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The Combination of Berberine and Irradiation Enhances Anti-Cancer Effects Via Activation of p38 MAPK Pathway and ROS Generation in Human Hepatoma Cells

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ABSTRACT

Berberine, an isoquinoline plant alkaloid, has been known to generate a wide variety of biochemical and pharmacological effects. In order to elucidate the molecular mechanism for the berberine-induced enhancement of radio-sensitization, the human hepatoma HepG2 cells were treated with berberine combined with irradiation. The anti-tumor effect of gamma radiation was found to be significantly enhanced by berberine. The evidences of apoptosis, such as apoptotic DNA fragmentation and annexin V staining, were observed in the cells treated with the combination of berberine and irradiation. Additionally, the levels of reactive oxygen species (ROS) and nitric oxide (NO) were apparently elevated in the combination system. The activations of p38, Bax, and caspase-3 were also detected in the irradiated cells pretreated with berberine. The productions of ROS and annexin V staining in the cells treated with the combination of berberine and irradiation were significantly inhibited by the specific inhibitor of p38 MAPK, SB203580. The cell death induced by berberine alone or the combination of berberine and irradiation was suppressed by the anti-oxidant, *N*-acetyl cysteine (NAC). Taken together, the present results clearly indicate that the combination of berberine and gamma-radiation enhance the anti-cancer effects through the p38 MAPK pathway and ROS generation. J. Cell. Biochem. 107: 955–964, 2009.

KEY WORDS: RADIATION; APOPTOSIS; BERBERINE; RADIOTHERAPY; ROS; p38

Many researchers have focused on human hepatocellular carcinoma (HCC), one of the global incidences of tumor that has increased extensively. HCC is the fifth most common cancer worldwide. Surgery and chemical treatments have been performed as general methods for cancer therapy. Though the positive effects are improved by many therapeutic tools, such as chemical treatment, radiotherapy, and liver transplantation, a large number of patients would still suffer from recurrence and metastasis [Huang et al., 2000; Keng and Sundram, 2003]. In order to increase therapeutic efficiencies and to reduce the side effects and complications for the treatment of liver cancer, the concurrent or sequential combination of radiotherapy and chemotherapy has been now considered as promising approaches [Jain, 2001; Choy and Kim, 2003].

Apoptosis is a highly regulated and organized death process controlling the development and homeostasis of multicellular organisms that occurs under a variety of physiological and pathological conditions [Arends and Wyllie, 1991]. It is important in normal cell development, and loss of control of the apoptotic program contributes to the occurrence of many diseases including the accumulation of unwanted cells through insufficient apoptosis and cell loss as a result of excessive apoptosis [Bratton and Cohen, 2001]. In recent years remarkable progresses have been made in defining the central pathways of apoptosis, since an increase in apoptotic cells may be observed after the exposure of cancer cells to ionizing radiation and/or anti-cancer drugs. Thus the enhancement of apoptosis in tumor cells is an important goal for setting up medical and radio-oncological tumor treatment strategies.

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The reactive oxygen species (ROS) play an important role in the apoptotic induction by anti-cancer drugs and chemopreventive agents [Simizu et al., 1998]. Excessive ROS lead to the impaired intracellular ionic homeostasis by damaging cellular macromolecules including DNA, proteins, and lipids. It has been reported that cell apoptosis or transformation into cancer may be caused by damaged DNA [Olive, 1998; Ogawa et al., 2003; Cadet et al., 2004]. Ionizing radiation gives rise to a variety of cellular lesions including both DNA and membrane damage.

Radiation therapy induces the expression of inducible NO synthase (iNOS), which has been reported in various tissues and cells such as ileum [MacNaughton et al., 1998], lung in rat [Tsuji et al., 2000], and murine embryonic liver cell line [Yoo et al., 2000]. The expression of iNOS has dual effects on tumor growth; that is, low concentrations of NO promote tumor growth mainly, whereas high concentrations of NO show anti-tumor action by inducing apoptosis of tumor cells themselves [Jenkins et al., 1995].

In recent studies, mitochondrion has been identified as the central control point of the lethal signal transduction cascade, which is eventually executed by the activation of effector caspases [Van Laethem et al., 2004, 2005]. The exposure of cells to ionizing radiation induces the simultaneous activation of multiple mitogen-activated protein kinase (MAPK) pathways in a cell-type dependent manner [Dent et al., 2003]. MAPK family includes the extracellular regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), and p38 kinase. The ERK activation has been implicated in cell proliferation and cell cycle progression, while JNK and p38 are more commonly activated in response to stress and cellular damage [Xia et al., 1995].

Berberine (BBR), an alkaloid (Fig. 1) isolated from *Hydrastis canadensis*, *Coptis chinensis*, *Berberis aquifolium*, and *Berberis vulgaris*, has been extensively studied on its multiple biological and pharmacological activities [Creasy, 1997; Schmeller et al., 1997; Kuo et al., 2004]. It has been reported that BBR can be used as an anti-diarrhea, anti-hypertension, anti-arrhythmia, and anti-inflammatory agent [Tai et al., 1981; Huang et al., 1989; Takase et al., 1993; Yamamoto et al., 1993; Fukuda et al., 1999; Lau et al., 2001]. Additionally, the natural product was demonstrated to possess an anti-tumor activity [Chi et al., 1994; Kuo et al., 1995; Miura et al., 1997; Chung et al., 2000; Iizuka et al., 2000; Hwang et al., 2006; Lin et al., 2006].

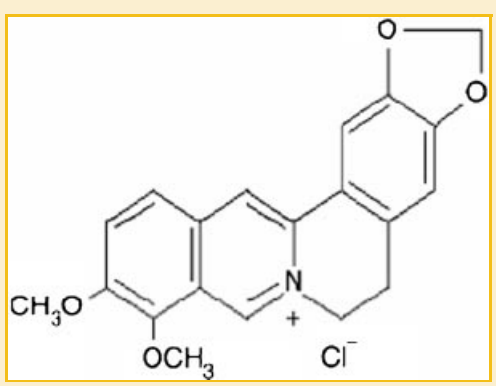

Fig. 1. The chemical structure of the berberine.

The anti-cancer effect of the combination of BBR and irradiation is little known, although BBR is a potential agent for caner therapeutics. In the present study, the therapeutic potential of BBR in combination with ionizing irradiation against human hepatoma HepG2 cells is described. In addition to that, a major role of ROS and MAPK axis in BBR plus irradiation-induced apoptosis is discussed.

MATERIALS AND METHODS

REAGENTS

BBR and SB203580 were purchased from Sigma Chemical Company (St. Louis, MO). Annexin V-fluorescein isothiocyanate was obtained from BD Biosciences (San Diego, CA). Polyvinylidene difluoride membrane was purchased from Bio-Rad. Antibodies against Bcl-2, Bax, NFκB p50, phosphor-IκBα, and caspase-3 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against total-p38/ERK and phospho-p38/ERK were obtained from Cell Signaling Technologies. All other chemicals were commercially available products of analytical grade.

CELL CULTURE

HepG2 human hepatoma cells were purchased from the American Type Culture Collection (Rockville, MD). The cells were cultured in a RPMI supplemented with 10% heat-inactivated fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2 in air.

BBR TREATMENT AND IONIZING IRRADIATION

BBR was purchased from Sigma Chemical Company and stored at −20°C. BBR stock solutions were prepared in a concentration of 100 μM in DMSO and diluted in RPMI medium prior to use. Exponentially growing HepG2 cells were incubated with BBR in a final concentration of 30 μM for 2 h before irradiation with 4 Gy of γ-radiation.

DETERMINATION OF CELL VIABILITY

To evaluate the cytotoxicity of BBR and irradiation, a 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed by determining cell viability. Cells were seeded in 24-well plates at a density of 4×10^4 cells/well and treated with BBR and irradiation. After the treatment, the medium was removed and the cells were washed with phosphate-buffered saline (PBS). Fresh medium was added and the cells were incubated with 100 μl of 1 mg/ml MTT for 3 h. The number of viable cells was determined by measuring spectrophotometrically the production of formazan at 570 nm.

DNA FRAGMENTATION

Apoptosis was confirmed by detecting the fragmentation of chromosomal DNA using the classic DNA ladder method. Briefly, 2×10^6 cells were immersed in nuclear lysis buffer with 200 μl/ml RNase A and incubated at 37°C for 30 min. Chromosomal DNA was extracted using the phenol–chloroform method and precipitated using ethanol. After washing in cold 70% ethanol, the resulting DNA pellet was resuspended in 40 μl of Tris–EDTA buffer (pH 7.5). The extracted DNA was examined by electrophoresis on 2.0% agarose gel.

ANNEXIN V STAINING

Cells were seeded onto 6-well plates at 4×10^5 cells/well, and pretreated with 30 μM BBR for 2 h then treated with 4 Gy of radiation. The cells were typsinized and gently washed with serum-containing culture medium followed by PBS. The cells were resuspended in binding buffer (10 mM HEPES, 140 mM NaCl, 25 mM $CaCl_2$) and incubated with annexin V-FITC and propidium iodide (PI) (MBL, Japan) at room temperature for 15 min. Fluorescence analysis was performed using a flow cytometer (Beckman Coulter FC500). The signals from annexin V-FITC were detected using the FL1 detector and the PI signals were detected using the FL3 detector.

HOECHST 33258 STAINING

The morphology of HepG2 cells exposed to BBR and irradiation was observed under fluorescence microscope. The Hoechst 33258 staining was used to observe the apoptotic morphology. Cells were fixed with 4% formaldehyde in PBS for 10 min, and then stained by Hoechst 33258 (10 mg/ml) for 15 min, and then subjected to fluorescence microscopy.

ROS ANALYSIS

The intracellular generation of ROS was measured using carboxy-H_2DCF-DA, which is a cell-permeable, non-fluorescent dye. Inside the cells, this compound is oxidized by ROS to form a fluorescent carboxydichlorofluorescein (DCF). Briefly, cells which were seeded in 6-well plates at 2×10^5 cells/well and treated with or without BBR were incubated with $5 \times$ μM carboxy-H_2DCF-DA at 37°C for 15 min. The cells were then washed twice with PBS, trypsinized, and resuspended in OptiMem I medium. The fluorescence resulting from the rate of oxidation of the dye in the cells was measured with a flow cytometer (Beckman Coulter FC500), using an excitation wavelength of 480 nm and an emission wavelength of 530 nm.

NITRIC OXIDE MEASUREMENT

Cells were cultured in 24-well plates with 1 ml of culture medium until confluence. They were pretreated with BBR for 2 h, and then exposure to ionizing radiation at 4 Gy, and then the culture media were collected after 72 h incubation. Control was performed without irradiation. Nitrite was measured by adding 100 μl of Griess reagent (1% sulphanilamide and 0.1% naphthylethylethylendiamine in 5% (v/v) phosphoric acid) to 100 μl samples of the culture medium. The absorbance at 550 nm (A550) was measured using a microplate reader, and the nitrite concentration was calculated by comparing with the absorbance produced using standard solution of sodium nitrite in the culture medium.

WESTERN BLOTTING

After BBR treatment, the medium was removed, and the cells were rinsed with PBS twice. After the addition of 0.6 ml of cold RIPA buffer (10 mM Tris [pH 7.5], 100 mM NaCl, 1 mM EDTA, 0.5% Na-deoxycholate [w/v], 0.1% SDS [w/v], and 1% Triton × 100 [w/v]) and protease inhibitors cocktail (Sigma Chemical Company), the cells were scraped at 4°C. The cell lysate was then centrifuged at 10,000*g* at 4°C for 10 min. The obtained proteins were separated by electrophoresis on 12% polyacrylamide gel and transferred onto nitrocellulose membranes. Loading samples were adjusted to equal amount of proteins. The protein was assayed by BCA kit. The membranes were stained with ponceau to confirm the uniform transfer of all samples and then incubated in a blocking solution of PBS with 0.05% Tween-20 (PBST) and 5% non-fat powdered milk at room temperature for 1 h. The membranes were reacted with the

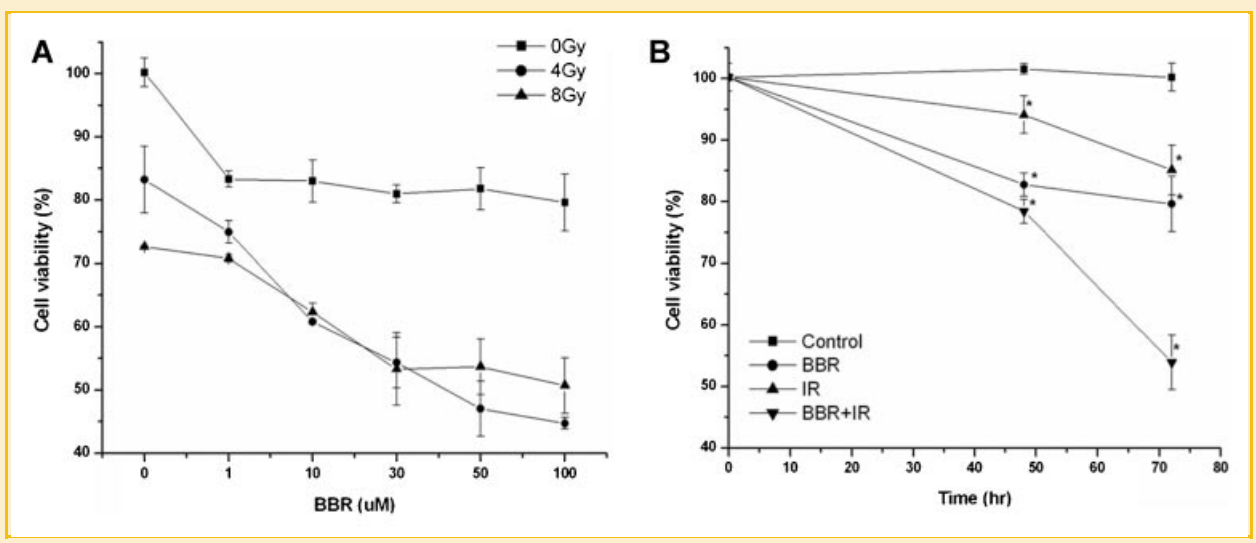

Fig. 2. Cytotoxicity of berberine, irradiation, and the combination of berberine and irradiation. HepG2 cells were pretreated with BBR for 2 h, exposed with 4 Gy, and then sampled for cell viability assays by dose-dependent manner (A). The cells were pretreated with 30 μM BBR for 2 h, exposed with 4 Gy, and then cells viability assay by time-dependent manner (B). $^*P < 0.05$.

following antibodies: caspase-3, phosphor-p38/ERK, total-p38/ERK, iNOS, and bax at a dilution of 1:1,000 for 90 min and extensive washes were performed followed with PBST. The membranes were then incubated with 1:1,000 horseradish peroxidase-conjugated secondary antibodies (Zymed) for 1 h, washed with PBST, and developed using the ECL kit. Western blots were developed using CCD camera. The ratios for the Western blot data were computed by imaging analysis program (Meta Morph).

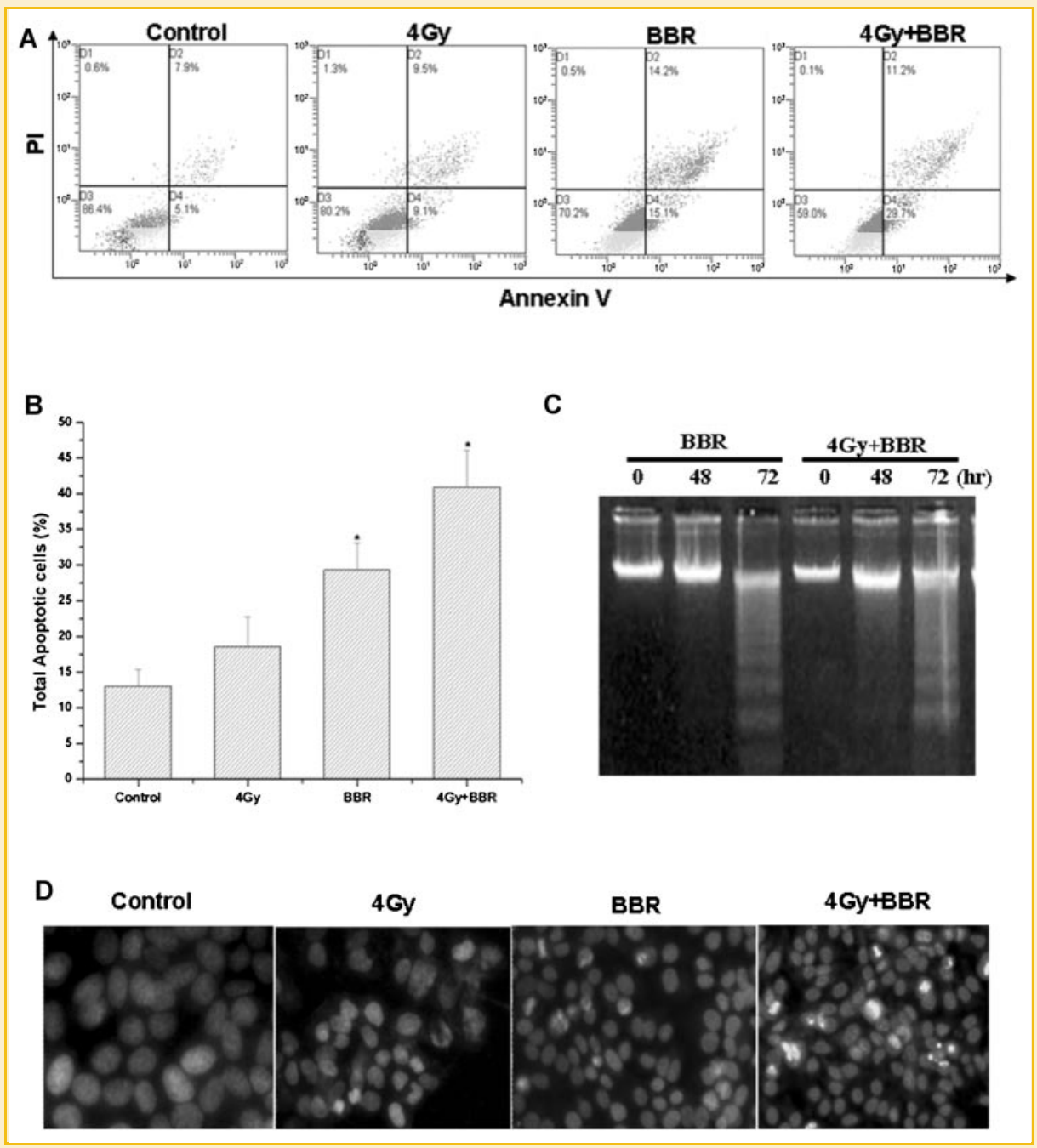

Fig. 3. The enhancement of apoptosis was increased by combination of berberine and irradiation. A: Assessment of cell death by flow cytometry. Lower right quadrant, early-apoptotic cells, that is, annexin V-FITC-positive/PI-negative cells; upper right quadrant, necrotic or late-apoptotic cells, that is, annexin V-FITC-positive/PI-positive cells. B: The ratio of total apoptotic cells (early-apoptotic cells and late-apoptotic cells) was presented. C: Cells were pretreated with 30 μM berberine for 2 h and irradiated with 4 Gy. DNA fragmentation was measured using 2.0% agarose gel electrophoresis. D: Changes in nuclei by Hoechst staining. HepG2 cells were treated with 4 Gy, BBR, or the combination of BBR and irradiation for 72 h. The cells were stained with Hoechst, and apoptotic cells were measured under fluorescence microscopy. Apoptotic cells showed condensed and fragmented nuclei.

STATISTICS ANALYSIS

All experiments were performed in triplicate and the results are expressed as the mean ± standard deviation. Statistical significance was analyzed using one-way analysis of variance (ANOVA), and the differences among means were determined using Duncan's multi-range tests. The value of $P < 0.05$ was considered statistically significant (STATSTICA 2.0, USA).

RESULTS

CELL VIABILITY

The HepG2 cell death was markedly induced by BBR in a time- and dose-dependent manner. The viability of the HepG2 cells irradiated with 4 Gy, which are the usual doses for a clinical treatment, was decreased by 83.24 ± 5.28% after 72 h (Fig. 2). The radiation-induced apoptosis of HepG2 cells was apparently increased by the pretreatment of BBR in a time-dependent manner. LD_{50} of BBR and irradiation was achieved at 30 μM and 4 Gy, respectively. The cell viability under the combination of 4 Gy-irradiation and 30 μM BBR treatment were significantly decreased to 53.91 ± 4.5% after 72 h, compared to the cells treated by 30 μM BBR alone (79.6 ± 4.5%) or 4 Gy irradiation alone (85.11 ± 4.1%), which indicated a significant enhancement of the cell death rate after 72 h ($P < 0.05$). These results indicate that the combination of BBR and radiation was approximately 1.5 times more effective than radiation alone for the HepG2 cell death rate.

APOPTOSIS BY BBR COMBINED WITH/WITHOUT IRRADIATION

As shown in Figure 3A, the annexin V-FITC-positive population of the cells (apoptotic cells; lower right quadrant) was not increased in the control cells. However, the apoptotic cell population was increased to 29.7%, and also the annexin V-FITC-positive/PI-positive population (necrosis and late apoptotic cells; upper right quadrant) was increased to 11.2% by the treatment of BBR plus radiation (Fig. 3A). The counterstaining of annexin V-RITC/PI proved to be an excellent probe to distinguish apoptotic cells from necrotic cells (Fig. 3B). An apparent DNA fragmentation was induced by the treatment of 30 μM BBR with/without radiation (4 Gy) (Fig. 3C). The morphological features of apoptotic cells were cell shrinkage, nuclear condensation, and genomic fragmentation down to the size of individual nucleosome units. The Hoechst 33258 staining was used to assess the nuclear condensation of apoptosis (Fig. 3D). The apoptotic features by the combination of BBR and irradiation was more increased than those by the treatment of BBR alone or 4 Gy alone.

The effects of BBR and irradiation treatment on the expression of Bax and Bcl-2 in HepG2 cells are shown in Figure 4. After the treatment with BBR, irradiation, or both combinations, the expression of Bax was appeared to be increased. In contrast, the expression of Bcl-2 was observed to be significantly decreased. The activation of caspase in response to BBR and irradiation was examined by analyzing the cell lysates with antibodies, which recognize the cleaved caspase-3 and caspase-3 proenzymes. The cleavage of caspase-3 in the cells exposed to BBR plus irradiation was detected after 6 h. On the other hand, the caspase-3 activation was induced by BBR or irradiation at later time point (48 h).

INDUCTION OF NITRIC OXIDE

The Bcl-2 family of proteins plays a central role in the regulation of apoptotic cell death induced by a wide variety of stimuli including NO. To evaluate whether NO was related with cell death by BBR, irradiation, or the combination of BBR and irradiation, the concentration of nitrite in culture media was examined by Griess reagent. Interestingly, the cells treated by the treatment of radiation and combination of BBR and radiation showed a significant increase of NO, compared to the only control cells (Fig. 5A). In order to investigate whether NO production in cells is related to iNOS, the expression level of iNOS was examined by Western blot analysis at different times (Fig. 5B). The level was increased in a time-dependent manner by the combination of BBR and radiation.

THE EFFECTS OF THE COMBINATION OF BBR AND RADIOTHERAPY ON MAP KINASES

The regulation of MAP kinase by irradiation or the combination of 4 Gy with BBR pretreatment was investigated using Western blot. The results indicated that the expression of p38 and ERK

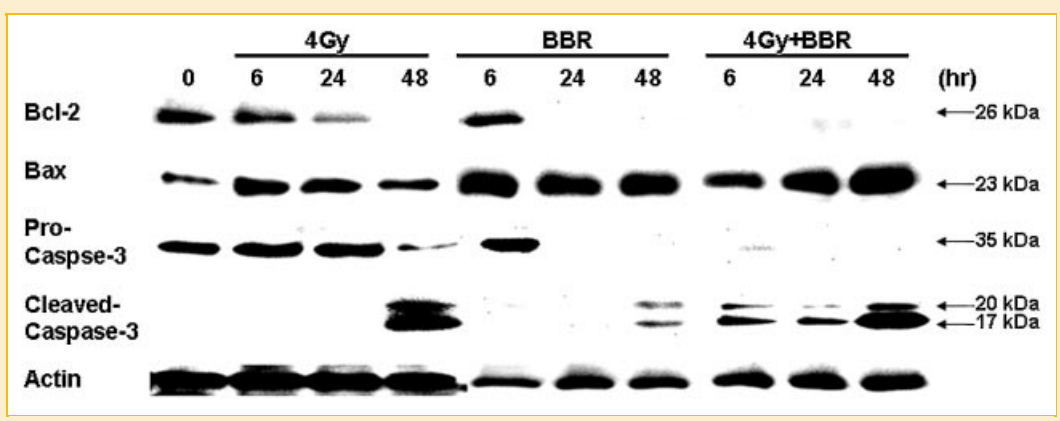

Fig. 4. Expression levels of Bcl-2 family proteins and caspase-3 by BBR, irradiation, and their combination. HepG2 cells were pretreated with 30 μM BBR for 2 h, then irradiated with 4 Gy, and examined over time. The expression levels of Bax, Bcl-2, procaspase-3, and cleavage caspase-3 protein were detected using immunoblotting.

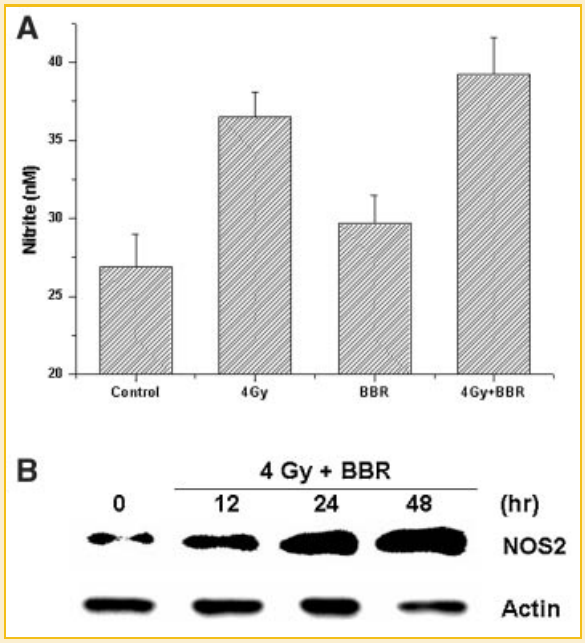

Fig. 5. Effects of berberine with or without radiation on the induction of nitric oxide (NO) in HepG2 cells. A: HepG2 cells were treated with BBR with or without radiation (4 Gy) and the concentration of NO in the cell culture medium was measured by Griess reagent. B: Cells were pretreated with BBR for 2 h and then treated with radiation (4 Gy).

phosphorylation were increased in the irradiated cells (Fig. 6A). And also, the activation of p38 was observed in HepG2 cells after the exposure to irradiation combined with/without BBR. In contrast, the activation of ERK was not induced by the pretreatment of BBR alone or the combination system.

HepG2 cells were pretreated with SB203580, an inhibitor of p38, to further examine the role of p38. As shown in Figure 6, the apoptotic cell death induced by BBR combined with irradiation was significantly reduced by the inhibitor (Fig. 6B). When the BBR plus irradiation treated cells were pretreated by SB203580, the expression of Bcl-2 protein was not decreased, and the activation of Bax, caspase-3, and iNOS were decreased (Fig. 6C).

THE COMBINATION OF BBR PLUS RADIOTHERAPY GENERATES ROS

It has been well known that ROS play an important role in apoptosis. The regulation of ROS generation was evaluated in the cells treated by irradiation with or without BBR pretreatment. The levels of ROS were measured using fluorescence sensitive probe (H_2DCF-DA) that detects various active oxygen species. As presented in Figure 7, the level of ROS was increased by BBR combined with/without irradiation. On the other hand, irradiation did not induce the generation of ROS. The induction of ROS by the combination treatment was only partially reduced by SB203580.

These results indicated that both p38 MAPK and ROS targets are essential for BBR combined with/without irradiation-induced

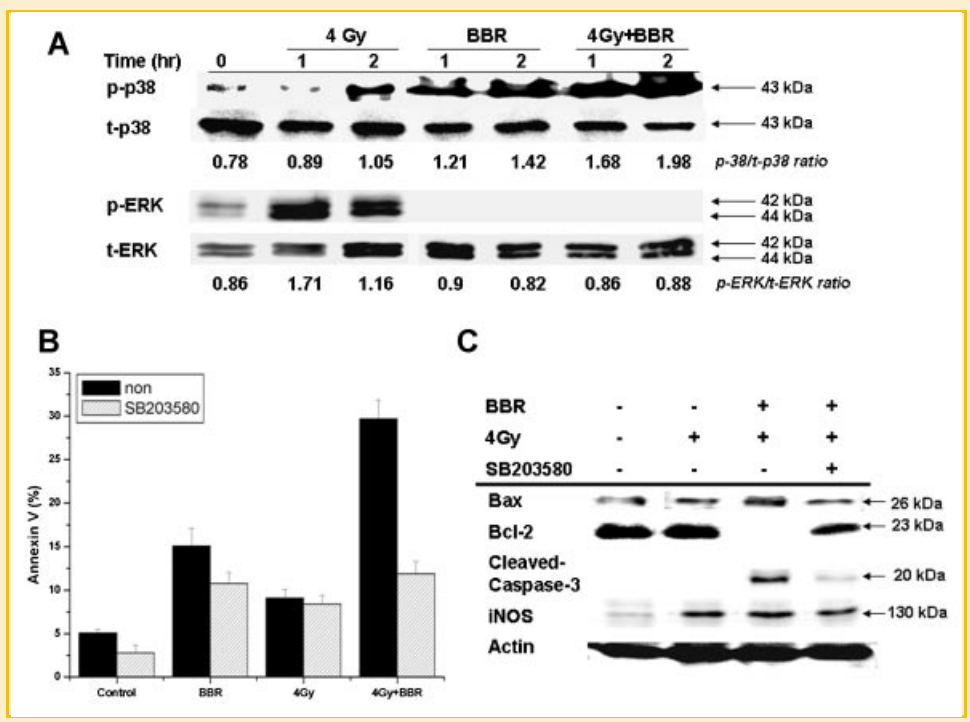

Fig. 6. A: Effect of the combination of BBR and irradiation on MAPK. Total cell lysates of cells by combined with/without BBR in irradiated cells for indicated time were extracted, and the phosphorylated (p-) and total proteins (t-) of p38 and ERK were immunoblotted. Densitometric analysis of the Western blots was determined by the ratio of phosphorylated proteins to total protein. B: Effects of p38 MAPK inhibition on berberine combined with irradiation-induced apoptosis in HepG2 cells. Apoptosis was determined by Annexin V/PI staining. The data represent averages of three independent experiments. C: The effects of p38 MAPK inhibitor, SB203580, on BBR, irradiation, and the combination of BBR and irradiation in HepG2 cells. The expression levels of the cleavage of Bax, Bcl-2, caspase-3, and iNOS proteins were detected using immunoblotting. The cells were treated with BBR and/or SB203580 for 2 h, and then exposed radiation or not irradiation. After treatment, they were incubated for 72 h.

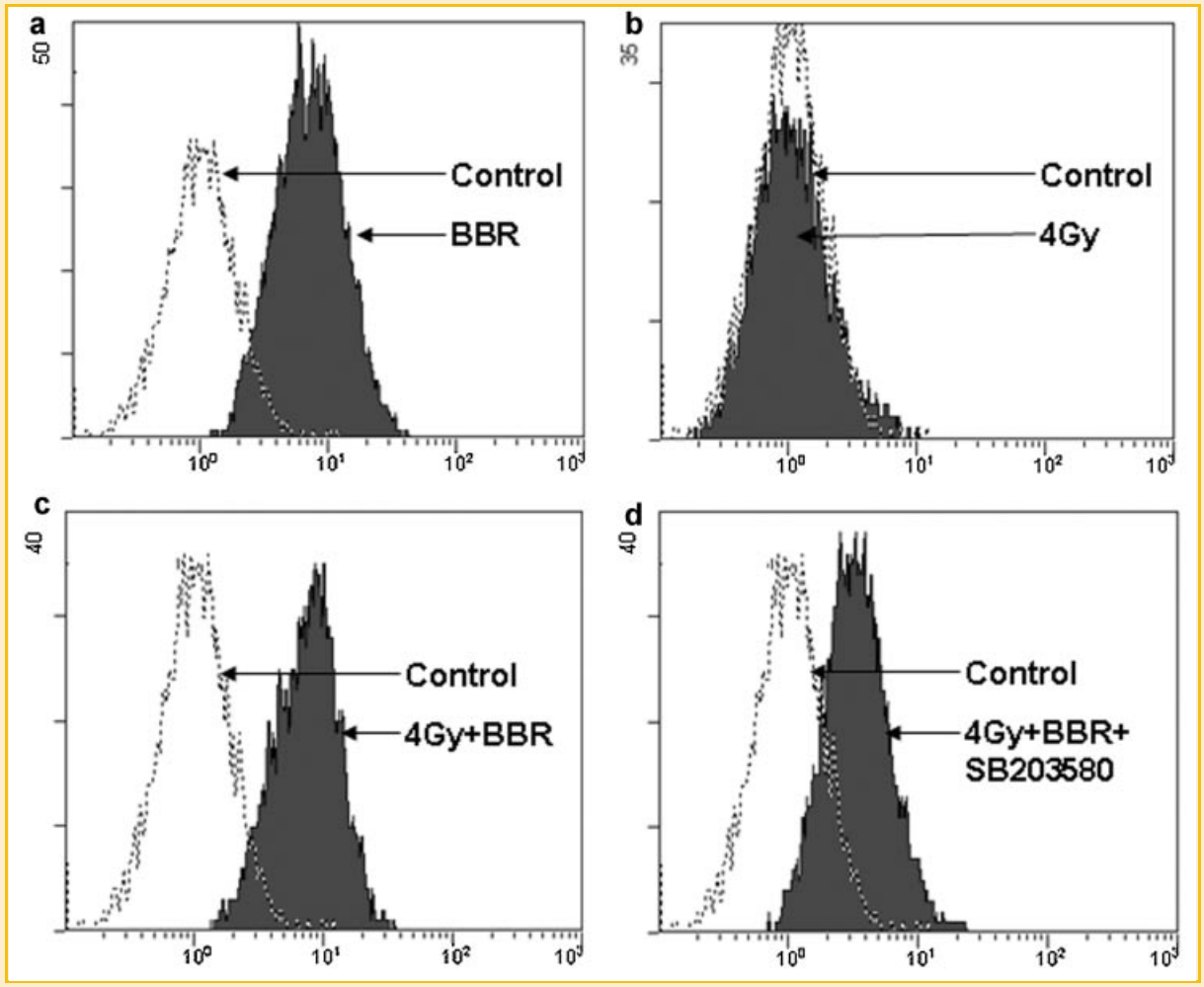

Fig. 7. The generation of ROS was induced by BBR and/or irradiation. The ROS generations by BBR, irradiation, the combination system, and the combination system plus SB203580 are shown. The intracellular ROS level was assayed using DCF-DA. Flow cytometry assay of ROS expression.

apoptosis, although the causal relation between p38 MAPK and ROS is unclear. Therefore, the roles of a ROS target in the activation of p38 MAPK was investigated in the apoptotic cells induced by the combination of BBR and irradiation (Fig. 8). The activation of p38 MAPK by the combination of BBR and irradiation was almost abolished by the pretreatment of *N*-acetyl cysteine (NAC), ROS scavengers. However, the activation of ERK by combination treatment was not decreased in the cells pretreated with NAC (Fig. 8A). In addition, when the cells pretreated with 100 μM NAC was exposed to the combination of BBR and irradiation, the expression of Bcl-2 protein was not decreased, but the activation of Bax and caspase-3 were decreased (Fig. 8B).

DISCUSSION

Radiation therapy has been commonly used in the treatment of human hepatoma. Unfortunately, the side-effects of radiotherapy such as radio-resistance, normal tissue injury, and inflammation have limited its usage. New therapeutic methods have been required to reduce side-effects and enhance therapy-effects. Thus, it is imperative to develop new and effective treatments, such as chemotherapy using herbal drugs for the treatment of cancer. Natural products have been shown to be excellent and reliable sources for the development of new therapy. BBR is one of the major components of Coptis chinesis, which has been frequently utilized in proprietary. The ability of BBR to induce apoptosis in cancer and non-cancer cells was previously described [Kuo et al., 1995; Yang et al., 1996]. BBR has many pharmacological effects including the inhibition of DNA and protein synthesis, arrest cell cycle progress, and anti-cancer effect [Zheng et al., 2003; Kuo et al., 2005; Lin et al., 2005; He et al., 2006]. Recently, it has been shown that BBR can cause apoptosis through a mitochondria-caspases-dependent pathway in human HepG2 cells [Hwang et al., 2006]. Hepatocellular carcinoma has been shown to be a highly resistant tumor to currently available chemotherapeutics and radiotherapy [Shimada et al., 1996]. Thus, we investigated the effect of BBR in a clinically feasible radiation dose range of 4 Gy using human hepatoma HepG2 cells. The HepG2 cells treated with BBR and exposed to 4 Gy showed

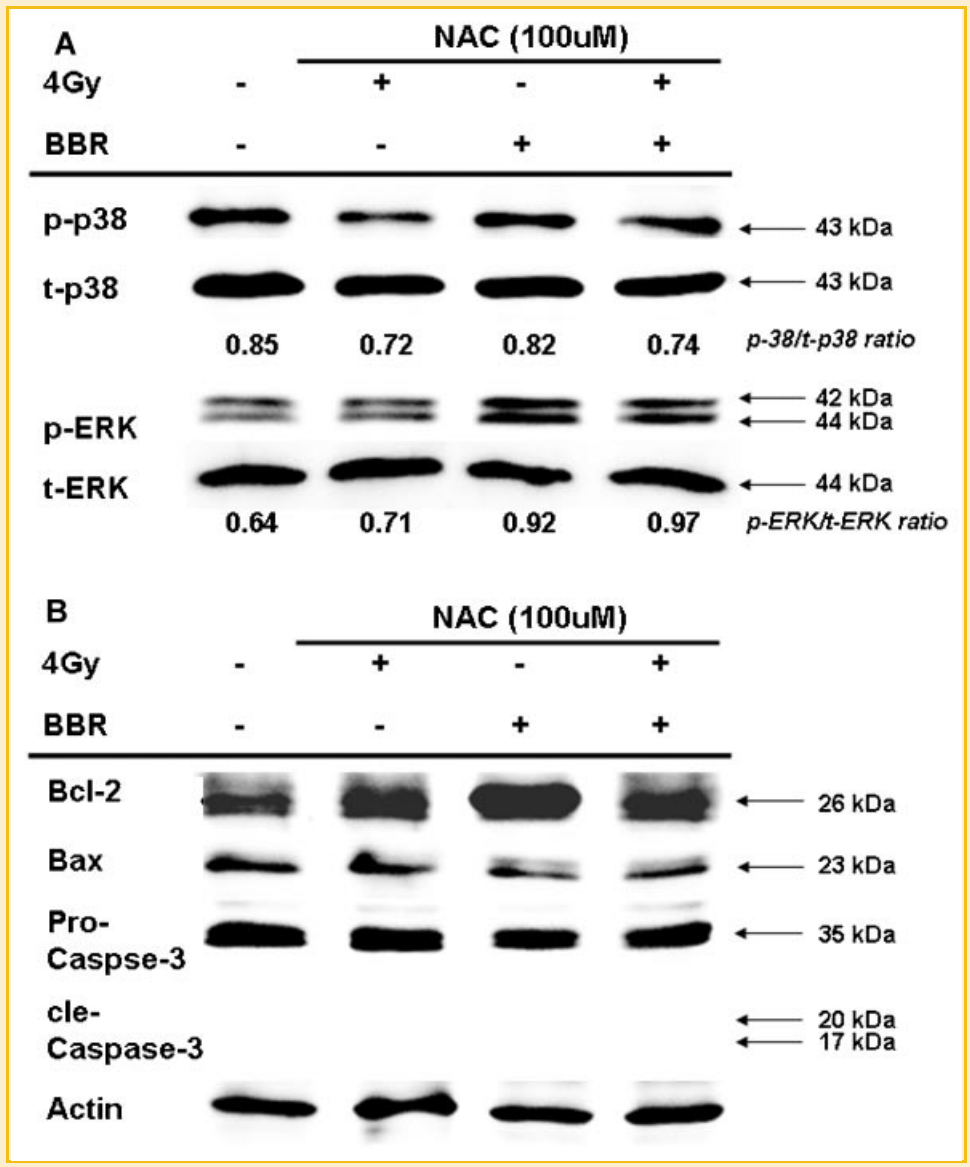

Fig. 8. Effect of NAC on MAPK, Bcl-2 family, and caspase-3. HepG2 cells were treated with NAC (100 μM) in combined with or without 4 Gy. Total cell lysates of cells treated with BBR, 4 Gy, and the combination of BBR and 4 Gy in the presence of 100 μM NAC were extracted, and the phosphorylated and total proteins of MAPKs (A), Bcl-2 family, and caspase-3 (B) were immunodetected by Western blot analysis.

an apparent enhancement of apoptosis, compared to the irradiation or BBR alone.

Mitochondria are involved in the apoptosis resulted from a variety of key events including the release of caspase activators, the changes in electron transport, the production of ROS, and participation in regulation of both pro- and anti-apoptotic Bcl-2 family proteins [Corbiere et al., 2004]. In this study, we have shown that the activation of caspase-3 and Bax was more significantly induced by the treatment of BBR combined with irradiation than the treatment of BBR or irradiation alone.

The present data are comparable to the previous study showing that down-regulation of endogenous Bcl-2 using Bcl-2 anti-sense reduced the survival rate of prostate cancer cells following irradiation, which was suggested to be a potentially important therapeutic approach for enhancing radiosensitivity in tumor via anti-sense oligonucleotide or other drug therapies that down-regulate Bcl-2 [Blumenstein et al., 1998]. In the present study, the expression of Bcl-2 was also observed in HepG2 cells exposed to radiation. However, the expression of Bcl-2 was strongly decreased by BBR combined with or without irradiation. Our results indicate that BBR is a functional inhibitor of Bcl-2 expression and enhances radiation-induced apoptosis in HepG2 cells.

NO is known to induce apoptosis via several pathways including the activation of a caspase cascade [Kim et al., 2000], the release of mitochondrial cytochrome *c* [Brown and Borutaite, 1999], and the regulation of cell survival or apoptosis [Kim et al., 1994]. Our results show that the production of NO was increased in HepG2 cells treated with BBR plus irradiation.

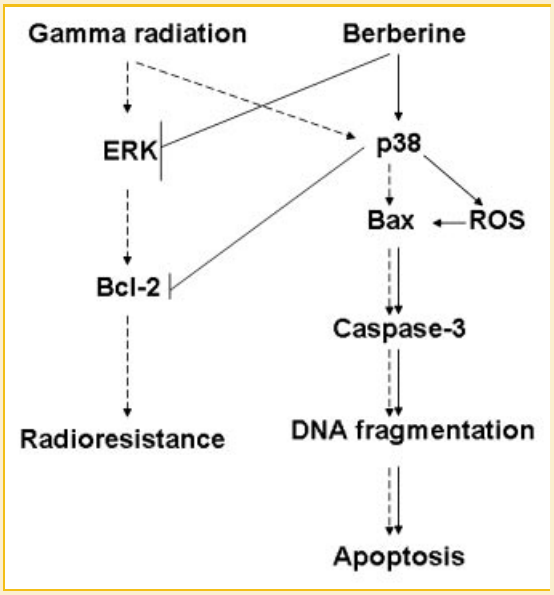

Fig. 9. Summary of a proposed mechanism for the enhancement of apoptosis produced by the combination of BBR and irradiation in human hepatoma HepG2 cells. BBR enhances radiosensitivity in cancer via the inhibition of radio-resistant proteins, such as Bcl-2 which is expressed by irradiation.

Irradiation of cells is known to activate the enzymes of MAPK family. The activation of caspase-3-like proteases, as well as the cell death, was suppressed via the inhibition of p38 activity with B202190 [Assefa et al., 2000; Tournier et al., 2000]. Also, the activation of p38 was accompanied by the generation of ROS in many cell lines treated with cytokines, anti-cancer drugs, or chemopreventive agents [Chen and Tan, 2000]. The ROS generated by irradiation reacts with nitric oxide to generate reactive nitrogen species [Reiter et al., 2000]. In this study, we observed a rapid phosphorylation of p38 in BBR combined with irradiation-treated HepG2 cells. However, the inhibition of p38 phosphorylation by a specific inhibitor, SB203580, blocks the generation of ROS, as well as the cell death in HepG2 cells treated by BBR combined with irradiation. In addition, SB203580 inhibit the expression of Bax induced by BBR combined with/without irradiation. It indicates that BBR is a functional inhibitor of Bcl-2 (radio-resistance) and enhances the radiation-induced apoptosis. Also, in SB203580-treated HepG2 cell, the activation of bax was inhibited by BBR combined with irradiation. Therefore, BBR-induced phosphorylation of p38 is dependent on the expression of Bcl-2 family in radiation-exposed HepG2 cells. In addition, our results show that NAC suppressed the apoptosis by the combination of BBR and/or irradiation and the activation of p38 MAPK and Bax. Thus, the combination of BBR and irradiation induced the apoptosis by the activation of p38 MAPK pathway and ROS generation. The present combination therapy may result in induction of apoptosis in other cancer cell types by similar mechanisms.

In summary, we suggest that BBR may be a potentially important therapeutic approach for enhancing radiosensitivity in cancer via the inhibition of radio-resistant proteins, such as Bcl-2 which is expressed by irradiation (Fig. 9).

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REFERENCES

Arends MJ, Wyllie AH. 1991. Apoptosis: Mechanisms and roles in pathology. Int Rev Exp Pathol 32:223–254.

Assefa Z, Vantieghem A, Garmyn M, Declercq W, Vandenabeele P, Vandenheede JR, Bouillon R, Merlevede W, Agostinis P. 2000. p38 mitogen-activated protein kinase regulates a novel, caspase-independent pathway for the mitochondrial cytochrome c release in ultraviolet B radiation-induced apoptosis. J Biol Chem 275:21416–21421.

Blumenstein M, Hossfeld DK, Duhrsen U. 1998. Indirect radiation leukemogenesis in DBA/2 mice: Increased expression of B2 repeats in FDC-P1 cells transformed by intracisternal A-particle transposition. Ann Hematol 76: 53–60.

Bratton SB, Cohen GM. 2001. Apoptotic death sensor: An organelle's alter ego. Trends Pharmacol Sci 22:306–315.

Brown GC, Borutaite V. 1999. Nitric oxide, cytochrome c and mitochondria. Biochem Soc Symp 66:17–25.

Cadet J, Bellon S, Douki T, Frelon S, Frelon S, Gasparutto D. 2004. Radiation-induced DNA damage: Formation, measurement, and biochemical features. J Environ Pathol Toxicol Oncol 23:33–43.

Chen YR, Tan TH. 2000. The c-Jun N-terminal kinase pathway and apoptotic signaling (review). Int J Oncol 16:16651–16662.

Chi CW, Chang YF, Chao TW, Chiang SH, Peng FK, Lui WY, Liu TY. 1994. Flow cytometric analysis of the effect of berberine on the expression of glucocorticoid receptors in human hepatoma HepG2 cells. Life Sci 26:2099–2107.

Choy H, Kim DW. 2003. Chemotherapy and irradiation interaction. Semin Oncol 30:3–10.

Chung JG, Chen GW, Hung CF, Lee JH, Ho CC, Ho HC, Chang HL, Lin WC, Lin JG. 2000. Effects of berberine on arylamine *N*-acetyltransferase. Activity and 2-aminofluorene-DNA adduct formation in human leukemia cells. Am J Chin Med 2:227–238.

Corbiere C, Liagre B, Terro F, Beneytout JL. 2004. Induction of antiproliferative effect by diosgenin through activation of p53, release of apoptosis-inducing factor (AIF) and modulation of caspase-3 activity in different human cancer cells. Cell Res 14:188–196.

Creasy WA. 1997. Biochemical effects of berberine. Biochem Pharmacol 28: 1081–1084.

Dent P, Yacoub A, Fisher PB, Hagan MP, Grant S. 2003. MAPK pathways in radiation responses. Oncogene 22:5885–5896.

Fukuda K, Hibiya Y, Mutoh M, Koshiji M, Akao S, Fujiwarw H. 1999. Inhibition by berberine of cyclooxygenase-2 transcriptional activity in human colon cancer cells. J Ethnopharmacol 2:227–233.

He ZW, Zhao XY, Xu RZ, Wu D. 2006. Effects of berbamine on growth of leukemia cell line NB4 and its mechanism. Zhejiang Da Xue Xue Bao Yi Xue Ban 35:209–214.

Huang WM, Wu ZD, Gan YQ. 1989. Effects of berberine on ischemic ventricular arrhythmia. Chin J Cardiovasc Dis 5:300–301.

Huang YH, Wu JC, Lui WY, Chau GY, Tsay SH, Chiang JH, King KL, Huo TI, Chang FY, Lee SD. 2000. Prospective case-controlled trial of adjuvant chemotherapy after resection of hepatocellular carcinoma. World J Surg 24:551–555.

Hwang JM, Kuo HC, Tseng TH, Liu YJ. 2006. Berberine induces apoptosis through a mitochondria/caspase pathway in human hepatoma cells. Arch Toxicol 2:62–73.

Iizuka N, Miyamoto K, Okita K, Tangoku A, Hayashi H, Yosino S, Abe T, Morioka T, Hazama S, Oka M. 2000. Inhibitory effect of Coptidis rhizoma and berberine on the proliferation of human esophageal cancer cell lines. Cancer Lett 1:19–25.

Jain RK. 2001. Normalizing tumor vasculature with anti-angiogenic therapy: A new paradigm for combination therapy. Nat Med 7:987–989.

Jenkins DC, Charles IG, Thomsen LL, Moss DW, Holmes LS, Baylis SA, Rhodes P, Westmore K, Emson PC, Moncada S. 1995. Roles of nitric oxide in tumor growth. Proc Natl Acad Sci USA 92:4392–4396.

Keng GH, Sundram FX. 2003. Radionuclide therapy of hepatocellular carcinoma. Ann Acad Med Singapore 32:518–524.

Kim YM, Talanian RV, Billiar TR. 1994. Nitric oxide inhibits apoptosis by preventing increased in caspase-3 like activity via two distinct mechanisms. J Biol Chem 272:31138–31148.

Kim YM, Chung HT, Simmons RL, Billiar TR. 2000. Cellular non-heme iron content is a determinant of nitric oxide-mediated apoptosis, necrosis, and caspase-inhibition. J Biol Chem 275:10954–10961.

Kuo CL, Chou CC, Yung BYM. 1995. Berberine complexes with DNA in the berberine-induced apoptosis in human leukemic HL-60 cells. Cancer Lett 93:193–200.

Kuo CL, Chi CW, Liu TY. 2004. The anti-inflammatory potential of berberine in vitro and *in vivo*. Cancer Lett 203:127–137.

Kuo CL, Chi CW, Liu TY. 2005. Modulation of apoptosis by berberine through inhibition of cyclooxygenase-2 and Mcl-1 expression in oral cancer cells. In Vivo 19:245–247.

Lau CW, Yao XQ, Chen ZY, Ko WH, Huang Y. 2001. Cardiovascular actions of berberine. Cardiovasc Drug Rev 3:234–244.

Lin SS, Chung JG, Lin JP, Chuang JY, Chang WC, Wu JY, Tyan YS. 2005. Berberine inhibits arylamine N-acetyltransferase activity and gene expression in mouse leukemia L 1210 cells. Phytomedicine 12:351–358.

Lin JP, Yang JS, Lee JH, Hsieh WT, Chung JG. 2006. Berberine induces cell cycle arrest and apoptosis in human gastric carcinoma SNU-5 cell line. World J Gastroenterol 1:21–28.

MacNaughton WK, Aurora AR, Bhamra J, Sharkey KA, Miller MJ. 1998. Expression, activity and cellular localization of inducible nitric oxide synthase in rat ileum and colon post-irradiation. Int J Radiat Oncol Biol Phys 74:255–264.

Miura N, Yamamoto M, Ueki T, Kitani T, Fukuda K, Komatsu Y. 1997. Inhibition of thymocyte apoptosis by berberine. Biochem Pharmacol 9:1315–1322.

Ogawa Y, Kobayashi T, Nishioka A, Kariya S, Hamasato S, Seguchi H. 2003. Radiation-induced reactive oxygen species (ROS) formation prior to oxidative DNA damage in human peripheral T cells. Int J Mol Med 11:149–152.

Olive PL. 1998. The role of DNA single and double strand breaks in cell killing by ionizing radiation. Radiat Res 150:42–51.

Reiter CD, Teng RJ, Beckman JS. 2000. Superoxide reacts with nitric oxide to nitrate tyrosine at physiological pH via peroxynitrite. J Biol Chem 275: 32460–32466.

Schmeller T, Latz-Brüning B, Wink M. 1997. Biochemical activities of berberine, palmatine and sanguinarine mediating chemical defence against microorganisms and herbivores. Phytochemistry 44:257–266.

Shimada M, Takenaka K, Kawahara N, Yamamoto K, Shirabe K, Maehara Y, Sugimachi K. 1996. Chemosensitivity in primary liver cancers: Evaluation of the correlation between chemosensitivity and clinicopathological factors. Hepatogastrology 43:1159–1164.

Simizu S, Takada M, Umezawa K, Imoto M. 1998. Requirement of caspase-3(-like) protease-mediated hydrogen peroxide production for apoptosis induced by various anticancer drugs. J Biol Chem 273:26900–26907.

Tai YH, Feser JF, Marnane WG, Desjeux JF. 1981. Antisecretory effects of berberine in rat ileum. Am J Physiol 3:253–258.

Takase H, Yamamoto K, Ito K, Yumioka E. 1993. Pharmacological studies on antidiarrheal effects of berberine and geranii herba, *Folia*. Pharmacol Jpn 2:101–112.

Tournier C, Hess P, Yang DD, Xu J, Turner TK, Nimnual A, Bar-Sagi D, Jones SN, Flavell RA, Davis RJ. 2000. Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. Science 288: 870–874.

Tsuji C, Shioya S, Hirota Y, Fukuyama N, Kurita D, Tanigaki T, Ohta Y, Nakazawa H. 2000. Increased production of nitrotyrosine in lung tissue of rats with radiation-induced acute lung injury. Am J Physiol 278:L719–725.

Van Laethem A, Van Kelst S, Lippens S, Declercq W, Vandenabeele P, Janssens S, Vandenheede JR, Garmyn M, Agostinis P. 2004. Activation of p38 MAPK is required for Bax translocation to mitochondria, cytochrome c release and apoptosis induced by UVB irradiation in human keratinocytes. FASEB J 18:1946–1948.

Van Laethem A, Claerhout S, Garmyn M, Agostinis P. 2005. The sunburn cell: Regulation of death and survival of the keratinocytes. Int J Biochem Cell Biol 37:1547–1553.

Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. 1995. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 270:1326–1331.

Yamamoto K, Takase H, Abe K, Saito K, Suzuki A. 1993. Pharmacological studies on antidiarrheal effects of a preparation containing berberine and Gerainin herba, *Folia*. Pharmacol Jpn 101:169–175.

Yang IW, Chou CC, Yung BYM. 1996. Dose-dependent effects of berberine on cell cycle pause and apoptosis in Balb/c 3T3 cells. Naunyn Schmiedebergs Arch Pharmacol 2:102–108.

Yoo JC, Pae HO, Choi BM, Kim WI, Kim JD, Kim YM, Chung HT. 2000. Ionizing radiation potentiates the induction of nitric oxide synthase by interferon-β (IFN-β) or IFN-γ and lipopolysaccharide in bnl cl.2 murine embryonic liver cells: Role of hydrogen peroxide. Free Radic Biol Med 28:390–396.

Zheng L, Zhou Z, Tao D, Lan T. 2003. Protective effect of berberine on cardiac myocyte injured by ischemia-reperfusion. Sichuan Da Xue Xue Bao Yi Xue Ban 34:452–454.