Tumor cycling hypoxia induces chemoresistance in glioblastoma multiforme by upregulating the expression and function of ABCB1

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Tumor cycling hypoxia is now a well-recognized phenomenon in animal and human solid tumors. However, how tumor cycling hypoxia impacts chemotherapy is unclear. In the present study, we explored the impact and the mechanism of cycling hypoxia on tumor microenvironment-mediated chemoresistance. Hoechst 33342 staining and hypoxia-inducible factor–1 (HIF-1) activation labeling together with immunofluorescence imaging and fluorescence-activated cell sorting were used to isolate hypoxic tumor subpopulations from human glioblastoma xenografts. ABCB1 expression, P-glycoprotein function, and chemosensitivity in tumor cells derived from human glioblastoma xenografts or in vitro cycling hypoxic stress-treated glioblastoma cells were determined using Western blot analysis, drug accumulation and efflux assays, and MTT assay, respectively. ABCB1 expression, P-glycoprotein function, and chemosensitivity in tumor cells derived from human glioblastoma xenografts or in vitro cycling hypoxic stress-treated glioblastoma cells were determined using Western blot analysis, drug accumulation and efflux assays, and MTT assay, respectively. ABCB1 expression and P-glycoprotein function were upregulated under cycling hypoxia in glioblastoma cells concomitant with decreased responses to doxorubicin and BCNU. However, ABCB1 knockdown inhibited these effects. Moreover, immunofluorescence imaging and flow cytometric analysis for ABCB1, HIF-1 activation, and Hoechst 3342 in glioblastoma revealed highly localized ABCB1 expression predominantly in potentially cycling hypoxic areas with HIF-1 activation and blood perfusion in the solid tumor microenvironment. The cycling hypoxic tumor cells derived from glioblastoma xenografts exhibited higher ABCB1 expression, P-glycoprotein function, and chemoresistance, compared with chronic hypoxic and normoxic cells. Tumor-bearing mice that received YC-1, an HIF-1α inhibitor, exhibited suppressed tumor microenvironment-induced ABCB1 induction and enhanced survival rate in BCNU chemotherapy. Cycling hypoxia plays a vital role in tumor microenvironment-mediated chemoresistance through the HIF-1–dependent induction of ABCB1. HIF-1 blockade before and concurrent with chemotherapy could suppress cycling hypoxia-induced chemoresistance.

Keywords: ABCB1, cycling hypoxia, glioblastoma, hypoxia-inducible factor-1, multidrug resistance (MDR) 1.

Glioblastoma multiforme (GBM) is the most common and aggressive form of brain tumor. Current therapy consists of surgical resection, followed by radiation therapy and concomitant chemotherapy. Despite these treatments, the prognosis for patients with GBM is poor. Although the use of chemotherapeutics plays an important role in the combined treatment of GBM, it remains a challenge because of tumor chemoresistance. The intrinsic chemoresistance of GBM is a critical factor that determines the effectiveness of chemotherapy. Many factors, including genetic alterations and microenvironments, affect tumor response to chemotherapeutic agents. One such factor is hypoxia, which is classified into 2 modes. The acute, intermittent, or cycling hypoxia is associated with inadequate blood flow, whereas chronic hypoxia is...
the consequence of increased oxygen diffusion distance resulting from tumor expansion. Although cycling hypoxia may be an important factor that limits tumor response to chemotherapy,9 direct evidence from endogenous tumor microenvironments supporting this notion is still lacking. Moreover, the underlying mechanism of cycling hypoxia-induced drug resistance remains undetermined.

Although the impacts and mechanisms of cycling hypoxia on drug resistance are still unclear, those of chronic hypoxia on drug resistance are well characterized. Chronic hypoxia is an independent prognostic indicator of poor clinical outcomes for patients with cancer and is correlated with increased tumor resistance to certain chemotherapeutic agents.10 Such resistance to anticancer drugs is attributed to intrinsic cellular mechanisms, including the lack of oxygen that is available for anti-tumor drugs to act, DNA over-replication, increased genetic instability, the anti-proliferative effect of hypoxia,11 and increased multidrug resistance (MDR) linked transporter ABCB112,13 which are mostly induced by hypoxia-inducible factor-1 (HIF-1). Unfortunately, these mechanisms were determined in vitro settings, which cannot be used to elucidate the details of MDR activity in vivo. It is known that anticancer drugs and oxygen are both taken in and circulate throughout the body and must diffuse from the bloodstream to individual tumor cells. Therefore, the establishment of pharmacological sanctuaries protecting tumor cells from therapeutic drugs by means of a diffusion barrier plays a vital role in MDR. Because of limited drug penetration in solid tumors, chronic hypoxic regions are often protected from the cytotoxic effects of chemotherapeutic agents, further reducing drug efficacy. Therefore, poor penetration of drugs through tumor tissue is the major mechanism of chronic hypoxia-mediated MDR in solid tumors.

Although the mechanisms involved in chemoresistance are complex, the most efficient way to mediate drug resistance is the use of efflux pump proteins that directly transport drug substrates out of cancer cells.7 ABCB1 protein is a well-known ATP-binding cassette drug efflux transporter that is often overexpressed in multidrug-resistant human cancer cells.14 Moreover, ABCB1 is expressed in the majority of brain tumors, including glioblastomas,15,16 which may affect the outcomes of chemotherapy in patients with glioblastoma.17 The ABCB1 gene is hypoxia responsive and can be induced via HIF-1 activation,18 further contributing to drug resistance in solid tumors.

In the present study, we determined the impact and mechanism of cycling hypoxia on drug resistance in GBM. We showed that cycling hypoxic stress significantly increases chemoresistance via HIF-1-mediated ABCB1 induction. We also showed that ABCB1 expression is predominantly localized in potentially cycling hypoxic areas with HIF-1 activation and blood perfusion in the tumor microenvironment. In summary, the cycling hypoxic tumor cells derived from glioblastoma xenografts exhibited higher ABCB1 expression, function, and chemoresistance, compared with chronic hypoxic cells or normoxic cells. HIF-1 blockade combined with BCNU chemotherapy in intracerebral U87 glioblastoma-bearing mice decreased tumor microenvironment-induced ABCB1 induction and increased overall therapeutic efficiency.

Materials and Methods

Cell Culture

U87, GBM8401, and U251 cells were cultured in DMEM (Life Technologies) supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES, and 1% penicillin-streptomycin.

In Vitro Hypoxic Treatments

The cells were treated in Biospherix C-Chamber (Biospherix) inside a standard culture chamber by means of exhausting and gassing with 95% N2 and 5% CO2 to produce oxygen concentrations of 0.5%–1% for 4 h at 37°C to achieve noninterrupted hypoxic condition. For cycling hypoxic treatments, cell cultures were exposed to 3 cycles consisting of 0.5%–1% O2 for 1 h interrupted by 5% CO2 and air for 30 min at 37°C in hypoxia chamber by timer-controlled regulator.

Western Blot Analysis

Cells were lysed and extracts were prepared as described previously.19 HIF-1α protein in human cells was detected in 150 μg of cell extract using monoclonal anti-HIF-1α antibody (diluted 1:750; Novus) and anti-ABCB1 antibody (1:500; Sigma-Aldrich). Western blots were normalized using a monoclonal anti-β-actin antibody (diluted 1:10 000; Santa Cruz Technology).

Real-time Quantitative Polymerase Chain Reaction (PCR)

Quantitative PCR analysis was performed as described previously.19 The primers for quantitative analysis of ABCB1 and the housekeeping gene 60S acidic ribosomal proteins were ABCB1 (F) 5′-CCCATCATTTGCAA TAGCAGG-3′ and (R) 5′-TGTTCAAACTTCTGCT CCTGA-3′; and the housekeeping gene 60S acidic ribosomal protein (F) 5′-ACGAGGTGTGCAAGGAGG GC-3′ and (R) 5′-GCAAGTCTGTCCTCCATC-3′.
Vector Constructions and Viral Transduction

The lentiviral vector pLKO AS2 (National RNAi Core Facility, Taiwan) was used as the backbone to generate a lentiviral reporter vector. The multiple cloning sites (MCS) of pTA-Luc vector (Clontech) was inserted with the cDNA fragment bearing −2028 to +4 bp ABCB1 promoter to drive the expression of firefly luciferase gene. The ABCB1 promoter driven reporter gene cassette was amplified from promoter to SV40 ployA on the constructed pTA-Luc vector with use of PCR and was inserted into pLKO AS2 by XhoI and MluI restriction enzymes. The full-length cDNA for human ABCB1 gene was amplified from the plasmid pHaMDRwt (Addgene) with use of PCR and was inserted into the pAS2 vector (National RNAi Core Facility, Taiwan), which was removed from the GFP gene as a lentiviral expression vector containing ABCB1 gene. The retroviral vector dxHRE-tk/eGFP-cmvRed2XPRT, kindly provided by Dr. Juri Gelovani (Experimental Diagnostic Imaging, The University of Texas M.D. Anderson Cancer Center) was used to generate glioblastoma reporter cells bearing HIF-1–inducible reporter gene (HSV1-tk/GFP fusion) and a constitutively expressed reporter gene (DsRed2/XPRT). Lentivirus or retrovirus production and cell transduction were performed according to protocols described elsewhere.20,21

The U87 and GBM8401 cells bearing the ABCB1 promoter-driven Luc reporter gene and the dual reporter gene cassette were termed U87-ABCB1-P-Luc, U87/hif-1-r, or GBM8401/hif-1-r.

Small Interfering RNA Transfection

Glioblastoma cells were transfected with ABCB1 or HIF-1α small interfering RNA (siRNA; Santa Cruz Biotechnology) using the OligofectAMINE transfection reagent (Invitrogen) according to the manufacturer’s instructions.

Cellular Assays

Several cellular assays were used in this study. These included the following: luciferase assays to determine the transcriptional activation of ABCB1, Rh123 uptake and efflux assays to assess ABCB1 function, and cytotoxicity assays to evaluate the cell survival rate in doxorubicin or BCNU treatment. Fluorescence-activated cell-sorting (FACS) analyses were used to isolate the subpopulations of tumor cells from glioblastoma xenografts and assay their ABCB1 expression. Tc-99m sestamibi uptake assay was used to determine the function of ABCB1 protein in tumor cell subpopulations derived from glioblastoma xenografts. The detail information is available as Supplementary Data.

Animal Models

Eight-week-old male NOD/SCID mice were used to establish animal tumor models. The procedure of orthotopic glioblastoma xenograft model was performed according to the published methods.22 In brief, 2 × 10⁵ U87-ABCB1-P-Luc, U87/hif-1-r, or GBM8401/hif-1-r was harvested using trypsinization and injected into the left basal ganglia of anesthetized mice. The tumors developed at 18 days after tumor implantation for immunofluorescence imaging, flow cytometry analysis, and cell sorting studies and at 14 days after tumor implantation for imaging in vivo ABCB1 induction and evaluating the efficiency of BCNU chemotheraphy studies. All animal experiments were conducted according to Institutional Guidelines of China Medical University after acquiring permission from the local Ethical Committee for Animal Experimentation.

Immunofluorescence Imaging

For the immunofluorescence imaging of glioblastoma xenografts, the perfusion marker Hoechst 33342 (1 mg/mouse; Sigma) was intravenously (i.v.) administered 30 min before tumor excision. Tumor tissues were frozen in the OCT embedding matrix (Shandon Lipshaw). Frozen tissue sections (10 μm) were obtained with an OTF cryomicrotome (Bright-Hacker), fixed in ice-cold methanol for 10 min, and washed with PBS. Tumor sections were co-stained for ABCB1 by including ABCB1 antibody (Sigma-Aldrich) at a final concentration of 10 μg/mL. Sections were washed 3 times in PBS, each wash lasting 5 min. For ABCB1 staining, sections were incubated with DyLight 649-conjugated goat anti-rabbit antibody (1:100; Molecular Probes) and washed again. For the immunofluorescence imaging of GBM specimens, fresh GBM operative specimens were obtained from patients undergoing a craniotomy at the China Medical University Hospital with ethical approval from the Research Ethics Board. Frozen primary tumor sections (10 μm) were incubated with primary antibodies, ABCB1 (1:500; Sigma-Aldrich), HIF-1α (1:100; Novus), or CD31 (1:100; Novus) overnight at 4°C and secondary antibodies, DyLight 649-conjugated goat anti-rabbit antibody (1:100; Molecular Probes), or Dylight 488–conjugated goat-anti-mouse antibody (1:100; Abcam). Tissue fluorescence was visualized with the Axio Observer A1 digital fluorescence microscope system (ZEISS).

Bioluminescent Imaging (BLI)

Intracerebral U87-ABCB1-P-Luc–bearing mice were received with YC-1 (15 mg/kg i.v.). BLI was performed before and at 24 h after YC-1 treatment. In BLI, mice were anesthetized with isoflurane and imaged 15 min after intraperitoneal injection of 50 mg/kg firefly D-Luciferin (Caliper Life Sciences). Luminescence from the animals was recorded with the IVIS Imaging System 200 Series (Caliper Life Sciences). Signal intensity was quantified in a region of interest over the head that was defined by the Living Image software.
Animal Survival Assay

Intracerebral U87-ABCB1-P-Luc–bearing mice were randomly assigned to 6 different therapeutic groups: control (DMSO 1 g/kg i.v.), pretreatment of YC-1 (15 mg/kg i.v.), BCNU (15 mg/kg i.p.), pretreatment of YC-1 (15 mg/kg i.v.) + BCNU (15 mg/kg i.p.), YC-1 (15 mg/kg i.v.), or YC-1 (15 mg/kg i.v.) + BCNU (15 mg/kg i.p.). The pretreatment of YC-1 was performed at day 13 after tumor cell injection. Systemic BCNU or YC-1 treatment was performed for 5 days, starting on day 14 after tumor cell injection. Animals were killed at the onset of neurologic signs or any type of distress.

Statistical Analysis

For multiple comparisons of nonparametric variables, Kruskal-Wallis ANOVA was used. For parametric variables, ANOVA was used along with Fisher’s least significant difference. For survival analysis, statistical software for Kaplan-Meier survival analysis with Tarone-Ware statistics (SPSS) was used. P < .05 was considered to be statistically significant. All analyses were 2-tailed.

Results

HIF-1α Is a Critical Mediator Involved in Cycling Hypoxia-Induced ABCB1 Induction

We first evaluated the abundance of ABCB1 at different times in the hypoxia-reoxygenation protocol in U87 cells. The expression level of ABCB1 was increased after each new cycle of hypoxia treatment (Fig. 1A). HIF-1α and ABCB1 protein and mRNA levels in U87 and GBM8401 cells under cycling hypoxia stress were significantly higher than those in U87 cells and GBM8401 under uninterrupted hypoxia stress (Fig. 1B and C). We then examined the role of HIF-1α in cycling hypoxia-mediated ABCB1 induction. U87 cells were stably transfected with a lentiviral vector bearing a 2028-bp ABCB1 promoter-driven luciferase reporter gene that allowed for the dynamic monitoring of the transcriptional activation of ABCB1. The transcriptional activation of ABCB1 in the cycling hypoxia-treated cells increased significantly after treatment, with maximal activation occurring 48 h after hypoxic stress in U87 cells (Fig. 1D). To verify that HIF-1α is a crucial transcription factor for cycling hypoxia-mediated ABCB1 induction, we observed the transcriptional activation of ABCB1 and ABCB1 expression in U87 cells with or without HIF-1α siRNA after in vitro cycling hypoxia treatment. The siRNA successfully knocked down HIF-1α expression in U87 cells under in vitro hypoxic treatment, whereas the negative control (Neg) siRNA did not (Supplementary Fig. S1). When HIF-1α induction was blocked by siRNA, cycling hypoxia-induced transcriptional activation of ABCB1 in U87 cells and ABCB1 expression in U87, and GBM8401 cells were inhibited in cells treated with HIF-1α siRNA (Supplementary Fig. S2 and Fig. 1C). These results indicate that HIF-1α is a crucial transcription factor for cycling hypoxia-mediated ABCB1 induction.

Cycling Hypoxia Mediates ABCB1 Function

Because ABCB1 functions by mediating drug efflux, which leads to reduced drug accumulation, we determined the uptake and efflux of anti-cancer drug Rhodamine 123 (Rh123) in U87 cells with or without in vitro hypoxic treatment. Rh123 uptake increased in a time-dependent manner in U87 cells after 90 min of incubation. In contrast, cycling hypoxia-pretreated cells did not accumulate substrate over this same period. Moreover, the efflux of Rh123 increased significantly in cycling hypoxia-treated cells, compared with that in the control cells (Fig. 2A and B). In addition, using ABCB1 siRNA to specifically knock down ABCB1 induction in glioblastoma cells, we examined whether the variation in intracellular drug concentrations is mediated by ABCB1. ABCB1 siRNA successfully knocked down cycling hypoxia-mediated ABCB1 expression, whereas the negative control (Neg) siRNA did not (Fig. 2C). In addition, the Rh123 accumulation in cycling hypoxia-treated cells increased significantly with ABCB1 siRNA treatment (Fig. 2D). These results indicate that cycling hypoxia increases ABCB1 function in glioblastoma.

Cycling Hypoxia-Enhanced ABCB1 Activation Promotes Chemoresistance in Glioblastoma Cells

Next, we investigated the effects of cycling hypoxia on drug sensitivity. MTT assays were used to determine the effects of cycling hypoxia on the cytotoxicity of U87 and GBM8401 cells to anti-cancer drugs doxorubicin and BCNU. Cycling hypoxia pretreatment significantly increased chemoresistance to doxorubicin and BCNU in glioblastoma cells, compared with that in normoxic controls (Fig. 3A and B). Then, we determined whether ABCB1 is a critical effector involved in cycling hypoxia-induced chemoresistance in glioblastoma cells by knocking down ABCB1 expression in glioblastoma cells under cycling hypoxic stress and examined their drug sensitivity to doxorubicin and BCNU. When ABCB1 induction was blocked by siRNA, the chemoresistance induced by cycling hypoxic stress decreased (Fig. 3C). We also verified the impact of ABCB1 on chemoresistance by overexpressing its expression in glioblastoma cells via lentiviral overexpression system (Fig. 3D). Overexpression of ABCB1 increased chemoresistance to doxorubicin and BCNU in glioblastoma cells, indicating ABCB1 is an effector involved in doxorubicin and BCNU sensitivity (Fig. 3C). Taken together, these results suggest that cycling hypoxia-mediated ABCB1 activation is a crucial mechanism involved in cycling hypoxia-induced chemoresistance in glioblastoma cells.
The Majority of ABCB1 Expression Occurred in Endogenous Cycling Hypoxic Areas in Glioblastoma Xenografts

To investigate the biosignature of ABCB1 expression and HIF-1 signal transduction activity in the tumor microenvironment, mice bearing orthotopic U87/hif-1-r xenografts developed after 18 days were injected intravenously with a perfusion marker (Hoechst 33342), and the tumors were excised for tissue immunofluorescence imaging. We observed tight co-localization of high GFP intensity and Hoechst 33342 signals (Fig. 4A–C and E), indicating that the majority of HIF-1 signal transduction activity occurs in areas with relatively high perfusion. The areas with positive Hoechst 33342 staining and GFP expression are also potential cycling hypoxic areas. However, some areas were positive for GFP expression but stained negative for Hoechst 33342, indicating that they were mostly chronic hypoxic areas. The ABCB1 staining indicated that ABCB1 expression tends to occur in the cycling hypoxic areas but not in the chronic hypoxic areas (Fig. 4D and F). Furthermore, the results from immunofluorescence staining of ABCB1, HIF-1α, and CD31 human glioblastoma specimens also demonstrate that the ABCB1 expressing cells were frequently positioned in perivascular or vascular areas (Supplementary Fig. S5). Moreover, ABCB1 expression co-localized with high expression of HIF-1α, suggesting that endogenous cycling hypoxia leads to the induction of ABCB1 in glioblastoma.

To better verify the endogenous tumor microenvironment-mediated HIF-1 activation and ABCB1 expression in the solid tumor, we identified subpopulations of tumor cells from U87/hif-1-r xenografts on the basis of differential Hoechst 33342 and GFP fluorescence and investigated ABCB1 expression in these cell subpopulations by flow cytometry. Flow cytometric analysis of tumor cells (DsRed-positive cells) after Hoechst 33342 and GFP gating revealed that approximately 56% ± 4% of cycling hypoxic cells (Hoechst 3342+ and GFP+), 25% ± 2% of chronic hypoxic cells (Hoechst 3342− and GFP+), and 15% ± 6% of normoxic cells (Hoechst 3342+ and GFP−) existed in...
the tumor cell suspensions (Supplementary Fig. S6). Moreover, cycling hypoxic cells exhibited significantly more ABCB1 expression than chronic hypoxic cells and normoxic cells (Supplementary Fig. S7). These results suggest that the majority of HIF-1 signal transduction activity and ABCB1 expression occurs in cycling hypoxic areas in solid tumors.

Increased ABCB1 Function and Chemoresistance in Cycling Hypoxic Cells Isolated from Glioblastoma Xenografts

To determine the function of ABCB1 and drug sensitivities of hypoxic cell subpopulations in glioblastoma xenografts, we used fluorescence-activated cell sorting (FACS) to isolate these subpopulations derived from disaggregated orthotopic GBM8401/hif-1-r and U87/hif-1-r xenografts. The function of ABCB1 and drug sensitivities of these tumor cells were further analyzed using radioisotope uptake and MTT assays, respectively. The uptake of Tc-99m sestamibi decreased significantly in cycling hypoxic cells (Hoechst 3342+ and GFP+) compared with that in normoxic cells (Hoechst 3342+ and GFP+) (Fig. 5A and B). The uptake of Tc-99m sestamibi in cycling hypoxic cells was lower than that in chronic hypoxic cells. Moreover, treatment with tariquidar (XR-9576), a specific ABCB1 drug efflux pump inhibitor, significantly enhanced Tc-99m sestamibi accumulation in cycling and chronic hypoxic cells. MTT assays also revealed that cycling hypoxic cells possessed stronger drug resistance to doxorubicin and BCNU, than did chronic hypoxic and normoxic cells (Fig. 5C and D). Tariquidar treatment significantly reduced chemoresistance in cycling and chronic hypoxic cells. Taken together, these results indicate that endogenous cycling hypoxia enhances the function of ABCB1 as a drug efflux pump and further promotes chemoresistance in glioblastoma xenografts.

HIF-1 Blockade Suppresses Tumor Microenvironment-Mediated ABCB1 Induction and Enhances the Efficiency of BCNU Chemotherapy

To investigate whether HIF-1 blockade suppresses tumor microenvironment-mediated ABCB1 induction...
and further improves the efficiency of chemotherapy, BLI was used to observe in vivo ABCB1 induction in the orthotopic U87 xenograft model. In vivo optical imaging revealed significantly enhanced luciferase activity levels in the animals 14 days after tumor implantation (Fig. 6A and B). However, this tumor microenvironment-mediated transcriptional activation of ABCB1 was inhibited in mice treated with YC-1 24 h before imaging. This short-term treatment with YC-1 did not significantly alter tumor size, suggesting that the decreased BLI signal intensity is reflected in the YC-1-inhibited transcriptional activation of ABCB1. Moreover, we examined whether YC-1 treatment enhances the therapeutic benefits of systemically administered BCNU chemotherapy. Mice that received a 1-day YC-1 treatment prior to the initiation of chemotherapy with BCNU had a better survival rate than did those not pretreated with YC-1 (Fig. 6C). Furthermore, combined YC-1 and BCNU treatment significantly prolonged the survival time of intracerebral glioblastoma-bearing mice, compared with those with BCNU or YC-1 treatment alone. These results suggest that HIF-1 blockade suppresses tumor microenvironment-mediated ABCB1 induction and enhances the efficiency of BCNU chemotherapy in glioblastoma xenografts.

**Discussion**

The causes of chemotherapy not being effective to treat solid tumors are complex and multifactorial in nature. In most cases, the ineffectiveness is attributable to pharmacokinetic resistance, tumor cell intrinsic resistance, and tumor microenvironment-related factors. The most investigated mechanisms in tumor cell intrinsic resistance are (i) activation of transmembrane proteins effluxing different chemical substances from the cells, (ii) activation of the enzymes of the glutathione detoxification system, and (iii) alterations of the genes and proteins involved in the control of apoptosis. The causes of chemotherapy not being effective to treat solid tumors are complex and multifactorial in nature. In most cases, the ineffectiveness is attributable to pharmacokinetic resistance, tumor cell intrinsic resistance, and tumor microenvironment-related factors. The most investigated mechanisms in tumor cell intrinsic resistance are (i) activation of transmembrane proteins effluxing different chemical substances from the cells, (ii) activation of the enzymes of the glutathione detoxification system, and (iii) alterations of the genes and proteins involved in the control of apoptosis. Most published studies on the resistance of cancers to chemotherapy address cellular and genetic mechanisms of
resistance, whereas only a small number of studies describe the role of the tumor microenvironment. This bias toward cellular and genetic mechanisms as the dominant influences that affect drug resistance reflects the overwhelming use of monolayer cultures as a model in cancer research, which is a model that does not reflect the microenvironment of solid tumors. For instance, cells in solid tumors are exposed to various microenvironments and to a wide range of drug concentrations as the drug diffuses through from blood vessels. In contrast, cells in monolayer cultures are generally exposed to a uniform environment and drug concentrations. Therefore, it is difficult to demonstrate MDR mechanisms entirely based on the monolayer culture models. It is critical to investigate tumor microenvironments, including tumor hypoxia, that truly cause environmental-mediated drug resistance.25

It is a well-known fact that hypoxia renders cells chemoresistant.26–29 However, most of the previous studies focus mainly on the effects of chronic hypoxia on chemosensitivity. These studies clearly demonstrate that chronic hypoxia protects tumor cells from apoptosis induced by radiotherapy and chemotherapy while increasing cell resistance against these treatments.30,31 Moreover, chronic hypoxia limits the ability of drugs to penetrate tumor tissue and reach all tumor cells in a potentially lethal concentration.32 In contrast to chronic hypoxia, cycling hypoxia is poorly understood. The reason is that it is difficult to clearly determine the repetition of hypoxia and reoxygenation cycles in

Fig. 4. The majority of ABCB1 expression occurred in endogenous cycling hypoxic areas in glioblastoma xenografts. Representative images of microscopic U87/hif-1-r xenografts. Top left, fluorescence image of DsRed reporter, indicating tumor cell localization within the brain. Top right, fluorescence image of GFP reporter, demonstrating HIF-1 transcriptional activity in tumor cells. Middle left, fluorescence image of Hoechst 33342 showing perfusion within the brain and tumor tissue. Middle right, fluorescence image of ABCB1 staining. Bottom left, fluorescence overlay image of Hoechst 33342 and GFP reporter, indicating chronic and cycling hypoxic areas. Bottom right, fluorescence overlay image of Hoechst 33342, GFP reporter, and ABCB1. Bars = 100 μm.
tumors, which characterize cycling hypoxia, and directly isolate or distinguish these cells from solid tumors. Therefore, there are many unaddressed questions and issues regarding the presence of hypoxia and reoxygenation cycles and the potential influence in the treatment of solid tumors. Here, we report that in vitro cycling hypoxia pretreatment significantly increases drug resistance in glioblastoma cells. Moreover, the cycling hypoxic tumor cells obtained from disaggregated U87/hif-1-r xenografts reproducibly exhibited significantly higher chemoresistance than did chronic hypoxic and normoxic tumor cells. Thus, for the first time to our knowledge, our data support the hypothesis that cycling hypoxia substantially contributes to resistance to chemotherapy in solid tumors and could therefore be responsible for tumor regrowth.

To directly isolate or distinguish cycling hypoxic tumor cells from solid tumors, we established a reliable protocol of cycling hypoxic cell identification that allows subsequent immunofluorescence imaging and flow cytometric analysis of the biosignature of these cells. This approach can also isolate subpopulations of tumor hypoxic cells via cell sorting and further assaying of the phenotypic characterizations. We modified a previously reported technique that uses the diffusion/consumption properties of Hoechst 3342 so that it can visibly separate tumor cells according to their distance from the blood supply when it passes through several cell layers. However, the original technique cannot be used to distinguish or isolate cycling or chronic hypoxic cells from a heterogeneous population of tumor cells in a solid tumor because of the lack of a cycling hypoxic biomarker. Therefore, we identified these cells according to the physiological and molecular characteristics of cycling hypoxia. Because cycling hypoxia tended to occur in highly vascular regions with relatively high permeability, cycling hypoxic areas still have blood perfusion after transient occlusion or narrowing of the vasculature. In contrast, chronic hypoxic areas do not have blood perfusion even when the blood perfusion of the areas proximal to the blood vessels has been restored. Therefore, the perfusion marker, Hoechst 33342, stains positive in both normoxic and cycling hypoxic cells in solid tumors when the marker is injected into living mice and allowed to circulate. Moreover, cells exposed to cycling hypoxia exhibit more robust HIF-1 activation than do cells exposed to chronic hypoxia. Therefore, reporter gene expression is induced by HIF-1 activation in both cycling and chronic hypoxic cells in solid tumors. Cells positive for

Fig. 5. ABCB1 function and chemoresistance were increased in cycling hypoxic cells isolated from glioblastoma xenografts. Functional uptake of Tc-99m sestamibi in normoxic tumor cells, cycling hypoxic tumor cells, and chronic hypoxic tumor cells isolated from disaggregated U87/hif-1-r xenografts (A) and GBM8401/hif-1-r xenografts (B). Cytotoxicity assay of doxorubicin and BCNU in normoxic tumor cells, cycling hypoxic tumor cells, and chronic hypoxic tumor cells isolated from disaggregated U87/hif-1-r xenografts (C) and GBM8401/hif-1-r xenografts (D). Error bars denote the standard deviation within triplicate experiments. *P < .01 compared to normoxia. #P < .01 compared to cycling hypoxia. †P < .01 compared to chronic hypoxia.
Hoechst 33342 staining and HIF-1 activation are potential cycling hypoxic cells. Therefore, the combination of Hoechst 33342 staining and HIF-1 activation labeling together with immunofluorescence imaging or flow cytometric analysis is an effective approach for identifying hypoxic heterogeneous populations in solid tumors.

The expression level of ABCB1 is reported to increase in patients with GBM. Previous studies have validated the role of ABCB1 expression as a prognostic marker in gliomas. The membrane-residing ABCB1 protein is an ATP-binding cassette transporter that acts as an efflux pump for various hydrophobic agents and reduces the intracellular concentrations of a wide range of chemotherapeutic drugs, including Vinca alkaloids, taxanes, etoposide, teniposide, colchicines, actinomycin D, camptothecins, imatinib mesylate, saquinavir, methotrexate, and mitoxantrone. However, whether ABCB1 contributes to tumor microenvironment-mediated drug resistance in GBM is still unclear. In the present study, we found that the bulk of ABCB1 expression and function occurs in tumor cycling hypoxic areas and contributes to drug resistance in human glioblastoma xenografts. A major regulator of cycling hypoxia-induced ABCB1 induction is HIF-1α. Earlier work on HIF-1 and ABC transporters showed that HIF-1α is able to activate ABCB1 transcription because of a functional hypoxia-responsive element that exists in the ABCB1 promoter. The contribution of HIF-1-mediated ABCB1 expression to hypoxia-induced drug resistance has been observed in several tumor cells, including glioma, gastric cancer, breast carcinoma, and colon cancer cells. However, these studies mainly focused on chronic hypoxia-mediated responses. Moreover, direct evidence from in vivo tumor microenvironment studies is still lacking. Therefore, this study is a significant step toward understanding the impact and mechanism of cycling hypoxia-mediated drug resistance in tumor microenvironments. Although some other mechanisms of microenvironment-mediated intrinsic tumor cell resistance may also be involved in cycling hypoxia-induced drug resistance, our results provide clear evidence that tumor cycling hypoxia enhances the expression and

Fig. 6. HIF-1 blockade suppressed the tumor microenvironment-mediated ABCB1 induction and enhanced the efficiency of BCNU chemotherapy. (A) In vivo bioluminescence images of the transcriptional activation of ABCB1 in U87 glioma xenografts before and after YC-1 treatment. (B) Quantitative data obtained from the BLI imaging of the transcriptional activation of ABCB1 in U87 xenografts. The data represent the mean ± standard deviations of average counts within the tumor region of interest in BLI from 6 mice. *P < 0.01 compared to before YC-1 treatment. (C) Kaplan–Meier survival curve of intracerebral U87 glioblastoma-bearing mice. Mice received DMSO (control), YC-1 pretreatment (YC-1-P), BCNU treatment (BCNU), YC-1 pretreatment combined with BCNU treatment (YC-1-P + BCNU), YC-1 treatment (YC-1), or YC-1 combined with BCNU treatment (YC-1 + BCNU). P < .001.
function of ABCB1 and further promotes drug resistance in glioblastoma xenografts. This finding is important in the selection of optimal genetic or pharmacological approaches to inhibit tumor microenvironment-mediated drug resistance via the blockade of this mechanism.

In the present study, we found that HIF-1α is a critical mediator involved in cycling hypoxia-induced ABCB1 induction and function, which contribute to tumor microenvironment-induced drug resistance. Therefore, we propose that HIF-1 blockade prior to chemotherapy could increase both the intracellular retention of cytotoxic drugs and overall tumor sensitivity to anticancer drugs. Moreover, several prior studies indicate that HIF-1 inhibitors synergize with many classes of cancer therapeutic agents to achieve a more robust tumor response.41,42 For this reason, HIF-1 blockade should be used before administering cytotoxic drugs and concurrent with chemotherapy. HIF-1 blockade should also be maintained throughout the course of chemotherapy to maximally increase tumor chemosensitivity, because such treatment will not only decrease cycling hypoxia-induced drug resistance, it will also inhibit HIF-1-dependent tumor progression. Our results also confirmed that such a treatment schedule suppressed tumor microenvironment-mediated ABCB1 induction and improved the tumor response to BCNU in the orthotopic U87 xenograft model. BCNU is widely used in the treatment of GBM. However, chemotherapy often fails because of drug resistance. Although it has not been proven that BCNU is a substrate of ABCB1, previous studies showed a relationship between increased ABCB1 expression in freshly resected samples of glioblastoma and in vitro resistance to BCNU.15,16 Moreover, inhibition of the function or expression of ABCB1 can sensitize glioma cells to chemotherapeutic drugs, such as doxorubicin etoposide (VP-16), carboplatin, and BCNU.43,44 Our results also indicate that ABCB1 gene overexpression confers resistance to BCNU in glioblastoma cells. Therefore, these results support the notion that ABCB1 mediates cancer cell resistance to BCNU.

YC-1 is widely used as an effective HIF-1 inhibitor both in vitro and in vivo and is also being developed as a novel anticancer drug. However, it needs to be indicated that it is not a specific HIF-1 suppressor. In addition to its effect on HIF-1, YC-1 regulates the intracellular concentration of cGMP though enhancing the activity of soluble guanylate cyclase.45 Recently, it has been reported that the function of human P-glycoprotein can be inhibited by this small molecule via cGMP-dependent pathway.46 Although YC-1 treatment enhanced the efficiency of BCNU chemotherapy in intracerebral glioblastoma-bearing mice in this study and may be associated with various mechanisms, our results suggest that the suppression of cycling hypoxia-induced ABCB1 expression and function is one of the mechanisms involved in this process. Moreover, YC-1 treatment may be a good clinical practice for blocking cycling hypoxia-mediated drug resistance and enhancing overall therapeutic efficacy in humans.

In summary, the present study highlights the importance and mechanism of cycling hypoxia on tumor microenvironment-mediated chemosensitivity. Thus, oncologists should be aware of cycling hypoxia-induced chemoresistance in cancer treatment. Furthermore, HIF-1 blockade should be considered to be used before and concurrent with chemotherapy, as well as maintained throughout the course of chemotherapy to maximally increase overall therapeutic efficiency.

Supplementary Material

Supplementary material is available online at Neuro-Oncology (http://neuro-oncology.oxfordjournals.org/).

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References