Identification of Hypoxia-Induced Genes in a Malignant Glioma Cell Line (U-251) by cDNA Microarray Analysis

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ABSTRACT

Introduction

Overcoming the metabolic restrictions of hypoxia may allow the progression of lower-grade tumors to glioblastoma multiforme. Our findings of up-regulation of HIF-1α and its downstream targets VEGF, GLUT-1, and CAIX in higher-grade gliomas support this hypothesis. We compared the gene expression profiles of the U-251 malignant glioma cell line under normoxic and hypoxic conditions to discover future research targets.

Methods

U-251 cells were grown to 75% confluence and exposed to either normoxic or hypoxic conditions for 24 hours. RNA was extracted, amplified, and hybridized to a cDNA microarray chip containing ~8,800 universal cellular genes. A threefold increase in mRNA expression was used as a threshold value for differential expression. Identified genes were divided into cell cycle control, stress response, and "newly connected" genes.

Results

Hybridization identified 11 hypoxia-induced genes: One involved with cell cycle control (*CCNG2*), six in stress response (*IGFBP3*, *SLC2A3, GSTT2*, *FOS*, *DDIT3, AKR1C3*), and two newly connected genes (*Depp*, *AKAP4*). One stress-related gene (*AKR1C3*) encodes for an enzyme that synthesizes progesterone. Of newly connected

genes, the gene *decidual protein induced by progesterone* (*Depp*) showed the highest expression (4.2-fold increase).

Conclusion

Possible future targeting for "hypoxic" glioma cells includes the targets for the AP-1 transcription factor complex (*FOS*), as well as blockade of the enzyme AKR1C3 with nonsteroidal anti-inflammatory drugs. Possible functions of the highly expressed gene *Depp* include tumor vascularization. Future studies will focus on the hypothesis that *Depp* is up-regulated in an autocrine fashion by the AKR1C3 enzyme in U-251 glioma cells under hypoxic conditions.

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INTRODUCTION

Astrocytomas are the most common primary central nervous system (CNS) tumor. Despite decades of research, the median survival after diagnosis of the most aggressive astrocytoma, glioblastoma multiforme (GBM) (WHO Grade IV), is still only 50 weeks (8, 23). GBM can occur as the result of progression from lower-grade astrocytomas (i.e., WHO Grade II or III) or can arise de novo (8). It has been suggested that a key step in astrocytoma tumorigenesis is the ability to adapt to hypoxic conditions (21, 22, 36).

Cells adapt to hypoxic conditions by activating multiple genes that control the metabolic switch to glycolysis, enhance oxygen delivery by stimulating angiogenesis, and enhance cellular survival (5). In gliomas, several key players have been identified, including hypoxia-inducible factor 1 α (HIF-1 α), vascular endothelial growth factor (VEGF), and multiple growth factors and other cytokines (2, 5, 8, 9, 22). Although much emphasis has been placed on the role of HIF-1 in this context, the role of additional mechanisms has not been adequately explored. In this study, we used cDNA microarray to identify possible future research targets by comparing the gene expression profiles of the U-251 malignant glioma cell line under normoxic and hypoxic conditions.

METHODS

U-251 hypoxia experiments

The malignant glioma cell line U251 was obtained from American Type Culture Collection. Cells were grown in Eagle's modified essential medium at 37°C in a

humidified incubator under 5% CO_2/95% air. Growth medium was changed twice weekly. U-251 cells were grown to 80% confluence and exposed to either normoxic or hypoxic conditions for 24 hours. Two cell culture plates per treatment groups were processed for RNA extraction.

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 the dishes were placed in a GasPak Plus anaerobic culture chamber containing hydrogen and a palladium catalyst (GasPak Plus Hydrogen-CO_2 generators, Becton Dickinson). Two palladium catalyst packets are used to diminish the oxygen concentration to 1–2%. Cells were treated for 24 hours. At the end of 24 hours, no change in media color was noted, indicating physiologic pH values between 7 and 8 were maintained throughout the experiment. To minimize the degradation of hypoxia-related molecules, the plates were placed in an ice-water bath and washed with ice-cold Dulbecco's phosphate-buffered saline and phosphatase inhibitor buffer (6.25 mM NaF, 1.25 mM Na_2V0_3). Cells were processed for RNA and cDNA microarray experiments as described below.

RNA isolation from U-251 cells in culture

Ribosomal nucleic acid (RNA) extraction was accomplished using a commercially available extraction kit, following the manufacturer's recommended protocol (RNeasy Midi Kit, Catalog #75142; Qiagen, Valencia, CA). Briefly, cells were lysed in vitro with

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supplied buffer RLT and β-mercaptoethanol and homogenized. Samples were then protein digested with Qiagen Proteinase K and incubated in a 55°C water bath for 20 minutes. Next, samples were centrifuged at 3000*g* for 5 minutes, after which the supernatant was kept and the pellet was discarded. Pure (100%) ethanol was added, this mixture was loaded into the supplied silica-gel membrane column and centrifuged at 3000*g* for 5 minutes, and the flow-through was discarded. Supplied buffer RW1 was loaded into the column and centrifuged at 3000*g* for 5 minutes and the flow-through was again discarded. Deoxyribonucleic acid (DNA) was digested on-column by placing DNase I (RNase-Free DNase Set, Catalog #79254, Qiagen) directly onto the nucleic acid purification silica-gel membrane and letting it stand at room temperature for 15 minutes. Buffer RW1 was again placed on-column and allowed to stand for 5 minutes before it was centrifuged at 3000*g* for 5 minutes and the flow-through discarded. Supplied buffer RPE was placed on-column and centrifuged at 3000*g* for 5 minutes and the flow-through was discarded. The buffer RPE rinse was repeated as above once. The column was then removed and placed into a new test tube. RNase free water was placed directly onto the silica-gel membrane and allowed to stand for one minute, followed by centrifuging at 3000*g* for 5 minutes. The elution (i.e., flow-through) was pipetted off and placed back onto the membrane and centrifuged at 3000*g* for 5 minutes. Running the elution through the silica-gel membrane a second time decreases the total yield but increases the concentration of isolated total RNA. Extracted RNA was stored in RNase free water at –80°C.

Hybridization to cDNA expression microarray blots

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RNA was extracted, amplified, and hybridized to a cDNA microarray chip containing ~8,800 universal cellular genes by the microarray core facility (Huntsman Cancer Institute, University of Utah, Salt Lake City, Utah) (Figure 1). A total of 4 samples (2 normoxic and 2 hypoxic) were analyzed by hybridizing one normoxic sample with one hypoxic sample for a total of two hybridization experiments.

Microarray gene analysis

A threefold increase in mRNA expression was arbitrarily used as a threshold value for differential expression (i.e., mRNA expression under hypoxic conditions divided by mRNA under normoxic conditions). Each microarray spot contains a unique unigene cluster identifier (e.g., Hs.93675). This unigene cluster number was checked against an on-line human genome database (http://informa.bio.caltech.edu/Hs_browser.html) and only those genes that have been identified in humans (i.e., hybridized cDNA genes from other species showing mRNA up-regulation were not included in analysis) were reported. Finally, genes were divided into “cell cycle,” “stress response,” and “newly connected” genes.

RESULTS

Hybridization identified 11 hypoxia-induced genes. One cell cycle control (*cyclin G2, CCNG2*), six stress response [*insulin-like growth factor binding protein 3* (*IGFBP3*); *solute carrier family 2, member 3 (SLC2A3); glutathione S-transferase, theta 2 (GSTT2); v-fos Finkel-Biskis-Jinkins (FBJ) murine osteosarcoma viral oncogene homolog (V-FOS); DNA damage-inducible transcript 3 (DDIT3), and aldo-keto*

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reductase family 1, member C3 (AKR1C3)], and two newly connected genes [*decidual protein induced by progesterone (Depp)* and *A-kinase anchor protein 4 (AKAP4)*] were found (Table 1).

DISCUSSION

Hypoxia in gliomas

Glioblastoma multiforme tumorigenesis is governed by alterations of complex pathways and cellular programs. Many mechanisms important to potential treatments of this cancer remain undiscovered. By using expression microarray on the glioma U-251 cell line under hypoxic conditions, we have identified 11 potentially new targets for future research efforts. Of these, *Depp* may have a role in GBM angiogenesis. We postulate that this protein may be up-regulated in an autocrine fashion by the protein AKR1C3.

Up-regulation of the cell cycle gene CCNG2 in hypoxia

The hypoxia-response gene involved with cell cycle control was *cyclin G2* (*CCNG2*). *CCNG2* is part of the cyclin family, which controls the cell cycle by binding cyclin-dependent kinases (CDKs) and regulating their activity (Table 1). Unlike classical cyclins that promote cell cycle progression, *cyclin G2* blocks cell cycle entry. *CCNG2* mRNA levels have been shown to be elevated in G_0 and decline as cells enter the cell cycle, elevating again in late S and G_2–M phases (1, 28). The role of *CCNG2* gene up-regulation in U-251 cells under hypoxic conditions is unclear, but it is probably necessary for conservation of cellular energy and ultimate survival.

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Up-regulation of stress genes in hypoxia

Hypoxia-induced stress-related genes included genes encoding for proteins promoting cell survival (*IGFBP3, SLC2A3, GSTT2, FOS, AKR1C3*), as well as genes promoting cell death (*DDIT3)* (Table 1).

Insulin-like growth factor-binding protein 3 (IGFBP3)

Insulin-like growth factor-binding protein 3 (*IGFBP3*) is located on chromosome 7p14-p12. Insulin-like growth factors (IGF), their receptors, and their binding proteins play key roles in regulating cell proliferation and apoptosis; however, this system becomes uncontrolled in cancer (6). *IGFBP3* has multiple functions, including acting as the major carrying protein for IGF1 and IGF2, modulating IGF bioactivity, directing growth inhibitor, and inducing apoptosis (12, 15). Interestingly, *IGFBP3* possesses both growth-inhibitory and potentiating effects on cells that are independent of IGF action mediated through specific *IGFBP3*-binding receptors located on cell membranes, cytosol, and nucleus. In brain tumors, the IGF system plays an important role in tumorigenesis, with apoptosis of tumor cells noted *in vivo* with the treatment of antisense-mediated down-regulation of IGF-1 receptors (14). The specific role that *IGFBP3* may hold in glioma tumorigenesis is unclear, but other researchers have shown that *IGFBP3* accumulates in human brain tumors (14).

Solute carrier family 2 (*facilitated transporter*), *member 3* (*SLC2A3*) (*aka GLUT3*)

Solute carrier family 2 (facilitated transporter), member 3 (SLC2A3) [also known as *glucose transporter 3* (*GLUT3*)] is located on chromosome 12p13.3 and encodes for a glucose transport protein. Hypoxia stimulates glucose transport by enhancing the function of the glucose transporters GLUT1, GLUT3, and GLUT4 (42). GLUT3 has been shown to be localized to the brain (i.e., "brain-type" glucose transporter) (32). Elevated GLUT3 levels have been identified in brain tumors (e.g., GBM), with increased levels correlating with biologically aggressive tumors (4, 31, 32). These studies provide evidence that up-regulation of the "brain-type" glucose transporter is a vital step in astrocytoma tumorigenesis.

Glutathione S-transferase, theta-2 (GSTT2)

Glutathione S-transferase, theta-2 (*GSTT2*) maps to chromosome 22q11.2. Glutathione S-transferase (GST) enzymes conjugate a vast number of substrates to glutathione for detoxification and are important in cellular detoxification of reactive oxygen metabolites (10, 26). Specifically, the enzyme GSTT2 dehalogenase activity toward halogenated compounds (e.g., dichloromethane). Furthermore, cancerous tumors expressing high levels of GST are associated with chemotherapy failure and poor patient survival (10). In U-251 glioma cell hypoxia, we hypothesize that *GSTT2* protects against hypoxia-induced reactive oxygen metabolites.

V-Fos FBJ Murine Osteosarcoma Viral Oncogene Homolog (V-Fos)

V-Fos FBJ murine osteosarcoma viral oncogene homolog (*V-Fos*) (also known as oncogene *FOS*) is located on chromosome 14q24.3 and is a major component of the

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activator protein-1 (AP-1) transcription factor complex. The AP-1 transcription factor complex regulates a multigenic invasion program, giving tumor cells the ability to invade surrounding tissue. Members of the Fos family (c-Fos, FosB, Fra-1, and Fra-2) dimerize with Jun proteins (JunB and JunD) to form the AP-1 transcription factor complex (33). AP-1 target genes include the invasion proteins in the matrix metalloproteinase family (extracellular proteases), as well as CD44 (cell surface hyaluronan-receptor) (33). Studies of human brain tumors showed a significant increase in v-fos mRNA in malignant tumors (e.g., GBM) when compared with benign tumors (e.g., cerebellar astrocytoma) (16, 17), suggesting that v-Fos may be a good molecular marker of aggressive brain tumors.

DNA damage-inducible transcript 3 (DDIT3) (aka CHOP)

DNA damage-inducible transcript 3 (*DDIT3*) [also known as C/EBP-homologous protein (CHOP)] is located on chromosome 12q13.1-q13.2 and is known to up-regulate under hypoxic conditions, functioning to induce death by promoting oxidation in the stressed endoplasmic reticulum (ER) (3, 7, 27). *DDIT3* has been implicated in the injury process of the peripheral nervous system and has been shown to induce neuronal apoptosis after neurotrophic factor deprivation and ischemia (30, 39, 40). Given the above evidence, *DDIT3* expression in U-251 cells under hypoxic conditions is probably detrimental to cell survival.

Aldo-keto reductase family 1, member C3 (AKR1C3)

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Aldo-keto reductase family 1, member C3 (*AKR1C3*) [also known as 3 α-hydroxysteroid dehydrogenase, Type II or hydroxysteroid (17-beta) dehydrogenase 5] is located on chromosome 10p15-p14 (18). The aldo-keto reductase (AKR) superfamily catalyzes the conversion of aldehydes and ketones to alcohols by utilizing NADH and/or NADPH as a cofactor. Human 3-α-hydroxysteroid dehydrogenase exists in four isoforms named AKR1C1–AKR1C4. AKR1C3 is a multifunctional enzyme that possesses 3α-, 3β-, and 20α-hydroysteroid dehydrogenase, as well as prostaglandin (PG) synthase activities, and it catalyzes androgen, estrogen, progestin, and PG metabolism (18, 24, 34). The functional plasticity of these isoforms highlights their ability to modulate the levels of active androgens, estrogens, and progestins (34). Specifically, AKR1C3 oxidizes 17β-oestradiol to oestrone, and 20α-hydroxyprogesterone to progesterone (34). Anti-inflammatory drugs highly inhibit this enzyme (29). The mRNA transcript of AKR1C3 is found in brain, kidney, liver, lung, placenta, and testis (24). The role of AKR1C3 up-regulation in U-251 hypoxic cells is unclear. However, we hypothesize that the AKR1C3 enzyme may be producing the progesterone necessary to up-regulate the "newly connected" gene *Depp* (Figure 2).

Newly connected hypoxia-induced genes

Decidual protein induced by Progesterone (Depp)

The *decidual protein induced by progesterone* (*Depp*) [also known as *fasting-induced gene (Fig)* and *fat-specific expressed gene (Fseg)*] is located on chromosome 10. *Depp* was cloned from human tissues and its sequence deposited into a public database in 1999 without further characterization. Its first description was in the context

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of embryo implantation into the uterine wall (41). During pregnancy the uterine lining (endometrium) prepares for implantation under the influence of progesterone. After embryo implantation, the uterine lining is termed the decidua and serves to support the embryo throughout gestation. Watanabe et al. (41) identified this protein from endometrial stromal cells (ESC) clone cells stimulated with progesterone. *In vitro* ESC experiments showed up-regulation of this protein with progesterone stimulation and inhibition of protein expression with the antiprogestin RU486 (41). Therefore, the name *decidual protein induced by progesterone* was given. *Depp* localizes to the nucleus more than the cytoplasm (41). *Depp* has been shown to be expressed in placenta, ovary, kidney, and arterial endothelial cells (35, 41). Studies suggest that Depp affects gene expression by activating the Elk-1 transcription factor, as well as by promoting tumor vascularization and wound healing (35, 41). The authors hypothesize that Depp may be acting as another mechanism by which astrocytomas promote blood vessel formation.

AKAP4

A-kinase anchor protein 4 (*AKAP4*) is located on Xp11.2 and encodes for an anchoring protein that tethers a cAMP-dependent protein kinase near its substrate (13). AKAP4 is found in the fibrous sheath of sperm flagellum and is involved in sperm motility (13). Other protein kinase A-binding proteins that are present in neuronal synapses and the hippocampus are involved with melatonin synthesis in the pineal gland and are also located near neuronal calcium channels (11, 25, 37, 38). It is unclear what role AKAP4 plays in hypoxia.

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Hypothesis: AKR1C3-derived progesterone up-regulates Depp in glioma hypoxia

On the basis of these findings, we hypothesize that hypoxia induces the up-regulation of the enzyme AKR1C3, which increases progesterone production. This increase in progesterone cellular levels drives Depp in an autocrine fashion, aiding glioma adaptation via increased angiogenesis (Figure 2). This hypothesis is further supported by studies showing that astrocytic tumors locally synthesize progesterone and that progesterone receptor expression increases with astrocytoma tumor grade, with 100% of GBMs exhibiting progesterone receptors (19, 20). We acknowledge that this hypothetical model is based on a single glioma cell line, but we are hopeful that these initial findings may ultimately lead to new GBM treatment targets.

CONCLUSIONS

Possible future targeting for “hypoxic” glioma cells includes the targets for the AP-1 transcription factor complex (*FOS*) and blockade of the enzyme AKR1C3 with nonsteroidal anti-inflammatory drugs. Possible functions of the highly expressed gene *Depp* may include angiogenesis and represent an interesting finding. Future studies will focus on the hypothesis that Depp is up-regulated in an autocrine fashion by the enzyme AKR1C3 in U-251 glioma cells under hypoxic conditions.

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TABLES AND FIGURES:

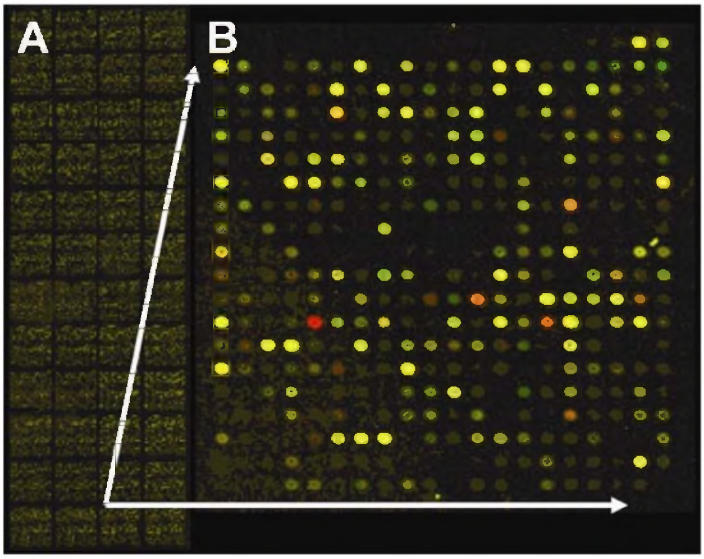

Figure 1: A-B. Representative microarray glass slide (A) with ~8,800 single-stranded DNA molecules attached at fixed locations (spots, enlarged view in B). RNA was extracted from normoxic U-251 (control) and hypoxic U-251 (sample) cells and labeled with fluorescent dye (green or red, respectively). Extracts are washed over the microarray. Gene sequences from the extracts hybridize to their complementary sequences in the spots. The dyes enable the amount of sample bound to a spot to be measured from the level of fluorescence emitted when excited by a laser. If the RNA from the sample population is abundant, the spot will be red; if the RNA from the control population is abundant, it will be green; if sample and control bind equally, the spot will be yellow; if neither binds, it will appear black. Thus, the relative expression levels of the genes in the sample and control populations can be estimated from the fluorescence intensities and colors for each spot.

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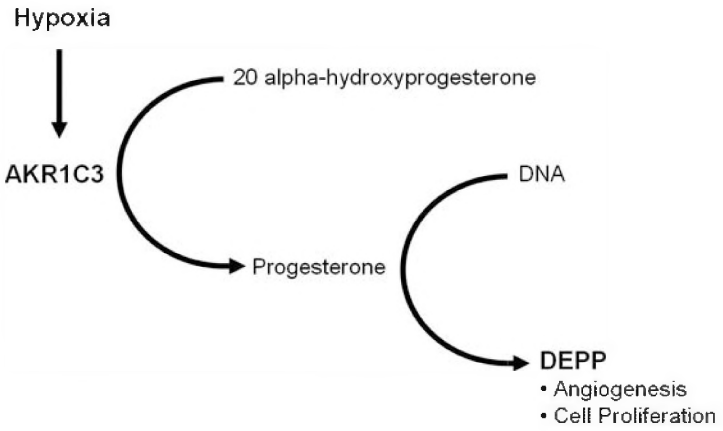

Figure 2: Hypothetical pathway by which *decidual protein induced by progesterone* (*Depp*) is up-regulated in an autocrine fashion in U-251 glioma cells under hypoxic conditions. This speculative diagram is based on the up-regulation of aldo-keto reductase family 1, member 3 (*AKR1C3*) mRNA and *Depp* mRNA under hypoxic conditions. The function of *Depp* is largely unknown, but it appears to be a key protein in the maturation of the uterine lining after embryo implantation (i.e., decidua), suggesting cell proliferative and/or angiogenic role(s).

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Table 1: Hypoxic genes identified in U-251 cells by cDNA microarray. Relative increase was calculated by dividing the gene expression of hypoxic cells with normoxic cells.

Gene symbol	Gene name	Possible role(s) under hypoxic conditions	Relative increase	Unigene No.
Cell Cycle				
CCNG2	Cyclin G2	G0 cell arrest	3.3	Hs.79069
"Stress" Response				
IGFBP3	Insulin-like growth factor binding protein 3	Both growth proliferative and inhibitory effects	4.4	Hs.77326
SLC2A3	Solute carrier family 2 (facilitated glucose transporter), member 3	"Brain-type" glucose transporter	3.9	Hs.7594
GSTT2	Glutathione S-transferase, theta 2	Cellular detoxification of reactive oxygen metabolites	3.8	Hs.1581
FOS	v-Fos FBJ murine osteosarcoma viral oncogene homolog	Forms AP-1 transcription factor	3.2	Hs.25647
DDIT3	DNA damage-inducible transcript 3	Promotes apoptosis	3.0	Hs.129913
AKR1C3	Aldo-keto reductase family 1, member C3	Modulate levels of androgen, estrogens and progestins	3.0	Hs.78183
Newly Connected Genes				
DEPP	Decidual protein induced by progesterone	Angiogenesis	4.3	Hs.93675
AKAP4	A-kinase anchor protein 4	Unclear	3.5	Hs.97633

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