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Curcumin induces radiosensitivity of *in vitro* and *in vivo* cancer models by modulating pre-mRNA processing factor 4 (Prp4)

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ABSTRACT

Radiation therapy plays a central role in adjuvant strategies for the treatment of both pre- and post-operative human cancers. However, radiation therapy has low efficacy against cancer cells displaying radio-resistant phenotypes. Ionizing radiation has been shown to enhance ROS generation, which mediates apoptotic cell death. Further, concomitant use of sensitizers with radiation improves the efficiency of radiotherapy against a variety of human cancers. Here, the radio-sensitizing effect of curcumin (a derivative of turmeric) was investigated against growth of HCT-15 cells and tumor induction in C57BL/6J mice. Ionizing radiation induced apoptosis through ROS generation and down-regulation of Prp4K, which was further potentiated by curcumin treatment. Flow cytometry revealed a dose-dependent response for radiation-induced cell death, which was remarkably reversed by transfection of cells with Prp4K clone. Over-expression of Prp4K resulted in a significant decrease in ROS production possibly through activation of an anti-oxidant enzyme system. To elucidate an integrated mechanism, Prp4K knockdown by siRNA ultimately restored radiation-induced ROS generation. Furthermore, B16F10 xenografts in C57BL/6J mice were established in order to investigate the radio-sensitizing effect of curcumin *in vivo*. Curcumin significantly enhanced the efficacy of radiation therapy and reduced tumor growth as compared to control or radiation alone. Collectively, these results suggest a novel mechanism for curcumin-mediated radio-sensitization of cancer based on ROS generation and down-regulation of Prp4K.

1. Introduction

Ionizing radiation (IR) has gained attention as a beneficial modality for cancer treatment, in which cancer patients undergo radiation therapy either alone or in combination with chemical agents, collectively known as radio-chemotherapy [1,2]. Radio-resistance remains a basic hurdle for achieving maximal therapeutic efficacy of radiotherapy for the treatment of different cancers [3]. Therefore, a valuable approach for the prevention of radio-resistance in patients undergoing radiotherapy is urgently needed. Studies have shown that concomitant treatment of radiotherapy with targeted anti-cancer agents improves the outcomes of cancer patients [4]. However, radio-sensitizers that augment tumor cell killing and reduce the radiation dose–response threshold for cancer cells with less deleterious effects on normal cells are lacking and require further development [5,6].

Reactive oxygen species (ROS) generation plays a central role in cell signaling and considered essential for various biological processes [7]. Biological systems have developed an efficient and complex structure of defense mechanisms for coping with lethal oxidative stress. The most commonly antioxidant enzymes involved in defense mechanisms include superoxide dismutase (SOD) that catalyzes the disproportion of superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2) and O_2, and peroxidases which decompose H_2O_2 and hydroperoxides [8,9]. Since ROS alterations mediate IR-induced cell death, elucidation of the factors modulating antioxidant enzymes may be of clinical importance in the protection of cells against IR-induced apoptosis [10]. NADPH is essential for the reconstruction of reduced glutathione (GSH) pool by glutathione reductase and for the activity of NADPH-dependent thioredoxin system [11], both provide protection to cells against oxidative damage. Furthermore, isocitrate dehydrogenase (ICDH) such as mitochondrial $NADP^+$-dependent ICDH (IDPm) and cytosolic ICDH (IDPc), mediate antioxidant function during oxidative stress. It has been reported that mitochondrial ICDH (IDPm) functioning to supply of NADPH needed for GSH production against mitochondrial oxidative damage [12]. Additionally, peroxiredoxins (Prx) is a group of non-seleno thiol-specific peroxidases composed of six isoforms (Prx1–Prx6), widely distributed in the cytosol, mitochondria, peroxisome and plasma, all of which inhibit ROS generation. In addition to their anti-oxidant role, these members also

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participate in various biological processes such as cell proliferation and apoptosis [13,14]. Therefore, use of compounds that induce ROS generation could be of primary importance in the treatment of cancer [15].

In recent decades, naturally occurring phytochemicals in plants have garnered considerable attention as mediators of cellular redox status as well as a strategy for the treatment of human diseases, including cancer. Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5dione], a ployphenolic turmeric constituent, inhibits cell proliferation and cell cycle transition in colon adenocarcinoma cell lines [16]. Curcumin has been reported to modulate several biological and biochemical signaling pathways, including inhibition of cell growth and induction of apoptosis through ROS generation [17]. Additionally, curcumin together with IR inhibited NF-κB activity in the ARMS cells, and reduced tumor volume in Rh30 and Rh41 ARMS xenografts [18]. Recently, a curcumin analogue T83 has been shown to sensitize human nasopharyngeal carcinoma cells to IR and induced apoptosis through inhibition of Jab1 as well as reduced cell growth and cell cycle progression [19]. Until now, the detailed mechanism of the sensitizing effects of curcumin on colorectal cancer is poorly understood.

Colorectal carcinoma and melanoma both are aggressive malignancies as well as resistant to therapeutic strategies, including chemotherapy and radiotherapy. We recently reported that curcumin-induced Prp4B-dependent apoptosis in human colorectal carcinoma (HCT-15) cells [20]. In the present study, we demonstrate that curcumin enhanced the radio-sensitivity of HCT-15 cell lines through ROS generation. Curcumin also inhibited the activation of anti-oxidant enzymes such as IDPm and IDPc, Prx1,-2, and -6, and Pre-mRNA processing factor 4 (Prp4) kinase (Prp4K). Curcumin combined with IR potentiated the efficacy of radiation against melanoma cell implanted-tumors in mice. It is our goal that elucidating the radio-sensitizing effects of curcumin on apoptosis in cancer cells will provide new insights into developing a therapeutic strategy for cancer radiotherapy.

2. Materials and methods

2.1. Materials

Curcumin, Hoechst 33342, and propidium iodide (PI) were obtained from Sigma–Aldrich (USA). 2′,7′-Dichlorofluorescin diacetate (DCFHDA) was purchased from Molecular Probes (Eugene, OR). Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), Cell Signaling (Beverly, MA) and Ab Frontier (South Korea). Electrophoresis reagents and Bio-Rad protein assay kit were provided by Bio-Rad Laboratories (USA). These chemicals were prepared according to the manufacturer's instructions.

2.2. Cell culture and treatment

HCT-15 cells (CCL-225) were obtained from ATCC, and grown at a density of 1×10^6 cells per dish in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) in an incubator containing a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Exponentially growing cells were exposed to radiation at room temperature with a 137Cs source (Cis Bio International, Gif-sur Yvette, France) at 1–5 Gy/min and treated with 30 μM curcumin.

2.3. Analysis of cell morphology

Morphological analysis of IR-induced apoptosis was performed with fluoresce microscopy after staining using Hoechst 33342 dye. After irradiation, HCT-15 cells were incubated with curcumin for 24 h, fixed in 4% paraformaldehyde and permeabilized with PBS/0.5% Triton X-100, after which nuclei were stained for 20 min using Hoechst 33342 dye.

2.4. Determination of apoptosis

Cell cycle analysis was performed by flow cytometric staining of permeabilized cells with PI to determine DNA content. Cells were collected by centrifugation, fixed with 70% ethanol, and then stained with PI solution containing 50 μg/mL of PI, 0.1% Triton X-100, 0.1 mM EDTA and 50 μg/mL of RNase for 20 min at 4 °C. Stained DNA was analyzed by a flow cytometer (Becton Dickinson), and the percentage of nuclei with sub-G1 content indicated apoptotic cells.

2.5. Measurement of ROS

Intracellular ROS concentration was assessed using the oxidant-sensitive fluorescent probe-DCFHDA, as shown previously [21]. Cells were exposed to 5 μM DCFHDA for 20 min and then washed with 1× PBS. DCF fluorescence (excitation, 480 nm; emission, 520 nm) was imaged using a laser confocal scanning microscope (DM/R-TCS, Leica) coupled to a microscope (Leitz DM REB).

2.6. Preparation of cytosolic protein fraction

Cytosolic fraction of cells was prepared as described previously [21]. Cells were sonicated in buffer A (20 mM Tris, pH 7.5, 250 mM sucrose, 10 mM EGTA, 2 mM EDTA, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL of aprotinin, 1 μg/mL of leupeptin, and 1 μg/mL of pepstatin) and centrifuged for 10 min at 1000×*g* to remove cell debris. Supernatants were then centrifuged at 13,000×*g* for 5 min and the resulting supernatant was saved as the cytosolic fraction.

2.7. Immunoblot analysis

Proteins ware extracted from whole cell lysates, fractionated by SDS–PAGE and electrotransferred onto nitrocellulose membranes. Membranes were then blocked using 5% non-fat dry milk and subsequently probed with primary antibody. Antibody–antigen complexes were visualized using horseradish peroxidase-labeled anti-rabbit or anti-mouse IgG, followed by detection using enhanced chemiluminescence (Amersham Pharmacia Biotech, UK).

2.8. Production of cDNA and siRNA-Prp4 HCT15 clones

Clone cDNA of Prp4K (sc117203) was obtained from OriGene (USA), whereas siRNA-Prp4K (SC-76257) and scrambled siRNA (sc-37007) pools were obtained from Santa Cruz Biotechnology and utilized according to the manufacturer's instructions. Briefly, HCT-15 cells were cultured at a density of 1×10^6 cells per dish to 45–55% confluence. Cells were then transfected by using both Lipofectamine LTX and Plus reagent for cDNA, whereas RNAiMAX was used for siRNA-Prp4K (Invitrogen, Carlsbad, CA, USA). After transfection, cells were exposed to radiation and curcumin. The effect of scrambled siRNA (sc-37007) was also evaluated.

2.9. Animal study protocol

Male C57BL/6J mice obtained from Hyochang Science (Daegu, Korea) were housed five mice per cage, under conditions of constant temperature of 22 °C, and a light/dark cycle of 12 h. Our experimental protocol was approved by the Institutional Animal Care and Use Committee, and is in agreement with international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, NIH publication No. 85-23, 1985). A total amount of