Berberine-Induced Apoptosis in Human Glioblastoma T98G Cells Is Mediated by Endoplasmic Reticulum Stress Accompanying Reactive Oxygen Species and Mitochondrial Dysfunction

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Berberine has a wide range of biochemical and pharmacologic effects, including antitumor activity, but the mechanisms involved in berberine-induced apoptosis remain unclear. The purpose of the present study was to investigate the changes in oxidative stress and endoplasmic reticulum (ER)-related molecules, which are closely associated with cell death-signaling transduction pathways, in human glioblastoma T98G cells treated with berberine. Berberine significantly decreased the cell viability of T98G cells in a dose-dependent manner. Berberine increased the production of reactive oxygen species (ROS) and level of intracellular Ca2+. Berberine induced ER stress as evidenced by the detection of ER stress-associated molecules such as phosphorylated protein kinase-like ER kinase, eukaryotic translation initiation factor-2α, glucose-regulated protein 78/immunoglobulin heavy chain-binding protein, and CCAAT/enhancer-binding protein (C/EBP)-homologous protein/growth arrest and DNA damage-inducible gene 153, which was associated with the activation of caspase-3. Furthermore, the administration of the antioxidants, N-acetylcysteine and glutathione, reversed berberine-induced apoptosis. Berberine also markedly enhanced apoptosis in T98G cells through the induction of a higher ratio of Bax/Bcl-2 proteins, disruption of the mitochondrial membrane potential, activation of caspase-9 and -3, and cleavage of the poly(ADP-ribose) polymerase (PARP). The inhibition of ER stress using salubrinal led to an increased level of Bel-2, whereas the level of Bax, cleavage of procaspase-9 and -3, and PARP were decreased when compared with cells treated with berberine alone, indicating that berberine-induced apoptosis is associated with mitochondrial dysfunction. These results demonstrate that berberine induces apoptosis via ER stress through the elevation of ROS and mitochondrial-dependent pathway in human glioblastoma T98G cells.

Key words berberine; endoplasmic reticulum; glioblastoma; reactive oxygen species; mitochondria

Glioblastoma is the most serious malignant type of primary brain tumor.1) With its highly invasive phenotype, glioblastoma diffusely infiltrates into the various regions of the brain, making its total surgical resection impossible; therefore patients with glioblastoma have a poor prognosis, even in response to multidisciplinary treatment strategies including surgery, radiotherapy, and chemotherapy.2,3) The successful eradication of cancer cells through apoptosis is the ultimate aim of chemotherapy. Apoptosis is a process that plays an important role in the control of the growth and development of organisms, and the perturbation of apoptosis is considered to be a crucial strategy for cancer prevention and therapy.4,5)

The endoplasmic reticulum (ER) is extremely sensitive to changes that affect its structure, integrity, and function. Changes in Ca2+ homeostasis, inhibitors of protein disulfide bond formation, oxidative stress, and inhibition of protein synthesis and folding, resulting in unfolded or misfolded proteins.5—9) The cell responds by initiating a cascade of quality control signaling mechanisms (termed “ER stress response”) that restore normal ER function.7) Several of these signaling molecules, which include the glucose-regulated protein family of chaperone proteins, also play critical roles in cytoprotection by triggering survival signals.5) Cancer cells adapt to their local cellular environments by triggering protective ER stress responses.9) Therefore, approaches to downregulate the protective ER stress response in cancer cells may significantly improve treatment outcome.6,8)

Berberine, a type of alkaloid, was initially isolated from a Chinese herbal medicine plant and has long been used as a broad-spectrum antibiotic.10) It is found in the root, rhizome, and stem bark of a number of important medicinal plants, including Berberis vulgaris (barberry), Berberis aquifolium (Oregon grape), Berberis aristata (tree turmeric), and Tinospora cordifolia.10,11) Berberine has been found to possess a wide variety of pharmacologic and biological activities such as antimicrobial, antihelmintic, antiinflammatory, and antioxidative effects.12—14) In recent years, berberine has also been examined for anticancer activity, following evidence of its antineoplastic nature. However, the mechanisms involved in berberine-induced apoptosis remain unclear. Therefore, we focused on the effects of berberine on human glioblastoma, the incidence of which has extensively increased worldwide and has become one of the most frequently observed malignant neoplasia. The purpose of the present study was to investigate the changes in oxidative stress and ER-related molecules, which are closely associated with cell death-signaling transduction pathways in human glioblastoma T98G cells treated with berberine.

MATERIALS AND METHODS

Chemical Reagents and Antibodies Minimal essential medium (MEM) and fetal bovine serum (FBS) were purchased from Gibco Life Technologies (Gaithersburg, MD, U.S.A.). Trypsin, streptomyacin, penicillin, N-acetylcysteine (NAC), glutathione (GSH), and berberine were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Salubrinal was purchased from Calbiochem (San Diego, CA,
U.S.A.). Rhodamine 123, 4′,6-diamidino-2-phenylindole (DAPI), and 5′,6′-tetrachloro-1′,3′,3′-tetraethylbenzimidazol-carbocyanineiodide (JC-1) were obtained from Molecular Probes (Eugene, OR, U.S.A.). The antibodies used in this study were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.). All chemicals were of the highest commercially available grade.

**Cell Culture** Human glioblastoma T98G cells were obtained from the Korean Collection for Type Cultures (KCTC) and cultured as monolayers in MEM supplemented with 10% heat-inactivated FBS and penicillin/streptomycin 100 µg/ml. Cells were maintained in an incubator with a humidified atmosphere of 5% CO₂ at 37 °C. All experiments were carried out 24 h after cells were seeded onto plates or dishes.

**Assessment of Cell Viability** Cell viability was measured in the crystal violet staining assay. In brief, cells were treated with varying concentrations of berberine (0, 50, 100, 150, 200 µg/ml). Exposure of the cell cultures was stopped after 24 h, the cells were stained with crystal violet solution (0.5% crystal violet in a 30% ethanol and 3% formaldehyde solution) for 15 min. The amount of crystal violet bound to the cells was dissolved with 1% sodium dodecyl sulfate (SDS) solution and optical densities (OD) were measured at 595 nm in a microplate reader (Molecular Devices Co., Sunnyvale, CA, U.S.A.). The OD in control cells was taken as 100% viability.

**Measurement of Reactive Oxygen Species Using Flow Cytometry** Cells were seeded overnight in 6-well plates in the presence or absence of berberine, and incubated with 10 µM of the fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCF-DA) for 30 min. After removing the culture supernant, the adherent cells were trypsinized and collected. After washing twice with phosphate-buffered saline (PBS, pH 7.4), the intensity of DCF-DA fluorescence was then determined using a FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA, U.S.A.). The OD in control cells were then incubated with 5 µg/ml of 4′,6-diamidino-2-phenylindole (DAPI) and observed with a laser scanning confocal microscope (Olympus Fluoview). The green fluorescence from the JC-1 monomer (with a 515-nm barrier filter) and red fluorescence from the aggregated form of JC-1 (with a 590-nm barrier filter) were visualized simultaneously.

**Western Blotting Analysis** Cytosolic protein extracts were prepared according to the previously described method. Brieﬂy, cells were collected by centrifugation at 3000×g for 5 min at 4 °C and washed with cold PBS. The cell pellet was resuspended in 500 µl of lysis buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES)-KOH 20 mM, pH 7.5, sucrose 250 mM, mannitol 70 mM, MgCl₂ 1.5 mM, KCl 10 mM, leupeptin 10 µg/ml, and digitonin 10 µg/ml). After incubation for 10 min at 25 °C, the sample was centrifuged at 14000×g for 15 min, and the supernatant containing cytosolic proteins was stored at −70 °C until use. The protein extract was subjected to standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto polyvinylidene difluoride membranes (Millipore), and probed with appropriate antibodies as described in the figure legends. The bound primary antibody was detected using an appropriate horseradish peroxidase-conjugated secondary antibody, and the reaction band was visualized using an enhanced chemiluminescence detection kit. Western blot analysis was performed using primary antibodies against PKR-like ER kinase (PERK), eukaryotic translation initiation factor-2α (eIF2α), glucose-regulated protein 78/immunoglobulin heavy chain-binding protein (GRP78/Bip), CCAAT/enhancer-binding protein (C/EBP)-homologous protein/growth arrest and DNA damage-inducible gene 153 (CHOP/GADD153), Bax, procaspase-9, procaspase-3, poly(ADP-ribose) polymerase (PARP), and β-actin (Santa Cruz Biotechnology) at the optimal dilution. The β-actin was used as an internal control to confirm the equal loading of proteins.

**Detection of Intracellular Ca²⁺ Concentration** The intracellular Ca²⁺ levels in T98G cells were determined using flow cytometry (Becton Dickinson FACS Calibur) with the Indo 1/AM loading solution (Calbiochem). Cells were treated with berberine (150 µg/ml) for 24 h, harvested, and washed twice. One group of cells as analyzed for apoptosis, and the other group was resuspended in Indo-1/AM (3 µg/ml), incubated at 37 °C for 30 min, and analyzed using flow cytometry.

**Measurement of Mitochondrial Membrane Potential** The loss of mitochondrial membrane potential (MMP) was monitored with the dye JC-1. The cells were treated with berberine, or berberine (150 µg/ml) and salubrinal (10 µM) for 24 h, and then incubated with JC-1 (10 µg/ml) at 37 °C for 15 min. After washing with PBS, the cells were resuspended in PBS and observed with a laser scanning confocal microscope (Olympus Fluoview). The green fluorescence from the JC-1 monomer (with a 515-nm barrier filter) and red fluorescence from the aggregated form of JC-1 (with a 590-nm barrier filter) were visualized simultaneously.

**DAPI Staining** The changes in nuclear morphology of DAPI-stained cells were examined using fluorescence microscopy. The monolayer of cells was fixed in PBS containing 3.7% paraformaldehyde for 15 min at room temperature. After fixation, the cells were washed three times with PBS and then incubated with DAPI (1 µg/ml) in PBS for 15 min at room temperature. After three washes, apoptotic nuclei were detected under ×200 magnification using a fluorescent microscope with a 340/380-nm excitation filter.

**Statistical Analysis** The data shown are a summary of the results from at least three independent experiments and are presented as the means±standard deviation (S.D.). A statistical evaluation of the results was performed with one-way analysis of variance (ANOVA). The results were considered significant at a value of p<0.01.

**RESULTS**

**Effects of Berberine on Cell Viability** To assess the effect of berberine on cell viability, T98G cells were cultured with a range of berberine doses (0, 50, 100, 150, 200 µg/ml) for 24 h. Cell viability was measured in the crystal violet staining assay. A significant reduction in cell viability was observed in a dose-dependent manner, and cell viability ranged from 25—61% after 24 h of berberine treatment. The IC₅₀ value of berberine was determined to be 160 µg/ml (Fig. 1).

**Effects of Berberine on Reactive Oxygen Species and Intracellular Ca²⁺ Levels** To determine whether treatment of T98G cells with berberine is associated with the generation of reactive oxygen species (ROS), T98G cells were incubated with DCF-DA. As shown in Fig. 2A, compared with control cells, treatment with 150 µg/ml of berberine for 18 h markedly increased ROS generation. Ca²⁺ is an important element in the human body and is present in cells and body fluids. Under normal conditions, the concentration of intracellular Ca²⁺ is much lower than that of extracellular Ca²⁺.
Free intracellular Ca\(^{2+}\) is involved in signaling pathways and correlates with early apoptotic signals. Ca\(^{2+}\) is an important messenger molecule in apoptosis,\(^{17}\) and there is a close relationship between Ca\(^{2+}\) and ROS in signal transduction pathways. Therefore, the induction of apoptosis by berberine was investigated to determine if it occurred via the increase in intracellular Ca\(^{2+}\). To accomplish this, T98G cells were incubated with berberine and the change in intracellular Ca\(^{2+}\) was measured using flow cytometry. As shown in Fig. 2B, treatment of T98G cells with berberine 150 \(\mu\)g/ml for 12 h led to a marked increase in the intracellular Ca\(^{2+}\) level. The effects of the antioxidants NAC and GSH on the increase in intracellular Ca\(^{2+}\) level in berberine-treated T98G cells were further examined. As expected, pretreatment of cells with NAC (10 mM) and GSH (10 mM) markedly decreased the levels of intracellular Ca\(^{2+}\).

**Effects of Antioxidants on Berberine-Induced Cytotoxicity and ER Stress**

The effects of antioxidants (NAC and GSH) on the berberine-induced cytotoxicity of T98G cells were further investigated. Cells were pretreated with either NAC (10 mM) or GSH (10 mM), followed by the addition of berberine 150 \(\mu\)g/ml for 12 h, and viability was measured using the crystal violet staining assay. As expected, the treat-
Treatment of T98G cells with berberine resulted in a significant decrease in cell viability (52% of control cells). However, pretreatment of cells with either NAC or GSH significantly increased the viability of berberine-treated cells. The data represent the mean ± S.D. of three independent experiments. *p < 0.01 compared with control cells, **p < 0.01 compared with berberine alone.

Effects of Berberine on Expression of Bcl-2, Bax, Caspases, and PARP and Inhibition of ER Stress by Salubrinal

Western blot analysis showed that treatment of T98G cells with berberine (150 μg/ml) for 24 h resulted in a marked reduction in the expression of Bcl-2 and an increase in the levels of Bax and cleaved PARP when compared with control cells not treated with berberine. Inhibition of ER stress by salubrinal (10 μM) led to the increased expression of Bcl-2 and the decreased expression of Bax and cleaved PARP. Western blot analysis showed that the treatment of T98G cells with berberine (150 μg/ml) for 24 h resulted in a marked reduction of procaspase-9 and procaspase-3 when compared with cells treated with salubrinal (10 μM).
reversible change in fluorescence emission from green to red as the MMP increases. Cells with high membrane potential promote the formation of dye aggregates (red fluorescence), and cells with low membrane potential contain monomeric JC-1 (green fluorescence). To investigate the loss of MMP during the early phase of apoptosis induced by berberine, cells were stained with JC-1 and monitored with a fluorescent microscope. As shown in Fig. 6, JC-1 was accumulated in untreated control cells, indicating a high membrane potential, which was comparable to the anti-apoptotic effect of salubrinal. In contrast, JC-1 was poorly accumulated in berberine-treated cells, indicating the disruption of MMP.

**Nuclear Morphology** To define the mode of cell death induced by berberine treatment, the nuclear morphology and DNA fragmentation of T98G cells were examined. After T98G cells were treated with berberine 150 μg/ml, their nuclear morphology was examined with DAPI staining. In contrast to the control group, the cells treated with berberine showed characteristic apoptotic phenotypes including heterogeneous staining, chromatin condensation, and DNA fragmentation under fluorescence microscopy (Fig. 7).

**DISCUSSION**

In the present study, the results demonstrated that berberine, a naturally occurring isoquinoline alkaloid, significantly decreased the viability of T98G cells (Fig. 1). Although the IC50 value of berberine on T98G cells was 160 μg/ml, which was a relatively high concentration, these observations suggest that berberine may be an effective chemotherapeutic agent against malignant glioma cells. Further studies were performed to elucidate the mechanism underlying the reduction in cell viability and the induction of cell death in T98G cells after berberine treatment. Previously, the inhibition of cell proliferation or induction of cell death in T98G cells by berberine was shown to be the result of the induction of G1 phase arrest and subsequent apoptotic processes. In this study, the evidence showed that berberine decreased the percentage of viable T98G cells through the induction of apoptosis via ER stress and the ROS-associated mitochondrial-dependent pathway.

Apoptosis is regulated by intracellular Ca2+ and ROS. ROS are highly reactive molecules that have the potential to cause cellular damage. Increases in intracellular Ca2+ levels lead to the activation of ROS and are commonly involved in chemical-induced apoptosis. Calcium is considered one of the second messengers strongly involved in ER stress signaling. In this study, treatment of T98G cells with berberine resulted in markedly increased levels of intracellular Ca2+ for 12 h and ROS for 18 h. The majority of Ca2+ remains in the ER, but ER stress will lead to the release of Ca2+ from the ER. Moreover, pretreatment of cells with either NAC or GSH markedly decreased the levels of intracellular Ca2+, suggesting that intracellular ROS were directly involved in the cytotoxic action of berberine (Fig. 2). These data indicate that berberine induces ER stress and the subsequent release of Ca2+ in T98G cells and this may cause the production of ROS in T98G cells.

The ER is a central organelle engaged in lipid synthesis, and protein folding and maturation. Due to the presence of a high concentration of protein, numerous chaperones exist in the ER which maintain proteins in a folding-competent state and prevent protein-folding intermediates from aggregating. However, a variety of toxic conditions including hypoxia, nutrient deprivation, protein synthesis failure, folding, transport, or degradation, and Ca2+ overload can disrupt the normal function of the ER, leading to the accumulation and aggregation of unfolded proteins in the ER. ER abnormalities are collectively called ER stress. There is increasing evidence that ER stress plays a crucial role in the regulation of apoptosis. That is, ER stress triggers several specific signaling pathways including ER-associated protein degradation and the unfolded protein response (UPR). In mammals, three ER transmembrane proteins, including inositol-requiring protein 1, activating transcription factor 6 (ATF6), and PERK may sense ER stress and eventually activate transcription factors for the induction of ER chaperones such as...
GRP78/Bip or eIF2α to inhibit the synthesis of new proteins.18,26] These processes form the UPR. The primary effects of UPR activation are designed to protect the ER, but they also serve to limit the damage to other organelles and protect the organism by eliminating cells undergoing prolonged stress.2,23] The UPR triggers apoptosis in cells that are unable to adapt to conditions of severe ER stress.2,26] In vitro data indicated that the treatment of T98G cells with berberine causes ER stress, as evidenced by elevation in the levels of GRP78/Bip and CHOP/GADD153, and the phosphorylation of PERK and eIF2α (Fig. 3).

The members of the Bcl-2 family of proteins are important regulators of apoptotic cell death. Although the involvement of Bcl-2 proteins in ER-stress-induced cell death is clear, the mechanism by which they are regulated by ER stress is less well understood. Until recently, Bcl-2 proteins were thought to regulate the mitochondrial-mediated apoptotic pathway exclusively. The first reports linking ER stress-induced cell death to the Bcl-2 family of proteins showed that overexpression of Bcl-2 or the deficiency of Bax and Bak conferred protection against lethal ER stress.28] Stress signals are relayed from the ER to mitochondria, and ER stress induced apoptosis, similar to mitochondrial-mediated apoptosis, is also regulated by the Bcl-2 family of proteins.29,30] The ratio of Bax/Bcl-2 is critical for the induction of apoptosis because it determines whether cells will undergo apoptosis.30] ER stress inducers including cellular stress inducers have also been shown to induce a change in the conformation of Bax, resulting in its accumulation on the mitochondria, and to induce the release of cytochrome c from the mitochondria into the cytosol.8,31] Cytosolic cytochrome c then binds to Apaf-1, leading to the activation of caspase-3 and PARP.32] The present results also indicate that the reduced expression of antiapoptotic Bcl-2 protein and the increased expression of the pro-apoptotic Bax protein facilitate the berberine-mediated cell death of T98G cells (Fig. 5A), which increases the ratio of Bax/Bcl-2. This may be responsible for the concomitant execution phase of apoptosis observed in these cells, which included the disruption of the mitochondrial membrane (Fig. 6A). As the level of cytochrome c increases in the cytosol, it interacts with Apaf-1, and ATP forms a complex with procaspase-9, leading to the activation of procaspase-9 and caspase-3.33] Activated caspase-3 is the key executioner of apoptosis and cleaved caspase-3 leads to the cleavage and inactivation of key cellular proteins such as PARP.33] The present results revealed that the treatment of T98G cells with berberine led to the activation of caspase-9, caspase-3, and PARP (Fig. 5B). Salubrinal was identified as a selective inhibitor of phosphatasets that act on eIF2α,20] thereby maintaining protein phosphorylation and offering protection from the adverse effects of ER stress. Inhibition of ER stress in T98G cells by salubrinal led to the increased expression of Bcl-2, procaspase-9, and procaspase-3, and the decreased expression of Bax and PARP (Fig. 5). These results suggest that the balance between anti-apoptotic Bcl-2 and pro-apoptotic Bax modulates ER/mitochondrial-dependent cell survival vs. death. Since DNA damage is a feature of apoptotic cell death, DNA damage using DAPI staining was further confirmed (Fig. 6B).

In the present study, berberine-induced apoptosis of T98G cells was mediated through the generation of ROS and intracellular Ca²⁺, ER stress, enhanced expression of Bax, disruption of the MMP, activation of caspase-9 and caspase-3, and cleavage of PARP. Further in vivo studies are required to determine the possibility that berberine could be an effective chemotherapeutic agent for the management of malignant gliomas.

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REFERENCES