Silencing of Elongation Factor-2 Kinase Potentiates the Effect of 2-Deoxy-β-Glucose against Human Glioma Cells through Blunting of Autophagy

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Abstract

2-Deoxy-β-glucose (2-DG), a synthetic glucose analogue that acts as a glycolytic inhibitor, is currently being evaluated in the clinic as an anticancer agent. In this study, we observed that treatment of human glioma cells with 2-DG activated autophagy, a highly conserved cellular response to metabolic stress and a catabolic process of self-digestion of intracellular organelles for energy use and survival in stressed cells. The induction of autophagy by 2-DG was associated with activation of elongation factor-2 kinase (eEF-2 kinase), a structurally and functionally unique enzyme that phosphorylates eEF-2, leading to loss of affinity of this elongation factor for the ribosome and to termination of protein elongation. We also showed that inhibition of eEF-2 kinase by RNA interference blunted the 2-DG-induced autophagic response, resulted in a greater reduction of cellular ATP contents, and increased the sensitivity of tumor cells to the cytotoxic effect of 2-DG. Furthermore, the blunted autophagy and enhanced 2-DG cytotoxicity were accompanied by augmentation of apoptosis in cells in which eEF-2 kinase expression was knocked down. The results of this study indicate that the energy stress and cytotoxicity caused by 2-DG can be accelerated by inhibition of eEF-2 kinase, and suggest that targeting eEF-2 kinase-regulated autophagic survival pathway may represent a novel approach to sensitizing cancer cells to glycolytic inhibitors.

[Introduction]

Cellular metabolism of malignant cells differs significantly from that of normal cells. Whereas normal cells rely on respiration, a process that consumes oxygen and glucose to produce energy-storing molecule ATP, malignant cells mainly depend on glycolysis, the anaerobic metabolism of glucose into ATP, even in the presence of sufficient oxygen. This increased dependency of malignant cells on glycolysis for ATP production is known as the so-called Warburg effect (1, 2). A number of molecular pathways have been revealed to be associated with this metabolic phenotype, including hexokinase 2 (3, 4), p53 (5), c-Myc (6, 7), HIF-1 (7), and defect in mitochondrial respiration (8). Due to the high dependence of malignant cells on glycolysis, interfering with this metabolic process has recently been proposed as a potentially useful approach for developing new selective cancer therapy (9). Treatment of cancer cells with 2-deoxy-β-glucose (2-DG), a synthetic glucose analogue that acts as a glycolytic inhibitor, has been shown to inhibit growth and viability of cancer cells (10–12), and enhance the efficacy of cancer chemotherapeutics and radiation regiments (13–18). In both in vitro and in vivo models, 2-DG was effective in the treatment of a variety of solid tumors (19–21). The pharmacologic basis of antitumor action of 2-DG is believed to be the high dependence of malignant cells, especially those hypoxic cells on glycolysis, the preferred ingestion and retention of 2-DG by tumor cells, and the blocking effect of 2-DG on glucose metabolic pathways. In addition, 2-DG causes oxidative stress through increasing pro-oxidant production and disrupting thiol metabolism, as evidenced by alterations in total glutathione content (16, 22). In the treatment of human brain malignancies, 2-DG has been shown to be effective in sensitizing tumor cells to radiation therapy (17, 23). Despite the demonstrations of the antitumor activity of 2-DG, large doses are usually needed to achieve a therapeutic effect, and cancer cells quickly become refractory to this agent. Therefore, approaches that can enhance the efficacy of 2-DG may make this agent more useful in the treatment of cancers.

Elongation factor-2 kinase (eEF-2 kinase; aka calmodulin-dependent protein kinase III), a unique calmodulin/calcium-dependent enzyme that inhibits protein synthesis, is overexpressed in several types of malignancies including gliomas (24, 25). eEF-2 kinase phosphorylates elongation factor-2, a 100-kDa protein that mediates the translocation step in peptide-chain elongation by inducing the transfer of peptidyl-tRNA from the ribosomal A to P site. Phosphorylation of EF-2 at Thr56 by eEF-2 kinase decreases the affinity of this elongation factor for ribosomes and terminates elongation, thereby inhibiting protein synthesis. Because protein synthesis requires a large proportion of cellular energy (26, 27), inhibition of protein synthesis by terminating elongation through activating eEF-2 kinase decreases energy use, and provides a survival mechanism against energy stress.

We have recently reported the critical role of eEF-2 kinase in the regulation of autophagy, a highly conserved cellular process that is activated in times of metabolic or environmental stress and leads to large-scale degradation of proteins (28). The process of autophagy involves formation of a double-membrane vesicle (“autophagosome”) in the cytosol that engulfs organelles and cytoplasm, then fuses with the lysosome to form the autolysosome, where the contents are degraded and recycled for protein and ATP synthesis (29). The formation of the autophagosome is mediated by a series of autophagy specific genes (ATG). This form of
self-digestion leads to self-preservation in times of nutrient deprivation; however, if left unchecked, autophagy has the potential of producing terminal self-consumption. Although apoptosis is known as type I cell death, autophagy is called type II cell death. We showed in human glioma cells that the activity of eEF-2 kinase is closely associated with the mammalian macro-autophagy pathway that is activated in response to nutrient, growth factor or oxygen deprivation. Furthermore, inhibition of eEF-2 kinase blunts autophagy and has deleterious effects on cell viability under nutrient starvation condition (28). These observations raise the possibility that blocking the activation of eEF-2 kinase may represent a potential therapeutic strategy to promote cell death induced by metabolic stress. As 2-DG inhibits cell growth and causes death of tumor cells through antagonizing glucose (20), we sought to determine the effects of this glycolytic inhibitor on eEF-2 kinase activity and autophagic cell survival pathway, and the effect of inhibiting eEF-2 kinase on sensitivity of tumor cells to 2-DG. We found that treatment of human glioma cells with 2-DG activated an eEF-2 kinase–dependent autophagic response, and inhibiting eEF-2 kinase by RNA interference (RNAi) blocked the induction of autophagy by 2-DG and increased the sensitivity of tumor cells to the cytotoxic effect of this glycolytic inhibitor. Our results suggest that targeting the eEF-2 kinase–regulated autophagic pathway could be an effective approach to augmenting the activity of 2-DG, and thus may have clinical implication in cancer treatment.

Materials and Methods

Cell lines and culture. The human glioblastoma cell lines, T98G and LN-229, were purchased from American Type Culture Collection. T98G cells were cultured in Ham’s F-10: DMEM (10:1) medium, and LN-229 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO2/95% air.

Reagents and antibodies. 2-Deoxy-D-Glucose, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and anti–eEF-2 kinase antibodies were purchased from Invitrogen Corporation. Chemiluminescence Western blot reagents were obtained from Pierce Biotechnology, Inc. The following antibodies were used in this study: anti–caspase-3 antibody, anti–eEF-2 kinase, anti–phospho-eEF-2 (Thr56), anti–phospho-AMPKα (Thr172); and anti–AMPKα, anti–phospho-S6 kinase (Thr389) antibodies (Cell Signaling Technologies); monoclonal anti–LC3 antibody (nanoTools, though Axxora; LLC).

siRNA preparation and transfection. siRNA sequence targeting eEF-2 kinase mRNA corresponded to the coding region 144 to 164 (5’-AAGCTGCAAGCCAGATGTC-3’) relative to the start codon (28). siRNA duplexes were prepared by Dharmacon Research, Inc. For transfection, cells in exponential phase of growth were plated in 60-mm tissue culture dishes at 5 × 10⁶ cells per dish, grown for 24 h, then transfected with siRNA (100 nmol/L) using Oligofectamine and OPTI-MEM 1-reduced serum medium, according to the protocol of the manufacturer. The concentrations of siRNAs were chosen based on dose-response studies. Twenty-four hours after transfection, the cells were harvested for further experiments.

Measurement of protein synthesis. The rate of protein synthesis was measured as described previously (30). Briefly, cells were seeded in 60-mm tissue culture dishes and labeled with 25 μCi/mL of EasyTag EXPRESS [35S] protein labeling mix (PerkinElmer) in RPMI 1640. After incubation at 37°C for 15 min, cells were washed 4 times with 4 mL of ice-cold PBS and lysed in 200 μL of Complete Lysis-M lysis reagent containing the Mini Protease Inhibitor Cocktail (Roche Diagnostics). Lysates were collected in a microfuge tube and clarified by centrifugation at 13,000 × g for 10 min at 4°C. The supernatants were precipitated with 20% trichloracetic acid and dissolved in 35S-methionine/cysteine permol of total protein per min. The rate of protein synthesis was determined as described previously (31). For detection of LC3-II, cell lysates were prepared by Dharmacon Research, Inc. Duplexes were prepared by Dharmacon Research, Inc. For transfection, cells in exponential phase of growth were plated in 60-mm tissue dishes at 5 × 10⁶ cells per dish, grown for 24 h, then transfected with siRNA (100 nmol/L) using Oligofectamine and OPTI-MEM 1-reduced serum medium, according to the protocol of the manufacturer. The concentrations of siRNAs were chosen based on dose-response studies. Twenty-four hours after transfection, the cells were harvested for further experiments.

Measurement of cellular ATP. Cells were plated in 96-well plates at 2.5 × 10⁵ cells per well and treated with 2-DG for 24 h. ATP contents were determined using the ATPlite Luminescence Assay kit (PerkinElmer) according to the manufacturer's protocol. The luminescence was measured by a Victor² Multi Label plate reader (PerkinElmer).

Measurement of autophagy. Autophagy was monitored by measuring the formation of LC3-II in the absence or presence of lysosomal protease inhibitors E64D (10 μg/mL) and pepstatin A (10 μg/mL), as described by Tanida and colleagues (31). For detection of LC3-II, cell lysates were prepared and 25 μg of total proteins were subjected to Western blot analysis using a monoclonal anti–LC3 antibody. GFP-LC3 cleavage assay was performed as previously described (32). Briefly, cells (1 × 10⁶) were
cotransfected with 3 µg of GFP-LC3 plasmid DNA and an eEF-2 kinase–targeted siRNA or a nontargeting RNA in Opti-MEM reduced medium (Invitrogen) and incubated overnight at 37°C. The cells were then treated with 2-DG in the presence of lysosomal protease inhibitors E64d (10 µg/mL) and pepstatin A (10 µg/mL). At the end of treatment, cells were fixed with 4% formaldehyde for 15 min and inspected at 60x magnification for numbers of GFP-LC3 puncta. Autophagy was also evaluated by electron microscopic examination of double or multimembrane vacuoles in the cytoplasm, as described below.

**Western blot analysis.** Cells were pelleted at 500 × g for 5 min and were lysed in cold lysis buffer [20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium Ppi, 1 mmol/L β-glycerol phosphate, 1 mmol/L Na3VO4, 1 µg/mL leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride] and sonicated for 5 s. The lysates were clarified by centrifugation at 12,000 × g for 30 min at 4°C. Identical amounts (25 µg of protein) of cell lysates were resolved by 8% or 15% SDS-PAGE, and proteins were transferred onto nitrocellulose or polyvinylidene difluoride. Membranes were incubated in blocking solution consisting of 5% powered milk in TBST [10 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, and 0.1% Tween 20] for 1 h, then immunoblotted with the respective antibodies. Detection by enzyme-linked chemiluminescence was carried out according to the protocol of the manufacturer.

**Figure 2.** Effect of 2-DG on ATP content (A), S6 kinase activity (B), and AMPK kinase activity (C) in glioma cells. T98G or LN-229 cells were treated with the indicated concentrations of 2-DG for 24 h. At the end of treatment, (A) ATP content was measured using the ATPlite Luminescence Assay kit. B, S6 kinase activity was determined by Western blot analysis of phospho-S6 kinase using an anti–phospho-S6 kinase antibody. C, AMPK activity was determined by Western blot analysis of phospho-AMPK using an anti–phospho-AMPK antibody, as described in Materials and Methods. Tubulin was used as a loading control. Results shown are the representative of three similar experiments; columns, mean of quadruplicate determinations; bars, SD. *, P < 0.05; **, P < 0.01.

**Electron microscopy.** Cells were harvested by trypsinization, fixed in 2.5% gluteraldehyde/4% paraformaldehyde in 0.1 mol/L cacodylate buffer, and then postfixed in 1% osmium tetroxide buffer. After dehydration in acetone, the cells were embedded in spur resin. Thin sections (90 nm) were cut on a Reichert Ultracut E microtome. Sectioned grids were stained with saturated solution of uranyl acetate and lead citrate. Sections were examined at 80 kV with a JEOL 1200EX transmission electron microscope.

**Cellular viability.** Cell viability was measured by MTT assay. Briefly, cells were plated at 5 × 10^3 per well in 96-well tissue culture plates and incubated at 37°C in a humidified atmosphere containing 5% CO₂/95% air. The formazan product, formed after a 4-h incubation with MTT, was dissolved in DMSO and read at 570 nm using a Victor3 Multi Label plate reader (PerkinElmer).

**Measurement of apoptosis.** Active caspase-3, which is present in cells undergoing apoptosis, was detected by Western blot using polyclonal anti–caspase-3 antibody that recognizes the cleaved, active caspase-3, as described previously (33). For apoptotic nuclei staining, pEGFP-transfected cells with different treatments were stained with Hoechst 33342 and observed under green fluorescence and UV. Green fluorescent protein (GFP)-positive cells were scored for the presence of apoptotic nuclei, and apoptotic rates were presented as the percentage of apoptotic cells out of the total number of GFP-positive cells assessed (34).
Results

Treatment with 2-DG activates eEF2 kinase and decreases protein synthesis in glioma cells. We previously reported that nutrient depletion caused activation of eEF2 kinase and autophagy in glioma cells, and blocking activation of eEF-2 kinase blunted the autophagic response (28). To determine the specific effect of glucosedeprivationontheactivityofeEF-2kinaseandautophagy, wetreatedT98GandLN229humangliomacellswithvarying concentrationsof2-DG, and then assayed the activity of the kinase. As shown in Fig. 1A, treatment of these glioma cells with 2-DG for 24 hours increased the activity of eEF-2 kinase in a dose-dependent manner, as evidenced by the increased phosphorylation of its substrate, EF2. To confirm the stimulatory effect of 2-DG on eEF-2 kinase activity, we compared the rate of protein synthesis in the cells treated with 2-DG to that of the cells treated with vehicle. Figure 1B shows that protein synthesis in the cells treated with 2-DG was markedly inhibited compared with that in vehicle-treated cells. These results were consistent with the role of eEF-2 kinase in regulating translation, i.e., activation of eEF-2 kinase inhibits elongation. Activation of eEF-2 kinase by 2-DG was accompanied by a reduction of cellular ATP contents (Fig. 2A), an inactivation (decreased phosphorylation) of the key translational governor and downstream effector of mammalian target of rapamycin (mTOR), S6 kinase (Fig. 2B), and an activation of AMPK, as shown by the increased phosphorylation of this intracellular energy sensor (Fig. 2C). These results indicate that treatment of glioma cells with 2-DG elicited an energy stress response.

Treatment with 2-DG induces autophagy in glioma cells. Given the effects of 2-DG on the activities of eEF-2 kinase (Fig. 1), S6 kinase, AMPK, and the cellular level of ATP (Fig. 2), we next determined whether treatment of tumor cells with this glycolytic inhibitor induced autophagy. LC3-II, a cleaved product of microtubule-associated protein 1 light chain 3, was used as a marker for autophagy. We found that both steady-state level (Fig. 3A) and turnover (Fig. 3B) of LC3-II were increased in the glioma cells treated with 2-DG, compared with the cells treated with the vehicle. The induction of autophagy by 2-DG was confirmed by electron microscopy (Fig. 3C), which visualized abundant double or multimembrane vacuoles in the cytoplasm of the cells treated with 2-DG. By contrast, these vacuoles were rarely observed in glioma cells treated with the vehicle (Fig. 3C). Although the basal level of autophagy in cells cultured in medium containing high concentration of glucose (25 mmol/L) was much lower than that in cells cultured in medium with low concentration of glucose (5.6 mmol/L), under either condition 2-DG activated autophagy in these tumor cells, and the levels of autophagy were correlated to the activity of eEF-2 kinase (Fig. 3D).

Silencing of EF-2 kinase expression blunts the 2-DG–induced autophagy. To further investigate whether induction of autophagy by 2-DG was mediated through eEF-2 kinase, we silenced the expression of eEF-2 kinase using RNAi approach and then determined the effect of eEF-2 kinase inhibition on the autophagic response to 2-DG treatment. Tumor cells were transfected with an eEF-2 kinase–targeted siRNA or nontargeting RNA.
for 24 hours, and then treated with various concentrations of 2-DG. Figure 4A shows that knockdown of eEF-2 kinase expression decreased the activity of the enzyme, as indicated by the dramatic decreases in the phospho-EF-2 at Thr56, and blunted the autophagic response in the cells treated with 2-DG, as manifested by the decreased formation of LC3-II. Electron microscopy and GFP-LC3 cleavage assay also showed that silencing of eEF-2 kinase by siRNA blocked the 2-DG–induced autophagy, as evidenced by the decreased formation of double-membrane vacuoles in the cytoplasm (Fig. 4B) and decreased numbers of cells with >20 GFP-LC3 punctae (Fig. 4C). Moreover, silencing of eEF-2 kinase also diminished the inhibitory effect of 2-DG on protein synthesis (Fig. 5A) and accelerated the 2-DG–induced reduction of cellular ATP contents (Fig. 5B), further indicating that inhibition of this kinase weakens the adaptive response of cells to metabolic stress and worsens the energy supply.

Silencing of eEF-2 kinase expression enhances the sensitivity of glioma cells to 2-DG. It has been shown that autophagy promotes cell survival under conditions of nutrient deprivation and that inhibiting eEF-2 kinase can abrogate this prosurvival response (28). To test whether blockade of 2-DG–activated autophagy via inhibiting eEF-2 kinase could enhance the cytotoxicity of this glycolytic inhibitor, we transfected tumor cells with a nontargeting RNA or a siRNA targeting eEF-2 kinase, then exposed the cells to various concentrations of 2-DG. As shown in Fig. 6A, the cytotoxicity of 2-DG was significantly increased by silencing eEF-2 kinase expression in T98G and LN-229 glioma cells. To explore the mechanism underlying the increased 2-DG cytotoxicity in cells with decreased eEF-2 kinase activity, we compared apoptosis in tumor cells with and without silencing of the enzyme after 2-DG treatment. Figure 6B shows that treatment of T98G cells with 2-DG not only activated autophagy, as measured by the formation of LC3-II, but also triggered apoptosis in a dose-dependent manner, as measured by the activation of caspase-3. More notably, inhibition of autophagy by silencing of eEF-2 kinase augmented apoptosis triggered by 2-DG, as evidenced by the increased appearance of the cleaved form of caspase-3 (Fig. 6B) and increased apoptotic nuclei (Fig. 6C), suggesting an increased death in cells with inhibition of eEF-2 kinase.

Discussion

eEF-2 kinase is a calcium/calmodulin-dependent enzyme that regulates protein elongation (35), and has been observed to be up-regulated in human and rat gliomas (25, 36). This kinase was subsequently found to have several unique characteristics including the ability to phosphorylate serines and threonines within α-helical turns (37). Further work by Proud’s group (38) showed the exquisite regulation of eEF-2 kinase by multiple enzymes involved in energy sensing and use, including mTOR, S6 kinase, and AMP kinase (39, 40). The role of eEF-2 kinase as an energy sensor was also shown in a study showing that this enzyme participates in the AMPK-mediated cardioprotection in response to metabolic stress (41). When exploring the role of eEF-2 kinase in malignant cells, we found that the activity of eEF-2 kinase and autophagy were rapidly increased by nutrient deprivation and that inhibiting the enzyme markedly diminished autophagic cell survival (28), suggesting that

Figure 4. Silencing of eEF-2 kinase expression blunts 2-DG–activated autophagy. T98G cells were transfected with a nontargeting RNA or an eEF-2 kinase–targeted siRNA (100 nM) for 24 h, and then treated with the indicated concentrations of 2-DG. A, phospho-EF-2, EF-2, eEF-2 kinase, and the autophagy marker, LC3-II, were detected as described in Fig. 3C. B, electron microscopic examination of autophagosomes was performed as described in Fig. 3C. Arrows, autophagic vacuoles. C, GFP-LC3–expressing cells were transfected with an eEF-2 kinase–targeted siRNA or a nontargeting RNA, and then treated with indicated concentration of 2-DG in the presence of lysosomal protease inhibitors E64d (10 μM) and pepstatin A (10 μM). At the end of treatment, cells were fixed with 4% formaldehyde for 15 min and inspected at ×60 magnification for numbers of GFP-LC3 puncta. At least 200 cells were scored in each treatment. Columns, mean of quadruplicate determinations; bars, SD. *, P < 0.01.

Results shown are the representative of three similar experiments.
this apparent relationship between eEF-2 kinase activity and cell survival might be linked directly to its role in protein synthesis.

In this study, we used the glycolytic inhibitor, 2-DG, to further explore the role of eEF-2 kinase in the autophagic response to energy stress. We show in human glioma cells that treatment with 2-DG activates autophagy (Fig. 3); the induction of autophagy by 2-DG seems to be dependent on the activity of eEF-2 kinase, as treatment with 2-DG also activates this kinase (Fig. 1); and knockdown of eEF-2 kinase expression blunts the autophagic response induced by 2-DG (Fig. 4). Furthermore, we show that silencing of eEF-2 kinase expression increases the cytotoxicity of 2-DG (Fig. 6A) and augments apoptosis (Fig. 6B and C). Because autophagy is known to favor cell survival under environmental and metabolic stress conditions (42–44), 2-DG-induced autophagy may represent an adaptive response to the reduction of energy supply caused by this glycolytic inhibitor, and explain, at least in part, the decreased sensitivity of tumor cells after exposure to this agent. Although the two glioma cell lines used in this study, T98G and LN-229, harbor aberrant and wild-type PTEN, respectively (45, 46), our results from the two cell lines were consistent, suggesting that different expression of PTEN does not affect the autophagic response to 2-DG treatment and the effect of inhibiting eEF-2 kinase on sensitivity to 2-DG.

The results showing that 2-DG–induced autophagy is associated with the activity of eEF-2 kinase are consistent with our previous observation that induction of autophagy by metabolic stress is regulated by this kinase (28), although the precise mechanism underlying this regulation is still unclear. The association of eEF-2 kinase activity with the regulation of autophagy is consistent with the function of this kinase, which acts as an inhibitor of peptide elongation. Protein synthesis is a process that accounts for a major percentage of energy consumption (26, 27); therefore, inhibition of protein synthesis may decrease cellular energy use to withstand nutrient starvation such as glucose deprivation. The activity of eEF-2 kinase is tightly regulated by signaling pathways involved in nutrient use and energy monitoring in the cell. For example, Proud’s group (38) reported that eEF-2 kinase is inhibited by mTOR and S6 kinase signaling pathways. They also show that eEF-2 kinase is activated by AMP kinase, an intracellular energy sensor that regulates cell metabolism (39, 40). Thus, the activation of eEF-2 kinase in 2-DG–treated cells could be a consequence of energy stress caused by this glycolytic inhibitor, as treatment of this agent leads to reduction of cellular ATP contents (Fig. 2A), decreased activity of S6 kinase (Fig. 2B), and increased activity of AMP kinase (Fig. 2C). A recent study in breast cancer cells also shows the activation of AMP kinase and deactivation of S6 kinase by 2-DG (47). These results support our hypothesis that induction of autophagy by 2-DG is a type of metabolic adaptation to energy stress because autophagy can increase ATP production through autophagic recycling of amino acids produced from digestion of cellular organelles and proteins, thus favoring cell survival in times of cellular stress. The stimulatory effect of 2-DG on autophagy may also contribute to the phenomenon that this agent protects glioma cells from glucose withdrawal–induced cell death (48). Additionally, our results showing that basal level of autophagy is higher in cells cultured in medium containing low concentration of glucose than in cells cultured in medium containing high concentration of glucose (Fig. 3D) also support the role of autophagy as a prosurvival mechanism in cells stressed by nutrient deprivation or metabolic alteration. Silencing of eEF-2 kinase does not completely suppress the autophagic response activated by 2-DG (Fig. 4), probably due to the fact that 2-DG treatment also causes oxidative stress (49) and this type of stress is a trigger for autophagy via other pathways (50). Thus, inhibiting multiple autophagic pathways might result in a more profound suppression of 2-DG–activated autophagy and hence increase the anticancer activity of this agent. It has been reported that inhibition of glutamate cysteine ligase, an enzyme involved in the glutathione synthetic pathway, sensitizes tumor cells to the cytotoxicity of 2-DG (22). We recognize that autophagy may also lead to cell death that is accompanied by caspase activation (51); however, based on our results, the role of autophagy is prosurvival but not prodeath in response to 2-DG treatment, as we observed that although silencing of eEF-2 kinase expression further increased caspase-3 activation in glioma cells treated with 2-DG (Fig. 6B), autophagy was inhibited. Moreover, cell viability was also decreased in siRNA-treated cells compared with the nontargeting RNA-treated cells (Fig. 6C).

Malignant cells use glucose at a higher rate than normal cells, and become more dependent on aerobic and anaerobic glycolysis. Hypoxic tumor cells are particularly dependent on anaerobic glycolysis, and are therefore more sensitive to 2-DG–induced cell cycle inhibition and cytotoxicity (12). Moreover, due to the up-regulation of glucose transporters in tumor cells and high affinity of 2-DG for glucose transporters, malignant cells show an enhanced uptake and retention of this agent (52). These factors are believed to contribute to the preferential toxicity of 2-DG in cancer cells. In this study, we show that targeting eEF-2 kinase, an inhibitor of protein elongation, can enhance the sensitivity of glioma cells to 2-DG (Fig. 6A). The potentiation of 2-DG cytotoxicity by inhibition...
of eEF-2 kinase seems to result from the metabolic catastrophe caused by the glycolytic inhibitor, as autophagy is markedly attenuated and ATP level is further decreased (due to recovery of protein synthesis) in tumor cells with silencing of eEF-2 kinase in comparison with the cells without the silencing of the enzyme (Figs. 4 and 5). Inducing metabolic catastrophe in cancer cells has been proposed as a new therapeutic approach awaiting for further investigation (53).

2-DG has entered into phase I clinical trials, but results of the clinical studies have not been well-documented in literatures. However, a recent study reported that 2-DG significantly prolongs survival of the mice bearing aggressive lymphoma with defective laforin expression (21). The results of the current study show that the cytotoxic effect of 2-DG on tumor cells can be potentiated by suppressing an eEF-2 kinase–dependent autophagic survival pathway, suggesting that 2-DG treatment in combination with the inhibition of eEF-2 kinase may stand for a new therapeutic strategy to improve the efficacy of glycolytic inhibitors such as 2-DG. Taken together, our study underscores the potential of eEF-2 kinase as a complementary target for sensitizing tumor cells to the glycolysis-targeted therapy.

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

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Figure 6. Silencing of eEF-2 kinase expression increases the sensitivity of glioma cells to the cytotoxicity of 2-DG. A, T98G or LN-229 cells transfected with a nontargeting RNA or an eEF-2 kinase–targeted siRNA were treated with the indicated concentrations of 2-DG for 60 h. At the end of treatment, cell viability was measured using MTT assay. Points, mean of quadruplicate determinations; bars, SD. *, P < 0.05. B, T98G cells with or without silencing of eEF-2 kinase expression were treated with the indicated concentrations of 2-DG for 24 h. Caspase-3, LC3-II, and eEF-2 kinase were detected by Western blot, as described in Materials and Methods. Tubulin was used as a loading control. C, T98G cells were cotransfected with a pEGFP expression vector and a siRNA targeting eEF-2 kinase or a nontargeting RNA, and then treated with the indicated concentrations of 2-DG. At the end of treatment, cells were fixed, stained with Hoechst 33342, and observed under green fluorescence and UV. GFP-positive cells were scored for the presence of apoptotic nuclei, and apoptotic rates were presented as the percentage of apoptotic cells out of the total number of GFP-positive cells assessed. Columns, mean of triplicate determinations; bars, SD. *, P < 0.05; **, P < 0.01.
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