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

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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-IL13QMQ. 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-IL13QMQ. 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 chemotherapy 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 sparing normal cells (11). Recombinant cytotoxins are very potent tumor cell-killing agents when compared with chemotherapeutic agents, because their IC50 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

Hyoxia 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 cytotoxins. We have found that hyoxia 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 cytotoxins 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 derivates, DT390, 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 DT390 by subcloning using HindIII and the resulted DT-IL13QM plasmid was used to transform DH5α cells. In-frame DNA sequence of DT-IL13QM was confirmed by automated sequencer before protein expression.

Recombinant DT-IL13QM fusion protein was expressed in BL21 (DE3) Escherichia coli cells and purified using fast protein liquid chromatography system (GE Healthcare) as detailed previously (22). In brief, BL21 E. coli cells were transformed with DT-IL13QM plasmid DNA and cultured in the LB medium supplemented with 100 μg/mL ampicillin in a 37°C shaker. When A600 value of bacterial culture reaches ~1.5, 1 mmol/L IPTG (Inalco) was added into bacterial culture for 90 min to induce the expression of recombinant protein. The inclusion bodies were isolated, denatured in 7 mol/L guanidine HCl/dithioerythritol, refolded in arginine/oxidized glutathione solution, and dialyzed against 10 mmol/L phosphate buffer (pH 7.4). DT-IL13QM protein was then purified on an ion exchange column, Q-Sepharose using fast protein liquid chromatography system. The resulting protein is >95% purity on SDS-PAGE and is recognized by anti-IL-13 antibody (Santa Cruz Biotechnology) in Western blot.
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 \( (1 \times 10^6) \) 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 \([10 \text{ mmol/L HEPES (pH 7.9)}, 50 \text{ mmol/L NaCl}, 0.5 \text{ mol/L sucrose}, 0.1 \text{ mmol/L EDTA}, 0.5\% \text{ Triton-X 100}, 1 \text{ mmol/L DTT plus protease inhibitors (Sigma)}]\) 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 \([10 \text{ mmol/L HEPES (pH 7.9)}, 10 \text{ mmol/L KCl}, 0.1 \text{ mmol/L EDTA}, 0.1 \text{ mmol/L EGTA}, 1 \text{ mmol/L DTT plus protease inhibitors}]\) and resuspended in \( 2/3 \text{ buffer C} \) \([10 \text{ mmol/L HEPES (pH 7.9)}, 500 \text{ mmol/L NaCl}, 0.1 \text{ mmol/L EDTA}, 0.1 \text{ mmol/L EGTA}, 0.1\% \text{ Igepal (NP-40), 1 mmol/L DTT plus protease inhibitors}]\) 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 placed back to normoxia. After reoxygenation for 24 h, different concentrations of DT-IL13QM were added to cells for another 48 h. In some experiments, glioblastoma multiforme cells were cultured for 30 min in the presence or absence of 100 μmol/L furin inhibitor, 1 Dec-RVKR-CMK (Calbiochem), followed by addition of DT-IL13QM into the wells. After 48 h incubation, 10 μL 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. 2. Cytotoxicity of DT-IL13QM in reoxygenated, previously anoxic glioblastoma multiforme cells. Glioblastoma multiforme cells were incubated with various concentrations of DT-IL13QM for 24 h. 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 maintained under hypoxia for 24 h followed by incubation under normoxia for 24 h before addition of various concentrations of DT-IL13QM for another 48 h. All assays were done in duplicates.
killing. Each point in proliferation curve represents 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.** IC_{50} values of DT-IL13QM on glioblastoma multiforme cells were analyzed with GraphPad Prism version 4.0 (GraphPad Software). The differences in IC_{50} 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 IC_{50} 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 IC_{50} values were higher than usual as a result of prolonged duration. In hypoxic SNB-19 cells, reoxygenation caused a restoration of the cytotoxicity without further improvement in killing efficiency (Fig. 1B). 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 anoxic/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 thus treated with DT-IL13QM under hypoxia (Fig. 1B). In all the tested glioblastoma multiforme cells, similarly to anoxic conditions, hypoxia significantly altered the potency of DT-IL13QM, reflected by a 4-fold (U-251 cells) to 24-fold (U-373 cells) decrease in the IC_{50} values (Fig. 1B).

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 IC_{50} 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 IC_{50} values were higher than usual as a result of prolonged duration. In hypoxic SNB-19 cells, reoxygenation caused a restoration of the cytotoxicity 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 cytotoxin on glioblastoma multiforme cells in the presence of an overexpressed IL-13Ra2. 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 cell killing glioblastoma multiforme cells. DT-IL13QM in reoxygenated glioblastoma multiforme cells killed more efficiently using a recombinant cytotoxin had their levels of both IL-13Ra2 and active 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-PE38QQQR, 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

Reoxygenation Potentiates Cell Killing by IL-13 Toxin

**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-13Ra2, 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-13Ra2 and activated furin also elevated when compared with normoxic cells. We have thus shown for the first time that anoxia/hypoxia 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-13Ra2 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-13Ra2 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-13Ra2 expression in 24 h anoxic cells notably decreased compared with that in normoxic cells (Fig. 5C). However, IL-13Ra2 expression rebounded in anoxic glioblastoma multiforme cells to at least basal, or even higher, levels after 6 to 48 h of reoxygenation (Fig. 5D).
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).

Fig. 5. Changes of IL-13Rα2 and active furin protein levels in glioblastoma multiforme cells during anoxia/hypoxia and consequent reoxygenation treatments. Glioblastoma multiforme cells were incubated either for up to 24 h under anoxia or up to 72 h under hypoxia. For reoxygenation studies, glioblastoma multiforme cells were maintained under anoxia or hypoxia condition for 24 h followed by incubation under normoxia for 2 to 48 h. Cell lysates were prepared at indicated time points for Western blot analyses of IL-13Rα2 or furin, respectively. Densitometry was done using Scion Image software. A, IL-13Rα2 expression in anoxic and hypoxic glioblastoma multiforme cells. B, furin expression and conversion in anoxic and hypoxic glioblastoma multiforme cells.
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 Targepeutics.

**References**

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