high, even under normoxic conditions, in a primary cultured GBM and the malignant glioma cell line U251 compared with two primary cultured meningioma cell lines (Figure 3a). VEGF production was increased significantly after 24 h of hypoxia for both glioma cell lines (Figure 3a, Student's *t*-test, $P < 0.001$). Examination of various glioma cell lines, U251, U87, U138, A172, T98G, showed increasing VEGF production over time even under normoxic conditions (Figure 3b). This was most dramatic in the U251 and U87 cell lines. It was even more evident under increasing time of hypoxia exposure (Figure 3c). Cobalt chloride (CoCl) induces HIF-1α in a manner similar to hypoxia [14]. Four hours of CoCl exposure increased VEGF secretion similar to 8 h of hypoxic stimulation for each of the cell lines (Figure 3c).

EGF and PDGF increased VEGF secretion in U251 cell lines in a dose-dependent manner at concentrations of 5, 10, and 20 ng/ml of growth factor (Figure 3d). VEGF secretion further increased under hypoxic conditions in addition to stimulation by the growth factors. In fact, hypoxia appears to be the most potent driving force behind VEGF secretion. Increasing growth factor dose enhanced HIF-1 expression, but once again, hypoxia appeared to be the more potent stimulus of HIF-1 expression (Figure 3e). Maximal expression of HIF-1α occurred at 5–6 h of exposure to either PDGF or EGF (data not shown).

Effects of alteration of HIF-1α expression on VEGF secretion and tumor growth

U251 glioma cells were stably transfected with plasmid vectors producing overexpression of WT HIF-1α or a DN HIF-1α. Wild-type HIF-1α overexpression in vector-transfected cell lines WT10, WT12, and WT6 is shown in Figure 4a. Cell lines WT10 and WT12 demonstrated greater VEGF secretion at various times under normoxic conditions than nontransfected and cells transfected with empty vector. This was also found in the WT6 cell line but to a lesser extent. Dominant-negative HIF-1α transfected cell line DN3 under hypoxic conditions demonstrated less VEGF secretion at 24 h than empty-vector transfected and nontransfected cell lines. DN5 did not demonstrate a similar effect (Figure 4b), although, under normoxic conditions, both DN cell lines had less VEGF secretion than the control transfected and untransfected cell lines (Figure 4c).

In vitro cell growth studies on U251 glioma cells stably transfected with WT or DN expression vectors showed no difference in growth rates compared with nontransfected cells. None of the three WT cell lines

(WT12, WT6, and WT10) nor the two DN cell lines (DN5 and DN3) showed any differences in growth compared with nontransfected U251 (data not shown).

In vivo growth studies were carried out using the same cell lines as those studied above. U251 cells transfected with WT, DN, or empty vector, or nontransfected cells were grown on the flanks of nude mice. Nontransfected U251 cells and cells transfected with empty vector showed similar growth rates (data not shown). Comparison of growth rates of DN transfected cells demonstrated a trend toward decreased growth compared with WT transfected cells but did not reach a consistent statistical difference in tumor size over time (Figure 5a). Tumors taken from these mice demonstrated persistent presence of the expression vector using polymerase chain reaction (data not shown).

Tumors transfected with the DN construct had a MIB-1 index of 6.1 ± 0.07 whereas the MIB-1 index of untransfected U251 cells was 15.0 ± 9.2 and that of tumors transfected with the empty vector construct was 13.6 ± 6.7; this difference was statistically significant ($P < 0.05$). The WT construct had a slightly higher

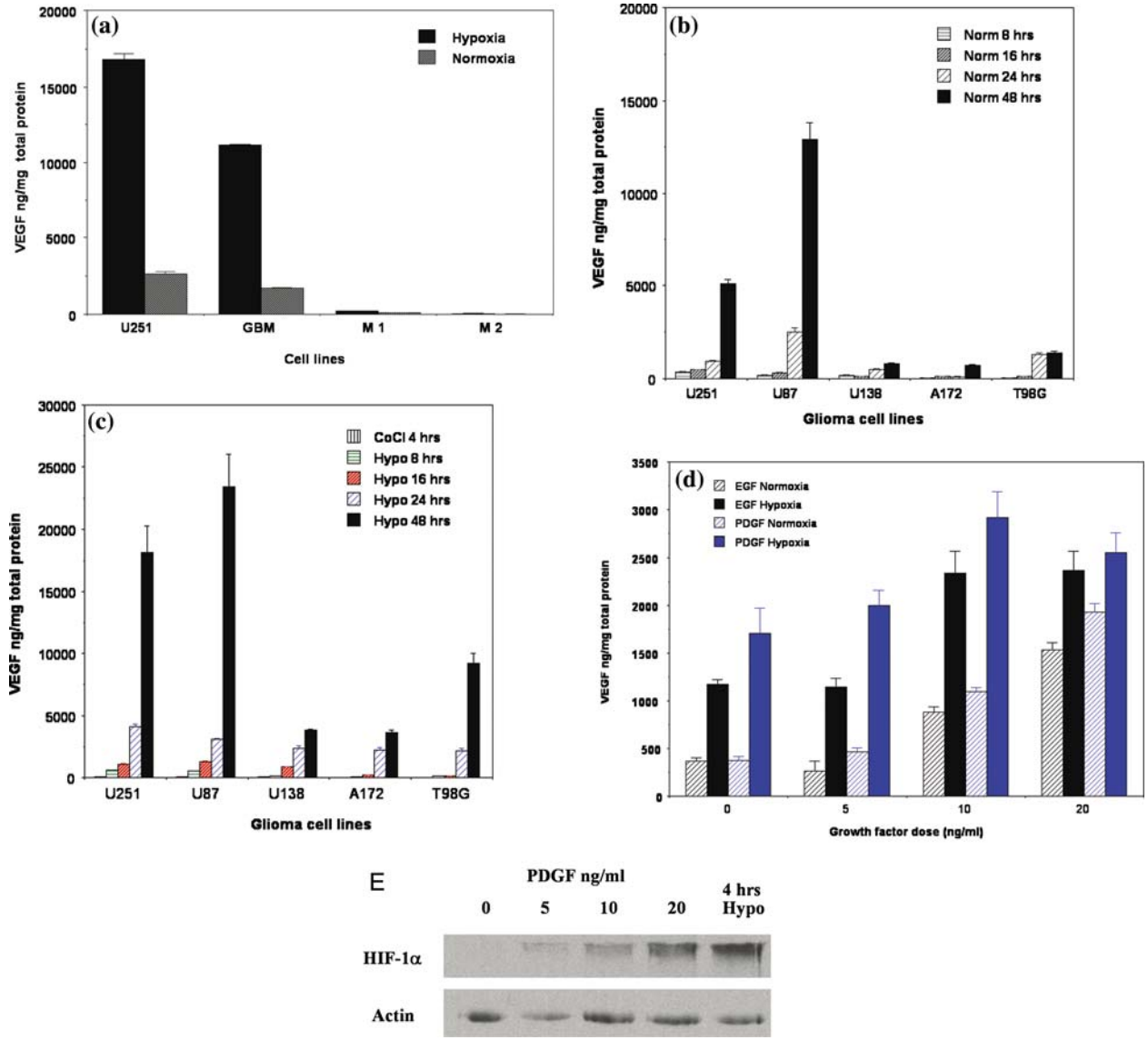
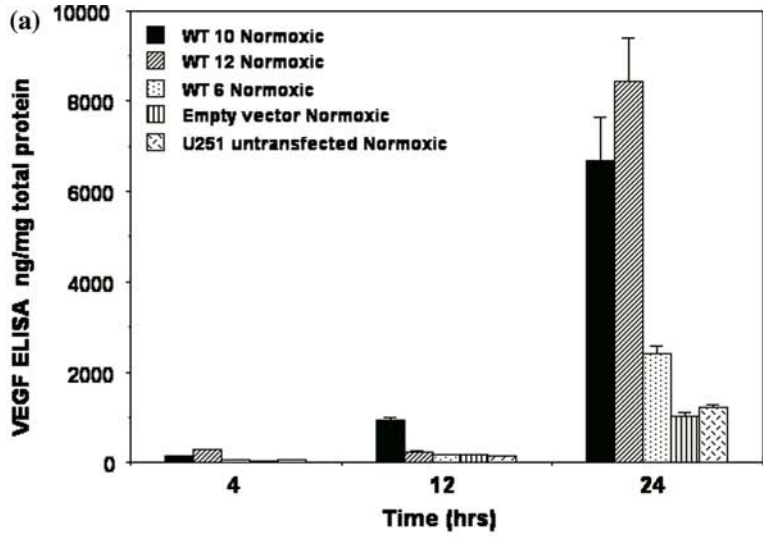
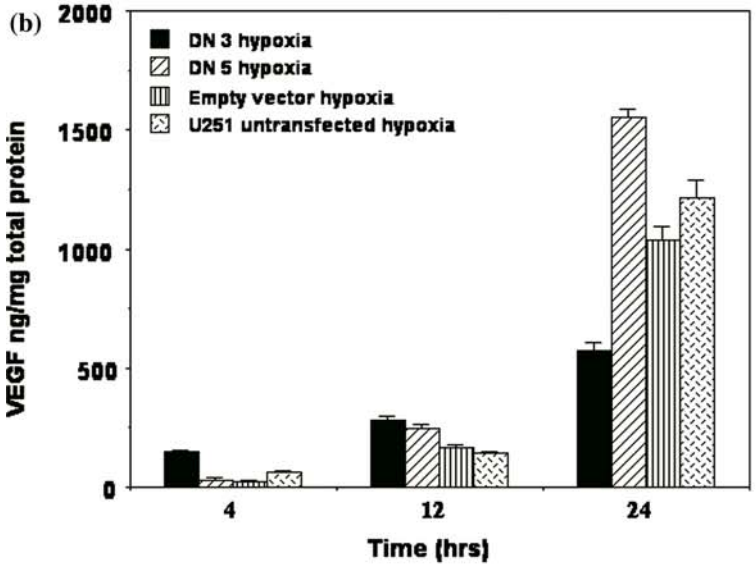


Figure 3. Measurement of VEGF secretion in primary glioma cell cultures and cell lines under conditions of normoxia or hypoxia. VEGF was measured using ELISA and normalized to total protein in the media. (a) Baseline VEGF was relatively high even under normoxic conditions in a primary cultured glioblastoma multiforme and the malignant glioma cell line U251 compared with two primary cultured meningioma cell lines (M1 and M2). VEGF production was increased significantly after 24 h of hypoxia for both glioma cell lines (Students *t*-test, $P < 0.001$). (b) Glioma cell lines U251, U87, U138, A172, and T98G showed increasing VEGF secretion over time even under normoxic conditions. This was most dramatic in the U251 and U87 cell lines. (c) VEGF was even more significantly increased with longer hypoxic exposure. Four hours of CoCl exposure increased VEGF secretion similar to 8 h of hypoxic stimulation for each of the cell lines. (d) Epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) increased VEGF secretion in a dose-dependent manner using concentrations of 5, 10, and 20 ng/ml of growth factor in U251 cells (hatched bars). VEGF secretion further increased under hypoxic conditions in addition to stimulation by the growth factors (solid bars). (e) Increasing growth factor dose (in this case 5, 10, and 20 ng/ml of PDGF) increased HIF-1 expression in U251 cells, as measured by immunoblot techniques but, once again, hypoxia appeared to be the more potent stimulus of HIF-1 expression.


(a)
WT 10 Normoxic
WT 12 Normoxic
WT 6 Normoxic
Empty vector Normoxic
U251 untransfected Normoxic
VEGF ELISA ng/mg total protein
10000
8000
6000
4000
2000
0
4
12
24
Time (hrs)


(b)
DN 3 hypoxia
DN 5 hypoxia
Empty vector hypoxia
U251 untransfected hypoxia
VEGF ng/mg total protein
2000
1500
1000
500
0
4
12
24
Time (hrs)

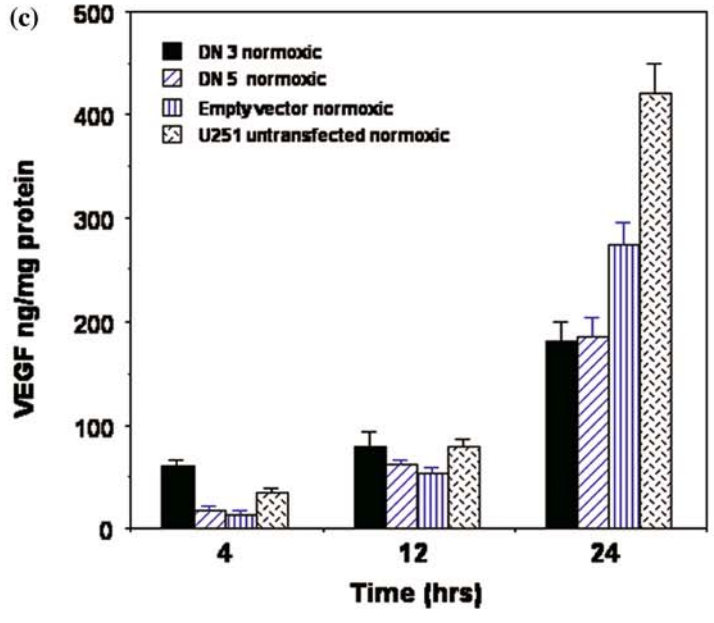

(c)
DN 3 normoxic
DN 5 normoxic
Empty vector normoxic
U251 untransfected normoxic
VEGF ng/mg protein
500
400
300
200
100
0
4
12
24
Time (hrs)

Figure 4. Measurement of VEGF secretion in U251 GBM cells transfected with plasmid vectors producing wild-type overexpression of HIF-1α (WT) or a dominant-negative HIF-1α (DN). VEGF was measured using ELISA and normalized to total protein in the media. (a) Wild-type HIF-1α overexpression of vector-transfected cell lines WT10, WT12, and WT6 under normoxic conditions are shown. Cell lines WT10 and WT12 demonstrated greater VEGF secretion than nontransfected cells and cells transfected with empty vector. This was found to a lesser extent in the WT6 cell line. (b) Dominant-negative HIF-1α (DN) transfected cell line DN3 (solid bars) under hypoxic conditions demonstrated decreased VEGF secretion at 24 h compared with empty-vector transfected and nontransfected cell lines. (c) Under normoxic conditions, both DN cell lines had decreased VEGF secretion compared with the control transfected and untransfected cell lines.

MIB-1 index at 17.5 ± 13.2, but this was not significantly different than the MIB-1 index of nontransfected or empty vector transfected tumors (Figure 5b).

Measurement of MVD between groups did not reach statistical significance. The DN group had a MVD of 56.03 ± 20.55 vessels/mm^2, that of WT was 59.25 ± 36.96 vessels/mm^2, that of U251 untransfected cells was 92.59 ± 25.92 vessels/mm^2, and that of empty vector transfected cells was 56.79 ± 15.42 vessels/mm^2. Because of the lack of convincing evidence that our DN vector or WT HIF-1 overexpression vector altered *in vitro* and *in vivo* glioma growth or angiogenesis

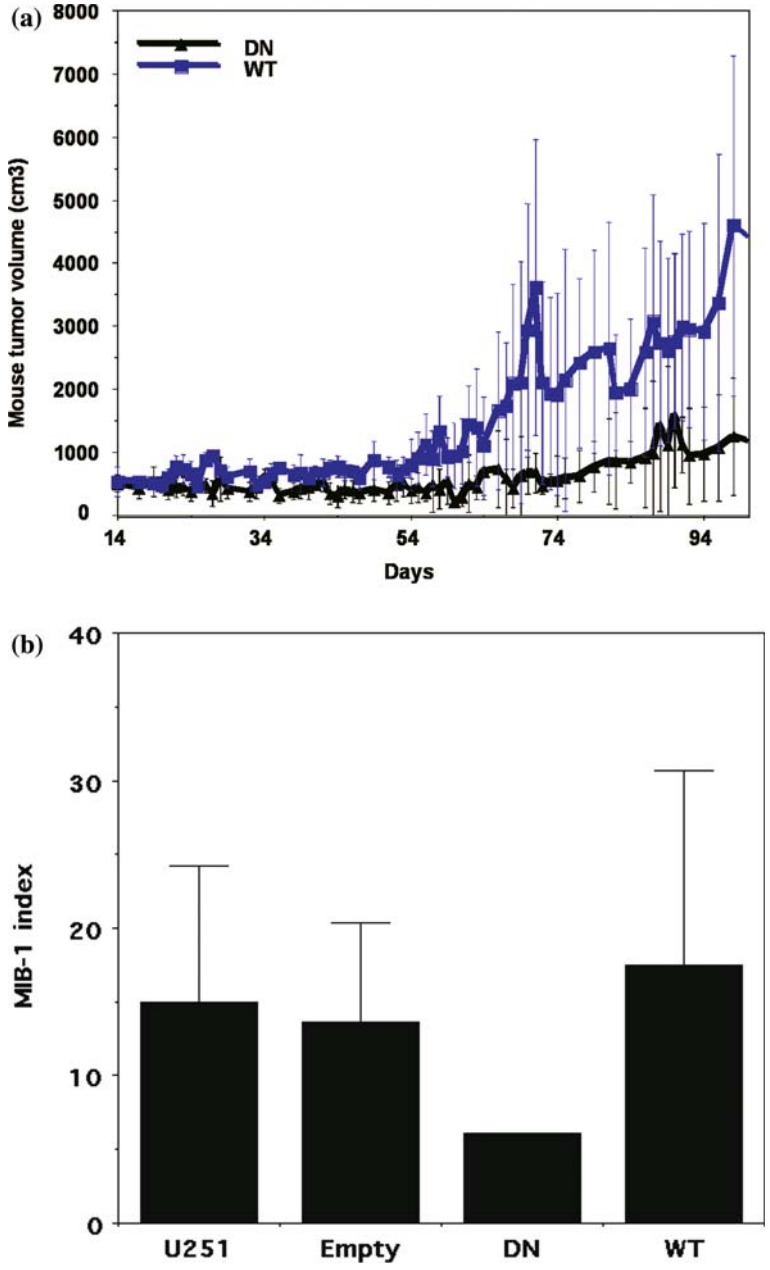


Figure 5. Growth studies on U251 transfected with wild-type (WT), dominant-negative (DN), empty vector, and nontransfected cells grown on the flanks of nude mice. (a) Comparison of growth rates of DN transfected cells demonstrates a trend toward decreased growth compared with WT transfected cells but does not reach a consistent statistical difference in tumor size over time. Nontransfected U251 cells and cells transfected with empty vector showed similar growth rates over time (data not shown). (b) Tumors transfected with the DN construct had an MIB-1 index of 6.1 ± 0.07 compared with 15.0 ± 9.2 for untransfected U251 cells and 13.6 ± 6.7 in cells transfected with the empty vector construct ($P < 0.05$). The WT construct had a slightly higher MIB-1 index at 17.5 ± 13.2, but this was not significantly different than MIB-1 indices for the nontransfected or empty vector transfected tumors.

significantly, we turned our attention to using siRNA techniques to study this question.

HIF-1 activity in siRNA transfected GBM cells

We developed siRNA constructs to inhibit HIF-1α function. Transfection of U251 glioma cells with either HIF-1α siRNA or nonsense control siRNAs was carried out and HIF-1 and VEGF were measured. Western blot analysis of transfected cells showed inhibition of HIF-1 activity by our siRNA constructs (Figure 6a). These constructs were transfected into a U251 cell line stably transfected with an HRE that drives luciferase production. This allows for a means of measuring HIF-1 activity directly within these cells. When these cells were transfected with HIF-1α siRNA, luciferase activity was decreased significantly under both normoxic and hypoxic conditions compared with untransfected cells or controls transfected with nonsense siRNA (Figure 6b). Cell lines stably transfected with the siRNA most efficient at HIF-1 inhibition (construct 1589, cell lines A–E) demonstrated decreased HIF-1 expression compared with cell lines F–I, which were transfected with a different siRNA (construct 2042) that is less efficient for HIF-1 expression inhibition, or with cells transfected with nonsense siRNA constructs (Figure 6c). HIF-1 function inhibition by siRNA (in this case construct 1589) decreases VEGF secretion compared with untransfected U251 cells or nonsense siRNA construct transfected cells (Figure 6d).

In vitro and in vivo growth studies of stably transfected siRNA U251 cells

Cell growth studies were performed on U251 cells transfected with the most effective anti-HIF-1 shRNA (construct 1589) found in the studies above. Growth in culture of cells transfected with control shRNA was increased over the anti-HIF-1 shRNA transfected cells when measured over approximately 4 days. The slopes of growth rate were significantly different ($P = 0.004$) and cell growth was decreased in the shRNA transfected cells ($P = 0.0001$) (Figure 7).

In vivo growth studies were performed using U251 cells transfected with either HIF-1 shRNA or control shRNA grown on the flanks of nude mice. The stable transfectants expressing shRNA with decreased HIF-1 activity had decreased growth compared with the control transfected U251 cells. Growth comparisons between siRNA transfected cells and control transfected

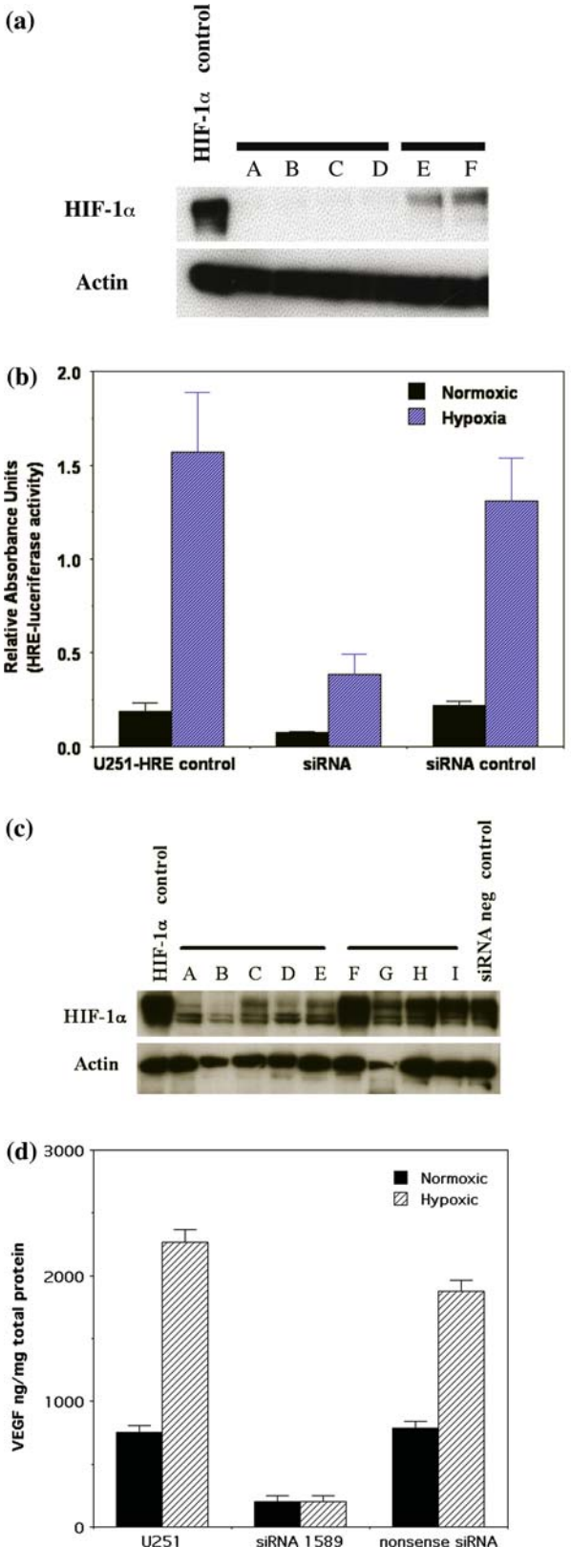


Figure 6. (a) Western blot analysis of U251 glioma cells stably transfected with HIF-1α siRNA or control scrambled siRNA. HIF-1 activity was significantly decreased by siRNA compared with control siRNA transfected cells. (b) Measurement of HIF-1 activity is measured by HRE-luciferase reporter gene activity. Nontransfected cells (U251-HRE control) and nonsense shRNA (siRNA control) showed normal hypoxia-activated luciferase activity and serve as a control. shRNA transfected U251-HRE (siRNA) showed statistically significantly less HIF-1 activity under both under normoxic and hypoxic conditions ($P < 0.001$). (c) Cell lines A–E stably transfected with siRNA (construct1589) directed against HIF-1 demonstrated decreased HIF-1 expression compared with cell lines F–I transfected with a different siRNA (construct 2042) that is less efficient for HIF-1 expression inhibition. Negative control using nonsense siRNA construct stably transfected into U251 cells showed no change in HIF-1 expression. (d) VEGF production by ELISA after siRNA inhibition. HIF-1 function inhibition by siRNA (in this case, construct 1589) decreases VEGF secretion compared with untransfected U251 cells or nonsense siRNA construct transfected cells.

cells were statistically different after 30 days ($P = 0.0012$), 40 days ($P = 0.0061$), 50 days ($P = 0.0027$), 60 days ($P = 0.0027$), 70 days ($P = 0.0076$), and 80 days ($P = 0.0019$) (Figure 8a). Tumors transfected with the 1589 siRNA construct had a MIB-1 index of 6.9 ± 3.8 compared with that of those transfected with the nonsense siRNA construct, which was 13.4 ± 3.3 ($P < 0.0001$) (Figure 8b).

The difference in the MVD between groups did not reach statistical significance. The shRNA inhibited group had a MVD of 40.98 ± 22.41 vessels/mm^2 whereas that of the nonsense shRNA transfected groups was 33.33 ± 25.07 vessels/mm^2. Tumors removed from the mice demonstrated decreased HIF-1 expression measured by Western blot and immunohistochemistry (data not shown). Polymerase chain reaction was used to confirm the continued presence of our expression vector in the transfected cells within the tumor taken from mouse flanks (data not shown).

Discussion

VEGF and HIF-1α in gliomas

A strong body of evidence suggests VEGF is the major mediator for glioma angiogenesis. Elevated expression of VEGF is found in GBM tumors and cell lines compared with expression in lower-grade tumors [23,24]. HIF-1α expression in gliomas is less well known but does seem to support our findings of increased expression in higher-grade human gliomas [21,25–28]. VEGF expression correlates with increasing grade of glioma malignancy, vascular proliferation, necrosis, and microvascular density [3]. This is less clear for HIF-1 [21] and although an association is suggested by our results further work is necessary. *In vivo* experiments involving various methods of VEGF inhibition demonstrate decreased GBM angiogenesis and subsequently decreased tumor growth in nude mice [29–31]. Diminished survival has been correlated with HIF-1 and VEGF expression in oligodendrogliomas with chromosome 1 loss [21].

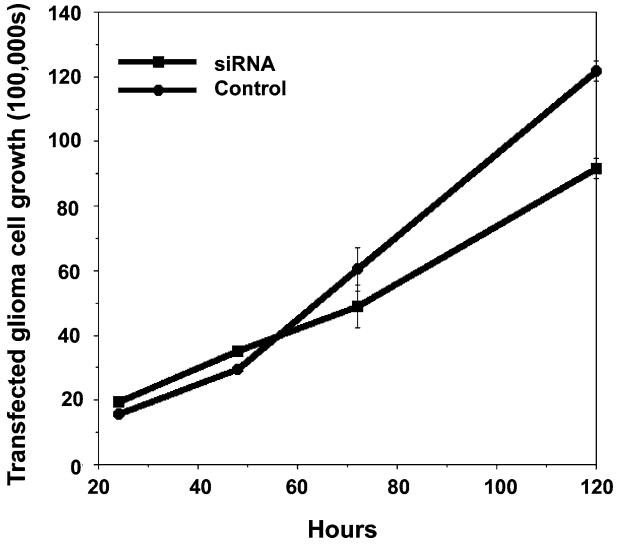


Figure 7. Cell growth studies were performed on U251 cells stably transfected with the most effective anti-HIF-1 shRNA (construct 1589) found in the studies above. Growth in culture of cells transfected with control shRNA was increased over the anti-HIF-1 shRNA transfected cells when measured over approximately 4 days. The slopes of growth rate were significantly different ($P = 0.004$) and cell growth numbers were less in the shRNA transfected cells ($P = 0.0001$).

The question that remains is why VEGF expression is up-regulated in the high-grade glioma. There are at least two possible explanations. One would be that VEGF is secreted in response to tumor hypoxia through the HRE and the transcription factor HIF-1α. This is supported by our data showing that glioma cell cultures subjected to hypoxic conditions produce HIF-1α and VEGF. The second explanation is that HIF-1α and/or VEGF expression is up-regulated inherently as part of the progression of gliomas from low-grade to higher-grade tumors. This might not necessarily require hypoxic stimulation. Our work demonstrates that both growth factor-induced and hypoxia-induced VEGF expression are preceded by an increase in HIF-1α.

HIF-1α and HIF-1α regulation

The transcription factor HIF-1 is composed of two heterodimeric subunits, HIF-1β and HIF-1α [32]. The amino-terminal domain is responsible for DNA binding but is altered in the DN HIF-1α constructs used in this study. HIF-1α and HIF-1β mRNA are both constitutively expressed and do not seem to be significantly modified by hypoxia [33]. In normoxic conditions (20% O_2), HIF-1α is rapidly degraded by proteasomal degradation through a mechanism involving targeting by the Von Hippel Lindau (VHL) protein. During low oxygen tension (1–2% O_2) conditions, this degradation is inhibited, leading to increased HIF-1α, which translocates to the nucleus to activate gene transcription [34,35]. HIF-1 binds a promoter known as the HRE that induces the transcription genes that help cells 'cope' with low oxygen conditions. In addition to VEGF, these genes include erythropoietin, transferrin and its receptor, and almost every gene in the glycolytic pathway including those examined in this study – carbonic anhydrase IX and glucose transporter-1 [5,36]. Activation of HIF-1 promotes cell survival via an adaptive modification of cellular metabolism that increases these glycolytic enzymes and hence the glycolysis rate.

Hypoxic regulation of HIF-1 and VEGF

Regions of hypoxia have been measured in glioma tumor models and GBM tumor in human patients [37–39]. Physiologic induction of VEGF expression in response to hypoxia seems to require HIF-1α regulation [7,9]. HIF-1α activation has been demonstrated in hypoxic regions of a number of tumors including GBM and is a major influence on VEGF expression, tumor angiogenesis, and xenograft tumor growth [8,40]. Both VEGF and HIF-1α expression are highest in the presumed hypoxic areas immediately adjacent to necrotic foci in a subpopulation of glioblastoma tumors known as palisading cells [3,41].

Glioma cell lines in culture have been shown to express HIF-1α and VEGF under hypoxic conditions [42–44]. As we have demonstrated and others have confirmed [4], U87 and U251 cell lines secrete a significantly higher level of VEGF than other glioma cell lines do. In fact, baseline HIF-1α is elevated in U251 cells under normoxic conditions, supporting the idea of loss of normal oxygen-dependent regulation of this protein. It has been suggested that this might be an artifact of cultured cells, which are in relatively hypoxic conditions because of the presence of overlying culture medium [32], but other cultured cells that we have studied, such as the primary meningioma cells, do not show this normoxic expression of HIF-1α. The more probable explanation is that high basal levels of HIF-1 may be an adaptive response in cells with increased metabolic demands or altered signal transduction pathways unrelated to hypoxia but govern HIF-1 cellular activity.

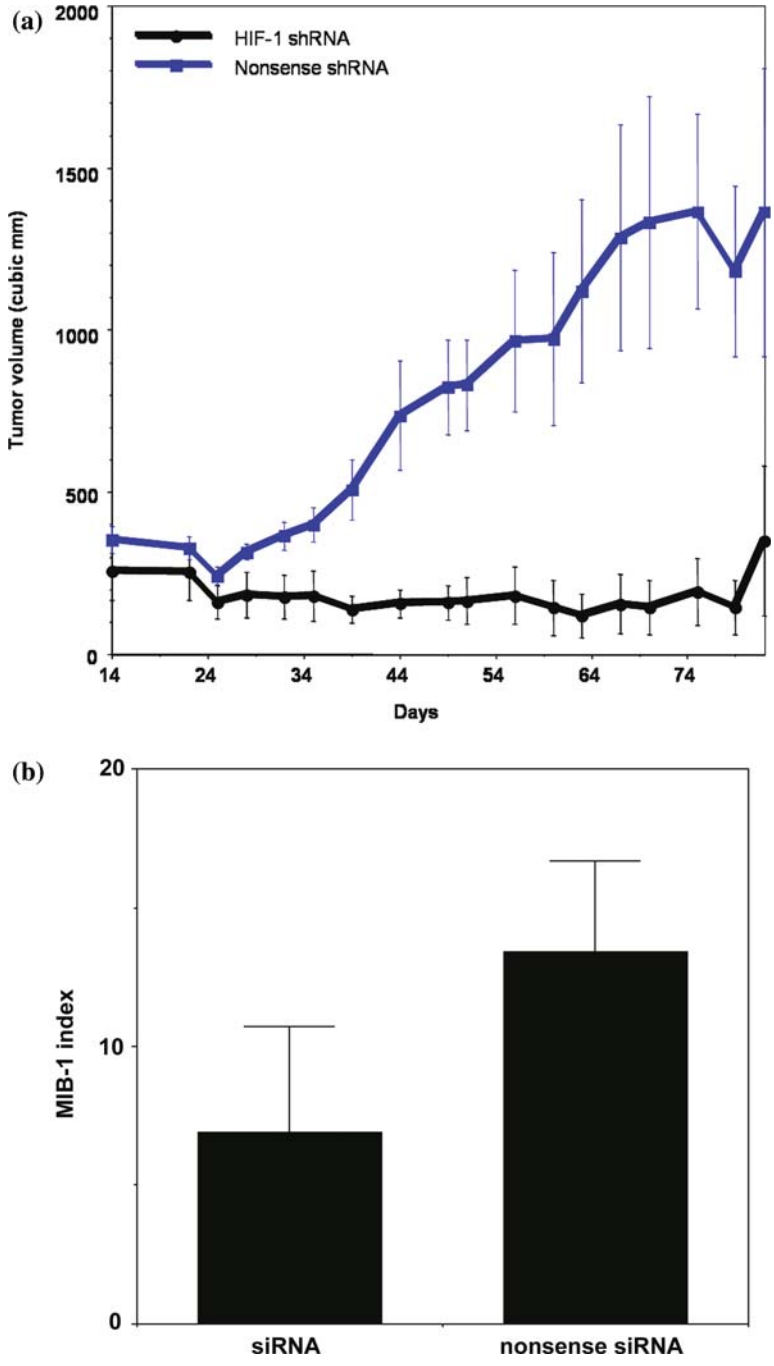


Figure 8. Growth studies on U251 cells transfected with shRNA and control shRNA transfected cells grown on the flanks of nude mice. (a) The stable transfectants expressing shRNA with decreased HIF-1 activity showed less growth than the control transfected U251 cells. Growth comparisons between siRNA transfected cells and control transfected cells were statistically different after 30 days ($P = 0.0012$), 40 days ($P = 0.0061$), 50 days ($P = 0.0027$), 60 days ($P = 0.0027$), 70 days ($P = 0.0076$), and 80 days ($P = 0.0019$). (b) Tumors transfected with the 1589 siRNA construct had an MIB-1 index of 6.9 ± 3.8 which was significantly lower than the MIB-1 index of those cells transfected with the nonsense siRNA construct (13.4 ± 3.3) ($P < 0.0001$).

Nonhypoxic regulation of HIF-1α and VEGF

Contrary to the studies above, however, others have shown that VEGF expression can be independent of hypoxia or HIF-1 regulation in a number of tumors and cancer cell lines [45–47]. Our immunohistochemical studies would support this based on HIF-1α and VEGF expression not being confined to presumed hypoxic regions in the perinecrotic areas of the GBM tumors studied. Loss of function of tumor suppressor genes or activating mutations in oncogenes is known to alter the regulation of various intracellular signal transduction pathways. Mutations of p53 correlate with VEGF or HIF-1α overexpression in some human cancers, including the few GBM tumors studied [48,49]. Evidence suggests that p53 inhibits HIF-1-mediated transcription, and loss of p53 can increase downstream HIF-1-related gene activity [14,50]. PTEN/MMAC1 (phosphatase and tensin homolog/mutated in multiple advanced cancers) tumor suppressor gene loss is demonstrated in 25–60% of GBM tumors studied [51,52]. PTEN negatively regulates the phosphatidylinositol 3-kinase (PI3K)-mediated signal transduction pathways, which are implicated in VEGF and HIF-1α expression and subsequent angiogenesis in nonglioma cells [40,48] and recently in a glioma cell line [43].

Growth factor-mediated or -activated oncogene activity is another potential source of VEGF/HIF-1α overexpression [53]. VEGF secretion is up-regulated in nonglioma cell lines expressing constitutively activated oncogenes [29,54]. VEGF secretion in human glioma cell cultures can be induced by physiological concentrations of EGF, PDGF-BB, and FGF [25,55]. In several nonglioma cell types, HIF-1α and, subsequently, VEGF expression can be induced by stimulation of growth factors such as PDGF, IGF1, and IGF2 to a level higher than that found after hypoxic stimulation [36,48,56]. Our data and that of others would suggest that HIF-1 and PDGF signaling are interrelated for glioblastoma [57]. Reciprocal positive regulation of both growth factors and HIF-1α has been suggested to form an autocrine loop that could promote tumor growth [58].

Targeting HIF-1α for inhibition of glioblastoma growth and angiogenesis

Tumors formed from HIF-1-deficient cells retarded in growth showed lower levels of vascularization and increased apoptosis [9]. Others have generated a tumor model using H-ras-transformed fibroblasts with the ability to genetically remove HIF-1α using a cre/loxP system that resulted in 'tumors' that were HIF-1α deficient [10]. As in our *in vivo* growth studies with the siRNA transfected cells, these researchers were able to show that the loss of HIF-1α resulted in decreased tumor growth. Surprisingly, they did not find any difference in MVD between the WT and null tumors, although VEGF induction under hypoxia was significantly reduced both *in vitro* and *in vivo*. In a similar study, a model of GBM was created using genetically engineered transformed astrocytes with loss of either HIF-1 or VEGF function [59]. Subcutaneously grown HIF-1-deficient tumors demonstrated reduced growth and angiogenesis. On the contrary, when grown intracranially these same cells demonstrate increased growth and invasion. Curiously, the VEGF-deficient cells had decreased growth and angiogenesis in both locations [59]. Inhibition of HIF-1 by small-molecule inhibitors and siRNA techniques similar to those used in this study has been employed to decrease *in vivo* tumor growth [60,61]. This work taken together suggests some form of direct growth enhancement or requirement of HIF-1α for solid tumor growth, but this story is far from clear. We plan to further our siRNA work on both subcutaneous and intracranial tumor models. Further work, directed toward inhibiting HIF-1 activity in gliomas, may provide a suitable method for future approaches for GBM treatment.

Conclusions

HIF-1 and its downstream-regulated proteins are overexpressed in higher-grade gliomas compared with lower-grade gliomas. HIF-1 expression and VEGF secretion are increased by both hypoxia and growth factor stimulation. Hypoxia appears to be the major driving force. Manipulation of HIF-1 activity using dominant-negative or WT overexpression vectors has no effect on *in vitro* growth but appears to decrease *in vivo* growth and proliferative index to a moderate extent. Transient transfection of glioma cells with siRNA constructs inhibiting HIF-1 activity inhibits VEGF secretion. HIF-1 siRNA-transfected glioma cell lines have decreased growth both *in vitro* and *in vivo* as well proliferative index. Microvascular density was unaffected by both siRNA and DN HIF-1 inhibition in these limited studies. This will be looked at in future studies in more detail.

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