Hyperthermia Sensitizes Glioma Stem-like Cells to Radiation by Inhibiting AKT Signaling

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Abstract

Glioma stem-like cells (GSCs) are a subpopulation of cells in tumors that are believed to mediate self-renewal and relapse in glioblastoma (GBM), the most deadly form of primary brain cancer. In radiation oncology, hyperthermia is known to radiosensitize cells, and it is reemerging as a treatment option for patients with GBM. In this study, we investigated the mechanisms of hyperthermic radiosensitization in GSCs by a phospho-kinase array that revealed the survival kinase AKT as a critical sensitization determinant. GSCs treated with radiation alone exhibited increased AKT activation, but the addition of hyperthermia before radiotherapy reduced AKT activation and impaired GSC proliferation. Introduction of constitutively active AKT in GSCs compromised hyperthermic radiosensitization. Pharmacologic inhibition of PI3K further enhanced the radiosensitizing effects of hyperthermia. In a preclinical orthotopic transplant model of human GBM, thermoradiotherapy reduced pS6 levels, delayed tumor growth, and extended animal survival. Together, our results offer a preclinical proof-of-concept for further evaluation of combined hyperthermia and radiation for GBM treatment. Cancer Res; 75(8); 1760–9. ©2015 AACR.

Introduction

Glioblastoma (GBM) is the most common malignant primary brain tumor (1). The median survival for patients with newly diagnosed GBM is about 1 year despite aggressive therapy with surgery, radio-, and chemotherapy (2). Radiation is the most efficacious, nonsurgical treatment but control rates remain poor (3). Glioma stem-like cells (GSCs) are increasingly recognized as playing important roles in tumor progression and therapeutic resistance (4–10). Therefore, treatments that improve targeting of GSCs are needed to provide durable tumor control. Hyperthermia is one of the oldest and most potent radio-sensitizers (11, 12) and has been shown to improve cancer control in numerous phase III clinical trials (13–17). When used in conjunction with brachytherapy, hyperthermia significantly improves the survival of patients with GBM (15). However, widespread adoption of hyperthermia has been limited because of technical challenges in its administration. In addition, brachytherapy has failed to demonstrate a clinical benefit over standard external beam irradiation (18, 19). Laser interstitial hyperthermia is emerging as an innovative, minimally invasive surgery that permits real-time three-dimensional thermometry within the operating theater. Initial studies of interstitial hyperthermia for patients with GBM have been promising, and this technique is gaining momentum as a new therapeutic option (20–22). In light of this developing treatment modality, we sought to determine the efficacy of hyperthermia and external beam radiation in treating GBM and to characterize the molecular mechanisms of radiosensitization by hyperthermia to uncover potential targets for improving GSC radiosensitivity.

Materials and Methods

Cell culture and treatment conditions

Glioma stem cells (specimens 3691 and 387) were isolated and functionally validated as previously described (23, 24) and cultured as neurospheres in neural basal medium enriched with B27 supplement (Gibco), 10 ng/mL basic fibroblast growth factor, and 10 ng/mL EGF (R&D Systems; ref. 24). Only low-passage GSCs were used (<5 passages). Non-stem tumor cells (NSTC) were cultured in DMEM containing 10% FBS. GSCs were sham-treated or treated with hyperthermia (HT, incubated in a humidified incubator, 5% CO2, temperature set at 42.5°C for 1 hour), radiation (RT, 2 Gy), or combined hyperthermia and radiation treatment (HT+RT, incubated in a 42.5°C incubator for 1 hour followed by 2 Gy irradiation within 30 minutes of completion of HT). We chose to use a clinically relevant dose of 2 Gy, the standard fraction size used for radiotherapy for patients with GBM (2) to investigate the effects of hyperthermia on GSCs. Cells were treated with a Cesium-137 irradiator. The pCDH-T2A-Puro-MSCV-GFP and pCDH-T2A-Puro-MSCV-MYR-AKT1 plasmids were introduced into GSCs as previously described (24). GSCs were treated with 0 or 5 μmol/L of the small-molecule inhibitor...
of PI3K, LY294002 (Cell Signaling Technology) for 2 hours before HT and/or RT.

Cell proliferation and colony formation
For cell proliferation, cells were plated in 6-well plates at $1 \times 10^4$ cells per well and incubated overnight in a humidified 37°C incubator. Treatment occurred 24 hours after plating and live cells were assessed via a hemocytometer and trypan blue exclusion. Cells were harvested and counted for each treatment group on days 0, 1, 3, 5, and 7. For GSC colony formation, cells were plated in 12-well plates at a density of $5 \times 10^3$ cells per well and incubated in a 37°C incubator. At day 7 after treatment, cells were induced to attach using 5% FBS (v/v) for 24 hours (5, 23). The media were removed and the attached colonies were then washed with PBS, fixed with 100% ice-cold methanol for 20 minutes, and stained with crystal violet.

Radiation dose–response analysis
Cells were plated in 10-cm plates at a density of $1.0 \times 10^4$ cells per plate and incubated overnight in a 37°C incubator. Treatment occurred 24 hours after plating with increasing doses of radiation (0 Gy, 2 Gy, 4 Gy, or 6 Gy) and hyperthermia plus radiation treatment (42.5°C for 1 hour followed by irradiation). At 7 days after treatment, a fraction of cells, 7.5% of cells (v/v), were transferred to a 96-well plate and incubated for 1 hour in a 37°C incubator allowing tumor spheroids to settle in the wells. All tumorspheres in each well ($n = 3$) were assessed via bright-field microscopy.

Cell-cycle analysis
Cells were collected and washed once with ice-cold PBS and dissociated with StemPro Accutase Cell Dissociation Reagent (Gibco). Cells were washed again with 1× PBS and stored at −20°C in 70% ethanol until used for analysis. Samples were washed and then suspended in 1× PBS. DNA extraction buffer was added to facilitate extraction of low molecular weight DNA. Cell pellets were incubated at room temperature in DNA staining solution (0.2% propidium iodide and 2% RNAase A) for 30 minutes. Cells were subsequently analyzed by flow cytometry.

Western blot analysis
Cells were washed with ice-cold PBS and lysed in M2 buffer (20 mmol/L Tris pH 7.6, 0.5% NP40, 250 mmol/L NaCl, 2.5 mmol/L EDTA, 3 mmol/L EGTA, and protease and phosphatase inhibitors). Protein concentration in the supernatants was measured using the Bio-Rad Protein Assay Dye Reagent (Gibco). Cells were washed again with 1× PBS and stored at −20°C in 70% ethanol until used for analysis. Samples were washed and then suspended in 1× PBS. DNA extraction buffer was added to facilitate extraction of low molecular weight DNA. Cell pellets were incubated at room temperature in DNA staining solution (0.2% propidium iodide and 2% RNAase A) for 30 minutes. Cells were subsequently analyzed by flow cytometry.

Immunofluorescence and immunohistochemistry
GSCs derived from patient specimens 3691 and 387 were plated on Matrigel-coated plate (BD Biosciences) and then treated as indicated. Cells were fixed with 4% PFA for 10 minutes at room temperature. Cells or sections were blocked with PBS containing 10% normal goat serum (Sigma) and 0.1% Triton X-100 (Sigma). Cells or sections were incubated overnight with the appropriate primary antibody at 4°C, and then washed with PBS three times before incubation with the appropriate secondary antibody for 1 hour at room temperature. Nuclei were counterstained with DAPI. TUNEL assay was performed using the ApopTag Red In Situ Apoptosis Detection Kit (Millipore) in accordance with the manufacturer’s protocol. For immunohistochemistry, tissue sections were deparaffinized and processed for immunoperoxidase staining with the primary antibody incubated at 4°C overnight, followed by the appropriate biotinylated secondary antibody and detected using the VECTASTAIN Elite kit (Vector Laboratories). Sections were counter stained with either Fast Red or hematoxylin. The following primary antibodies were used: 53BP1 (Bethyl Laboratories), pS6 (Ser240/Ser244; Cell Signaling Technology), and Ki67 (Vector Laboratories). Cells with any detectable Ki67 staining were scored as being positive.

Human phospho-kinase array
The Human Phospho-Kinase Array Kit was purchased from R&D Systems (ARY003B) and the protocol was followed as recommended in the vendor’s handbook.

Animal procedures and treatments
All animal experiments were performed in accordance with protocols approved by the Cleveland Clinic Institutional Animal Care and Use Committee and in agreement with the NIH animal welfare guidelines. The 20,000 GSCs derived from human GBM and passaged as xenografts were intracranially injected into the right cerebral hemisphere of four to six-week-old athymic nude mice as previously described (24). Hyperthermia was administered using the FDA-approved BSD 500 commercial hyperthermia unit. Heating was provided by 915 MHz microwaves emanating from two antennae on the left and right aspects of the tumor-bearing area. A thermistor was placed between the antennae that controlled the microwave power to monitor real-time temperature. A circulating water bolus set at 42°C was placed on top of the mice, antennae, and thermistors. Tumors received an average T90 of 42.4°C for 1 hour, in which T90 represented 90% of all measured intratumoral temperatures exceeding 42.4°C. Radiotherapy of 2 Gy in 1 fraction was delivered to the mouse head using a Pantak X-ray cabinet irradiator (300 kVp, 10 mA). Lead shielding was used to ensure that only the head was irradiated. For mice receiving hyperthermia and radiation, radiation was delivered within 30 minutes of completion of hyperthermia. The IVIS 100 bioluminescence imaging system was used to assess tumor growth. Five to 7 mice were included in each treatment group ($N = 26$). Animal experiments were confirmed using independent groups of mice ($N = 38$).

Statistical analyses
All graphs, statistical significances and Kaplan–Meier survival curves were generated using GraphPad Prism software. One-way ANOVA or t test assuming unequal variances was used for all continuous measures and χ² or Fisher exact testing for proportion comparisons. Mouse survival comparisons were determined by log-rank analysis.

Results
Hyperthermia improves the radiosensitivity of GSCs
GSCs contribute to tumor progression by resisting radiotherapy (5, 7, 25). We first investigated the ability of hyperthermia to improve the radiosensitivity of GSCs by assessing tumorsphere
formation and colony formation, two indicators of the self-renewal capacity of GSCs in vitro (5, 26). Two different GSC specimens were treated with escalating doses of radiation (0-6 Gy) or combined hyperthermia and radiation (42.5°C for 1 hour followed by radiation). With increasing doses of radiation, thermoradiotherapy decreased the surviving fraction of GSCs significantly more than radiation alone (Fig. 1A and B). The dose-modifying factor (ratio of radiation dose with hyperthermia to radiation alone to achieve 90% cell kill) was 0.66 and 0.62 for GSC 387 and GSC 3691, respectively, showing a considerable enhancement in cell killing with hyperthermia. There were no appreciable differences in GSC death treated with hyperthermia alone (42.5°C for 1 hour) or the control, sham-treated cells (data not shown).

**Figure 1.** Hyperthermia sensitizes GSCs to radiation and impairs self-renewal. A and B, GSC specimens 3691 (A) and 387 (B) were treated with increasing doses of radiation (RT) or combined hyperthermia and radiation (HT+RT; 42.5°C for 1 hour followed by the indicated radiation dose), and spheres were counted on day 7. C and D, representative images (left) of colony formation in GSC specimens 3691 (C) and 387 (D) treated with hyperthermia (HT; 42.5°C for 1 hour), radiation (RT, 2 Gy) or combined hyperthermia and radiation (HT+RT; 42.5°C for 1 hour followed by 2 Gy) and quantification of colonies (right). E and F, representative images of tumorspheres (left) of GSCs 3691 (E) and 387 (F) treated as indicated with quantification of tumorspheres (right). Graphed data, means ± SD (n = 3); *P < 0.05; **P < 0.01; ***P < 0.001.
The fractions of Ki67 alone (Fig. 2A and B).

assays. Treatment with combined hyperthermia and radiotherapy
consistent with colony formation and tumorsphere formation
after treatment, radiation reduced cell counts by about 50%,
eration compared with control cells (Fig. 2A and B). Seven days
hyperthermia had little impact on cell number or prolif-

Thermoradiotherapy reduces GSC viability and proliferation. A and B, GSCs were treated as described in Fig. 1 and absolute cell counts are shown. Cell numbers were counted every 2 days. Data, means ± SD (n = 3). C and D, quantification of relative fraction of Ki67+ cells on days 0 and 3 after treatment is shown. The fractions of Ki67+ cells were normalized to the control group. E and F, representative staining of Ki67 in GSCs is shown. Nuclei were counterstained with DAPI (blue). HT, hyperthermia; RT, radiation; HT+RT, hyperthermia and radiation. *P < 0.05.

We next determined the effect of hyperthermia on GSC colony formation. Hyperthermia alone had little effect on GSC colony formation (Fig. 1C and D) or tumorsphere formation (Fig. 1E and F). Radiation alone attenuated GSC colony formation and tumorsphere growth by about 50%, but a significant number of GSCs remained and were able to form tumorspheres. The most effective treatment was thermoradiotherapy, which reduced GSC self-renewal by an additional 50% compared with radiation alone (Fig. 1C–F). Together, these studies demonstrate that thermoradiotherapy is more effective than radiation alone in reducing the clonogenic capacity of GSCs.

The addition of hyperthermia to radiation reduces GSC proliferation

We next assessed changes in cell survival and proliferation. Hyperthermia alone had little impact on cell number or proliferation compared with control cells (Fig. 2A and B). Seven days after treatment, radiation reduced cell counts by about 50%, consistent with colony formation and tumorsphere formation assays. Treatment with combined hyperthermia and radiotherapy significantly reduced cell number compared with radiotherapy alone (Fig. 2A and B).

To determine whether the reduction in cell number reflected changes in the rate of proliferation, we assessed immunofluorescence staining of Ki67, a marker of proliferation, after the indicated treatments. Ki67 staining revealed that hyperthermia or radiation alone had little effect on GSC proliferation compared with control-treated cells. Compared with radiation alone, thermoradiotherapy efficiently reduced by about 5-fold GSC proliferation (Fig. 2C–F).

Thermoradiotherapy reduces DNA repair and promotes cell death

GSCs preferentially activate their DNA repair machinery after radiation and resist apoptosis compared with NSTCs (6). Inhibition of DNA repair improves the radiosensitivity of GSCs (27). Hyperthermia is thought to act as a radiosensitizer primarily by interference of DNA damage repair (28). We examined whether combined thermoradiotherapy could reduce the efficiency of double-strand break (DSB) repair in GSCs and induce cell death. Cell-cycle analysis by flow cytometry showed an increase in the fraction of dying cells, as indicated by the sub-G1 population, in GSCs treated with thermoradiotherapy compared with those that were treated only with radiation. Furthermore, GSCs treated with thermoradiotherapy exhibited higher levels of apoptosis as assessed by TUNEL staining (Fig. 3C).

We next assessed the efficiency of DNA strand break repair by 53BP1 staining. Cells that underwent thermoradiotherapy showed similar levels of 53BP1 staining 30 minutes and 3 hours after treatment compared with those that were treated only with radiation (Fig. 3D and E). At 10 hours after treatment, although 53BP1 levels decreased for GSCs treated only with radiation, it remained elevated in GSCs treated with thermoradiotherapy, suggesting that hyperthermia impairs DNA strand break repair (Fig. 3D and E).
Hyperthermia itself had minimal effect on 53BP1 foci staining or apoptosis compared with control sham-treated cells. Together, these data suggest that inhibition of DNA repair and enhanced cell death contribute to hyperthermic radiosensitization.

**Hyperthermia reduces AKT activation after irradiation**

To study the molecular events underlying thermoradiotherapy, we performed a human phospho-kinase array (Fig. 4A). There was no significant difference in phospho-kinase profile in GSCs treated with hyperthermia alone compared with the control GSCs. Radiation treatment increased p3 and CHK2 phosphorylation as expected (Fig. 4A). Radiation alone also induced the phosphorylation of multiple kinases, including, ERK, AKT, RSK1/2, and p70 S6K. However, thermoradiotherapy reduced phosphorylation of AKT and its downstream kinases RSK1/2 and p70 S6K (Fig. 4A). pERK levels did not appear to differ between radiation and thermoradiotherapy-treated cells. We did not detect significant changes in the phosphorylation of multiple other proteins on the array, including p38 MAPK, AMPK, Src, JNK, or STAT (refer to Supplementary Fig. S1 for map of array components).

We next confirmed these results by immunoblot analysis in paired GSCs and NSTCs derived from the same patient specimen. Hyperthermia alone had no obvious effect on the phosphorylation of AKT, RSK1/2, or p70 S6K (Fig. 4B and Supplementary Fig. S2). However, radiotherapy significantly increased levels of phosphorylated AKT, RSK1/2, p70 S6K, and ERK in GSCs. Radiation-induced activation of AKT appeared significantly greater in GSCs but not in NSTCs. These results are consistent with increased AKT signaling mediating radiation resistance (29–31). Combined thermoradiotherapy reduced radiation-induced phosphorylation of AKT, RSK1/2, and p70 S6K in GSCs, but had no demonstrable effect on ERK phosphorylation (Fig. 4B).

We next tested the ability of constitutively active AKT to rescue hyperthermic radiosensitization of GSCs using cells that stably expressed myristoylated AKT (MYR-AKT) or control GFP (Fig. 4C). In GFP-expressing cells, thermoradiotherapy reduced colony formation compared with radiation alone. However, in MYR-AKT-expressing cells, radiation and thermoradiotherapy groups exhibited similar numbers of colonies (Fig. 4D). Treatment of GSCs with the PI3K inhibitor 1Y294002 further reduced GSC colony formation (Fig. 4E). Together, these data suggest that a major mechanism by which thermoradiotherapy mediates GSC radiosensitization is through downregulation of AKT signaling.

**Thermoradiotherapy extends survival in a mouse model of GBM**

To further investigate the potential therapeutic benefit of thermoradiotherapy in GBM, we examined the effects of thermoradiotherapy on tumor growth of GSC-derived GBM xenografts.
GSCs stably expressing luciferase were transplanted into the forebrains of immunocompromised mice. When tumors reached similar levels of luminescence, mice were randomized to receive sham treatment, hyperthermia, radio-, or thermoradiotherapy. Mice bearing tumors treated with thermoradiotherapy consistently displayed reduced tumor size and significantly increased animal survival relative to tumors treated with hyperthermia or radiation alone (HT+RT vs. RT alone, *P* = 0.0231; Fig. 5A and F). This survival advantage was confirmed with an independent cohort of 38 mice (data not shown). Consistent with our *in vitro* data, thermoradiotherapy reduced levels of phosphorylated S6, an effector of AKT (Fig. 5B and D), and proliferation (Fig. 5C and E) in tumors. These data suggest that the combination of hyperthermia and radiation reduces tumor growth and improves survival potentially by abrogation of radiation-induced AKT signaling.

**Discussion**

The PI3K–AKT pathway is aberrantly regulated in more than 40% of GBM, and is associated with poor patient prognosis.
Figure 5.
Thermoradiotherapy suppressed GBM growth and increased survival. A, representative images of cross-sections (hematoxylin and eosin stained) of mouse brains harvested on day 5 after treatment. B and C, immunohistochemical staining for phospho-S6 (B) and Ki67 (C) in GBM xenografts treated as indicated are shown. D and E, quantification of fraction of phospho-S6\(^+\) and Ki67\(^+\) tumor cells to total tumor cells in the indicated group is shown. F, Kaplan–Meier analysis of neurologic sign-free survival of mice (n = 26) treated as indicated revealing improved survival in mice treated with thermoradiotherapy versus radiation alone, P = 0.0231. Mouse survival data were confirmed in an independent cohort of 38 mice. Graphed data, means ± SD (n = 3). HT, hyperthermia; RT, radiation; HT+RT, hyperthermia and radiation. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
This pathway is frequently overactivated in brain tumor stem cells to mediate radiation resistance (29, 31, 35). The proposed stem cell marker CD133/Prominin directly interacts with the p85 subunit of PI3K to facilitate AKT signaling in GSCs (36), and GSCs are particularly sensitive to AKT pathway inhibition (31, 37, 38). Therefore, targeting this pathway may improve therapy for patients with GBM.

Our study reveals that hyperthermia can abrogate radiation-induced activation of AKT in GSCs and this translated into reduced tumor growth and improved animal survival in a preclinical model of GBM. We found that thermoradiotherapy reduced levels of phosphorylated AKT and its downstream kinases, p70 S6K and RSK1/2, but had minimal effect on other pathways, including ERK, p38 MAPK, Src, JNK, or STAT. In tumors, combined hyperthermia and radiation diminished phospho-S6 levels and decreased proliferation to baseline levels. Introduction of constitutively activated AKT rescued GSCs from cell death induced by thermoradiotherapy. In addition, inhibition of AKT signaling by a PI3K inhibitor further sensitized GSCs to radiotherapy and thermoradiotherapy. Together, these data suggest that hyperthermia may improve the radiosensitivity of GSCs primarily by inhibition of AKT proliferative and prosurvival signaling. These results are consistent with results of gold nanoshell-mediated hyperthermia in improving the radiosensitivity of breast cancer stem cells (39), and suggest that maximizing PI3K–AKT inhibition with hyperthermia and pharmacologic inhibition may further improve radiosensitization of cancer stem cells.

In our preclinical model, tumors exhibited suppression of AKT signaling that persisted for over 5 to 7 days after a single hyperthermia treatment and low-dose radiation. If hyperthermia were followed immediately by fractionated radiation, then we anticipate that GSCs would remain radiosensitive for at least the first few fractions of radiotherapy. Currently in clinical practice, a delay of over 3 to 4 weeks occurs between surgery, including interstitial hyperthermia, and the start of radiochemotherapy. This delay in treatment allows for healing after surgery and devising a custom radiation plan. Because interstitial hyperthermia is minimally invasive and delivered through small burr holes, less time is needed for healing. Furthermore, radiotherapy may be preplanned as there is less tissue distortion with interstitial hyperthermia compared with traditional craniotomy and tumor resection. Our studies suggest that decreasing the time interval between these treatment modalities would maximize hyperthermic radiosensitization of GSCs. Additional studies are needed to determine the optimal timing between interstitial hyperthermia and radiotherapy in patients.

Impairment of DNA damage repair is one of the major mechanisms attributed to hyperthermic radiosensitization (11, 40, 41). The PI3K–AKT pathway is increasingly recognized as a modulator of DNA DSB repair (42–45). Radiation facilitates activation of AKT via ATM or DNA-dependent protein kinase (DNA-PK; refs. 46, 47). PTEN, a negative regulator of the PI3K–AKT signaling pathway, regulates DNA damage response by regulating CHK1 localization (48), and nuclear PTEN regulates sensitivity to radiation damage in an ATM-dependent manner (49). We found that hyperthermia reduced the efficiency of DNA damage repair in GSCs after clinically relevant doses of radiation.

Historically, monotherapy against one signaling pathway in cancer is often ineffective in the clinic due to redundant pathways. Our data suggest that maximizing AKT inhibition with pharmacologic inhibitors and hyperthermia may enhance cancer control and potentially overcome resistance mechanisms. In summary, our studies reveal that hyperthermia improves the radiosensitivity of GSCs by suppressing radiation-induced AKT activation and proliferation. Our preclinical mouse models further support that combined thermoradiotherapy impairs tumor growth and extends animal survival. These studies support clinical translation of combined hyperthermia and radiation for patients with GBM.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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