Berberine Induces Senescence of Human Glioblastoma Cells by Downregulating the EGFR-MEK-ERK Signaling Pathway

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Abstract

Glioblastoma multiforme (GBM) is the most common primary malignant brain tumor and has a poor prognosis. We, here, report a potent antitumor effect of berberine, an isoquinoline alkaloid, on GBM. Berberine was found to have an IC₅₀ that is much lower than temozolomide in vitro in U87, U251, and U118 glioblastoma cells. Although previous studies showed that berberine primarily exerts its anticancer effect by inducing cell-cycle arrest, apoptosis, and autophagy, we observed that the antitumor effect of berberine on glioblastoma cells was primarily achieved through induction of cellular senescence. In glioblastoma cells treated with berberine, the level of epidermal growth factor receptor (EGFR) was greatly reduced. Examination of the activities of the kinases downstream of EGFR revealed that the RAF-MEK-ERK signaling pathway was remarkably inhibited, whereas AKT phosphorylation was not altered. Pharmacologic inhibition or RNA interference of EGFR similarly induced cellular senescence of glioblastoma cells. Furthermore, the cellular senescence induced by berberine could be rescued by introduction of a constitutively active MKK. Berberine also potently inhibited the growth of tumor xenografts, which was accompanied by downregulation of EGFR and induction of senescence. Our findings thus revealed a new route by which berberine exerts its anticancer activity. Because EGFR is commonly upregulated in glioblastoma, the demonstration of effective inhibition of EGFR by berberine points to the possibility of using berberine in the treatment of patients with glioblastoma. Mol Cancer Ther; 14(2); 355–63. ©2014 AACR.

Introduction

Malignant gliomas are the most common primary malignant brain tumors, with an annual incidence of 5.26 per 100,000 population (1). These tumors usually have a poor prognosis, with a 5-year survival rate of WHO grade 4 tumors (glioblastoma and variants) less than 5% (2, 3). Radiotherapy combined with DNA alkylating agent such as temozolomide is considered the first-line of adjuvant treatment for all patients (1, 3, 4). However, although this regimen can increase the 2-year survival rate from 10% to 27%, its benefit is still limited. Moreover, it causes serious side effects such as lymphopenia and nausea, and nearly all patients with glioblastoma experience disease progression (1). More efficacious measures against malignant glioma thus remain to be explored.

Berberine, an isoquinoline alkaloid present in many medicinal herbs, including Huanglian (Coptis chinensis), is one of the most commonly used herbal medicines (7–9). It is shown to possess anticancer activities against various types of cancer cells. Berberine can induce apoptosis and cell-cycle arrest of tumor cells (10–14). In this report, we studied the effect of berberine on glioblastoma cells in vitro and on tumor xenografts. We found that berberine possesses a more potent antitumor effect on glioblastoma cells than temozolomide in U87, U251, and U118. Interestingly, the antitumor effect of berberine was attributable to its ability to induce cellular senescence. We further demonstrated that berberine can greatly reduce the levels of EGFR, leading to a downregulation of the ERK signaling pathway.

Materials and Methods

Cells

U-87 MG, U251 MG, U-118 MG, and SHG-44 human glioma cell lines were obtained from the Cell Bank of Chinese Academy of
cells were harvested, washed twice in PBS, and resuspended in 1×EDTA, centrifuged (300×g) for use in oral gavage. The cells were maintained in RPMI-1640, MEM, or DMEM (Gibco, Invitrogen) supplemented with 10% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified 5% CO₂/95% air atmosphere at 37°C.

Chemicals
Berberine chloride, hereinafter as berberine, and temozolomide were from Sigma-Aldrich. The structure of berberine is as shown in a review (9). Berberine was dissolved in DMSO for use in cell culture and in carboxymethylcellulose sodium (7 g/L) for use in oral gavage. MTT (3-[4, 5-dimethyl-2-yl]-2, 5-diphenyl tetrazolium bromide) and all other chemicals were of analytic grade and were also purchased from Sigma Chemical.

Cell viability assay
Cells were trypsinized and plated in 96-well cell culture plates at the concentration of 2×10⁴ cells per well. Twenty-four hours later, the medium was removed and replaced with fresh medium with or without berberine or temozolomide. Cell density was measured on day 1, 2, and 3 by using the MTT following the manufacturer’s instructions. The absorbance of converted dye is directly proportional to cell viability. All experiments were repeated at least three times.

Analysis of cell cycle and apoptosis by flow cytometry
Control and treated cells were harvested using 0.25% Trypsin–EDTA, centrifuged (300 × g), and washed once with cold PBS. The pellet was resuspended in ice-cold 70% ethanol and stored at −20°C. Samples were incubated with 20 μg/mL propidium iodide/0.1% Triton X-100 staining solution with 0.1 mg/mL RNase A. Cell-cycle distribution was determined using the BD Biosciences FACSCanto II Analyzer. At least 20,000 cells were counted in three independent samples to score the number of senescent cells.

Western blot analysis
Cell senescence was examined by SAβ-gal activity as described above. The samples were loaded on 6% SDS-PAGE and transferred to the membrane (Millipore) and blocked with 5% nonfat dry milk in TBS-Tween 20 (0.1%, v/v) for 1 hour at room temperature. The membrane was incubated with the appropriate horse-radish peroxidase secondary antibody (diluted 1:5,000; Amer sham Pharmacia Biotech) for 1 hour. Following several washes, the blots were developed by enhanced chemiluminescence (Millipore).

Plasmids and transfection
MKK2-CA (a constitutively active form of MKK2, ΔN4/S222E/S226D) was as previously described (15). The plasmids were transiently transfected into U87 and U251 cells using the Lipofectamine 2000 transfection reagent (Invitrogen) following the manufacturer’s protocol. The cells were stably selected with hygromycin B at a final concentration of 50 μg/mL. Cells stably expressing MKK2-CA were treated with berberine to determine the effect of MKK1/2–ERK1/2 signaling on cell senescence.

Small interfering RNA
EGFR siRNA duplex 1, 2, and 3 (cat. nos. 1523827, 1523828, and 1523829) and UNIV NEGATIVE CONTROL small interfering RNA (siRNA; #2) were synthesized (Sigma) and transfected into U87 and U251 cells using Lipofectamine 2000 for 24 hours. EGFR protein levels were determined by Western blotting analysis and cell senescence was examined by SAβ-gal activity as described above.

cDNA synthesis and real-time PCR
DNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. cDNA was synthesized by reverse transcription of 1 μg of total RNA with random hexamers. The total volume of reverse transcription reaction was 20 μL. Real-time quantitative reverse transcription PCR was performed using the LightCycler 480 sequence Detection System (Roche Applied Science). Human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified as an internal control. The levels of EGFR and GAPDH mRNA were measured by the SYBR Green I assay. EGFR was amplified by using the primers with the sequence 5'-AGGACAGCTAACAAGGATAC-3' (forward) and 5'-ATGAG-GCATAACCCGACCACC-3' (reverse). The GAPDH primer was 5'-CAGAACAATCATCTCGCTCTAC-3' (forward) and 5'-TGAGAG-TCAGAGGACACCCTGT-3' (reverse). The samples were loaded

Senescence-associated acidic β-galactosidase staining
A senescence β-Galactosidase Staining Kit purchased from Cell Signaling Technology was used. Four high-power fields per sample were counted in three independent samples to score the number of senescent cells.

Liu et al.
in quadruple, and the results of each sample were normalized to GAPDH.

**In vivo tumor xenograft study**
Six- to 8-week-old BALB/c nude mice (Vital River Laboratories) were anesthetized with phenobarbital sodium (60 mg/kg, i.p.). Approximately 1.5 × 10^5 U87 cells (>95% viability) in a volume of 3 μL were stereotactically injected into the right caudate nucleus: bregma (anatomic point on the mouse skull at which the coronal suture is intersected perpendicularly by the sagittal suture) 0.5 mm; lateral, 1.75 mm. The needle was initially advanced to a depth of 3.5 mm and then withdrawn to a depth of 3 mm to limit reflux up the needle tract during injection of cells. Mice were gavaged with the vehicle alone (control), or berberine (50 and 100 mg/kg body weight) daily, and euthanized after 5 weeks (n = 5–6 per group). Brains were fixed with 4% paraformaldehyde in phosphate buffered saline at 4°C overnight, dehydrated in 20% sucrose until the tissue sinks, embedded in OCT (Fisher), and then sectioned for hematoxylin and eosin (H&E) staining, immunofluorescence, senescence and TUNEL assay. Tumor volume was measured as L × W^2/2, where L is the length and W is the width. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Shandong University.

**Fluorescence immunohistochemistry**
Frozen sections (7 μm) were washed with PBS twice, and then blocked with 10% goat serum in PBS at 37°C for 1 hour, following which rabbit anti-Ki67 antibody (Cell Signaling Technology) or rabbit anti-EGFR antibody (Abcam) was added at a dilution of 1:400 and 1:300 in 5% bovine serum albumin in PBS respectively and incubated overnight at 4°C. Sections were then washed three times in PBS before incubating in the dark with a Rhodamine-labeled or FITC-labeled secondary antibody at a dilution of 1:300 in 5% bovine serum albumin in PBS for 60 minutes. The secondary antibody solution was then aspirated and the sections were washed four times in PBS. Sections then were incubated in the dark with DAPI (1 μg/mL) in PBS for 5 minutes and coverslips were mounted with an antifade solution (Molecular Probes). Slides were then examined on a fluorescence microscope. Negative controls were performed by omitting the primary antibodies.

**TUNEL assay**
TUNEL assay was performed using the In Situ Cell Death Detection Kit, TMR red (Roche) following the manufacturer’s recommendations. After labeling, the slides were counterstained with DAPI and visualized under a fluorescence microscope. As positive controls, frozen sections were treated with DNase (Life Technologies) at a concentration of 1.0 μg/mL and incubated for 10 minutes at room temperature to induce DNA strand breaks.

**Statistical analysis**
For each measurement, three or four independent experiments were performed. Results were expressed as mean ± SD. Statistical calculations were performed using the SigmaPlot 2000 software (Systat Software). Differences in measured variables between experimental and control groups were assessed using the t test. P < 0.05 was considered statistically significant. Statistical significance was also taken as *P* < 0.05 and **P* < 0.01.

**Results**
Berberine potently inhibits the proliferation of glioblastoma cells
The cytotoxic effects of berberine on four glioblastoma cell lines were first evaluated by the MTT cell viability assay. As shown in Fig. 1A, the cell viability was reduced in a dose-dependent and time-dependent manner in three of the four cell lines. Importantly, berberine possesses a more potent effect than temozolomide in the three cell lines. The IC_{50} values of temozolomide were at least 6-fold lower than those of temozolomide (Fig. 1B). SHG-44 cells appeared to be resistant to both berberine and temozolomide. Because its fluorescent nature, the intracellular accumulation of berberine can be visualized under fluorescence microscopy or detected by flow cytometry, and the cytotoxic effect of berberine depends on its intracellular accumulation (16). We observed that while the berberine-sensitive U87, U251, and U118 cells all exhibited remarkable intracellular accumulation of berberine, SHG-44 cells were devoid of berberine staining (data not shown), which may explain why the SHG-44 cells were more resistant to berberine than the other three cell lines.

Because MTT assay is based on MTT reduction by the mitochondrial succinate dehydrogenase (complex II), and temozolomide-resistant glioma cells tend to have increased activities of complexes II–III and IV in the electron transport chain (17), there is the possibility that the MTT assay may have underestimated the cytotoxicity of temozolomide. We, therefore, next used the EdUrd incorporation assay to compare the effects of berberine and temozolomide on glioma cells. As shown in Fig. 1C, berberine again showed a much more potent effect than temozolomide. Furthermore, flow cytometric analysis of cell-cycle distribution indicated that the berberine-treated glioblastoma cells were primarily arrested at G1–G0 phase (Fig. 1D). Taken together, our data suggest that berberine can potently arrest the proliferation of glioblastoma cells.

Berberine induces cellular senescence of glioblastoma cells
Berberine can induce apoptosis in a variety of cancer cells (12, 13, 18–20). We, therefore, determined whether berberine could also induce apoptosis of glioblastoma cells. We observed that the basal level of early apoptotic cells was approximately 1%. When treated berberine, there was hardly any increase in the percentage of apoptotic cells (Fig. 2A), suggesting that the antitumor effect of berberine on the glioblastoma cells tested in this study was not mediated by induction of apoptosis.

Glioblastoma cells were recently shown to undergo either cellular senescence or apoptosis in response to ionizing radiation (IR) and the switch between senescence and apoptosis is determined by PTEN (21). Lack of PTEN, as in U87 and U251 cells, renders glioblastoma cells resistant to IR-induced apoptosis, but predisposes them to cellular senescence. We, therefore, tracked the fates of U87 and U251 cells treated with berberine using SA-β-gal staining assay. As shown in Fig. 2B and C, berberine treatment led to foci of SA-β-gal positive cells, whereas the percentages of EdUrd–positive cells were greatly reduced 7 days after treatment with berberine (Fig. 2D), suggesting that very few of the berberine-treated cells were in S phase. Taken together, these results suggest
that induction of cellular senescence is the major mechanism by which berberine exerts its antitumor effect.

We previously reported that berberine may exert its antitumor effect by functioning as a genotoxicant, especially as an inducer of DNA double-strand breaks (DSB; refs. 12, 16). Because genotoxic stress can induce premature cellular senescence, we speculated that the induction of senescence by berberine might be mediated by DNA damage response. We
tested this notion by measuring the level of γ-H2AX, a marker of DSBs, in berberine-treated glioblastoma cells. However, while γ-H2AX-positive cells were common in U251 cells in response to berberine treatment, they were rare in berberine-treated U87 cells (Supplementary Fig. S1). This result suggests that DNA damage response may not be required during berberine-induced cellular senescence. This notion is substantiated by results shown below.

EGFR-RAF-MEK-ERK signaling pathway was inhibited by berberine

Amplification and/or point mutation of EGFR are one of the most common genetic alterations in glioblastoma. Gain of function of EGFR can lead to the upregulation of RAS and PI3K–AKT signaling pathways, and thereby drives cell survival and proliferation (22–24). We, therefore, tested whether EGFR-initiated pathways are altered in glioblastoma cells treated with berberine. Interestingly, in both U87 and U251 cells treated with berberine, we observed a significant reduction in the level of EGFR (Fig. 3). Furthermore, the levels of phosphorylated (active) form of RAF, MEK, and ERK were all decreased (Fig. 3). This result indicated that accompanying the declined level of EGFR, the RAF–MEK–ERK pathways was significantly downregulated in berberine-treated glioma cells. However, the PI3K–AKT pathway, the other pathway downstream EGFR, was not affected (Fig. 3). It should be noted that while the amount of EGFR was reduced by berberine at protein level, the mRNA level instead showed an increase (Supplementary Fig. S2), suggesting that the downregulation of EGFR by berberine was not due to decreased EGFR transcription.

Downregulation of the EGFR-RAF-MEK-ERK signaling pathway mediates berberine-induced senescence

We next determined whether the downregulation of EGFR is responsible for the induction of cellular senescence in berberine-treated cells. We first tested whether erlotinib, an inhibitor of EGFR, can also induce senescence in glioma cells. We noted that more than 60% of the U87 cells became senescent after they were treated with 10 μmol/L erlotinib for 7 days (Fig. 4A). Like berberine, erlotinib also failed to induce apoptosis in U87 cells (Supplementary Fig. S3). We also examined the fate of EGFR-knockdown U87 cells by SA-β-gal staining. As shown in Fig. 4B, EGFR knockdown in U87 cells also led to a great increase in the percentage of senescent cells. These results indicate that inhibition or downregulation of EGFR alone is sufficient to induce cellular senescence in U87 cells. They also substantiate that notion that the berberine can induce cellular senescence independent of its genotoxic effect. Furthermore, U0126, an MEK inhibitor, also induced senescence in U87 and U251 cells (Fig. 4C). We next obtained U87 and U251 cells that stably express MKK2-CA and tested whether those cells could resist berberine-induced senescence. As shown
in Fig. 4D, berberine-induced senescence was greatly attenuated in MKK2-CA cells. Correspondingly, inhibition of ERK activation by berberine was remarkably relieved in the presence of MKK2-CA. Together, these results indicate that berberine-induced senescence in glioma cells was mediated by the downregulation of the ERK–MEK–ERK signaling pathway.

**Berberine induces cellular senescence of glioblastoma xenografts**

The *in vitro* data shown above clearly demonstrated a potent antitumor effect of berberine against glioblastoma cells. We next determined whether berberine could inhibit the growth of glioma *in vivo*. For this purpose, U87 cells were inoculated into the right striatum of mouse brains and the tumor-bearing mice were administered with berberine (50 and 100 mg/kg body weight) daily for 5 weeks. Examination of brain sections revealed the presence of tumor mass in all 5 mice in the control group, in 4 out of 6 in the 50 mg/kg group, but only in 2 out of 6 in the 100 mg/kg group (Fig. 5A). Furthermore, the tumor volume was greatly reduced in berberine-treated cells and that pharmacologic inhibition or RNA interference of EGFR similarly was greatly reduced in berberine-treated cells. Because the cellular senescence induced by berberine could be rescued by introduction of constitutive active MKK, we conclude that the induction of cellular senescence in berberine-treated glioblastoma cells is likely mediated by the downregulation of the ERK–MEK–ERK signaling pathway. Because EGFR amplification is characteristic of classic subtype of glioblastoma multiforme (GBM) and sustains the proliferation of GBM (27–29), the effective downregulation of EGFR by berberine suggests that use of berberine could be considered in the treatment of GBM.

It was recently reported that glioblastoma cells may either undergo cellular senescence or apoptosis in response to ionizing radiation (IR) depending on the status of PTEN (21). Upon treatment with IR, the PTEN-deficient glioblastoma cells invariably underwent senescence, whereas those with wild-type PTEN all took the fate of apoptosis. Interestingly, the PTEN-deficient glioblastoma cells were not intrinsically resistant to apoptosis, because they could become apoptotic when treated with doxorubicin (21). Thus, the effect of berberine on GBM cells resembles that of IR, but differs from that of doxorubicin. However, although berberine can inflict DNA DSBs and thus mimic the effect of IR (12, 16), it should be pointed out that the DNA damage response pathway, including p53 activation, that usually results in cellular senescence or apoptosis is not required for the induction of senescence in berberine-treated GBM cells. This notion is supported by the fact that an increase in the level of DSBs was not detected in U87 cells treated with berberine at the concentration that induced cellular senescence. Although it can be argued that the p53 activation in GBM cells bearing DSBs may accelerate senescence, it does not appear to be required for berberine-induced senescence in GBM cells. Importantly, inhibition of EGFR alone, by RNAi or by erlotinib, could effectively induce senescence in GBM cells. Thus, our results indicated that the downregulation of EGFR alone might be sufficient for the induction of senescence in some GBM cells. Our findings are in contrast with the report that inhibition of EGFR could radiosensitize non–small cell lung cancer cells by inducing senescence in cells sustaining DSBs (30).

**Discussion**

Our results showed that berberine possesses a potent antitumor effect against some glioblastoma cells. It can effectively induce glioblastoma cells to undergo cellular senescence. Interestingly, while berberine has been reported to exert its cytotoxic effect by inducing apoptosis in numerous types of cancer cells (13, 16, 18, 19), none of the glioblastoma cell lines we tested in this study showed signs of increased apoptosis in response to berberine treatment. Therefore, induction of cellular senescence is probably the major mechanism by which berberine exerts its antitumor effect against glioblastoma cells. Indeed, induction of senescence has recently been recognized to be an important therapeutic strategy for various types of cancer (25, 26). We further showed that the level of EGFR was greatly reduced in berberine-treated cells and that pharmacologic inhibition or RNA interference of EGFR similarly induced cellular senescence of glioblastoma cells. Accompanying the downregulation of EGFR, the RAF–MEK–ERK signaling pathway downstream of EGFR was remarkably inhibited in berberine-treated cells. Because the cellular senescence induced by berberine could be rescued by introduction of constitutive active MKK, we conclude that the induction of cellular senescence in berberine-treated glioblastoma cells is likely mediated by the downregulation of the ERK–MEK–ERK pathway. Because EGFR amplification is characteristic of classic subtype of glioblastoma multiforme (GBM) and sustains the proliferation of GBM (27–29), the effective downregulation of EGFR by berberine suggests that use of berberine could be considered in the treatment of GBM.
Multiple signaling pathways, including PI3K–AKT and MEK–ERK, can be initiated from EGFR. Although we detected a downregulation of the MEK–ERK pathway in berberine-treated glioblastoma cells, the PI3K–AKT pathway, which is upregulated in U87 and U251 cells due to loss of PTEN, appeared to be unaffected by berberine. Interestingly, application of U0126, an MEK inhibitor, similarly induced senescence in GBM cells, suggesting that the downregulation of the MEK–ERK pathway is responsible for the induction of cellular senescence. We confirmed this notion by using GBM cells that express constitutively active MKK2 (MKK2-CA). Berberine-induced senescence was greatly attenuated in MKK2-CA cells. Correspondingly, inhibition of ERK activation caused by berberine was remarkably relieved in those cells. These findings establish the EGFR–RAF–MEK–ERK signaling pathway as the mediator of berberine-induced senescence in GBM cells.

Whereas the protein level of EGFR was greatly reduced in berberine-treated GBM cells and in xenografts, the mRNA level of EGFR even exhibited a modest increase, suggesting that downregulation of EGFR by berberine was not due to decreased transcription. It was recently reported that berberine could stimulate the activation of ubiquitin ligase Cbl and promote EGFR ubiquitylation and degradation in colon tumor cells (31).
Figure 5.
Efficacy of berberine against human glioma cells in vivo. U87 cells were inoculated into the right striatum of mouse brain (n = 5–6 per group). Mice were gavaged with the vehicle alone (Cont), berberine (50 and 100 mg/kg body weight) daily, and euthanized after 35-day treatment. Brains were fixed, frozen, and systematically sectioned throughout the tumor injection site. Frozen sections (7 µm) were obtained and immunohistochemical detection was performed. A, representative pictures of H&E staining of tumor sections from mice of three groups (x10). Arrows, edge of tumor. Scale bar, 1 mm. B, berberine-administered mice exhibited decreased tumor volumes. C, representative images of IHC showing the positive SA-β-gal staining of tumor sections from berberine-administered mice (x400). Scale bar, 20 µm. D, staining of transferase dUTP nick end labeling (TUNEL; x400). E and F, representative IHC images showing the cell proliferation marker Ki67 (E; x400) and EGFR (F; x1,000). DAPI was used for the nuclear staining. G, schematic model of signaling pathways in berberine-induced cell senescence.
Future studies may reveal whether berberine promotes EGFR degradation by a similar mechanism.

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

Authors’ Contributions
Conception and design: Q. Liu, Y. Gong, C. Shao
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References
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Qiao Liu, Xiuhua Xu, Minnan Zhao, et al.