RESEARCH ARTICLE

Hyperthermia Promotes Apoptosis and Suppresses Invasion in C6 Rat Glioma Cells

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Abstract

Gliomas are a group of heterogeneous primary central nervous system tumors. Hyperthermia has proven to be a potential therapeutic tool for cancers in the clinic. However, the molecular mechanisms of hyperthermia remain unclear. The objective of this study was to investigate the effects of hyperthermia on the invasiveness in C6 glioma cells and related molecular pathways. Here our data show hyperthermia stimulated the release of tumor necrosis factor-alpha (TNF-α) and decreased C6 glioma cell migration and invasive capability at 30, 60, 120 and 180 min; with increased spontaneous apoptosis in C6 glioma cells at 120 min. We also found mitogen-activated protein kinase (P38 MAPK) protein expression to be increased and nuclear factor-kappa B (NF-κB) protein expression decreased. Based on the results, we conclude that hyperthermia alone reduced invasion of C6 glioma cells through stimulating TNF-α signaling to activate apoptosis, enhancing P38 MAPK expression and inhibiting the NF-κB pathway, a first report in C6 rat glioma cells.

Keywords: Hyperthermia - glioma invasiveness - TNF-α - P38 MAPK - NF-κB

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Introduction

Gliomas are a group of heterogeneous primary central nervous system (CNS) tumors arising from the glial cells. Malignant gliomas account for a majority of malignant primary CNS tumors and are associated with high morbidity and mortality (Stupp et al., 2007). Despite recent advances in treatment strategies, the prognosis of patients remains poor. Therapeutic treatment protocols using surgery, radiation and/or chemotherapy did improve survival rates, but the median overall survival length after first-line therapy does not exceed 15 months (Stupp et al., 2005). Treatment of glioma has therefore been among the most challenging fields in oncology. More recently, various minimally invasive treatments, including thermotherapy, have become available for cancer therapy. There are three types of thermotherapy, including thermocoagulation, hyperthermia and cryotherapy. Among them, hyperthermia, which selectively targets cancer cells, is the most conservative one. In randomized control studies, hyperthermia has been reported as an effective therapy for many cancers, including pelvic tumors (van der Zee et al., 2000), breast cancer (Vernon et al., 1996), malignant melanoma (Overgaard et al., 1995), and glioblastoma (Sneed et al., 1993, 1998; Fiorentini et al., 2006), breast cancer (Vernon et al., 1996), malignant melanoma (Overgaard et al., 1995), and glioblastoma (Sneed et al., 1993, 1998; Fiorentini et al., 2006; Silva et al., 2011). In combination with radiotherapy. There has been much interest in studying the effects of heat on the brain (Sminia et al., 1994; Haveman et al., 2005). A number of well-localized, mainly interstitial, invasive hyperthermia combination therapies have been applied for gliomas (Tanaka et al., 1987; Sneed et al., 1991, 1992, 1998; Stea et al., 1990, 1994; Moran et al., 1995; Fiorentini et al., 2006; Silva et al., 2011). The temperatures of interest for treating cancers are in the range of 40-46 °C. This maximizes the tumor damage while preserving the surrounding normal tissue (Yoo and Lee., 2007). Hyperthermia has a synergistic effect when combined with chemotherapeutic agents in treating cancer, but it also works just as well when combined with tumor necrosis factor related apoptosis-inducing ligand (TRAIL), tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), or TNF-α + IFN-γ (Lee et al., 1993). TRAIL is well known to play a critical role as an inducer of apoptosis in a variety of cancer cells in vitro and has been shown to limit tumor growth efficiently in vivo, with minimal damage to normal tissues (Lee et al., 1993; Ashkenazi et al., 1999; Walczak et al., 1999; Kato et al., 2007; Yoo and Lee, 2008). TRAIL is expressed as a type II integral membrane protein belonging to the tumor necrosis factor (TNF) superfamily (Lee et al., 1993;
Ashkenazi et al., 1999; Walczak et al., 1999; Kato et al., 2007; Yoo and Lee, 2008).

The pro-inflammatory cytokine tumor necrosis factor-a (TNF-a) regulates various signaling pathways in proliferation, apoptosis, and inflammation through the activation of phospholipases, mitogenactivated protein kinases (MAPK) and nuclear factor kappa B (NF-kB) (Baud and Karin, 2001; Dorsett and Tuschl, 2004). It has been reported that hyperthermia has a synergistic effect with TRAIL in causing cytotoxicity in CX-1 human colorectal cancer cells (Yoo and Lee, 2007, 2008). It was observed that TRAIL-induced apoptotic death can be enhanced with mild hyperthermia (41 – 42 °C) through caspase activation and cytochrome c release in mitochondria of CX-1 cancer cells (Yoo & Lee., 2007). In another study, hyperthermia increases the efficacy of chemotherapeutic agents such as oxaliplatin or melphalan when used in combination with TRAIL, killing the CX-1 colorectal cancer cells more effectively (Yoo and Lee, 2008). Furthermore, it promoted an antitumor immune response by activation of HSP70 (Tulapurkar et al., 2009).

Based on these previous studies of hyperthermia and TNF-α, we raised the hypothesis that hyperthermia alone could reduce the invasion of C6 glioma cells through stimulation of TNF-α signaling pathway. To test our hypothesis, we first examined the effects of hyperthermia on cell released TNF-α concentration after treatment. We found that hyperthermia stimulated the cell released TNF-α level, activate apoptosis, attenuated the cell migration and invasion in C6 gliomas cells. To investigate the mechanism, the effects of TNF-α on the expression levels of P38 MAPK, a key regulator whose pathways regulate diverse processes ranging from proliferation and differentiation to apoptosis (Baud and Karin, 2001; Dorsett and Tuschl, 2004), were examined. We found that hypoxia enhanced the expression of P38 MAPK protein in C6 glioma cells. We also demonstrated that the hyperthermia-based inhibited in NF-xB protein expression, which playing important roles in cancer development and progression, is mediated by the accumulation of TNF-α in C6 rat glioma cells.

Materials and Methods

Antibodies, cell culture and treatment

Primary antibodies used were as follows: anti-GFAP, anti-P38MAPK anti-NF-KB, and anti-GAPDH were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). TNF-α radioimmunoassay kit was obtained from Boster (Boster Corp, Wuhan, China). TNF-α level, a are measured using the radioimmunoassay kit (Boster Corp, Wuhan, China).

Cell apoptosis assay

The C6 glioma cells were synchronized by 24 h of serum starvation. Cells were exposed to hyperthermia (42 °C) in different time groups (0, 10, 30, 60, 120, 180 and 240 min), and the cells were maintained for 24 h. To determine the extent of spontaneous apoptosis, 5×10^5 cells were stained with fluorescein isothiocyanate (FITC)-conjugated Annexin V and propidium iodide using the Annexin V-FITC Apoptosis Detection kit (Jingmei Co., China) following the manufacturer’s instructions. Cell spontaneous apoptosis was determined using FACS caliber II sorter and Cell Quest FACS system. The experiments were repeated three times.

Transwell chamber assay C6 glioma migrative and invasive capability

The in vitro migrative capability of C6 glioma cells were measured in a transwell chamber assay. 100 μl diluted Matrigel solution was added to upper chamber of the transwell insert (6.5 mm, 12.0 μm pore size). The inserts were incubated for 4 hrs at 37 °C for gelling; they were then pretreated with serum-free DMEM medium for 1 h at 37°C. C6 glioma cells were added at a density of 2×10^5 cells in 100 μl medium with 1% FCS. The lower chamber was filled with 600μl DMEM containing 10% FCS. The transwell inserts were incubated for different time groups (0, 10, 30, 60, 120, 180 and 240 min) on hyperthermia (42 °C), then the inserts were incubated for 24 hrs at 37 °C to allow cells to migrate. Cells on the upper side of the insert filter were then removed with a cotton swab. Cells that had invaded through the Matrigel coated filter were fixed with 10% methanol for 30 sec and stained with 0.1% crystal violet (prepared in ethanol) for 20 min. The number of migrated cells was counted under a microscope in five predetermined fields. The experiment was repeated three times.

Immunocytochemical staining

Glial fibrillary acidic protein (GFAP) expression was determined with immunocytochemistry. C6 glioma cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂ in Dulbecco’s modified Eagle’s medium, supplemented with 10% (v/v) fetal calf serum. C6 glioma cells were grown on cover slips for 24h before fixation with 95% ethanol for 30 min. After fixation, the cells were permeabilized with 0.1% Triton X-100 for 20 min. After blocking with 3% H₂O₂ in methanol for 20 min and 0.5% bovine serum albumin (BSA) in PBS for 30 min, the cells were incubated for 1h at 37 °C with monoclonal mouse anti-GFAP. After washing, a HRP-conjugated secondary antibody (Pierce, USA) was added and incubated for 1h at 37 °C. DAB was used to detect the secondary antibodies. GFAP staining was used for identification.

Radioimmunoassay

C6 glioma cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% (v/v) fetal calf serum. Then cells were exposed to hyperthermia (42 °C) in different time groups (0, 10, 30, 60, 120, 180 and 240 min), 1 ml culture medium of each group was collected and saved in tube under -70 °C, and the culture medium concentrations of TNF-α were measured using the radioimmunoassay kit (Boster Corp, Wuhan, China).
times.

The in-vitro migrative capability of C6 glioma cells were measured in a transwell chamber assay. C6 glioma cells were added at a density of 2x10^5 cells in 100 μl medium with 1% FCS to upper chamber of the transwell insert (6.5 mm, 8 μm pore size). The lower chamber was filled with 600 μl DMEM containing 10% FCS. The transwell inserts were incubated for different time groups (0, 10, 30, 60, 120, 180 and 240 min) on hyperthermia (42 °C), then the inserts were incubated for 8 hrs at 37 °C to allow cells to migrate. Cells on the upper side of the insert filter were then removed with a cotton swab. Cells that had migrated through the transwell insert were fixed with 10% methanol for 30 sec and stained with 0.1% crystal violet (prepared in ethanol) for 20 min. The number of migrated cells was counted under a microscope in five predetermined fields. The experiment was repeated three times.

Protein lysates and western blot analysis

Cultured cells were treated by hyperthermia (42 °C) in different time groups (10, 60, 120, 180 and 240 min), scraped into radio-immunoprecipitation (RIPA) buffer, and lysed on ice for 30 min. Protein concentrations were determined using the Pierce Micro BCA protein assay system. From each sample, 45 μg of protein was loaded onto 8% SDS-PAGE gel for electrophoresis and transferred onto nitrocellulose membrane. The membranes were blocked with 5% non-fat milk (diluted in TTBS) at room temperature for 1 h. All target proteins were immunoblotted with appropriate primary and horseradish peroxidase-conjugated secondary antibodies. Primary antibodies were incubated for 1 h at 37 °C. After the membranes were washed with TTBS three times, the bound antibodies were then detected by using horseradish peroxides (HRP)-conjugated goat anti-rabbit or mouse anti-goat antibody (Pierce, USA) and Protein signals were visualized by enhanced chemiluminescence using ECL Western blotting detection reagents for 1 min and exposed to Kodak Biomax XAR film. The experiment was repeated three times.

Statistical analysis

The results are presented as the mean ± standard deviation (SD). Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison tests with SPSS v12.0 (SPSS Inc., Chicago, IL) to determine the level of significance between the different groups. p values < 0.05 were considered as statistically significant and indicated with * in the tables and figures. p values<0.01 were considered as statistically highly significant and indicated with ** in the tables and figures.

Results

Identification of C6 glioma cells

The GFAP protein was localized in the cytoplasm area and the cytoplasm area was stained brown, indicating the glioma cells as shown in Figure 1.

Hyperthermia stimulates release of TNF-α in C6 cells

The concentrations of TNF-α in the C6 glioma cell culture medium after different hyperthermia times were measured by radioimmunoassay. The maximum level of TNF-α of C6 glioma cells was observed at 120 min after hyperthermia treatment, and there were significant differences among that at 30min, 60min 120 min and 180min (p<0.05) (Table 1), which suggest that hyperthermia can increase the release of TNF-α in C6 glioma cells.

Hyperthermia induces C6 glioma cell apoptosis

Annexin V/PI staining was used to determine the level of spontaneous apoptosis by hyperthermia in C6 glioma cells at different time points. The data showed that hyperthermia increased the level of C6 glioma spontaneous apoptosis when compared to control; C6 glioma cells spontaneous apoptosis reached at the peak level after 120 minutes of hyperthermia. There were statistically significant differences between experimental group and control group (p<0.05) (Figure 2).

Hyperthermia suppresses C6 glioma cell migration

Transwell migration chamber assay was employed to determine the effect of hyperthermia on the migrative capability of C6 glioma cells. The average number of cells passing through the insert filter decreased from 123 to 23 when cells were treated with hyperthermia for 120 minutes under the condition offered. There were significant differences in the number of cells passing through the insert filter compared with control at 30min, 60 min 120 min and 180 min, respectively (p<0.05). However, no statistically significant difference was found between

Figure 1. Glial Fibrillary Acidic Protein (GFAP) Expression was Determined with Immunocytochemistry in C6 Glioma Cells in this Microscope Image with 40x Magnification. The brown nucleus indicates the glioma cells

Figure 2. Histogram Shows the Level of Apoptosis of C6 Glioma Cells Induced by Hyperthermia at Different Time Points. * p < 0.05 Compared to Control
Hyperthermia inhibited C6 glioma cell invasion

With Transwell invasion chamber assay, we found that the average number of cells passing through the insert filter decreased from 237 to 37 when cells were treated with hyperthermia for 120 minutes under the condition offered. There were statistically significant differences in the number of cells passing through the insert filter compared with control at 30 min, 60 min 120 min and 180 min, respectively (p<0.05). Also that there was no statistically significant differences found between the experimental and the control group at 240 min (Figure 4). These results indicated that hyperthermia can decrease the C6 glioma cells invasive capability.

Effect of Hyperthermia on the expression of P38 MAPK and NF-κB

The effects of hyperthermia on P38 MAPK and NF-κB expression were determined by Western Blot. The P38 MAPK protein expression increased in the C6 glioma cells after hyperthermia treatment for 60, 120 and 180 min, and the relative expression level of P38 MAPK was 0.22, 0.61 (p<0.05) and 0.85, respectively (Figure 5). However there was no difference of P38 MAPK protein between experimental and control groups were detected. After 120 min of hyperthermia treatment, the relative expression level of NF-κB was 0.28 (p<0.05). The relative expression level of P38 MAPK and NF-κB were both significantly different at 120 min, and the amount of NF-κB protein at this time point was much less when compared with other experimental groups (Figure 5).

Discussion

Hyperthermia acts as a promoter in cytotoxicity of human cancer cells when combined with other modalities. Previous studies have shown that mild hyperthermia (41-42 °C) rather than acute hyperthermia (44-46 °C) effectively promotes the effectiveness of TNF-α-induced cytotoxicity by facilitating activation of caspases through mitochondria-dependent cytochrome c release in human colorectal cancer cells (Yoo and Lee, 2007). It was demonstrated that hyperthermia works synergistically with chemotherapeutic agents like melphalan and oxaliplatin combined with TNF-α in killing cancer cells (Yoo and Lee, 2008). Few researches targeted on the relationship between hyperthermia and glioma metastasis. In this study we found that hyperthermia stimulated the release of TNF-α in C6 glioma cells (Table 1) followed by the
Several lines of evidence have suggested that TNF-α releases through the phosphorylation of NF-κB, p38 mitogen-activated protein (MAP) kinase and stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) in rat C6 glioma cells (Yamaguchi et al., 2009). In this study, we demonstrated that hyperthermia worked synergistically alone to elevate TNF-α-induced apoptosis by activation of c-Jun N-terminal kinase (p38MAPK) and p38MAPK promoted TNF-α-induced rat glioma apoptosis. In this study, we were able to demonstrate that hyperthermia works synergistically alone by enhancing TNF-α-induced apoptosis by activation of p38 MAPK as well as inhibition of NF-κB. This provided similar results in cell death as well as showing a delay in growth the in vivo mouse model of human colorectal tumors.

Given the mechanistic data regarding how hyperthermia functions, our results now profiled that hyperthermia decreased C6 glioma cells invasive and migrative capability (Figure 3, 4) resulted from TNF-α-induced apoptosis. This agreed with the report of Satoshi Kokura’s study (Kokura et al., 2003) that hyperthermia can inhibit the TNF-α-induced NF-κB activation and that hyperthermia renders human gastric cancer cells susceptible to the ROS. It is well known that NF-κB acts as a suppressor of intracellular ROS formation in TNF-α treated cells (Shen and Pervaiz, 2006). Crosstalk occurs between JNK and NF-κB, and a role for ROS in TNF-α signaling has emerged. The intermediacy of ROS in the crosstalk between JNK and NF-κB is that hyperthermia can inhibit the TNF-α-related activation of NF-κB and that hyperthermia renders human gastric cancer cells susceptible to the TNF-α-induced ROS path. Zhang et al. (2003) reported that TNF-α-induced activation of Mitogen-activated protein kinase (p38MAPK) and p38MAPK promoted TNF-α-induced rat glioma apoptosis. In this study, we were able to demonstrate that hyperthermia works synergistically alone by enhancing TNF-α-induced apoptosis by activation of p38 MAPK as well as inhibition of NF-κB. This provided similar results in cell death as well as showing a delay in growth the in vivo mouse model of human colorectal tumors.

Several lines of evidence have suggested that TNF-α activates spontaneous apoptosis, enhanced expression of TNF-α-induced MAPK, and inhibited the expression of NF-κB (Figure 5). Our findings imply that the molecular mechanism of hyperthermia attenuated glioma metastasis in C6 glioma cells were including: hyperthermia stimulated TNF-α accumulation following by apoptosis, TNF-α triggered the expression of MAPK and inhibited the NF-κB pathway.

TNF-α is a potent pro-inflammatory cytokine with a major role in initiating a cascade of activation of other cytokines and growth factors in inflammatory responses (Li and Verma, 2002). TNF-α is synthesized by microglia, astrocytes and some populations of neurons and has several important functions in the CNS, including injury-mediated microglial and astrocyte activation, regulation of blood-brain barrier permeability, febrile responses, glutamatergic transmission, and synaptic plasticity (Mc Coy and Tansey, 2008). TNF-α leads to activation of inhibitory kappa B (IκB) by the IκB kinase (IκK) complex, which in turn leads to IκB polyubiquitination and subsequent degradation by proteasome (Hayden and Ghosh, 2004). Consequently, nuclear factor kappa B (NF-κB) is phosphorylated, liberated from IκB and translocates to the nucleus, where it binds to the promoter regions of NF-κB responsive genes and initiates transcription of genes such as those for the proinflammatory cytokines interleukin (IL)-6, IL-1, and TNF-α (Li and Verma, 2002; Hayden and Ghosh, 2004). It is thought that TNF-α induces apoptosis by binding to the death receptors DR4 and DR5, members of the tumor necrosis factor (TNF) receptor superfamily, which results in conformational changes that expose a binding surface for Fas-associated death domain (FADD), an adaptor protein (Kischkel et al., 2000; Thomas et al., 2004; Park et al., 2005). The adaptor molecule recruits the initiators caspase-8 and -10 to promote formation of the death-inducing signaling complex (DISC). The activation of caspases has been documented by several observations, providing evidence that caspase-8, an initiator caspase, cleaves Bid and triggers mitochondrial damage and subsequently induces the release of cytochrome c from the mitochondria (Li et al., 1997; Gong et al., 2004; Oh et al., 2005).

Table 1. Concentrations of TNF-α in the Nutrient Fluid of C6 Cells After Hyperthermia Intervention (µg/L, n=15/group)

| Group             | 0             | 30            | 60            | 120           | 180           | 240           |
|-------------------|---------------|---------------|---------------|---------------|---------------|---------------|
| Control (µg/L)    | 1.2±0.04      | 0.89±0.03     | 1.35±0.10     | 1.08±0.10     | 1.20±0.07     | 1.19±0.06     |
| Hyperthermia (µg/L) | 1.54±0.03    | 7.65±0.82*    | 9.53±0.94*    | 13.43±0.76**  | 5.84±0.36*    | 1.52±0.05     |
| Release TNF-α (µg/L) | 0.33±0.02    | 6.76±0.79     | 8.20±0.84     | 12.35±0.66    | 4.64±0.29     | 0.33±0.04     |

*p<0.05, **p<0.01 compared to control
transduced though its receptors to simultaneously elicit two opposing effects: the induction of apoptosis and transcription of antiapoptotic genes, such as the gene that encode NF-xB (Beg and Baltimore, 1996; Liu et al., 1996; Van Antwerp et al., 1996; Wang et al., 1996; Ryu et al., 2011; Zhang et al., 2012). At the same time, NF-xB regulates the expression of many genes that are essential for cell growth, differentiation, angiogenesis and metastasis. Hagemann et al. (2005) reported that the TNF-α induced tumor cell invasion though NF-xB and JNK-mediated up-regulation of migration-inhibitory factor (MIF) in macrophages and through enhanced matrix metalloproteases (MMPs) production in tumor cells. Kulbe et al. (2005) reported TNF-α enhance cells migration and metastasis though NF-xB -dependent induction of the chemokine receptor CXCR4, and intercellular adhesion molecule-1. Monesano et al (2005) reported that TNF-α enhanced the invasiveness of tumor cells through induction of MMPs. It is possible that hyperthermia promotes caspase enzyme activity and subsequently enhances JNK activity through promoting Mst1 cleavage (activation) and TNF-α-induced apoptotic death. We believe that many critical questions still remain to be answered to understand the mechanism of the promotion of JNK activation during hyperthermia. However, this model will provide a framework for future studies. Further exploration into the mechanisms of hyperthermia on glioma would surely provide reliable evidence for clinical practice.

In summary, our data in the present study demonstrated that hyperthermia attenuated invasion of C6 glioma cells due to the increased apoptosis, enhanced p38MAPK protein expression and inhibition of the NF-xB expression regulated by stimulation of TNF-α signal transduction pathway. The effects of external hyperthermia along with TRAIL could lend another approach to our future studies. We have come to the conclusion that our data will serve as the backdrop to these future studies.

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