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Berberine Inhibited Radioresistant Effects and Enhanced Anti-Tumor Effects in the Irradiated-Human Prostate Cancer Cells

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The purpose of this study was to elucidate the mechanism underlying enhanced radiosensitivity to ^{60}Co γ-irradiation in human prostate PC-3 cells pretreated with berberine. The cytotoxic effect of the combination of berberine and irradiation was superior to that of berberine or irradiation alone. Cell death and Apoptosis increased significantly with the combination of berberine and irradiation. Additionally, ROS generation was elevated by berberine with or without irradiation. The antioxidant NAC inhibited berberine and radiation-induced cell death. Bax, caspase-3, p53, p38, and JNK activation increased, but activation of Bcl-2, ERK, and HO-1 decreased with berberine treatment with or without irradiation. Berberine inhibited the anti-apoptotic signal pathway involving the activation of the HO-1/NF-κB-mediated survival pathway, which prevents radiation-induced cell death. Our data demonstrate that berberine inhibited the radioresistant effects and enhanced the radiosensitivity effects in human prostate cancer cells via the MAPK/caspase-3 and ROS pathways.

Key words: Berberine, Radiation, Apoptosis, Radioresistant

INTRODUCTION

Prostate cancer is one of the most prevalent malignancies and the second leading cause of death from cancer in older men worldwide (Baldwin, 1996). Various studies have consistently identified the anti-apoptotic protein Bcl-2 in the recurrence of tumors after radiotherapy (Baldwin, 1996). In recent studies, Bcl-2 overexpression was greater in the prostate tumors of patients undergoing radical prostatectomy after failed radiotherapy than in tumors of those whose initial treatment was surgery alone (Bergman *et al*., 1997). More recently, Bcl-2 overexpression was found to be associated with increased radiation resistance in human prostate cancer PC-3 cells, and down-regulation was associated with radiation sensitization (Blumenstein *et al*., 1998).

Improving the overall therapeutic effects of prostrate cancer depends on combined therapies whose purpose in prostate cancer is to enhance their therapeutic efficiency and to reduce side effects. Because most of the widely used chemotherapeutic agents and γ-irradiation employ apoptosis as the main death pathway, some overlap may exist between the causes of chemoresistance and radioresistance (Bohnke *et al*., 2004); however, how an anticancer drug can influence radioresistance remains unclear. Ionizing radiation and a variety of other toxic stresses induce simultaneous compensatory activation of multiple mitogen-activated protein kinase (MAPK) pathways and nuclear factor-κB (NF-κB), whose ratio controls cell survival and death (Bristow et al., 1996). Three MAPK subfamilies have been identified: extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK. ERK is important for cell survival, whereas JNK and p38 MAPK are involved in apoptosis, although the regulation of apoptosis by MAPK is more complex than initially thought and often controversial (Carson, 2006). NF-κB is a transcription factor that prevents apoptosis by inducing several anti-apoptotic proteins, including FLIP, Bcl-2, and Bfl/A1 (Colombo *et al*., 2006).

Ionizing radiation induces DNA and membrane damage. DNA damage activates a coordinated network of signal transduction pathways involved in cell cycle arrest, apoptosis, the stress response, and DNA repair processes. Remarkable progress has recently been made defining the central pathways of apoptosis because an increase in apoptotic cells may occur after exposure of cancer cells to ionizing radiation and/or anticancer drugs. Thus, enhanced apoptosis in tumor cells is an important goal for developing medical and

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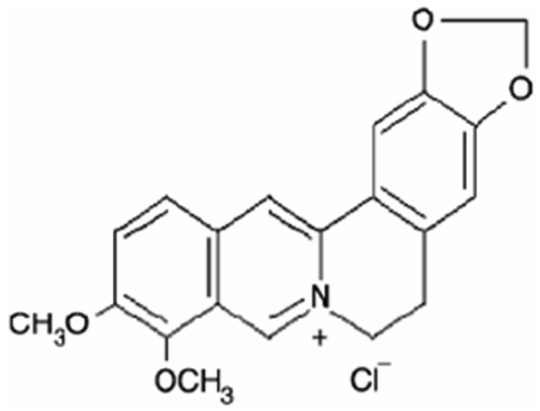

Fig. 1. The chemical structure of the berberine.

radio-oncological tumor treatment strategies (Kaminski *et al*., 2003).

Berberine (BBR) (Fig. 1), an alkaloid isolated from *Hydrastis canadensis*, *Coptis chinensis*, *Berberis aquifolium*, and *Berberis vulgaris*, has been extensively studied for its multiple biological and pharmacological activities (Corbiere *et al*., 2004). BBR can be used as an antidiarrheal, antihypertensive, antiarrhythmic, and anti-inflammatory agent (Greenlee *et al*., 2000). Additionally, the natural product was demonstrated to possess antitumor activity (He *et al*., 2006). The purpose of this study was to investigate the effects of the combination of BBR and irradiation on PC-3 cells and to examine the molecular mechanisms of radiosensitivity induced by BBR and γ-irradiation in human prostrate cancer cells, focusing on the possibility that it might act, at least in part, by inhibiting the radioresistance protein in irradiated PC-3 cells.

MATERIALS AND METHODS

Reagents. BBR was purchased from Sigma Chemical Company (St. Louis, MO). Annexin V-fluorescein isothiocyanate was obtained from BD Biosciences (San Diego, CA). Polyvinylidene difluoride membranes were purchased from Bio-Rad (Hercules, CA). Antibodies against Bcl-2 (DC-21), Bax (P-19), phosphor-IκBα (Ser32), and IκBα were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibodies against p38, phospho-p38, ERK, phosphor-ERK, JNK, and phosphor-JNK were obtained from Cell Signaling Technology (Danvers, MA). All other chemicals were commercially available analytical grade products.

Cell culture. PC-3 human prostate cancer cells were purchased from the American Type Culture Collection (Rockville, MD). The cells were cultured in RPMI medium 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 stock solutions were prepared at a concentration of 100 μM in dimethyl sulfoxide and diluted in RPMI medium prior to use. Exponentially growing PC-3 cells were incubated with BBR at a final concentration of 30 μM for 2 h prior to 6 Gy γ-irradiation.

Determination of cell viability. To evaluate the cytotoxicity of BBR and irradiation, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to determine 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 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 μ*l* of 1 mg/m*l* MTT for 3 h. The number of viable cells was determined by spectrophotometrically measuring the production of formazan at 570 nm.

Annexin V-FITC staining. Cells were seeded onto six-well plates at 4×10^5 cells/well, pretreated with 30 μM BBR for 2 h, then treated with 6 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, Tokyo, Japan) at room temperature for 15 min. Fluorescence analysis was performed using a flow cytometer (Beckman FC500; Beckman Coulter, Fullerton, CA). The signals from annexin V-FITC were detected using an FL1 detector, and the PI signals were detected using an FL3 detector.

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

Measurement of caspase-3 activity. After treatment under various conditions, cells were collected, washed with PBS, and lysed in lysis buffer [1% Trypton X-100, 0.32 M sucrose, 5 mM ethylenediaminetetraacetic acid (EDTA), 10 mM Tris-HCl (pH 8), 2 mM dithiothione, 2 mM phenylmethanesulfonyl fluoride, 10 mg/m*l* pepstatin A, and 10 mg/

m*l* leupeptin) for 20 min at 4°C followed by centrifugation (10,000 ×*g*) for 30 min. Caspase-3 activity was assayed in 1 m*l* reaction mixtures with a fluorogenic report substrate peptide specific for caspase-3. The substrate peptide (200 mM) was incubated at 37°C with cytosolic extracts (50 mg of total protein) in reaction buffer (100 mM HEPES, 10% sucrose, 10 mM dithiothreitol, 0.1% 3-[3-chloroamidopropyldimethylamonio]-1-propanesulfonate). Fluorescence was measured after 2 h (excitation wavelength, 485 nm; emission wavelength, 505 nm) with a FLUOstar galaxy fluorescence plate reader (BMG Lab Technologies, Offenberg, Germany).

Western blotting. After BBR treatment, the medium was removed, and the cells were rinsed twice with PBS. After adding 0.6 m*l* of cold RIPA buffer [10 mM Tris (pH 7.5), 100 mM NaCl, 1 mM EDTA, 0.5% Na-deoxycholate (w/v), 0.1% sodium dodecyl sulfate (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 proteins were separated by electrophoresis on 12~15% polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were stained with Ponceau-S 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% nonfat powdered milk at room temperature for 1 h. The membranes were incubated overnight at 4°C with primary antibody, and extensive washes were performed followed by PBST. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (Zymed, San Francisco, CA) for 1 h, washed with PBST, and developed using the ECL kit.

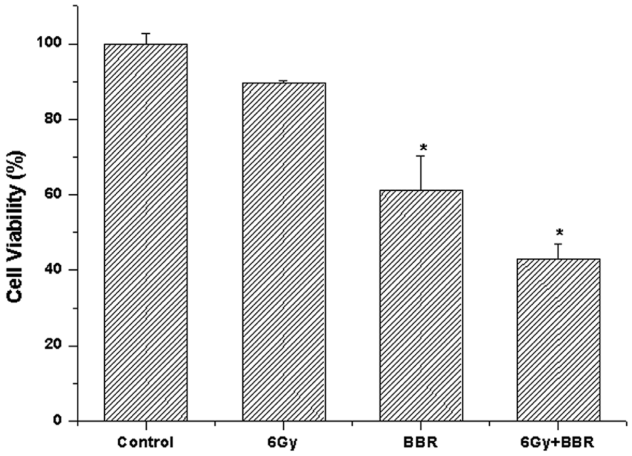

Fig. 2. The effects of berberine (BBR) and irradiation on cell growth. Cells were incubated with 30 μM BBR and then exposed to 6 Gy of irradiation. The percent cell viability was determined by the MTT assay after 72 h. Control cells were considered 100% viable (*p < 0.05).

Statistical analysis. All experiments were performed in triplicate, and the results are expressed as the mean ± standard deviation. Statistical significance was analyzed using a one-way analysis of variance (ANOVA), and the differences among means were determined using Duncan's multiple-range test. A value of p 0.05 was considered statistically significant (Statistica 2.0; StatSoft, Tulsa, OK).

RESULTS

Induction of apoptosis by BBR with or without irradiation. PC-3 cell death was markedly induced by BBR. The viability of the PC-3 cells irradiated with 6 Gy decreased by 88.2 ± 1.3% after 72 h (Fig. 2). Cell viability with the combination of 6 Gy-irradiation and 30 μM BBR decreased sig-

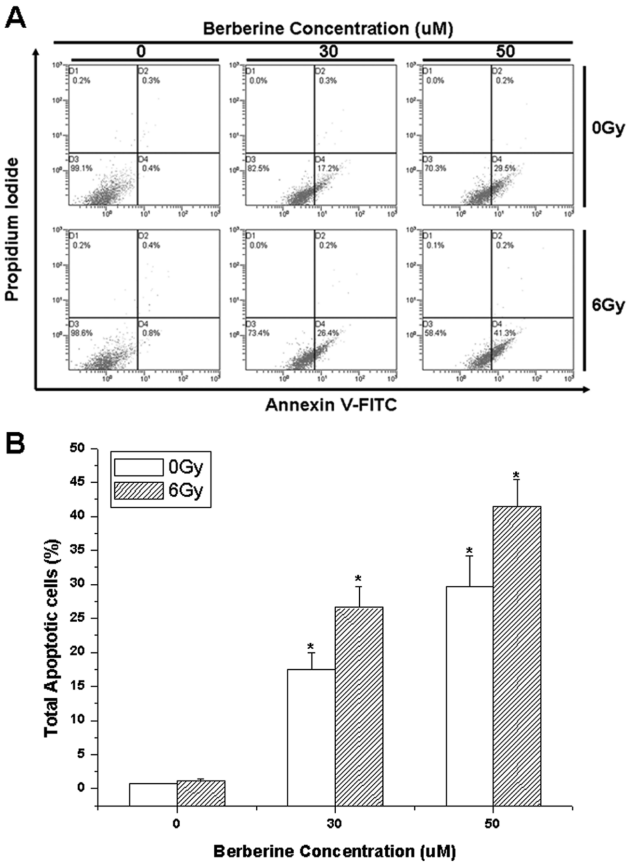

Fig. 3. Induced apoptosis in PC-3 cells following berberine (BBR) treatment and irradiation. Cells were incubated with 30 μM BBR and irradiated with 6 Gy. A: The cell death assessment was conducted by flow cytometry. Early apoptotic cells, which comprise the annexin V-FITC-positive/PI-negative cell population, are reported in the lower-right quadrant. Necrosis or late apoptotic cells, which comprise the annexin V-FITC-positive/PI-positive cell population, are reported in the upper-right quadrant. B: Apoptotic cell expression is the sum of early apoptotic cells and late apoptotic cells (*p < 0.05).

nificantly to 49.9 ± 4.5% after 72 h compared to cells treated with 30 μM BBR alone (61.4 ± 9.1%) or 6 Gy irradiation alone (88.2 ± 1.28%), indicating a significant enhancement of the cell death rate after 72 h ($p < 0.05$). These results showed that the combination of BBR and radiation was approximately 1.7 times more effective than radiation alone for inducing PC-3 cell death.

Counterstaining with annexin V-FITC/PI proved to be an excellent probe to distinguish apoptotic cells from necrotic cells (Fig. 3A). The annexin V-FITC-positive population of cells (apoptotic cells; lower right quadrant) did not increase with 6 Gy alone or in the vehicle control. However, BBR treatment for 72 h resulted in a significant dose-dependent enhancement in the total number of apoptotic cells: 0 μM (vehicle control, 0.7%), 30 μM (17.5%), and 50 μM (29.7%). The combination of BBR and 6 Gy resulted in a significantly greater number of apoptotic cells than BBR alone: 0 M (6 Gy alone, 1.2%), 30 μM (26.6%), and 50 μM (41.5%) (Fig. 3B).

BBR plus radiotherapy induces ROS generation. ROS play an important role in apoptosis. The regulation of ROS generation was evaluated in cells treated by irradiation with or without BBR pretreatment. ROS levels were measured using a fluorescence sensitive probe (H_2DCF-DA) that detects various active oxygen species. The ROS level increased with BBR combined with or without irradiation (Fig. 4A); however, irradiation did not induce ROS generation. We investigated the role of ROS generation by combining 30 μM BBR and irradiation in PC-3 cells (Fig. 4B). The ROS

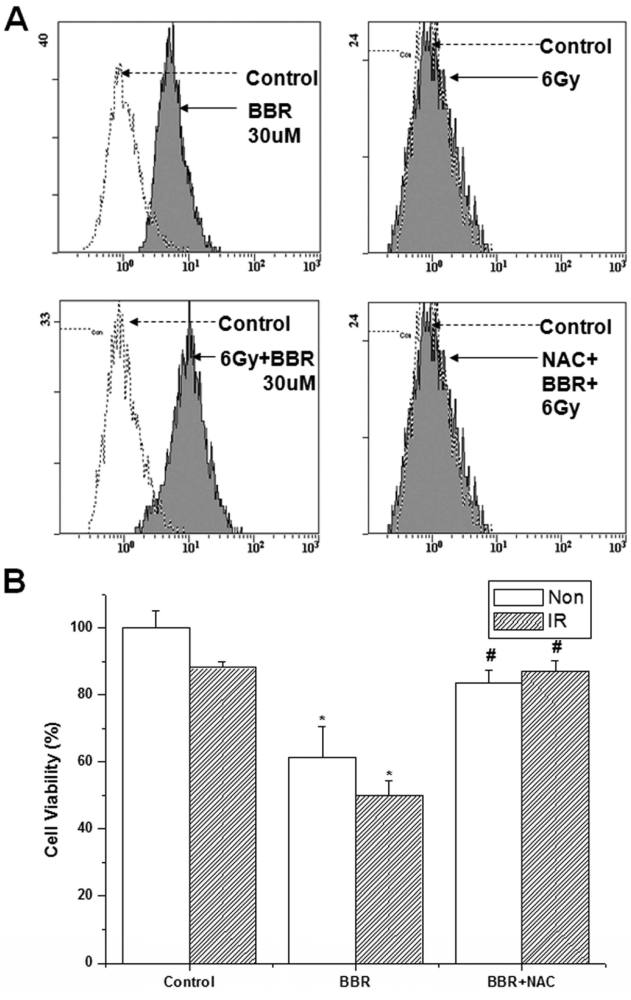

Fig. 4. The generation of reactive oxygen species (ROS) in PC-3 cells treated with 30 and 50 μM berberine (BBR) for 2 h with or without 6 Gy of radiation. A: Flow cytometry assay of ROS generation under various conditions. B: The antioxidant NAC with 30 μM BBR alone or the combination of BBR and 6 Gy-induced cell death. *; $P < 0.05$ versus control. #; $P < 0.05$ versus BBR alone.

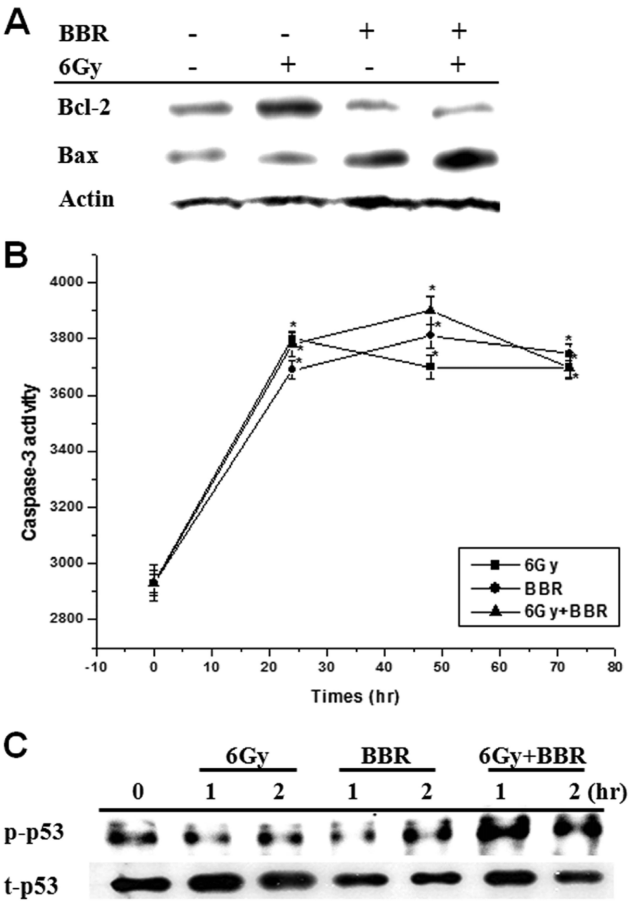

Fig. 5. Activation of pro- and anti-apoptotic proteins. A: After treatment with 30 μM berberine (BBR) and irradiation for 48 h, a Western blot analysis was used to detect Bcl-2 and Bax. B: Caspase-3 activity by proteolytic fluorogenic substrates. Ac-DEVED-pNA was used as the caspase-3 substrate, and peptide cleavage was monitored at 405 nm. C: Western blot analysis of p53 expression in PC-3 cells exposed to BBR and/or irradiation. *; $P < 0.05$ versus control.

inhibitor NAC revealed a decrease in cell death using BBR plus irradiation and BBR alone.

The expression of apoptosis-related proteins using BBR with or without irradiation. The effects of 30 μM BBR and irradiation treatment on the expression of Bax and Bcl-2 in PC-3 cells are shown in Fig. 5A. After treatment with BBR, irradiation, or both, Bax expression appeared to increase. In contrast, Bcl-2 expression decreased significantly. Caspase-3 plays a pivotal role in the terminal execution phase of apoptosis induced by diverse stimuli. Caspase-3 activation in response to 30 μM BBR and irradiation was examined using a specific fluorogenic peptide substrate (Ac-DEVD-MCA) to detect caspase-3 activity. Treatment with BBR, 6 Gy, or a combination of BBR and 6 Gy induced a dramatic increase in DEVD-specific caspase activity in PC-3 cells (Fig. 5B). A Western blot p53 expression analysis disclosed a significant increase in protein with 6 Gy plus BBR treatment (Fig. 5C).

Inhibition of radioresistance by BBR. We examined the inhibitory effect of 30 μM BBR on IκBα phosphorylation. Irradiation of PC-3 cells resulted in IκBα phosphorylation expression and degradation (Fig. 6). However, the BBR-treated PC-3 cells prior to irradiation did not show signals for IκBα phosphorylation, indicating a remarkable inhibition effect on radiation-induced IκBα phosphorylation. HO-1 expression indicates cell protective effects by oxidative stress. Irradiated cells induced HO-1, but BBR treatment reduced HO-1 in irradiated PC-3 cells (Fig. 6).

The combination of BBR plus radiotherapy influences MAP kinase. The regulation of MAP kinase using 6 Gy with or without 30 μM BBR pretreatment was investigated using Western blotting. The results indicated that the expression of p38, JNK, and ERK phosphorylation increased in

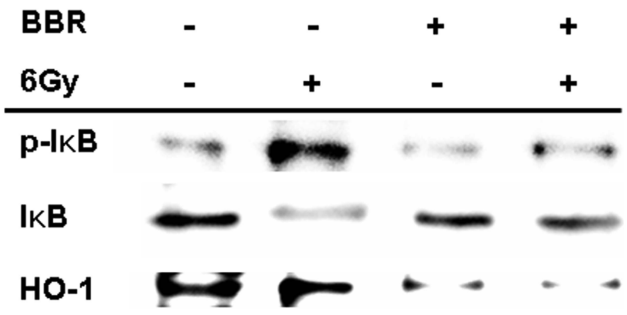

Fig. 6. The expression of radioresistant-related proteins. The effects of berberine (BBR) on irradiation-induced p-IκBα activation in PC-3 cells. Cells were pretreated with 30 μM BBR for 2 h, followed by irradiation. The cell IκBα and p-IκBα lysate levels were determined by Western blotting. Inhibition of HO-1 in BBR-treated cells. PC-3 cells were incubated with 30 μM BBR for 2 h and then irradiated (6 Gy) for 48 h.

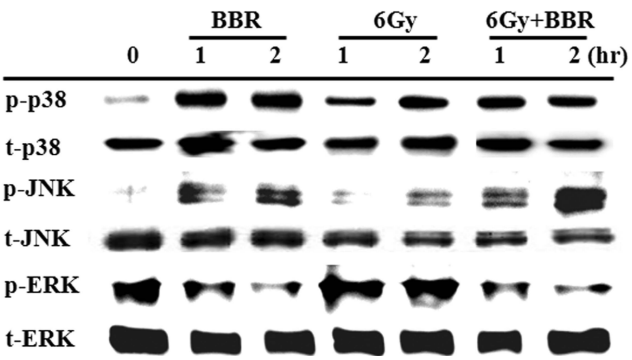

Fig. 7. Effects of berberine (BBR) and/or irradiation on MAPK phosphorylation in PC-3 cells. The cells were incubated in 30 μM BBR with or without irradiation for the indicated times and then subjected to Western blot analysis using phosphor-specific antibodies to p38, ERK, and JNK.

irradiated cells (Fig. 7). A significant increase in activated p38 and JNK was observed in PC-3 cells after exposure to irradiation with or without BBR. In contrast, when BBR pretreatment was applied alone or combined with irradiation, no ERK activation was induced.

DISCUSSION

Prostate cancer remains one of the most difficult tumors to treat (Hwang *et al*., 2006), and surgical resection has been the only curative therapy for primary prostate cancer (Keyse *et al*., 1989). Unfortunately, most patients are not candidates for surgery, and are sometimes recommended to receive nonsurgical therapies, including radiotherapy, radiofrequency hyperthermia, gene therapy, or a combination (Baldwin, 1996). Radiotherapy has been commonly used in the treatment of human prostate cancer, but the cause of radiosensitization has yet to be elucidated. New approaches that may reduce side effects and provide good quality of life are required. Thus, developing new and effective treatments, such as combined chemotherapy and radiotherapy, is imperative to effectively treat this disease (Kucharczak *et al*., 2003). We determined the effect of enhanced radiotherapy and BBR in a human prostate cancer cell line. BBR has many pharmacological effects including the inhibition of DNA and protein synthesis, arrest of cell cycle progress, and an anticancer effect (Lau *et al*., 2001; Kuo *et al*., 2004, 2005). Recently, BBR was shown to cause apoptosis through a mitochondria-caspase-dependent pathway in human hepatoma cells (He *et al*., 2006). Thus, we examined the effect of BBR using a feasible radiation dose range of 4~6 Gy and human prostate cancer cells. The PC-3 cells treated with BBR and exposed to 4 and 6 Gy showed an apparent enhancement of apoptosis, as compared to irradiation or BBR alone.

ROS play pivotal roles in DNA damage, apoptosis, and cell death induced by irradiation (Lau *et al*., 2001). In this

study, irradiation alone did not increase ROS, but BBR or a combination of BBR and irradiation elevated ROS. The ROS inhibitor NAC decreased cell death induced by BBR plus irradiation or BBR alone. Therefore, BBR enhanced the radiotherapy via ROS generation in prostate cancer cells. Mitochondria are involved in apoptosis resulting from a variety of key events including the release of caspase activators, changes in electron transport, ROS production, and participation in the regulation of both pro- and anti-apoptotic Bcl-2 family proteins (Rosser *et al*., 2003). Our results indicated that in cells pretreated with BBR prior to irradiation, the fraction of apoptotic cells increased significantly through the release of Bax and caspase-3 activation. The present data are comparable to those of a previous study reporting that downregulation of endogenous Bcl-2 using Bcl-2 antisense reduces the survival rate of prostate cancer cells following irradiation; this was suggested to be a potentially important therapeutic approach to enhance radiosensitivity in tumors via antisense oligonucleotide or other drug therapies that downregulate Bcl-2 (Rosser *et al*., 2004).

The effect of a specific molecule on radiation response often depends on the genetic background of the tumor cell, e.g., p53 (Bristow *et al*., 1996; Bohnke *et al*., 2004). Thus, using the drugs, BBR, and irradiation, we evaluated how BBR affects the radiation response. In this study, the combined treatment significantly increased p53 activation, but irradiation or BBR alone slightly increased p53 activation in PC-3 cells. Thus, p53 may play a central role mediating the induction of senescence through the combination of BBR and irradiation.

NF-κB is activated by ionizing radiation (Szotowski *et al*., 2007) and protects cells from radiation-induced apoptosis (Vink *et al*., 2006). NF-κB is inactive in the cytoplasm in a complex with IκB proteins. NF-κB is activated by phosphorylation of the nuclear factor κα (IκBα) inhibitor at two serine residues (Ser-32 and Ser-36) by IκB kinases, leading to its subsequent ubiquitination and proteasome degradation (Wada *et al*., 2004). NF-κB probably exerts its anti-apoptotic function by upregulating anti-apoptotic gene expression (Wang *et al*., 1998). In this study, we inhibited IκB degradation and phosphorylation using a combined BBR and irradiation treatment, indicating that BBR enhanced irradiation-induced apoptosis by inhibiting the anti-apoptotic pathway and radiation-induced NF-κB activation.

HO-1 is a 32-kDa inducible heat shock protein, which is found at low levels in most mammalian tissues, but is highly induced by a variety of stress stimuli, including heat shock, UV irradiation, hydrogen peroxide, heavy metals, hypoxia, and cytokines (Wilson *et al*., 1993). HO-1 and its products possess anti-inflammatory and anti-apoptotic functions (Xia *et al*., 1995). Here we showed that HO-1 expression increased following irradiation but a combined treatment of BBR and radiation did not increase HO-1 expression. HO-1 seemed to have protected the irradiated prostate cancer cells, which potentially makes cells more resistant to irradiation. However, BBR inhibited irradiation-induced HO-1 expression, indicating that BBR enhances radiosensitivity effects by inhibiting HO-1 activation.

The MAPK family includes ERK, JNK, and p38 kinase. The activation of ERK has been implicated in cell proliferation and cell cycle progression, whereas JNK and p38 are more commonly activated in response to stress and cellular damage (Zhang *et al*., 2004). In this study, the combination of BBR and/or irradiation induced p38 MAPK and JNK activation in PC-3 cells. ERK activation increased with irradiation, but BBR and BBR plus irradiation decreased ERK activation, demonstrating that activation of the MAPKs pathway was mediated by the combination of BBR and/or irradiation.

In summary, out findings suggest that BBR may be a potentially important therapeutic agent for enhancing radiosensitivity in prostate cancer via the inhibition of radioresistant proteins such as Bcl-2, NF-κB, HO-1, and ERK, which are expressed following irradiation.

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