Reoxygenation of Hypoxic Glioblastoma Multiforme Cells Potentiates the Killing Effect of an Interleukin-13-Based Cytotoxin

Tie Fu Liu, Jiaozhong Cai, Denise M. Gibo, and Waldemar Debinski

Abstract

Purpose: Hypoxia is a cause for resistance to cancer therapies. Molecularly targeted recombinant cytotoxins have shown clinical efficacy in the treatment of patients with primary brain tumors, glioblastoma multiforme, but it is not known whether hypoxia influences their antitumor effect.

Experimental Design: We have exposed glioblastoma multiforme cells, such as U-251 MG, U-373 MG, SNB-19, and A-172 MG, to either anoxia or hypoxia and then reoxygenated them while treating with an interleukin (IL)-13-based diphtheria toxin (DT)-containing cytotoxin, DT-IL13QM. We measured the levels of immunoreactive IL-13Rα2, a receptor that mediates IL-13-cytotoxin cell killing, and the levels of active form of furin, a protease that activates the bacterial toxin portion in a cytotoxin.

Results: We found that anoxia/hypoxia significantly alters the responsiveness of glioblastoma multiforme cells to DT-IL13QM. Interestingly, bringing these cells back to normoxia caused them to become even more susceptible to the cytotoxin than the cells maintained under normoxia. Anoxia/hypoxia caused a highly prominent decrease in the immunoreactive levels of both IL-13R and active forms of furin, and reoxygenation not only restored their levels but also became higher than that in normoxic glioblastoma multiforme cells.

Conclusions: Our results show that a recombinant cytotoxin directed against glioblastoma multiforme cells kills these cells much less efficiently under anoxic/hypoxic conditions. The reoxygenation brings unexpected additional benefit of making glioblastoma multiforme cells even more responsive to the killing effect of a cytotoxin.

Glioblastoma multiforme is a high-grade astrocytoma and represents the most common form of primary brain tumors. The successful treatment of patients with glioblastoma multiforme is still a major challenge, and a median survival rate is 14.5 months since diagnosis (1). In addition to the invasive nature of glioblastoma multiforme tumors, hypoxia, a unique property of solid tumors, has also been considered as an important factor affecting the efficacy of current treatments in glioblastoma multiforme (2, 3). Hypoxia is an alteration of balance between cellular proliferation and oxygen supply, resulting in significantly lower oxygen levels in focal regions than those encountered in surrounding both malignant and normal tissues (4). Evidence suggests that hypoxia influences the behavior of human tumor cells and endows hypoxic tumor cells a higher resistance to radiotherapy and certain chemotherapies and a higher mutation rate and potential for a more metastatic and malignant phenotype (2). The tumor oxygenation is negatively associated with increasing grade of human astrocytomas (5). Similarly to other solid tumors, glioblastoma multiforme tumors exhibit resistance to radiotherapy and chemotherapy largely in part due to the hypoxic tumor microenvironment (6). Several clinical trials have been done with hyperbaric oxygen or hypoxic cell radiosensitizers intending to overcome the problem of the radioresistance of hypoxic tumor cells (7–9). The results of these trials have shown benefit of proper oxygenation for glioblastoma multiforme radiotherapy.

Introduction of specific molecular targeted therapy using cytotoxins has offered new hopes for the successful treatment of glioblastoma multiforme (10). A typical recombinant cytotoxin is a single-chain fusion protein consisting of a ligand with high specificity for the overexpressed tumor-associated receptors and a potent bacterial toxin, such as the derivatives of diphtheria toxin (DT390) and Pseudomonas exotoxin A (PE38QQR; ref. 10). Cytotoxins are designed to take an advantage of the difference in receptor/target expression between normal and tumor cells, so that cytotoxins kill targeted tumor cells while sparring normal cells (11). Recombinant cytotoxins are very potent tumor cell-killing agents when compared with chemotherapeutic agents, because their IC_{50} values can reach the femtomolar range (10). Clinical studies using convection-enhanced delivery have employed several cytotoxins administered directly to glioblastoma multiforme tumors, such as...
Translational Relevance

Hypoxia is very frequent in glioblastoma multiforme and, in its severe form, leads to necrosis, a hallmark of this brain tumor. There are few effective modalities of treatment of glioblastoma multiforme, and one of them is recombinant cytoxins. We have found that hypoxia has a detrimental effect on the efficacy of recombinant IL-13 mutant-based cytotoxin on glioblastoma multiforme cells. However, improving oxygenation status not only brings the effectiveness of the cytotoxin back to the basal levels but also makes the killing by the cytotoxin more effective. Therefore, the clinical maneuvers that either normalize tumor circulation, and subsequently oxygenation, or apply direct hyperbaric approach should be considered when recombinant cytoxins are used in patients with glioblastoma multiforme.

Materials and Methods

Construction, expression, and purification of DT-IL13QM fusion protein. Recombinant IL-13 quadruple mutant (IL-13QM; IL-13.E13K/R66D/S69D/K105R) was generated by site-directed mutagenesis as detailed previously (21). PCR products of IL-13QM containing HindIII/EcoRI sequences was subcloned into a TA vector using TA cloning kit (Invitrogen) and transformed to TOP-10 cells. IL-13QM-containing plasmid was amplified in TOP10 cells, digested with HindIII and EcoRI (all restriction enzymes were from Fermentas), and gel purified. Vector DNA containing DT derivatives, DT389, was obtained by removing the IL-2 fragment in our previously generated DT-IL2 plasmid. The NH2-terminal end of IL-13QM was fused to the COOH-terminal of PE toxin (27). The expression levels, especially the conversion of pro-form to active form, of furin in targeted tumor cells are likely contributory determinants of tumoricidal efficacy of cytoxins.

Given that glioblastoma multiforme are extensively hypoxogenated and necrotic tumors, this condition may also affect the efficacy of anti-glioblastoma multiforme recombinant cytoxins. In the present study, we compared the cytotoxicity of IL-13-based cytoxins in hypoxic and then reoxygenated glioblastoma multiforme cells. We also analyzed the expression of both IL-13Ra2 and furin under these conditions. Our data show that hypoxia significantly impairs the response of glioblastoma multiforme cells to the killing of the cytotoxin. Reoxygenation of hypoxic glioblastoma multiforme cells reversed this phenomenon and, surprisingly, even further increased the sensitivity of glioblastoma multiforme cells to cytotoxin. These changes in susceptibility of glioblastoma multiforme cells to an IL-13-based cytotoxin in response to hypoxia and reoxygenation were paralleled by the changes in the expression of IL-13Ra2 and in the conversion of pro-furin.

Some of the most potent bacterial toxins known, such as DT and PE, possess ADP-ribose transferase enzymatic activity, which inactivates elongation factor-2 and subsequently inhibits protein synthesis that leads to cell death (24). However, the toxins and their portions in cytoxins require proteolytic activation by a calcium-dependent serine endoprotease, furin (25–28). Furin is a primary pro-protein convertase responsible for the post-translational endoproteolysis of inactive precursor proteins including most secretory proteins and membrane type I matrix metalloproteinases (29). The cleavage sites in target pro-proteins are usually paired or multiple, basic residues, such as Arg-X-Lys/Arg-Arg sequences (30). An up-regulated expression and an increased activity of furin in tumor cells have been associated with tumor invasiveness (31, 32). Furin itself is synthesized in endoplasmic reticulum as 96-kDa pro-furin and exits endoplasmic reticulum after rapid conversion to a 90-kDa active form by an intramolecular autocatalytic process (33). DT, PE, and cytoxins based on these toxins contain a furin cleavage site that must undergo proteolysis during their entry into the host cells (25–28, 34). The peptide motif for furin cleavage is found in the 14-amino acid loop constricted by a disulfide bond between Cys186 and Cys201 in the DT toxin (34), and it is found in Cys265/Cys267 conserved loop of domain II of PE toxin (27). The expression levels, especially the conversion of pro-form to active form, of furin in targeted tumor cells are likely contributory determinants of tumoricidal efficacy of cytoxins.


Downloaded from clincancerres.aacjrournals.org on May 13, 2015. © 2009 American Association for Cancer Research.
analysis. Glioblastoma multiforme cells were significantly more sensitive and normal cells less sensitive to DT-IL13QM than to IL-13-PE38QQR (23), the first generation of IL-13-based cytotoxin (19), one of the most potent anti-glioblastoma multiforme agents.

**Cell culture.** Human glioblastoma multiforme cell lines U-251 MG, U-373 MG, SNB-19, and A-172 MG were obtained from the American Type Culture Collection. U-251 MG cells were maintained in DMEM (Invitrogen) supplemented with 1× nonessential amino acid (Invitrogen) and 10% FCS (Hyclone). U-373 MG and A-172 MG cells were cultured in DMEM containing 10% FCS. SNB-19 cells were cultured in RPMI 1640 (Invitrogen) supplemented with 1× nonessential amino acid, 1 mmol/L sodium pyruvate (Invitrogen), and 10% FCS.

**Anoxia/hypoxia treatment of glioblastoma multiforme cells.** Glioblastoma multiforme cells (1 × 10^6) per 100 mm² dish or 1,000 per well in 96-well plates were incubated in an Invivo2 hypoxia workstation (Ruskinn) under anoxia (0% O₂, 95% N₂, and 5% CO₂) or hypoxia (0.1% O₂, 94.9% N₂, and 5% CO₂). For recovery study, glioblastoma multiforme cells, which were preincubated in hypoxia chamber for 24 h, were placed back into normoxic incubator. Cells in dishes were immediately placed in cold PBS at indicated time points to prevent hypoxia-inducible factor-1α protein from degradation. Cell lysates were prepared by incubation of cell pellets in harvesting buffer [10 mmol/L HEPES (pH 7.9), 50 mmol/L NaCl, 0.1 mmol/L EDTA, 0.5% Triton-X 100, 1 mmol/L DTT plus protease inhibitors (Sigma)] for 5 min on ice followed by centrifugation. The supernatants were collected and stored at -80°C. The nuclei pellets were washed once with buffer A [10 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L DTT plus protease inhibitors] and resuspended in 2× buffer C [10 mmol/L HEPES (pH 7.9), 500 mmol/L NaCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 0.1% Igepal (NP-40), 1 mmol/L DTT plus protease inhibitors] for 15 min on ice. After centrifugation, supernatants were collected as nuclear extracts and stored at -80°C until use for Western blotting analysis.
**Colorimetric MTS/PMS cell viability assay.** Cytotoxicity of DT-IL13QM on glioblastoma multiforme cells was tested using colorimetric MTS/PMS cell viability assay (Promega) as described previously (21). Cycloheximide-treated (Calbiochem) wells served as a background control. To study the sensitivity of glioblastoma multiforme cells to IL-13-cytotoxin under anoxia or hypoxia, glioblastoma multiforme cells in 96-well plate wells were pretreated for 6 h under anoxia or hypoxia followed by incubation of cells with different concentrations of DT-IL13QM for additional 24 h in hypoxia chamber before the addition of MTS/PMS. For recovery study, glioblastoma multiforme cells were pretreated in hypoxia chamber for 24 h. Anoxic or hypoxic glioblastoma multiforme cells were then placed back to normoxic CO\(_2\) incubator for another 24 h. All assays were done in duplicates.

Fig. 2. Cytotoxicity of DT-IL13QM in reoxygenated, previously anoxic glioblastoma multiforme cells. Glioblastoma multiforme cells were cultured for 30 min in the presence or absence of 100 \(\mu\)mol/L furin inhibitor, 1 Dec-RVKR-CMK (Calbiochem), followed by addition of DT-IL13QM into the wells. After 48 h incubation, 10 \(\mu\)L MTS/PMS mixture (1:20) was added into each well and incubated for 90 min at 37°C. The absorbance at 490 nm was recorded using a microplate reader (SpectraMax 340PC; Molecular Devices). A replicate plate was set under normoxia for comparison of sensitivity with that of anoxic/hypoxic or reoxygenated glioblastoma multiforme cells to DT-IL13QM.

Fig. 3. Cytotoxicity of DT-IL13QM in reoxygenated, previously hypoxic glioblastoma multiforme cells. Glioblastoma multiforme cells were cultured for 30 min in the presence or absence of 100 \(\mu\)mol/L furin inhibitor, 1 Dec-RVKR-CMK (Calbiochem), followed by addition of DT-IL13QM into the wells. After 48 h incubation, 10 \(\mu\)L MTS/PMS mixture (1:20) was added into each well and incubated for 90 min at 37°C. The absorbance at 490 nm was recorded using a microplate reader (SpectraMax 340PC; Molecular Devices). A replicate plate was set under normoxia for comparison of sensitivity with that of anoxic/hypoxic or reoxygenated glioblastoma multiforme cells to DT-IL13QM.
All points in proliferation curve represent mean ± SD of duplicates.

**Immunoblotting.** Proteins (20 μg/well) of cell lysates or nuclear extracts was loaded onto 12% SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane (Perkin-Elmer). Blots were blocked with 5% milk-PBS for 1 h at room temperature. Immunoreactive IL-13Ra2 was recognized by 2 μg/ml goat anti-human IL-13Ra2 (R&D Systems). Furin was recognized by 1 μg/ml rabbit anti-human furin polyclonal antibody (Santa Cruz Biotechnology). β-Actin was used as protein loading control and probed with 0.04 μg/ml anti-human β-actin monoclonal antibody (Sigma). The blots were incubated with primary antibodies diluted in blocking buffer overnight at 4°C. The primary antibody-protein complexes were detected by incubation for 1 h at room temperature with secondary antibody conjugated with horseradish peroxidase (Sigma) diluted 1:5,000 in blocking buffer. The detection was done using an Enhanced Chemiluminescence Plus kit (GE Healthcare).

**Statistical analysis.** IC50 values of DT-IL13QM on glioblastoma multiforme cells were analyzed with GraphPad Prism version 4.0 (GraphPad Software). The differences in IC50 values of DT-IL13QM in normoxic, hypoxic, and reoxygenated glioblastoma multiforme cells were analyzed using Student's paired t-test for all data points. P values < 0.05 were considered significant.

**Results**

Both anoxic and hypoxic glioblastoma multiforme cells are significantly less sensitive to IL-13-cytotoxin than normoxic cells. It is well known that the initial molecular response to hypoxia is an increased level of hypoxia-inducible factor 1 protein, a multisubunit protein consisting of α and β helix-loop-helix subunits (35–37). To examine whether anoxia or hypoxia affects susceptibility of glioblastoma multiforme cells to IL-13-cytotoxin killing, we employed a modified 24 h cell viability assay. We found that anoxia had a dramatic effect on the potency of DT-IL13QM in all glioblastoma multiforme cell lines studied. Anoxic glioblastoma multiforme cells were up to 16-fold less sensitive to DT-IL13QM than normoxic cells (Fig. 1A). The normoxia/anoxia ratios of IC50 values for DT-IL13QM on U-251 MG, U-373 MG, SNB-19, and A-172 MG glioblastoma multiforme cells were 8/74, 12/200, 0.2/1.3, and 16/127 pmol/L, respectively. In some of these assays, the IC50 values were higher than usual as a result of prolonged duration. In hypoxic SNB-19 cells, reoxygenation caused a restoration of the cytotoxic potential without further improvement in killing efficiency (Fig. 3). Interestingly, SNB-19 cells were by far the most responsive to DT-IL13QM among other studied cells to start with (Figs. 1–3).

Reoxygenation of previously hypoxic glioblastoma multiforme cells restores and even further increases the sensitivity of glioblastoma multiforme cells to DT-IL13QM. Both anoxia and hypoxia significantly decreased a response of glioblastoma multiforme cells to IL-13-cytotoxin killing (Fig. 1). We next tested whether reoxygenation may reverse this effect. U-251, U-373, SNB-19, and A-172 glioblastoma multiforme cells were cultured for 24 h either under anoxia/hypoxia conditions. The cells were then reoxygenated for 24 h in normoxic incubator before the addition of DT-IL13QM. We have found that reoxygenation for 24 h not only restored the susceptibility to the cytotoxin fully but also statistically significantly increased the sensitivity of previously anoxic U-251, U-373, or SNB-19 glioblastoma multiforme cells to the killing by DT-IL13QM when compared with cells maintained in normoxia (Fig. 2). The reoxygenated anoxic glioblastoma multiforme cells were 2- to 10-fold more sensitive to DT-IL13QM killing than normoxic glioblastoma multiforme cells. The IC50 ratios of normoxic/recovered anoxic U-251, U-373, SNB-19, and A-172 glioblastoma multiforme cells were 63/6, 49/7.8, 7/1.8, and 11/6.7 pmol/L, respectively.

An increase in susceptibility to DT-IL13QM in previously anoxic and subsequently reoxygenated glioblastoma multiforme cells was unexpected and we continued these experiments with cells kept first under hypoxic conditions. Reoxygenation of previously hypoxic U-251, U-373, and A-172 cells for 24 h significantly improved DT-IL13QM killing when compared with normoxic cells (Fig. 3). The normoxia/hypoxia ratio of IC50 values for DT-IL13QM in 24 h reoxygenated U-251 and U-373 cells were 100/12 and 337/51 pmol/L, respectively. In some of these assays, the IC50 values were higher than usual as a result of prolonged duration. In hypoxic SNB-19 cells, reoxygenation caused a restoration of the cytotoxin potency without further improvement in killing efficiency (Fig. 3). Interestingly, SNB-19 cells were by far the most responsive to DT-IL13QM among other studied cells to start with (Figs. 1-3).

**Inhibition of furin protease activity diminishes cytotoxicity of DT-IL13QM on glioblastoma multiforme cells.** An overexpression of IL-13Ra2 in glioblastoma multiforme cells determines
the susceptibility of glioblastoma multiforme cells to IL-13-cytotoxin (19, 38, 39) and furin protease is responsible for the intracellular process of cytotoxin to exert cytotoxicity (25–28, 34). We now tested directly whether furin protease activity plays a role in the killing potency of IL-13-based cytotoxins on glioblastoma multiforme cells in the presence of an overexpressed IL-13Rα2. As shown in Fig. 4A, >90% of U-251 cells were killed by DT-IL13QM alone. However, the potent cytotoxic effect of DT-IL13QM on U-251 cells was completely blocked by 100 μmol/L furin inhibitor, I-Dec-RVKR-CMK (Fig. 4A). Furin inhibitor alone did not change cell viability when compared with control. A similar result was observed in another glioblastoma multiforme cell line, SNB-19 cells. DT-IL13QM alone killed SNB-19 cells potently and the inhibition of furin protease activity prevented this cell killing (Fig. 4B). These experiments further documented an essential role of furin in bacterial toxins’ processing and subsequent targeted cytotoxin potency.

**IL-13Rα2 expression in glioblastoma multiforme cells is dependent on oxygenation status.** We reasoned that the regulation of IL-13Rα2 expression in glioblastoma multiforme cells by anoxia or hypoxia may be responsible, at least in part, for relative insensitivity of anoxic or hypoxic glioblastoma multiforme cells to DT-IL13QM. We have thus begun analyzing the levels of immunoreactive IL-13Rα2 in U-251, U-373, SNB-19, and A-172 glioblastoma multiforme cells kept under anoxia or hypoxia conditions. In response to anoxia or hypoxia, IL-13Rα2 expression in glioblastoma multiforme cells gradually decreased starting even before 6 h of anoxia/hypoxia with dramatically decreased levels found after 24 h (anoxia) or 72 h (hypoxia) in all four glioblastoma multiforme cell lines studied (Fig. 5A).

**Levels of mature furin change dramatically under anoxia/hypoxia in glioblastoma multiforme cells.** Inhibition of furin activity using a specific inhibitor blocked the glioblastoma multiforme cell killing by IL-13-cytotoxin (Fig. 4). Hence, we analyzed the effect of anoxia or hypoxia on the levels of mature form of furin in U-251, U-373, SNB-19, and A-172 glioblastoma multiforme cells. Immunoreactive activated furin (90 kDa) protein levels in glioblastoma multiforme cells started to decrease even after 2 h of anoxia until almost undetectable levels at and past 24 h, although the levels of 96-kDa pro-furin also changed and became close to the detection limit in some cell lines after 24 h of anoxia (Fig. 5B). Similar patterns of furin protein expression and pro-furin conversion in glioblastoma multiforme cells was observed in response to hypoxia stress (Fig. 5B).

**Reoxygenation causes a rebound or even a further increase in protein levels of IL-13Rα2 and active furin in glioblastoma multiforme cells subjected to anoxia or hypoxia.** We next investigated the effect of reoxygenation of anoxic glioblastoma multiforme cells on IL-13Rα2 expression and the conversion of pro-furin to mature furin, in view of previous results showing restoration or even better killing activity of DT-IL13QM in glioblastoma multiforme cells (Figs. 2 and 3). The U-251, U-373, SNB-19, or A-172 cells were kept under anoxia or hypoxia for 24 h. Cells were then reoxygenated in 5% CO2 incubator under normoxia from 2 to 48 h. IL-13Rα2 expression in 24 h anoxic cells notably decreased compared with that in normoxic cells (Fig. 5C). However, IL-13Rα2 expression rebounded in anoxic glioblastoma multiforme cells to at least basal, or even higher, levels after 6 to 48 h of reoxygenation (Fig. 5C).

The protein levels of activated furin in reoxygenated anoxic glioblastoma multiforme cells exhibited a similar pattern to that by IL-13Rα2. Activated form of furin in anoxic glioblastoma multiforme cells (90 kDa) started to rebound after 4 h of reoxygenation and steadily increased until 48 h of reoxygenation. Reoxygenation of hypoxic cells resulted again in a similar pattern of changes in activated furin levels with tendency to recover furin to at least basal if not higher levels (Fig. 5D).

**Discussion**

We have found that anoxia/hypoxia altered prominently the responsiveness of glioblastoma multiforme cells to the killing by IL-13-based cytotoxins. This alteration was associated with a significant decrease in the levels of IL-13Rα2, targeted by the cytotoxin plasma membrane receptor, and of active furin, a protease that activates the toxin portion of a cytotoxin. Interestingly, the cells that were subjected to anoxia/hypoxia first and then brought back to normoxic conditions became better responders to IL-13-cytotoxin than the cells maintained continuously in normoxia. These reoxygenated glioblastoma multiforme cells that were killed more efficiently using a recombinant cytotoxin had their levels of both IL-13Rα2 and activated furin also elevated when compared with normoxic cells. We have thus shown for the first time that anoxia/hypoxia negatively affects the potency of recombinant cytotoxins in killing glioblastoma multiforme cells. Furthermore, reoxygenation offers even better efficacy of the cytotoxins.

Recombinant cytotoxins have been already tested in the clinic showing considerable promise (10) and human IL-13-PE38QQR, the first generation of IL-13-based cytotoxins, showed a highly significantly better progression-free survival in patients with recurrent glioblastoma multiforme when compared with standard of care (16). Our current study clearly indicates that anoxia/hypoxia, which is so characteristic of glioblastoma multiforme tumors, has hampered the efficacy of the cytotoxins in glioblastoma multiforme cells. However, when cells were reoxygenated, the cytotoxin became even more potent. For example, reoxygenation reversed the diminished sensitivity of hypoxic glioblastoma multiforme cells to IL-13-cytotoxin resulting in a 100-fold lower IC50 values for DT-IL13QM in reoxygenated glioblastoma multiforme cells than that in hypoxic glioblastoma multiforme cells. This strongly implies that an effort should be made to diminish possible tumor anoxia/hypoxia in glioblastoma multiforme patients before cytotoxin treatment initiation. One of the possible means to overcome the hypoxia-associated resistance of glioblastoma multiforme therapies is to prenormalize oxygen status with hyperbaric oxygen before therapy. It has been shown that hyperbaric oxygen therapy can efficiently improves oxygen supply to hypoxic cells (7, 8). The results of clinical trials with combination of hyperbaric oxygen therapy and radiotherapy in glioblastoma multiforme patients encourage its application to other therapies such as targeted therapy with recombinant cytotoxins. Being that the hyperbaric oxygen is not widely available clinically, combination of antiangiogenic therapy with cytotoxins therapy provides another opportunity to overcome hypoxia-associated resistance to drugs. Recent attempts at antiangiogenic therapy (e.g., with
AZD2171, a pan-vascular endothelial growth factor receptor tyrosine kinase inhibitor) have shown “normalization” of hypoxia and favored radiotherapy of glioblastoma multiforme patients (40, 41).

Hypoxia regulates expression of a panel of genes during the adaptation period to stress conditions with subsequent functional changes (35). Overexpressions of genes and proteins of vascular endothelial growth factor and chemokine (C-X-C motif) receptor 4 in glioblastoma multiforme cells have been associated with a well-developed neovascularization and invasive nature of glioblastoma multiforme tumors (42, 43). The regulation of expression of tumor marker proteins and enzymes by hypoxia that are crucial to recombinant cytotoxins therapy of glioblastoma multiforme has not been illustrated previously. The cytotoxic effect of a cytotoxin parallels the number of receptor molecules in targeted cells (19, 38). For example, turning off IL-13Rα2 gene in IL-13-PE38QQR-sensitive glioblastoma multiforme cells resulted in the resistance to IL-13-PE38QQR killing (39). In the present study, we found that the changes in IL-13Rα2 protein levels correlated with DT-IL13QM cytotoxicity on glioblastoma multiforme cells in response to anoxia/hypoxia stress and subsequent reoxygenation. IL-13Rα2 protein levels dramatically decreased in glioblastoma multiforme cells after 24 h anoxia and steadily rebounded to even higher than the background levels during the reoxygenation. A similar pattern in IL-13Rα2 protein levels change was seen in hypoxic and reoxygenated glioblastoma multiforme cells. It is thus likely that the changes in expression levels of a target molecule under anoxic and/or hypoxic tumor cells are in part responsible for the changes of their sensitivity to cytotoxin killing. Currently, some models of glioblastoma multiforme reflect hypoxia and necrosis, especially when large tumors are developed in rodents (44).

In addition to the levels of targeted receptor molecule on tumor cells that are important to the cytotoxin efficacy, the intracellular processing of cytotoxin by furin is another significant determinant of cytotoxin potency. Furin is encoded by fur gene, which is overexpressed in glioblastoma multiforme cells to start with (31), similarly to IL-13Rα2. fur gene decodes the full-length pro-furin followed by intramolecular autocatalytic cleavage in endoplasmic reticulum (33).
Only the mature form of furin can release an active domain of the toxin into the cytosol by cleavage at the furin recognition sequence RXK/RR that is located in the translocation domain of DT and PE toxins (25–28, 34). Therefore, not only the fur gene transcription but also the conversion of pro-furin to active furin could be factors determining the sensitivity of targeted tumor cells to cytotoxin therapy. We observed that the inhibition of furin activity using a specific inhibitor significantly blocked the cytotoxicity of IL-13-cytotoxin in glioblastoma multiforme cells. The protein levels of active furin gradually declined to almost undetectable levels when cells were exposed to anoxia/hypoxia. The levels of furin, however, rebounded to even higher than the background levels after reoxygenation. These changes were closely associated with the cytotoxic potency of DT-IL13QM in glioblastoma multiforme cells and thus indicate on an indispensable role of furin in the potency of cytotoxins in glioblastoma multiforme cells.

In summary, prenormalization of tumor hypoxia status should be attempted to further improve the results of targeted therapy of glioblastoma multiforme using IL-13-based cytotoxins.

**Disclosure of Potential Conflicts of Interest**

W. Debinski has an ownership interest in and has served as a consultant for Targetpeutics.

**References**


Cancer Therapy: Preclinical


Reoxygenation of Hypoxic Glioblastoma Multiforme Cells Potentiates the Killing Effect of an Interleukin-13-Based Cytotoxin

Tie Fu Liu, Jiaozhong Cai, Denise M. Gibo, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/15/1/160

Cited Articles
This article cites by 44 articles, 24 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/15/1/160.full.html#ref-list-1

Citing articles
This article has been cited by 4 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/15/1/160.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.