PERK Regulates Glioblastoma Sensitivity to ER Stress Although Promoting Radiation Resistance

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Abstract

The aggressive nature and inherent therapeutic resistance of glioblastoma multiforme (GBM) has rendered the median survival of afflicted patients to 14 months. Therefore, it is imperative to understand the molecular biology of GBM to provide new treatment options to overcome this disease. It has been demonstrated that the protein kinase R–like endoplasmic reticulum kinase (PERK) pathway is an important regulator of the endoplasmic reticulum (ER) stress response. PERK signaling has been observed in other model systems after radiation; however, less is known in the context of GBM, which is frequently treated with radiation-based therapies. To investigate the significance of PERK, we studied activation of the PERK–eIF2α–ATF4 pathway in GBM after ionizing radiation (IR). By inhibiting PERK, it was determined that ionizing radiation (IR)-induced PERK activity led to eIF2α phosphorylation. IR enhanced the pro-death component of PERK signaling in cells treated with Sal003, an inhibitor of phospho-eIF2α phosphatase. Mechanistically, ATF4 mediated the prosurvival activity during the radiation response. The data support the notion that induction of ER stress signaling by radiation contributes to adaptive survival mechanisms during radiotherapy. The data also support a potential role for the PERK/eIF2α/ATF4 axis in modulating cell viability in irradiated GBM.

Implications: The dual function of PERK as a mediator of survival and death may be exploited to enhance the efficacy of radiation therapy.

Visual Overview:
http://mcr.aacrjournals.org/content/16/10/1447/F1.large.jpg.

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Introduction

Glioblastoma multiforme (GBM), the most common primary malignant brain tumor in adults, is characterized by a high propensity for invasion and proliferation although being resistant to therapy (1). Although slight improvements in GBM therapy outcomes were achieved with the advent of temozolomide and radiotherapy, the 5-year survival rate remains under 10% (2). In efforts to improve therapy for patients afflicted with GBM, oncologists have sought to identify novel molecular targets involved in promoting the viability and therapeutic resistance. One approach to therapy for GBM involves targeting elements of the endoplasmic
reticulum stress response (ERSR). The ERSR has been increasing-
ly shown to be highly deregulated in cancer, where pro-
survival aspects of the stress response are preferentially acti-
vated and involved in oncogenesis (3). In GBM, upregulation of a
downstream prosurvival mediator of the ERSR, GRP78, has been
linked to tumor grade, temozolomide resistance, and prognosis (4–6). The ERSR is a broad signaling network
involving three major pathways: activating transcription factor
6 (ATF6), inositol-requiring enzyme 1 (IRE1), and protein
kinase R-like endoplasmic reticulum kinase (PERK; ref. 7).

During ER stress, ATF6 translocates to the Golgi where it
is cleaved by proteases (7). The cleaved ATF6 cytosolic fragment
can then act as a transcription factor and drive the synthesis of ER
chaperones, including GRP78 (8). IRE1 spllices X-box binding
protein 1 (XBP1) mRNA to yield XBP1-S, which then upregulates
transcription of genes involved in protein degradation (7, 8). PERK
phosphorylates initiation factor 2α (eIF2α) and transiently
halts the global protein translation although upregulating ATF4
expression (7). ATF4 then triggers expression of genes involved in
amino acids metabolism and suppression of oxidative stress (8).
However, during prolonged ER stress, ATF4 promotes CHOP
expression, which is involved in promoting ER stress–induced
cell death (7). Characterization of the physiologic roles of ATF6,
IRE1, and PERK in the radiation response may lead to the
identification of novel molecular targets to improve therapeutic
efficacy.

Previously, we have shown that radiation can induce ER
stress and downstream signaling associated with the ERSR
(8). Studies have shown that PERK signaling is also induced
by radiation in endothelial cells (9). In this study, we found that
radiation activates the PERK pathway in GBM. We show
that radiation induction of VEGF-A, a recently identified target
of ATF4 (10), occurs in GBM and can be attenuated by inhi-
bition of PERK. We also found that radiation can sensitize
GBM to chemical inducers of ER stress in a PERK-dependent
manner. Furthermore, we found that knockdown of ATF4 in
combination with radiation led to reduced proliferation and
colony formation. This study supports a potential role for
the PERK/eIF2α/ATF4 axis in modulating cell viability in irra-
diated GBM.

Materials and Methods
Cell cultures and chemicals
The human glioblastoma cell line D54 was a gift from
Dr. Yancey Gillespie (University of Alabama at Birmingham,
Birmingham, AL). Human glioblastoma cell line LN827 was
a gift from Dr. Joshua Rubin (Washington University in St. Louis,
St. Louis, MO). Mouse glioblastoma cell line GL261 was obtained
from ATCC. GL261 cells were cultured in DMEM/F12
media containing 10% FBS and 1% penicillin-streptomycin.
LN827 cells were cultured in DMEM containing 10% FBS and
1% penicillin–streptomycin. 293T cells were cultured in DMEM
low-glucose media containing 10% FBS and 1% penicillin–streptomy-
cin. All cell cultures were grown in a humidified incubator at
37°C with 5% CO2. 2-Deoxy-glucose (2DG) was purchased from
Sigma. GSX2066414 and Sal003 were purchased from EMD
Millipore. Radiation of cells was performed at a dose rate of
2.5 Gy/minute with RS2000 160 kV X-ray Irradiator using a 0.3-
mm copper filter (Rad Source Technologies).

Lentiviral transduction
The pLenti-CHOP-mCherry plasmid was a gift from
Dr. Fumihiko Urano (Washington University in St. Louis,
St. Louis, MO). Lentiviruses were generated by cotransfecting
pLenti-CHOP-mCherry, the envelope plasmid (pCMV-VSV-G),
and the packaging plasmid (pCMV-dR8.2) into 293T cells using
FuGENE 6. The target cells were incubated with virus supernatant
for 18 hours, after which they were allowed to recover for 24 hours
before selection with 2 µg/mL puromycin for 72 hours. Puromycin-resistant cells were then used in downstream assays.

Transfection of siRNA
Silencer-select predesigned siRNAs against ATF4 and nontar-
geting control siRNA were purchased from Life Technologies/
Ambion. Lipofectamine RNAiMax transfection reagent (Life
Technologies/Ambion) was used to deliver siRNAs, according to
manufacturer’s protocol. Gene silencing was confirmed 48 hours
after transfection by qRT-PCR.

Quantitative real-time PCR analysis
One microgram of RNA was used to produce cDNA with High
Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems).
Primer sequences were as follows: PERK primers 5′-ACGATGA-
CAGAGGTGCGAC-3′ (forward), 5′-ATAAGGAGCAGATATCC-
TCCG-3′ (reverse); VEGF-A primers 5′-AGGCCAGAAATGATCG-
GAGT-3′ (forward), 5′-AGGGTCTCGAGTTGGGAG-3′ (reverse);
actin primers 5′-CATGATACGGTGTACCAAGC-3′ (forward), 5′-
CTCTTTAATGCAGCCAGAT-3′ (reverse); GAPDH primers 5′-
GGAGCGAGATCCCTCCAAAAT-3′ (forward), 5′-GTTCCTGTTTGC-
TAACGTCAG-3′ (reverse). The ΔΔCt method for quantitation
of relative gene expression was used to determine the mean
expression of each target gene normalized to the geometric mean
of actin and GAPDH.

Flow cytometry
Cells (D54) were transfected with ATF4 specific and nontar-
geting control siRNA and radiated with 3 Gy. Ninety-six
hours after IR, cells (1 × 105) were collected and stained with
Cleared-PARP PE antibody (BD Biosciences) according to the
manufacturer’s protocol. Stained cells were analyzed by flow
cytometry. Similarly, cells transduced with CHOP-mCherry
were treated with specified doses of radiation prior to collection
after 96 hours for analysis by flow cytometry.

Western immunoblot analysis
Protein extracts (40 µg) were analyzed using antibodies for the
detection of phospho-eIF2α, total-eIF2α (Cell Signaling Technol-
y and, ATF4 (ProteinTech). Antibody against tubulin (Sigma)
or GAPDH (Cell Signaling Technology) was used to normalize
protein loading in each lane. Blots were imaged with ChemiDoc-
MP Imaging System (Bio-Rad), and analyzed with Image Lab
Software (Bio-Rad).

Colony formation assays
Cells were seeded at defined cell densities depending on the
radiation dose and allowed to attach overnight. Cells were then
irradiated with 0 or 3 Gy. After incubating for 7–10 days, plates
were stained with 0.5% crystal violet. Colonies comprised of
50 cells or more were counted as a colony. The colony counts
were normalized to plating efficiency and represented as a
surviving fraction relative to control (sham/nontargeting
siRNA).
Cell proliferation assays
Glioma cells (3,000 per/well) were seeded in 96-well plates 48 hours after siRNA transfection. Cells were irradiated with 3 Gy and allowed to grow for 96 hours. Proliferation was determined by reading the optical density by adding 10 μL of PrestoBlue cell viability reagent (Life Technologies) to each well. The cells with presto blue were incubated at 37°C for 15 minutes; the absorbance was measured at 560nm/590nm with a microplate reader.

Statistical analysis
Where indicated, statistical analyses were performed using the Student t test and one-way or two-way ANOVA. Bonferroni’s multiple comparisons test was applied where necessary. These analyses were performed in Prism 6 (GraphPad Software), and statistical significance was indicated in each graph where appropriate.

Results
Irradiation of glioma cells induces phosphorylation of eIF2α
PERK-mediated phosphorylation of eIF2α occurs during the ER stress response (11). Thus, to begin our study of PERK signaling in the radiation response, we investigated the effect of radiation on the phosphorylation status of eIF2α. We observed that the levels of phospho-eIF2α were elevated in a dose-dependent fashion in D54 and GL261 cell lines 24 hours post 3 Gy and 6 Gy IR (Fig. 1A). These observations support the IR induction of the eIF2α component of the ERSR.

Radiation induction of p-eIF2α and VEGF-A is downstream of PERK
Phosphorylation of eIF2α is downstream of several other kinases, including controlled nonrepressed kinase (CGN2), heme-regulated eIF2α kinase (HRI), and protein kinase R (PKR; ref. 12). To determine whether PERK is involved in radiation-induced eIF2α phosphorylation, we used an inhibitor of PERK, GSK2606414 (PERKi). PERKi specifically inhibits PERK over other eIF2α kinases with an IC50 of 0.4 nmol/L (13). PERKi has been used in animal models to study Alzheimer’s disease (14) and Parkinson disease (15). PERKi was found to be neuroprotective with minimal systemic neurotoxicity (15).

Figure 1.
Radiation induction of p-eIF2α and VEGF-A is downstream of PERK. A, D54 and GL261 cells were irradiated with 3 Gy or 6 Gy and harvested 24 hours after IR. Shown are the immunoblot, showing eIF2α phosphorylation in D54 and GL261 cell lines. B, Cells were incubated with 1 μmol/L PERKi for 1.5 hours before 6 Gy IR and harvested 24 hours after IR. Shown are the immunoblots of eIF2α phosphorylation in D54 and LN827 cells. C, D54 cells were incubated with 1 μmol/L PERKi for 1.5 hours before 6 Gy IR, and RNA was analyzed 24 hours after IR. Shown are the qRT-PCR analysis of PERK and VEGF-A gene expression in D54 cells. In all graphs, data shown are the means ± SD (n = 4). ***, P < 0.0001,
We treated D54 cells with 1 μmol/L PERKi prior to 6 Gy IR and measured phospho-eIF2α levels 24 hours after IR. Pretreatment of D54 and LN827 cells with PERKi resulted in attenuation of baseline phosphorylation of eIF2α, and abrogation of radiation-induced phosphorylation of eIF2α (Fig. 1B). VEGF-A is a recently identified downstream target of the PERK/ATF4 axis (16). We examined the impact of PERK inhibition on VEGF-A RNA expression by qRT-PCR. We found that radiation-induced PERK 4.1-fold and VEGF-A 3.9-fold compared with untreated control (DMSO; P < 0.0001; Fig. 1C). Inhibition of PERK-attenuated induction of both PERK and VEGF-A by IR (Fig. 1C). These results suggest that PERK plays a role in radiation-induced eIF2α phosphorylation and associated downstream signaling.

IR potentiates ER stress which reduced proliferation in a PERK-dependent manner
PERK is a well-characterized switch between survival and death during persistent ER stress and is known to mediate cell death through induction of CCAAT/enhancer–binding protein homologous protein (CHOP; ref. 11). Because we observed radiation-induced activation of the PERK and phospho eIF2α, we hypothesized that IR could affect the sensitivity of cells to chemical ER stressors. To test this hypothesis, we irradiated D54 and LN827 cells with PERK inhibitor (PERKi), ER stress inducer (2DG) and GADD34 phosphatase inhibitor (Sal003). We found that treatment with 5 mmol/L 2DG or 10 μmol/L Sal003 reduced cell viability by 23% and 33%, respectively, in D54, and 36% and 40%, respectively, in LN827 (Fig. 2A). Combination of 3 Gy IR
and 5 mmol/L 2DG resulted in 49% and 51% attenuation in cell viability in D54 and LN827, respectively (Fig. 2A). Similarly, the combination of 3 Gy IR and 10 μmol/L Sal003 leads to a 48% and 51% decrease in cell viability in D54 and LN827, respectively (Fig. 2A). In both D54 and LN827, treatment with PERKi abrogated the additive effect of IR and 2DG or Sal003 on cell viability. To determine whether IR was enhancing prodeath signaling when combined with chemical induction of ER stress, we transduced D54 and LN827 with a CHOP-mCherry promoter reporter. IR (3 Gy) led to 1.13- and 1.2-fold increase in CHOP levels in D54 and LN827, respectively (Fig. 2B). Combining IR (3 Gy) with Sal003 lead to an increase of 6.8- and 1.8-fold expression of CHOP in D54 and LN827, respectively (Fig. 2B). These results indicate that IR can potentiate the effect of ER stressors on prodeath signaling downstream of PERK.

Knockdown of ATF4 and combination of radiation lead to decreased proliferation and colony formation

Although the prodeath functions of PERK are activated during prolonged ER stress, prosurvival signaling is also known to occur via ATF4 (17). Translation of ATF4 is promoted by phosphorylation of eIF2α and results in upregulation of genes involved in mitigation of oxidative stress (18). To determine whether IR also induces ATF4, we treated D54 with IR (3 or 6 Gy) and analyzed for ATF4 levels by Western immunoblot analyses. We found that ATF4 levels increased in a dose-dependent manner 24 hours after IR (Fig. 3A). To determine the role of ATF4 in proliferation and colony formation we silenced ATF4 using siRNA (Fig. 3B). Knockdown of ATF4 in D54 prior to IR with 3 Gy resulted in a 23% decrease in proliferation (P < 0.0001; Fig. 3C) and 12% decrease in colony formation (P < 0.05; Fig. 3D) when compared with IR alone. To assess the role of ATF4 in apoptosis, we analyzed PARP cleavage by flow cytometry. D54 cells following the ATF4 knockdown, when treated with 3 Gy IR, showed a 2.3-fold increase in PARP cleavage when compared with IR alone (Fig. 3E). These results suggest that ATF4 could play an important role in glioma cell viability during the radiation response.

Discussion

Earlier, we found that radiation can induce ER stress and downstream signaling associated with the ERSR (8). Induction of ER stress appears to be linked to changes in ROS balance secondary to irradiation. Our interest in the PERK pathway of the ER stress response stems from its role in mediating oxidative stress responses in various cell models (17, 18). Reactive oxygen species primarily mediate the effects of radiation on cell physiology. We therefore hypothesized that PERK signaling may play a role in the radiation response. In cancers such as GBM,
which involve radiation as an essential component of therapy, studying PERK signaling may reveal molecular targets for drug development.

First, we studied PERK activation by measuring the abundance of phospho-eIF2α. Our observation that radiation-induced phosphorylation of eIF2α, together with our finding that PERK activity is required for this phosphorylation, indicates that radiation may be activating the ER stress response in GBM. Activation of PERK signaling by radiation has been observed in vascular endothelial cells following high dose (15 Gy) IR (9). The fact that lower doses of radiation (3 Gy and 6 Gy) could activate this pathway in GBM suggests that there may be differential sensitivity of GBM cells to the effects of radiation. We speculate that GBM cells may be less adapted to managing oxidative stress, and may require induction of PERK signaling to mitigate radiation-induced oxidative damage.

PERK has been implicated in the regulation of angiogenesis via downstream binding of ATF4 to the VEGF-A promoter (16, 19). To evaluate whether PERK inhibition is sufficient to disrupt downstream signaling events, we analyzed expression of PERK and VEGF-A mRNAs. Data showing that IR induced both PERK and VEGF-A mRNAs, and that PERK inhibition abrogated this response, suggests that signaling pathways downstream of PERK are intact during the radiation response. Because PERK is known to promote cell death during chronic ER stress, we tested the hypothesis that radiation-induced PERK activity could modulate the sensitivity of GBM to agents that induce ER stress. We used 2DG to induce ER stress and found that inhibition of PERK partially rescued GBM cells from ER stress. A similar response was observed in irradiated GBM cells, suggesting that radiation-induced PERK activity may potentiate the effects of ER stress-inducing agents. This was further supported by our finding that inhibition of GADD34 phosphatase–enhanced CHOP transcription in irradiated GBM cells.

Sal003, a GADD34 phosphatase inhibitor is a positive regulator of eIF2α, and functions by inhibiting dephosphorylation of eIF2α (20), thereby simulating persistent PERK activity. These results highlight the potential of utilizing the prodeath functions of PERK to enhance therapeutic efficacy.

During the early phase of the ERSR, PERK activity can promote survival by inducing translational arrest, upregulating chaperones, and enhancing expression of antioxidant genes (11). We explored the prosurvival functions of PERK signaling by targeting ATF4 with siRNA. We found that ATF4 knockdown had reduced proliferation and colony formation in GBM. This suggests that ATF4 is downstream of PERK, and that PERK activation may be important in promoting cell viability. The PERK–eIF2α–ATF4 pathway has been shown to confer protection from oxidative stress by promoting glutathione production (18). In the context of the radiation response, we postulate that the antioxidant aspect of ATF4 signaling may account for its observed influence on cell survival. It remains to be shown how the dynamics of ATF4 expression may lead to different effects on cell viability. Given the dual roles of PERK–eIF2α–ATF4 in cell death and survival, it is conceivable that transient induction of ATF4 during the radiation response may be sufficient to enhance survival. Induction of ATF4 however, as observed during chemical induction of ER stress, promotes CHOP transcription that leads to reduced proliferation and colony formation. This model could explain PERK-dependent potentiation of cell death when radiation was combined with 2DG. Furthermore, this model highlights the potential for radiation to trigger adaptive signaling in GBM that could contribute to tumor recurrence.

In conclusion, the data from this study show that induction of PERK signaling occurs in irradiated GBM and that the dual function of PERK as a mediator of survival and death may provide multiple approaches to enhancing the efficacy of radiation therapy. Furthermore, this study supports the notion that induction of ER stress—signaling by radiation may contribute to adaptive survival mechanisms during radiation therapy. Beyond the implications discussed regarding potentiation of cell death and adaptive survival, the observed influence of PERK on VEGF-A may indicate a connection between therapeutic stress and a proangiogenic response. Further investigation is needed to characterize the functional role of radiation-induced VEGF-A in GBM.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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