Resveratrol and piperine enhance radiosensitivity of tumor cells

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The use of ionizing radiation (IR) is essential for treating many human cancers. However, radioresistance markedly impairs the efficacy of tumor radiotherapy. IR enhances the production of reactive oxygen species (ROS) in a variety of cells which are determinant components in the induction of apoptosis. Much interest has developed to augment the effect of radiation in tumors by combining it with radiosensitizers to improve the therapeutic ratio. In the current study, the radiosensitizing effects of resveratrol and piperine on cancer cells were evaluated. Cancer cell lines treated with these natural products exhibited significantly augmented IR-induced apoptosis and loss of mitochondrial membrane potential, presumably through enhanced ROS generation. Applying natural products as sensitizers for IR-induced apoptotic cell death offers a promising therapeutic approach to treat cancer. [BMB reports 2012; 45(4): 242-246]

INTRODUCTION

Ionizing radiation (IR) has long been used to treat patients with cancer (1). However, dose-limiting normal tissue toxicity and radioresistant tumors are still linked to life-threatening radiation treatment failure. Much interest has developed to augment the effect of radiation on tumors by combining it with targeted tumor therapeutics to improve the therapeutic ratio of these two factors (2). Therefore, efforts of many researchers have been focused on radiosensitizers, which lower the radiation dose-response threshold for cancer cells without enhancing the radiosensitivity of normal cells (1, 3).

The induction of apoptosis in cancer cells has become an indicator of the cancer treatment response and reduced mortality in patients with cancer (4). IR enhances ROS generation in a variety of cells (5). ROS, such as superoxide, hydroxyl radicals,

singlet oxygen, and hydrogen peroxide, damage critical cellular components such as DNA, proteins, and lipids, eventually causing physical and chemical damage to tissues that subsequently leads to apoptosis or neoplastic transformation (6). Because ROS are responsible for triggering cell death induced by IR, the production of additional ROS leads to irreversible oxidative stress (7). A unique antitumor strategy named oxidative therapy or pro-oxidant cancer therapy has been developed by inducing ROS generation directly on tumor cells or by preferentially inhibiting the antioxidative defense systems of tumor cells (7-9).

Here, the radiosensitizing effects of two natural products on tumor cells were investigated. Several natural products act as antioxidants in normal cells but are prooxidants in cancer cells. Resveratrol, a polyphenol found in berries, nuts, and red wine, has a variety of cancer chemopreventive activities including anti- inflammatory, pro-apoptotic, anti-angiogenic, and chemosensitizing properties in a variety of cultured cells and *in vivo* systems (10). Although resveratrol is redox active and has been claimed to be an antioxidant (11), reports also show the pro-oxidant capacity of resveratrol (12, 13). Piperine, a main component of *Piper longum Linn.* and *Piper nigrum Linn.*, is a plant alkaloid with a long history of medicinal use. Piperine exhibits a variety of biological activities, including anti-pyretic, anti-inflammatory, anti-depressant, hepatoprotective, and antitumor activities (14).

The results of this study showed that resveratrol and piperine enhanced the radiosensitivity of cancer cell lines by increasing ROS generation. Applying natural products as sensitizers for IR-induced apoptotic cell death offers a promising therapeutic approach for treating cancer.

RESULTS AND DISCUSSION

ROS initiate several cellular signal transduction pathways that may either aid the cell in coping with the excess oxidative stress resulting from the IR or activate pathways that lead to cell destruction beyond repair (15, 16). Naturally occurring compounds that enhance ROS production in cancer cells under IR may be developed as promising radiosensitizers from the perspective of the cancer cell killing potential of ROS. In the present study, the sensitizing effects of resveratrol and piperine on IR- induced apoptosis were evaluated.

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Enhanced cell death and a sensitizing effect of resveratrol and piperine on apoptosis in cancer cells were observed when cultured mouse colon carcinoma CT26 (Fig. 1) and mouse melanoma B16F10 cells (Fig. 2) were exposed to γ-irradiation. Previous studies have identified caspases as important mediators of apoptosis induced by a range of apoptotic stimuli (17). Cleavage of procaspase-3 induced by IR was more pronounced in cells treated with the natural products. Cleaved poly (ADP-ribose) polymerase products increased markedly when the cells were exposed to the combination of IR and the natural products compared to those in either treatment alone. The abundance of Bcl-2, an anti-apoptotic protein, decreased significantly following the combined treatment of IR and the natural products. Bid, a death agonist member of the Bcl-2/Bcl-X$_L$ family, is a specific proximal substrate of caspase-8 in the Fas signaling pathway (18). When cells were exposed to IR, Bid cleavage was enhanced in cells treated with natural products compared to that in untreated cells.

Alterations in mitochondrial integrity and function may play an important role in the apoptotic cascade. The mitochondrial membrane potential (MMP), associated with the opening of large pores in mitochondrial membranes, is a very important event in apoptosis, and ROS are one of the major stimuli that alter the MMP (19). The change in the MMP was assessed by measuring the intensity of fluorescence emitted from the lipophilic cation dye rhodamine 123 to evaluate whether the natural products modulated the MMP upon exposure to IR. In this assay, high fluorescence indicates healthy mitochondria. Significantly less rhodamine 123 dye was taken up by the mitochondria of cells treated with natural products compared to
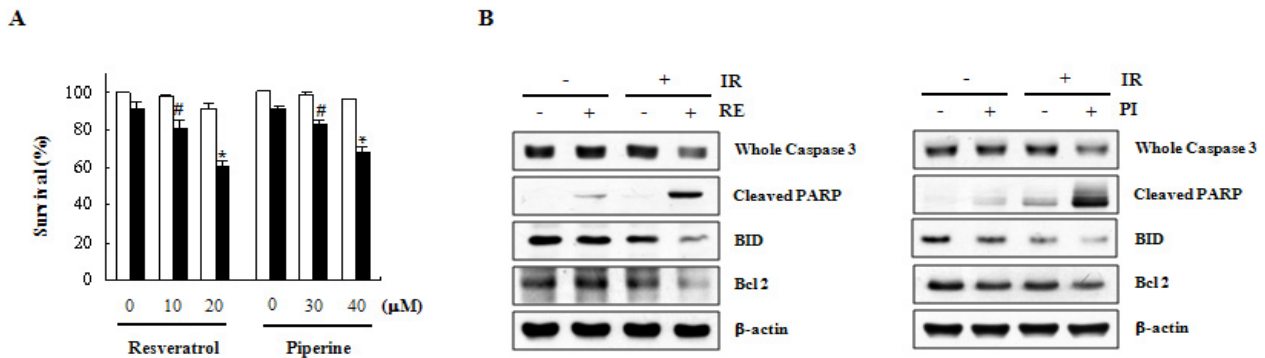

Fig. 1. Effects of natural products on viability and apoptosis of CT26 cells exposed to ionizing radiation (IR). (A) Viability of CT26 cells exposed to 15 Gy of γ-irradiation and resveratrol (RE) or piperine (PI). White and shaded bars represent cancer cells unexposed and exposed to IR, respectively. Each value represents the mean ± SD from three independent experiments. #P < 0.05 and *P < 0.01 vs. IR irradiated cells untreated with natural products. (B) Western blot analysis of apoptotic marker proteins in CT26 cells exposed to 15 Gy of γ-irradiation and 20 μM RE or 40 μM PI for 24 hr. β-Actin was run as an internal control.
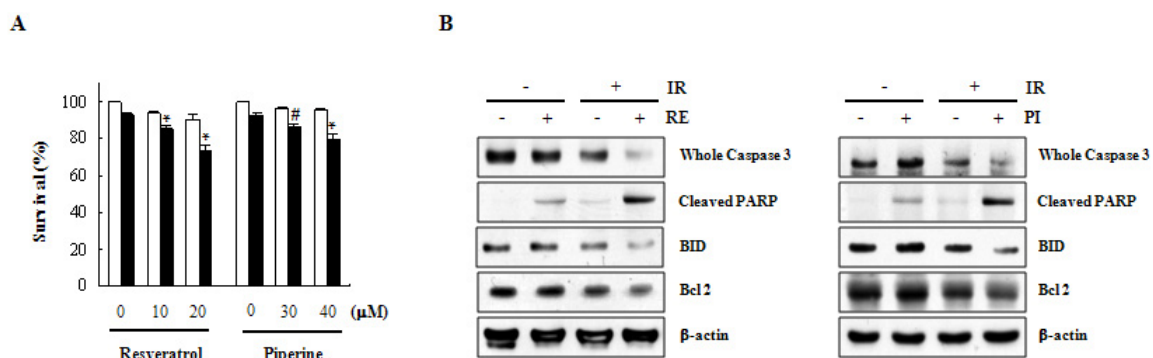

Fig. 2. Effects of natural products on viability and apoptosis of B16F10 cells exposed to ionizing radiation (IR). (A) Viability of B16F10 cells exposed to 5 Gy of γ-irradiation and resveratrol (RE) or piperine (PI). White and shaded bars represent cancer cells unexposed and exposed to IR, respectively. Each value represents the mean ± SD from three independent experiments. #P < 0.05 and *P < 0.01 versus IR irradiated cells untreated with natural products. (B) Western blot analysis of apoptotic marker proteins in B16F10 cells exposed to 5 Gy of γ-irradiation and 10 μM RE, or 30 μM PI for 24 hr. β-Actin was run as an internal control.

that in untreated cells when the cells were exposed to IR (Fig. 3A). To determine if changes in MMP were accompanied by changes in mitochondrial ROS, the levels of peroxides in the mitochondria of cancer cells were evaluated by fluorescence microscopy with the oxidant-sensitive probe dihydrorhodamine (DHR) 123. As shown in Fig. 3B, fluorescence intensity was significantly higher in cells treated with natural products compared to that in the mitochondria of untreated cells when cancer cells were exposed to IR.

To determine whether the difference in apoptotic cell death of the untreated and treated cells was associated with ROS formation, the levels of intracellular ROS in cancer cells were evaluated by fluorescence microscopy with the oxidant-sensitive probe 2′,7′-dichlorofluorescin diacetate (DCFH-DA). As shown in Fig. 4A, an increase in 2′,7′-dichlorofluorescein (DCF) fluorescence was observed in cancer cells when they were exposed to IR, and the increase in fluorescence was significantly enhanced in cells treated with the natural products. Glutathione (GSH) is one of the most abundant intracellular antioxidants, and identifying changes in its concentration provides an alternative method to monitor oxidative stress within

cells. Cellular GSH levels determined with the GSH-sensitive fluorescent dye t-butoxycarbonyl-Leu-Met-7-amino-4-chloromethylcoumarin (CMAC) in cancer cells treated with natural products decreased significantly compared to those in untreated cells (Fig. 4B).

In conclusion, the results of the current study demonstrate that the natural products resveratrol and piperine lead to the radiosensitiztion of cancer cells by increasing apoptotic cell death. Sensitization of IR-induced apoptosis by natural products is likely to be relevant to cancer treatment. The development of resistance to apoptosis in cancer cells is a major cause of treatment failure during consecutive radiotherapy. In this regard, natural products could play a role as a radiosensitizer in combined cancer therapy.

MATERIALS AND METHODS

Materials
DCFHDA, CMAC, DHR 123, and rhodamine 123 were purchased from Molecular Probes (Eugene, OR, USA). Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Cell Signaling Technology (Beverly, MA, USA).

Cell culture
Mouse colon carcinoma CT26 and mouse melanoma B16F10 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/ streptomycin in a humidified atmosphere containing 95% air and 5% CO_2 at 37°C. Cells were treated with vehicle (DMSO) or a natural product dissolved in DMSO and irradiated at room temperature with a ^{137}Cs source at a dose rate of 1 Gy/min. Cell viability following exposure to IR was determined by trypan blue exclusion after 48 hr.

Cellular redox status
ROS generation was measured by the oxidative-sensitive fluorescent probe DCFH-DA. Cells were grown at 2×10^6 cells per 100-mm plate containing a slide glass coated with poly-L-lysine and maintained in a growth medium for 24 hr. Cells were treated with 10 μM DCFH-DA for 15 min, and then the cells on the slide glass were washed with PBS and a cover glass was placed over the slide glass. DCF fluorescence (excitation, 488 nm; emission, 520 nm) was imaged by fluorescence microscopy. The intracellular GSH level was determined using the GSH-sensitive fluorescence dye CMAC. Cells (1×10^6 cells/ml) were incubated with 5 μM CMAC cell tracker for 30 min. CMAC cell tracker fluorescence images by GSH were analyzed using a Zeiss Axiovert 200 inverted microscope in the fluorescence DAPI region (excitation, 351 nm; emission, 380 nm) (20).

Western blot analysis
Whole cell lysates were fractionated by SDS-PAGE, the proteins were electrotransferred to nitrocellulose membranes, and
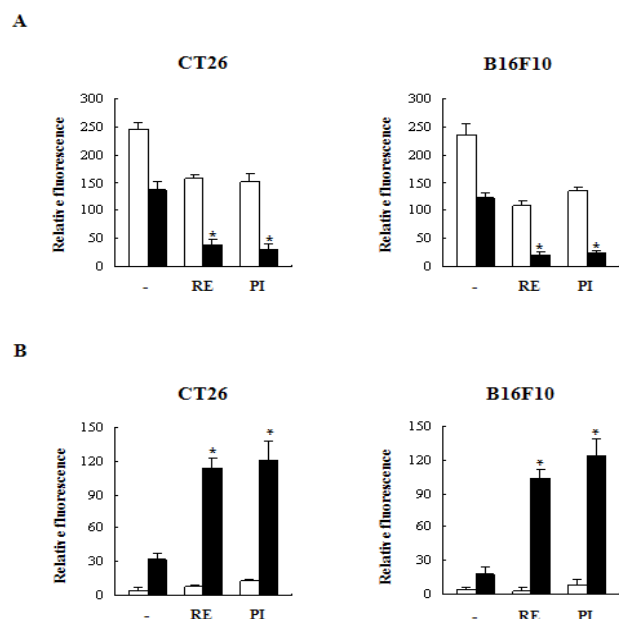

Fig. 3. Effects of natural products on mitochondrial dysfunction and mitochondrial redox status of cancer cells exposed to ionizing radiation (IR) (A) The mitochondrial membrane potential (MMP) of cancer cells was measured by rhodamine123 dye incorporation into mitochondria. (B) DHR 123 was employed to detect mitochondrial reactive oxygen species (ROS). (A, B) Fluorescence images were obtained by microscopy. Average fluorescence intensity was calculated as described previously (22). White and shaded bars represent cancer cells unexposed and exposed to IR, respectively. Each value represents the mean ± SD from three independent experiments. *P < 0.01 vs. IR irradiated cells untreated with natural products.
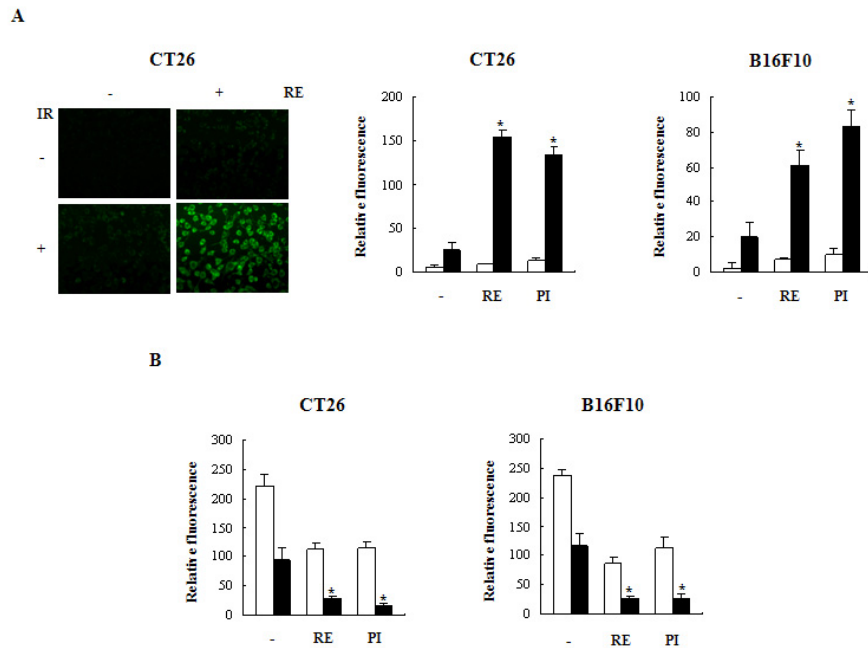
A

B

Fig. 4. Intracellular reactive oxygen species (ROS) levels in cancer cells. (A) DCF fluorescence was measured in cancer cells exposed to ionizing radiation (IR) and natural products using a fluorescence microscope. Representative images of irradiated CT26 cells untreated and treated with resveratrol are shown. (B) Glutathione (GSH) levels reflected by fluorescence images of CMAC-loaded cells were obtained under microscopy. (A, B) Average fluorescence intensity was calculated as described previously (22). White and shaded bars represent cancer cells unexposed and exposed to IR, respectively. Each value represents the mean ± SD from three independent experiments. *P < 0.01 vs. IR irradiated cells untreated with natural products.

probed with the appropriate antibodies. Proteins were visualized by using horseradish peroxidase-labeled anti-rabbit IgG and an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Mitochondrial redox status and damage

Cells in PBS were incubated for 20 min at 37°C with 5 μM DHR 123 to evaluate the levels of mitochondrial ROS, and cells loaded with the fluorescent probes were imaged with a fluorescence microscope. The change in MMP was estimated using the fluorescent cationic dye rhodamine 123, as reported previously (21). Cells were exposed to IR and then treated with 5 μM rhodamine 123 for 15 min. Fluorescence was measured at an excitation wavelength of 488 nm and an emission wavelength of 510 nm.

Quantitation of relative fluorescence

The averages of the fluorescence intensity from fluorescence images were calculated as described previously (22).

Statistical analysis

All data are presented as mean ± SD. Significance was determined using Student's *t*-test. A P value of < 0.05 was considered statistically significant.

Acknowledgements

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