Involvement of reactive oxygen species and caspase-dependent pathway in berberine-induced cell cycle arrest and apoptosis in C6 rat glioma cells

TING-CHING CHEN1, KUANG-CHI LAI2,3, JAI-SING YANG4, CHING-LUNG LIAO5, TE-CHUN HSIA6, GUANG-WEI CHEN7, JEN-JYH LIN8, HUI-JU LIN9, TSAN-HUNG CHIU10, YIH-JING TANG11 and JING-GUNG CHUNG1,12

1Department of Biological Science and Technology, 2School of Medicine, China Medical University, Taichung; 3Department of Surgery, China Medical University Beigang Hospital, Beigang, Yunlin; 4Department of Pharmacology, 5Graduate Institute of Chinese Medical Science, China Medical University; 6Department of Internal Medicine, China Medical University Hospital, Taichung; 7Department of Traditional Chinese Medical, Chung-Ho Memorial Hospital, Kaohsiung Medical University, Kaohsiung; 8Division of Cardiology, Departments of 9Ophthalmology and 10OBS/GYN, China Medical University; 11Family Medicine Department, Taichung Veterans General Hospital; 12Department of Biotechnology, Asia University, Wufeng, Taichung, Taiwan, R.O.C.

Received January 9, 2009; Accepted March 12, 2009

DOI: 10.3892/ijo_00000299

Abstract. The cytotoxicity of berberine on C6 rat glioma cells indicated that berberine induced morphological changes and caused cell death through G2/M arrest and apoptosis. While undergoing apoptosis, there was a remarkable accumulation of G2/M cells with the upregulation of Wee1 but it also inhibited cyclin B, CDK1 and Cdc25c that led to G2/M arrest. Along with cytotoxicity in C6 cells, several apoptotic events including mitochondrial cytochrome c release, activation of caspase-9, -3 and -8 and DNA fragmentation were induced. Berberine increased the levels of GADD153 and GRP 78 in C6 cells based on the examination of Western blotting and this is a major hallmark of endoplasmic reticulum (ER) stress. We also found that berberine promoted the production of reactive oxygen species and Ca2+ in C6 cells. Western blotting assay also showed that berberine inhibited the levels of anti-apoptotic protein Bcl-2 but increased the levels of pro-apoptotic protein Bax before leading to a decrease in the levels of mitochondrial membrane potential (Δϕm) followed by cytochrome c release that caused the activations of caspase-9 and -3 for apoptotic occurrence. The caspase-8, -9 and -3 were activated by berberine in C6 cells based on the substrate solution (PhiPhiLux-G,D1, CaspaLux 8-L,D2, CaspaLux 9-M,D for caspase-3, -8 and -9, respectively) and analyzed by flow cytometer and each inhibitor of caspase-8, -9 and -3 led to increase the percentage of viable C6 cells after exposure to berberine. This finding was also confirmed by Western blot assay which showed that berberine promoted the active form of caspase-8, -9 and -3. These results demonstrate that the cytotoxicity of berberine in C6 rat glioma cells is attributable to apoptosis mainly through induced G2/M-arrested cells, in an ER-dependent manner, via a mitochondria-dependent caspase pathway regulated by Bax and Bcl-2.

Introduction

Anti-cancer agents cause anti-cancer activities through cell death by i) interfering with the processes of the cell cycle and ii) through apoptosis. It still remains to be confirmed whether or not chemotherapeutic agents induce apoptosis in cancer cells, and what is the determinant of the response of tumor cells to these agents. Glioma tumor, one of the most threatening brain malignant cancers, is sensitive to anti-cancer chemotherapeutic reagents through either interfering with the cell cycle or causing apoptosis. Therefore, the best strategies for chemoprevention or chemotherapy are to induce apoptosis in cancer cells. It was reported that the mitochondrial production of reactive oxygen species (ROS) seems to play a role in cell death (1) and it also known that the exogenous and endogenous ROS, such as H2O2 and O2-, respectively, cause apoptosis through mitochondrial permeability transition. Other investigators also point out that some of the anti-cancer drugs can induce the generation of ROS in apoptosis (2,3) and induce apoptosis through a direct mitochondrial effect leading to the activation of caspases (4).

Many cancer treatment drugs are obtained from natural plant sources and ~74% of these chemotherapeutic drugs were...
discovered by investigating a folklore claim (5,6). Berberine, an isoquinoline alkaloid, is present in the roots, rhizome and stem bark of Berberis aquifolium, Berberis vulgaris and Berberis aristata (7). Berberine has been reported to exhibit a wide range of biological activities such as anti-diarrheal, anti-arrhythmic and anti-tumor activities (7-10). Berberine also inhibits cyclooxygenase-2 transcriptional activity in human colon cancer cells (11,12), inhibits tumor promoting activity of teleocidin in two-stage chemical carcinogenesis on mouse skin (13), inhibits RNA topoisomerase I and II in a biochemical system (14,15) and induces cell cycle arrest and apoptosis in human epidermoid carcinoma A431 cells (16). Our previous studies showed that berberine inhibits WEHI-3 leukemia cells in vivo (17), induces apoptosis in human HSC-3 oral cancer cells via simultaneous activation of the death receptor-mediated and mitochondrial pathway (18) and induces down-regulation of matrix metalloproteinase-1, -2 and -9 in human gastric cancer cells (SNU-5) in vitro (19). However, there is no information to address berberine-induced apoptosis in C6 rat glioma cells. Therefore, we investigated the inhibition of growth, proliferation and apoptosis induction in C6 cells. Our study also provides insight into the mechanism of berberine-induced apoptosis in C6 cells.

Materials and methods

Chemicals and reagents. Berberine, dimethyl sulfoxide (DMSO) and propidium iodide (PI) were purchased from Sigma Chemical (St. Louis, MO). The primary antibodies were obtained as follows: antibodies for Fas/CD95, Bax, Bcl-2, caspase-8, -9 and -3, pro-caspase-8, -9 and -3, GADD153, GRP 78, pro-caspase-12 and β-actin were purchased from Cell Signaling Technology (Beverly, MA, USA); antibodies for cytokeratin c, Cyclin B, CDK1, Wee1, Cdc25c and the secondary antibodies, which were horseradish peroxidase (HRP)-linked goat anti-mouse IgG and goat anti-rabbit IgG, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The protein assay kit was purchased from Bio-Rad (Hercules, CA, USA).

Cells, culture conditions and treatments. C6 rat glioma cells were purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan, R.O.C.). The culture medium consisted of KGM's modification of Ham's F-12 medium (KMHF) (Gibco Life Technologies, Gaithersburg, MD, USA), 10% fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY, USA), 1% penicillin-streptomycin (100 U/ml penicillin and 100 μg/ml streptomycin). C6 cells were cultured as monolayers in KMHF medium and maintained in an incubator with a humidified atmosphere of 95% air and 5% CO2 at 37°C. In all treatments, berberine was initially dissolved in a small amount of DMSO and made up to the maximum final concentration of 0.2% (v/v) in the complete cell culture medium. The sub-cultured cells were treated with either varying concentrations of berberine or vehicle alone (0.2% DMSO in media) that served as control (20-22).

Assessment of cell morphology and viability. Approximately 2x10^5 cells/well of C6 cells were plated onto 12-well plates in KMHF culture medium and incubated at 37°C for 24 h then treated individually with 0, 50, 500 and 500 μM berberine and incubated for 24 h or treated with 100 μM berberine for 0, 12, 24, 48 and 72 h. Vehicle alone (DMSO) was used as control. For cell morphological examination, cells onto the plate were directly examined and were photographed under a phase-contrast microscope. For percentage of cell viability, cells were collected, PI was added and then they were counted by flow cytometric analysis, as previously described (20,21).

Determination of cell cycle and apoptosis by flow cytometry. Approximately 2x10^5 cells/well of C6 cells were grown in a 12-well plate in KMHF culture medium for 24 h then treated individually with 100 μM berberine and cells incubated at 37°C, 5% CO2 and 95% air for 0, 12, 24, 48 and 72 h. For cell cycle arrest with sub-G1 (apoptosis), isolated cells were fixed by 70% ethanol then kept at 4°C overnight and then re-suspended in PBS containing 40 μg/ml PI and 0.1 mg/ml RNase and 0.1% Triton X-100 in dark room for 30 min at 25°C. The cells were analyzed for the cell cycle distribution, then determined using FACScalibur instrument (BD Biosciences, San Jose, CA, USA) equipped with CellQuest 3.3 software. The cell cycle with sub-G1 was then determined and analyzed (20,21,23).

Comet assay for DNA damage. The Comet assay was used for examining the DNA damage and was done according to the procedures of Wang et al (23) with some modifications. Approximately 2x10^5 cells/well of C6 cells were grown in a 12-well plate in KMHF culture medium for 24 h then then treated individually with 0, 50, 100 and 200 μM berberine and/or 0.5% H2O2 then cells incubated at 37°C, 5% CO2 and 95% air for 24 h. Cells were harvested by centrifugation for the examination of DNA damage using the Comet assay as described elsewhere (23,24).

DAPI staining for apoptosis. Approximately 2x10^5 cells/well of C6 cells were grown in a 12-well plate in KMHF culture medium for 24 h then treated individually with 0, 50, 100 and 200 μM berberine and cells incubated at 37°C, 5% CO2 and 95% air for 48 h. Cells were harvested from each sample for 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining, as described previously (9). After staining, the cells were examined and were photographed under a fluorescein microscope (20,21).

DNA laddering fragmentation. Approximately 1x10^6 cells/well of C6 cells were grown in 6-well plates and treated with 100 μM berberine for 0, 3, 6, 12, 24, 48 and 72 h. DNA was isolated from each sample and examined in 0.8% agarose gel electrophoresis, then photographed under a fluorescence microscope, as described previously (25).

Reactive oxygen species (ROS), Ca2+ concentrations and mitochondrial membrane potential (ΔΨm) determinations. Approximately 2x10^5 cells/well of C6 cells were grown in a 12-well plate in KMHF culture medium for 24 h then either pretreated or not with NAC, treated individually with 100 μM berberine and incubated at 37°C, 5% CO2 and 95% air for 0,
0.25, 0.5, 2 and 4 h to detect the changes of ROS, Ca^{2+} concentrations and ΔΨ_m. Cells were harvested by centrifugation then re-suspended in 500 μl of DCFH-DA (10 μM) for ROS determination, re-suspended in 500 μl of Indo 1/AM (3 μg/ml) for Ca^{2+} concentrations and suspended in 500 μl of DiOC6 (4 μmol/l) for incubation at 37˚C for 30 min and analyzed by flow cytometry (18,20,21).

Figure 1. The effects of berberine on the morphological changes and percentage of viable C6 cells. Cells were plated in KMHF medium + 10% FBS with various concentrations of berberine for 24 h, or treated with 100 μM berberine for 0, 12, 24, 48 and 72 h and the morphological changes were examined under phase contrast microscope (a). Total percentage of viable cells dose-dependent (b) and time-dependent (c) were determined by flow cytometry. Each point is mean ± SD of three experiments. ***P<0.001.

Caspase-3, -8 and -9 activity determination by flow cytometer. Approximately 2x10^5 cells/well of C6 cells were grown in a 12-well plate in KMHF culture medium for 24 h then treated individually with 100 μM berberine and incubated at 37˚C, 5% CO₂ and 95% air for 0, 24, 48 and 72 h. Cells were harvested from each sample by centrifugation and 50 μl of each substrate solution (PhiPhiLux-G, CaspaLux 12-L1, CaspaLux 9-M, D2 for caspase-3, -8 and -9, respectively) was added to the cell pellet (1x10^5 cells per sample) and cells were incubated at 37˚C for 60 min in the dark, washed twice with 1 ml of ice-cold PBS and re-suspended in 1 ml fresh PBS, as described previously (9,10).
Caspase-3, -8 and -9 activities were determined and analyzed according to the manufacturer’s instructions (20,21,24).

Examination by Western blotting of cell cycle and apoptosis-associated proteins. Approximately 5x10^5 cells/well of C6 cells were grown in a 12-well plate in KMHF culture medium for 24 h then treated individually with 100 μM berberine and cells incubated at 37˚C, 5% CO2 and 95% air for 0, 6, 12, 24, 48 and 72 h. Cells were harvested from each sample by centrifugation for the total protein determination, and for Western blotting examination. Cyclin B, CDK1, Wee1 and Cdc25c, Fas/CD95, pro-caspase-8 and -3, cytochrome c, pro-caspase-9, Bcl-2 and Bax, GADD153, GRP 78 and pro-caspase-12 expression levels were examined using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting, as described previously (19-22).

Results

The effects of berberine on the morphological changes and the percentages of C6 rat glioma cells. In order to examine the cytotoxic effects of berberine, C6 cells were treated with 0, 50, 100, 200 and 500 μM berberine for 24 h or treated with 100 μM berberine for 0, 12, 24, 48 and 72 h and both cell morphological changes and cell death were assayed. The results indicated that berberine induced cell morphological changes and cell death at >50 μM and this effect is dose- and time-dependent (Fig. 1a). Based on these pilot observations, berberine caused strong cell morphological changes (Fig. 1a) and cell death induction mostly dose- (Fig. 1b) and time-dependent (Fig. 1c) in the examined time periods. We selected the 100 μM dose and next assessed whether the growth-inhibitory and cell death effects of berberine are accompanied by an effect on cell cycle progression and/or apoptotic cell death.

The effects of berberine on the cell cycle and sub-G1 group of C6 rat glioma cells. Berberine showed statistically significant cell cycle arrest at 100 μM berberine following its treatment for 0, 12, 24, 48 and 72 h (Fig. 2a and b). At 12, 24, 48 and 72 h-treatment intervals, compared with DMSO controls, it caused an arrest of G2/M phase (5 vs. 2, 22, 36 and 34% P=0.001, respectively), which were at the expense of a strong decrease in S-phase cell population (Fig. 2b). In cell cycle and sub-G1 studies assessing whether berberine also causes apoptotic cell death, as shown in Fig. 2a, the treatment at 100-500 μM dose for 24-72 h resulted in a strong and statistically significant apoptotic cell death. Based on the sub-G1, it appears that increased dose of berberine led to
increase percentage of sub-G1 and this indicates that it increased the percentage of apoptosis (Fig. 2a) and these effects were time-dependent. Collectively, the results shown in Fig. 2 clearly show the cytotoxic effects of berberine on C6 cell growth inhibition and death, as well as cell cycle arrest and induction of apoptosis.

**Berberine-induced DNA damage and apoptosis in C6 rat glioma cells.** Although other studies have shown that berberine induced DNA damage in some cell lines, here, in order to examine whether or not berberine induced DNA damage in C6 cells, Comet assay was used and results indicated that berberine induced DNA damage based on the DNA damage tail production which is shown in Fig. 3a. Increasing the dose of berberine led to an increase of DNA damage and those effects were dose-dependent. To determine whether cytotoxic effect of berberine was due to apoptosis induction, the membrane blebbing and DNA ladder were observed in C6 cells treated with the examined dose of berberine and photographed under a fluorescence microscope. As shown in

Figure 3. Berberine-induced DNA damage and apoptosis in C6 cells. Cells were incubated with various concentrations of berberine and 0.5% H$_2$O$_2$ for 24 h. DNA damage was determined using Comet assay (a) and apoptotic cells were determined by DAPI staining (b) and DNA gel electrophoresis (c) by fluorescence microscopy.
Fig. 3b and c, the photographs from DAPI staining and DNA gel electrophoresis assays revealed that apoptotic cells were observed in berberine-treated C6 cells compared with intact control cells and this effect was dose-dependent (Fig. 3b and c).

Berberine affected the levels of reactive oxygen species (ROS) and Ca\(^{2+}\) and mitochondria membrane potential (Δψ\(_{m}\)) in C6 cells. In order to examine whether or not berberine induced DNA damage and apoptosis in C6 cells due to the effects of ROS, Ca\(^{2+}\) and Δψ\(_{m}\), C6 cells were pre-exposed with NAC, then treated with 100 μM berberine for various time periods. ROS production was analyzed and quantified by flow cytometry. The data demonstrated that berberine induced ROS production quite early and time-dependently (Fig. 4a) and pretreatment with NAC decreased the ROS after treatment with berberine. The data demonstrated that berberine induced Ca\(^{2+}\) production was slightly increased up to 4 h to produce high level of Ca\(^{2+}\) (Fig. 4c). The results also showed that berberine promoted the loss of mitochondrial Δψ\(_{m}\) in C6 cells and this effect also was time-dependent (Fig. 4b).

Berberine affects the levels of caspase-3, -8 and -9 activity in C6 cells. In order to examine whether or not berberine induced apoptosis in C6 cells through caspase-activation, cells were treated with or without 100 μM berberine, the activities of caspase-8, -9 and -3 were determined by flow cytometry. The results are shown in Fig. 5a-c, which indicates that berberine promoted the activation of caspase-8, -9 and -3 in C6 cells and these effects were time-dependent.

Berberine affects the apoptosis associated proteins in C6 cells. Based on our results, which show that berberine causes strong apoptotic death (Fig. 2a and b), in order to examine whether or not berberine induced apoptosis in C6 cells through caspase-dependent or -independent pathways, cells were treated with 100 μM berberine for various time periods before harvesting for Western blotting examination. The results are shown in Fig. 6a-d. Results indicated that berberine promoted the expression of Wee1 (Fig. 6a), Fas/CD95 (Fig. 6b), cytochrome c and Bax (Fig. 6c), GADD153 and GRP78 (Fig. 6d), but it decreased the levels of cyclin B, CDK1 and cdc25c (Fig. 6a), pro-caspase-8 and pro-caspase-3 (Fig. 6b), pro-caspase-9 and Bcl-2 (Fig. 6c) and pro-caspase-12 (Fig. 6d).

As shown in Fig. 6b and c, treatment of cells with berberine resulted in a time-dependent decrease in pro-caspase-9, -3 and -8 with the strongest effect at 24, 48 and 72 h, suggesting a possible involvement of caspase activation in the apoptotic effect of berberine. Fig. 4b shows that berberine decreased the levels of Δψ\(_{m}\) and we also found that the pro-apoptotic protein Bax was increased and the anti-apoptotic protein Bcl-2 was decreased. The ratio of Bax/Bcl-2 is associated with the levels of Δψ\(_{m}\) and the results showed that berberine promoted the levels of Bax but decreased the levels of Bcl-2 which led to changes of Δψ\(_{m}\).

Discussion

Although berberine is widely used as an anti-inflammatory, anti-diarrhea medicine and it may be used as a clinical application in cancer therapy in future, a clear understanding of its mechanisms of action is not known. Therefore, we showed that berberine induced cell cycle arrest and apoptosis in C6 rat glioma cells in a dose- and time-dependent manner.
Berberine only induced G2/M arrest when berberine concentration was increased to 50 μM. Berberine acts as a potent genotoxicant as it induces a remarkable accumulation of DNA damage based on the results from Comet assay (Fig. 3a).

It is well known that berberine exhibits a tumor-inhibitory effect. We also gained some understanding of how berberine caused the cell cycle arrest and apoptosis in some cancer cells. Those effects are cell type-dependent. Other previous studies showed that berberine had no cytotoxic, mutagenic or recombinogenic activity on non-dividing cells from Saccharomyces cerevisiae, but it induced significant cytotoxic and cytostatic effects on dividing cells (26).

It remains to be determined how berberine induces DNA damage. Some studies suggest that berberine may either directly bind duplex DNA by intercalation (27-30) or inflict DNA damage indirectly by its interaction with topoisomerasers I and II (28,31-33). Other investigators also showed that berberine at 150 mg/ml was able to induce the rate of sister chromatid exchanges, which reflects homologous recombination repair, by several fold (34).
Figure 6. Berberine affects the apoptotic associated proteins in C6 cells. A total of 5\times10^5 C6 cells/ml were treated with 100 \mu M berberine for 0, 6, 12 and 24 h. Cells were harvested from each sample and associated proteins were determined by Western blotting. Cyclin B, CDK1, Wee1 and Cdc25c (a), Fas/CD95, pro-caspase-8 and -3 (b), cytochrome c, caspase-9, Bcl-2 and Bax (c), GADD153, GRP 78 and pro-caspase-12 (d) expression levels were examined using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting as described in Materials and methods.

Figure 7. The possible signaling pathway of berberine induced cell cycle arrest and apoptosis in C6 rat glioma cells. Schematic diagram shows berberine-induced signaling concentration on activation of the apoptotic machinery in C6 cells.
Our results showed that berberine induced G2/M arrest in C6 cells through the inhibition of cyclin B, CDK1 and cdc25c but promoted the levels of Wee1 (Fig. 6a) and it seems to be p53-dependent because our Western blotting also showed that berberine promoted the levels of p53 (data not shown). However, other studies showed that berberine caused p53-dependent G1 arrest and it can also induce p53-independent G2/M arrest. G2/M arrest also occurred in other cell lines in response to berberine and other treatments (35,36). It has also been reported that the down-regulation of cyclin B1 and up-regulation of Wee1 are associated with berberine-induced G2/M arrest in leukemia cells (36), and indicated that p27KIP1 plays a critical role in the G2 restriction point (37,38). Thus, proteins responsible for berberine-induced G2 arrest in C6 cells remain to be identified.

Our findings showed that berberine can cause remarkable DNA damage in cultured cells via ROS production and it also showed ER stress due to the increased levels of GADD153 and GRP78 which are the hallmarks of ER stress followed by the release of Ca^{2+} from ER. This study also showed that berberine changes the ratio of Bax/Bcl-2 which led to dysfunction of mitochondria followed by the cytochrome c release, caspase-9 and -3 activations, causing apoptosis. Overall, this is summarized in Fig. 7. Berberine may, through the Fas/CD95 receptor, cause the signaling pathway for apoptosis. An examination of tissue distribution of berberine in vitro and its correlation to the level of DNA damage, apoptosis and cell death in a corresponding tissue would provide invaluable insight into its genetic safety.

Acknowledgements

This study was supported by grants CMU96-087 and CMU97-086 and CMU97-087 from China Medical University, Taichung, Taiwan.

References