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Original Article

The Effect of Potassium Cyanate (KCN) on Radiation Treatment of the Colorectal Cancer Cell Line, HCT 116

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Radiation is one of the major therapy for the removal of cancer cells. The results of the radiation therapy depend on the radio-resistance of cancer cells. For the effective treatment in these radio-resistant cancers, the use of chemicals that act on cancer cells is known to enhance the cytotoxic effects of radiation therapy. In this study, I investigated the effect of potassium cyanate (KCN) on the irradiated-colorectal cancer cell line, HCT 116 cells. KCN induces the carbamylation of proteins and can change the biological activity of various human cells. To understand the effect of KCN on the radiosensitivity of HCT 116 cells, I examined alteration of the cell cycle, generation of reactive oxygen species (ROS), cell viability, apoptosis and intracellular signaling proteins in the irradiated cells with/without KCN treatment. Combination treatment caused significant increase in sub G_0/G_1 and ROS generation in HCT 116 cells. KCN inhibited the proliferation and cell viability in irradiated HCT 116 cells. KCN-induced apoptosis of irradiated cells was processed via the activation of caspase 3 and caspase 9. Apoptosis-associated signal proteins, including Bax and Bcl-2 were regulated by irradiation with KCN treatment. Taken together, these results may indicate that KCN enhances the radiosensitivity of radio-resistant cell and then has a synergistic effect on radiation therapy in colorectal cancer.

Key Words: Potassium cyanate, Colorectal cancer, Radiosensitivity, Radio-resistant cell

INTRODUCTION

Irradiation is integral to the treatment of many tumors including colorectal, breast, lung and gastrointestinal cancers. The radiation therapy is particularly important as an initial therapy for colorectal cancer. In the colorectal cancer, the surgeon's ability to obtain tumor clearance is often challenged by the anatomic constraints of a large tumor in a narrow pelvis. Radiation has the potential to decrease tumor size before surgery, enabling a greater chance of obtaining a tumor-free surgical margin (Blumberg and Ramanathan, 2002). Unfortunately, many tumors, including colorectal cancers, are radio-resistant (Mehta et al., 2001; Read et al., 2001; Bouzourene et al., 2002). For the effective treatment in the radio-resistant cancers, the use of chemicals that have the cytotoxic effect on cells is known to enhance the inhibitory effects of radiation therapy. Although many studies have been reported for the chemicals that enhance the radiosensitivity, the chemicals that induce carbamylation of cellular protein have not been fully investigated in radio-resistance of cancer cells.

Potassium cyanate (KCN) is an inorganic compound and is the conjugate based of cyanic acid. Cyanic acid reacts irreversibly with the N-terminal groups of amino acid, peptides within proteins and this process is known as carbamylation (Fluckiger et al., 1981; Kraus and Kraus, 2001). Carbamylation can change the structure of proteins, and modify the activity of enzymes, cofactors, hormones and antibodies (Kuckel et al., 1993; Inoue et al., 2001). Recently, the carbamylated proteins induce cell death in various diseases, including chronic kidney disease, athero-

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sclerosis and coronary artery disease (OK et al., 2005; Apostolov et al., 2011). These evidences indicate that KCN has potential cytotoxic effect against tumor cells.

In the present study, I investigate the effect of KCN on the radiosensitivity of colorectal cancer cells. To understand the anti-cancer effect of KCN, I examined alteration of the cell cycle, generation of reactive oxygen species (ROS), cell viability, apoptosis and intracellular signaling proteins in the irradiated cells with/without KCN treatment.

MATERIALS AND METHODS

Reagents

Potassium cyanate (KCN) (Sigma Aldrich, St. Louis, MO) was dissolved in sterile distilled water to prepare the stock solutions (10 mg/ml). RPMI 1640 medium and FBS were purchased from Life Technologies, Inc. (Gaithersburg, MD). Trypan blue stain solution, anti-Bcl-2, anti-Bax, Anti-β-actin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). 2', 7'-dichlorofluorescein diacetate (DCFDA) was purchased from Fluka Chemie GmbH (Steinheim, Switzerland). Cell proliferation kit was purchased from Roche (Penzberg, Germany). Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit was purchased from BD biosciences (San Diego, CA). Bcl-2 human ELISA kit and Bax ELISA kit were purchased from Abcam (Massachusetts, USA). Caspase-Glo® 3 assay kit and caspase-Glo® 9 assay kit were purchased from Promega (Madison, WI).

Cell culture and KCN treatment

HCT 116 cells were the human colorectal cancer cell line and were purchased from American Type Culture Collection (Rockville, MD, USA). These cells were cultured in RPMI 1640 medium supplemented with 10% heatinactivated FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml), and were incubated at 37℃ in 5% CO_2 incubator.

For KCN treatment, HCT 116 cells were incubated for 12 hours in the absence and presence of KCN (0.1, 1, 2.5 and 5 mM).

Radiation experiments

Cells cultured in 90Φ cell culture dish were treated either with KCN and irradiation (1, 2 and 4 Gy) or combinations. Irradiation was administered by a Leksell Gamma Knife B2 type. Immediately after irradiation, cells were replenished with fresh media containing no drugs and left in the incubator for 24 hours for experiments as described below.

Cell cycle

Following treatment, cells were fixed in ice cold 70% ethanol and stored at -20℃. For DNA staining, cells stained with 50 μg/ml propidium iodide (PI). Cell cycle analysis was performed using a FACScan flow cytometry (BD bioscience) and the percentage of cell in sub G_0/G_1 phase was determined using the CellQuest software (BD bioscience).

ROS production

After each treatment, the cells were washed and were resuspended at 1×10^7 cells/ml in prewarmed PBS, respectively. The cells were 5 μM of DCFDA to label the intracellular ROS and were incubated for 10 min at room temperature. Labeled cells were immediately observed using fluorescence activated cell sorting (FACS) analysis (BD Biosciences).

MTT assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole) assay was performed to determine cell proliferation using the cell proliferation kit (Roche, Penzberg, Germany). After KCN treatment or irradiation, HCT 116 cells in 100 μl of the culture medium were plated into a 96-well culture plate. 10 μl of MTT solution was added in each well. After incubation of the plate at 37℃ for 4 h, 100 μl of solubilization solution was added to each well. After 24 h incubation, the absorbance was measured using an ELISA reader (Bio-Tek Instruments, Winooski, VT) at 550 nm.

Trypan blue exclusion test

Cell viability was determined by the dye exclusion test.

A cell suspension was simply mixed with dye and then visually examined to determine whether cells take up or exclude dye. A viable cell will have a clear cytoplasm whereas a nonviable cell will have a blue cytoplasm. The cell survival index was calculated as follows: viable cells (%) = total number of viable cells per ml of aliquot/total number of cells per ml of aliquot × 100.

Cell apoptosis

For measurement of the apoptosis, the cells were incubated with the FITC-labeled annexin V and propidium iodide (PI) for 15 min at room temperature. Annexin V is a marker for phosphatidylserine (PS), which exposed on plasma membrane at the initial stage of apoptosis. PI is a marker for membrane permeability in the late-apoptosis and necrotic cells. Apoptotic cells were analyzed by flow cytometry using CellQuest software and were defined as the cells in the right quadrant that stained positive for annexin V with/without PI. To analyze, 10,000 events were collected for each sample.

Enzyme linked immunosorbent assay

The cells were washed with ice-cold PBS and lysed with lysis buffer (10 mM HEPES, 10 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 1% NP-40, 0.5 mM PMSF, 0.1 mM DTT, 0.1 mM Na_3VO_4, and protease inhibitors). To determine the concentrations of Bcl-2 and Bax in the cell lysates, I was performed a sandwich ELISA using Bcl-2 human ELISA kit and Bax ELISA kit according to the manufacturer's instructions. All assays were performed tin triplicate. The concentration of each protein was calculated from standard curve.

Caspase-3/7 and 9 activities

Caspase-3/7 and -9 activities were performed in triplicates using assay kits Caspase-Glo 3/7 and 9 on white 96-well microplate. Then, caspase activity was investigated according to manufacturer's protocol. Briefly, 100 μL of the caspase-Glo reagent was added and incubated at room temperature for 30 minutes. The presences of active caspases from apoptotic cells will cleave the synthetic tetrapeptide, labeled with aminoluciferin in the reagent. The released amino-luciferin acts as a substrate for the luciferase enzyme in the reagent to produce light, which is measured using Tecan Infinite 200 Pro (Tecan, Männedorf, Swizerland) microplate reader.

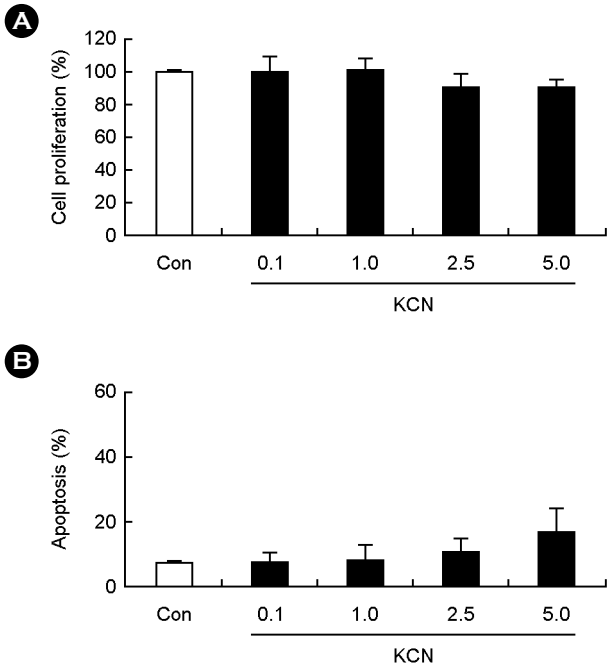

Fig. 1. The effect of KCN on cell proliferation and apoptosis of HCT 116 cells. HCT 116 cells were incubated for 12 h in the absence and presence of KCN (0.1, 1.0, 2.5 and 5.0 mM). (A) The cell proliferation of these cells was measured by performing MTT assay. The data are expressed as the relative ratio to the absorbance of the untreated cells (Con), which was set at 100%. (B) Apoptosis of these cells was analyzed by measuring the binding of annexin V-FITC and PI using flow cytometry. Apoptotic cells were defined as the cells that stained positive for annexin V with/without PI. Data are expressed as the means ± SD in three individual experiments.

Statistical analysis

All data were expressed as mean ± SD. Data were analyzed by Student's *t*-test using SPSS statistical software package (Version 10.0, Chicago, IL). A *P* values less than 0.05 was considered statistically significant.

RESULTS

KCN has no cytotoxic effect on HCT 116 cells

Before the effect of KCN on the irradiated HCT 116 cells was examined, I first confirmed the KCN-induced effects on the cell proliferation and the apoptosis of HCT

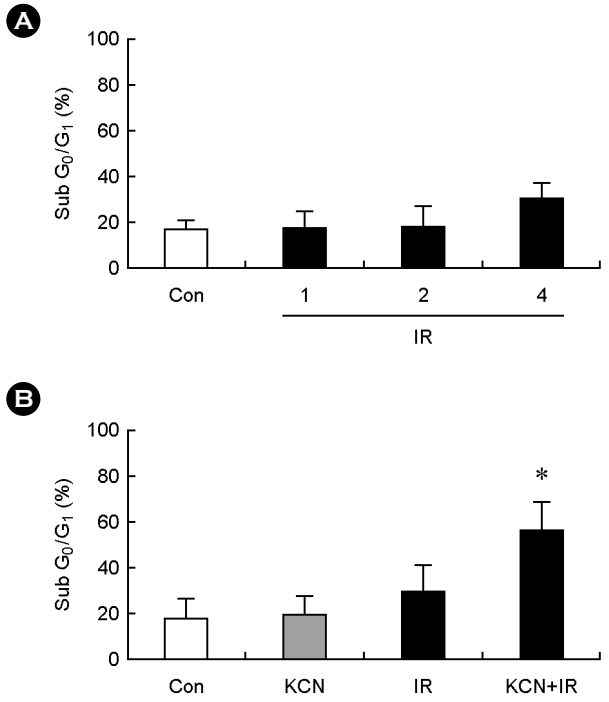

Fig. 2. The effect of KCN, irradiation and the combined treatment on cell cycle regulation in HCT 116 cells. (A) HCT 116 cells were treated with irradiation (IR) (1, 2 and 4 Gy) or with out IR (Con). After radiation, cells were incubated for 24 h. (B) HCT 116 cells were treated either with KCN (KCN), irradiation of 4 Gy (IR) or combinations (KCN + IR). Sub G_0/G_1 phase in cell cycle of these cells was assessed using PI stain with flow cytometry. Data are expressed as the means ± SD in three individual experiments. $*P < 0.05$ was considered as significant difference between the control group and treatment group.

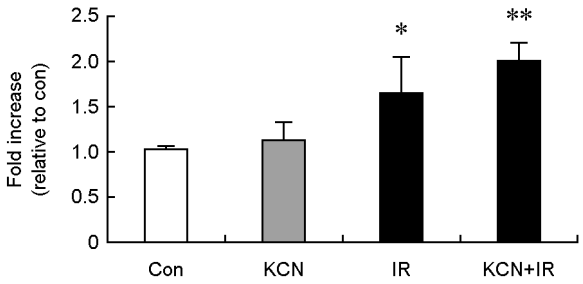

Fig. 3. The effect of KCN, irradiation and the combined treatment on ROS generation in HCT 116 cells. HCT 116 cells were treated either with KCN (KCN), irradiation of 4 Gy (IR) or combinations (KCN + IR). The ROS generation was determined by the CDFCA fluorescence with flow cytometry. Data are expressed as the means ± SD in three individual experiments. $*P < 0.05$ and $**P < 0.01$ were considered as significant difference between the control group and treatment group.

116 cells. After addition of KCN in HCT 116 cells, cell proliferation was slightly decreased without statistical significance (Fig. 1A). KCN also has no effect on the apoptosis after KCN treatment (Fig. 1B). For the treatment of non-toxic chemicals, KCN was used at concentration of 5 mM in the subsequent experiments of this study.

KCN increases sub G_0/G_1 phase and ROS generation in the irradiated HCT 116 cells

I initially measured the dose necessary for HCT 116 cells to have radio-resistant activity. HCT 116 cells were irradiated at doses of 1, 2 and 4 Gy. 24 hours after irradiation, cells were harvested for flow cytometry analysis and the percentage of cells in sub G_0/G_1 phase was plotted as these phases were the most modulated. The percentage of cells in sub G_0/G_1 phase was not significantly altered by irradiation at 1, 2 and 4 Gy (Fig. 2A). In subsequent irradiation experiments, HCT 116 cells were irradiated at a dose of 4 Gy. To determine the cell cycle modulation by irradiation with KCN treatment, HCT 116 cells were incubated with 5 mM KCN for 12 hours, treated with 4 Gy of irradiation or carried out combined treatment. As a result, HCT 116 cells showed a significant increase in sub G_0/G_1 phase when combination treatment (Fig. 2B).

To investigate the further mechanism of KCN-induced effect on irradiated HCT 116 cells, intracellular ROS level was measured by DCFDA labeling of the cells. As shown in Fig. 3, the exposure to KCN induced the generation of ROS in the irradiated cells.

HCT 116 cells enhance cancer radioresponsiveness by promoting apoptosis

To determine the other effects of KCN on the radio-resistant cells, I examined the cell proliferation, viability and apoptosis. The levels of proliferation and viability of cell-pretreated with KCN were inhibited by 4 Gy of irradiation (Fig. 4A and 4B). Apoptosis of HCT 116 cells was also considerably increased by the combination of KCN and irradiation (Fig. 4C). These facts confirmed that the increased in sub G0/G1 phase induced by the combined treatment of KCN and irradiation (Fig. 2B) is due to apoptosis.

To confirm that the cells are undergoing apoptosis, the

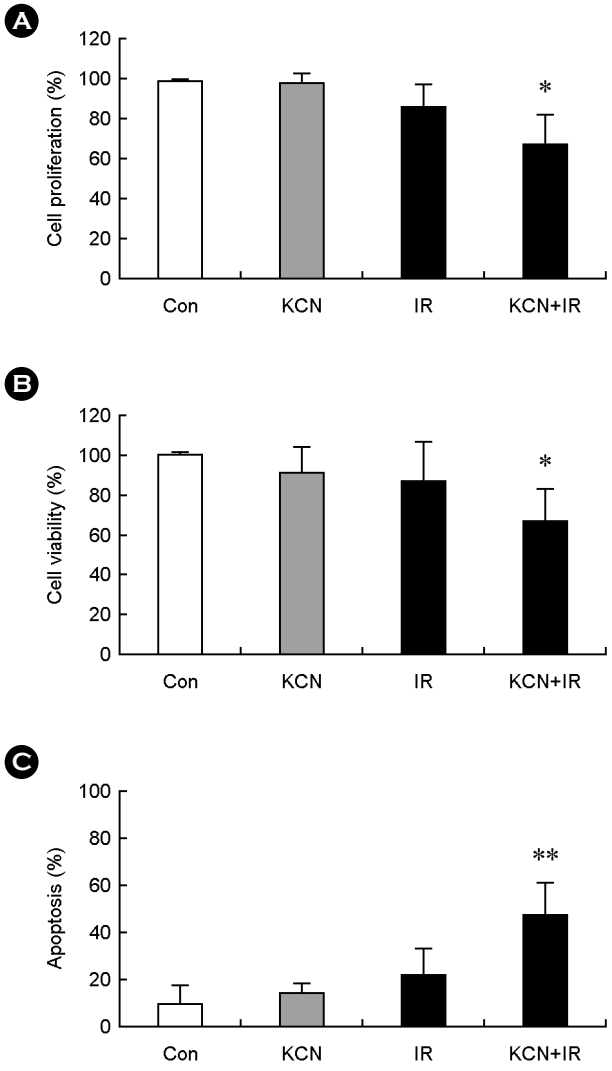

Fig. 4. The effect of KCN, irradiation and the combined treatment on survival and death of HCT 116 cells. HCT 116 cells were treated either with KCN (KCN), irradiation of 4 Gy (IR) or combinations (KCN + IR). (A) The cell proliferation of these cells was measured by performing MTT assay. The data are expressed as the relative ratio to the absorbance of the untreated cells (Con), which was set at 100%. (B) Cell viability was determined by trypan blue exclusion test. (C) Apoptosis of these cells was analyzed by measuring the binding of annexin V-FITC and PI using flow cytometry. Apoptotic cells were defined as the cells that stained positive for annexin V with/without PI. Data are expressed as the means ± SD in three individual experiments. $*P < 0.05$ and $**P < 0.01$ were considered as significant difference between the control group and treatment group.

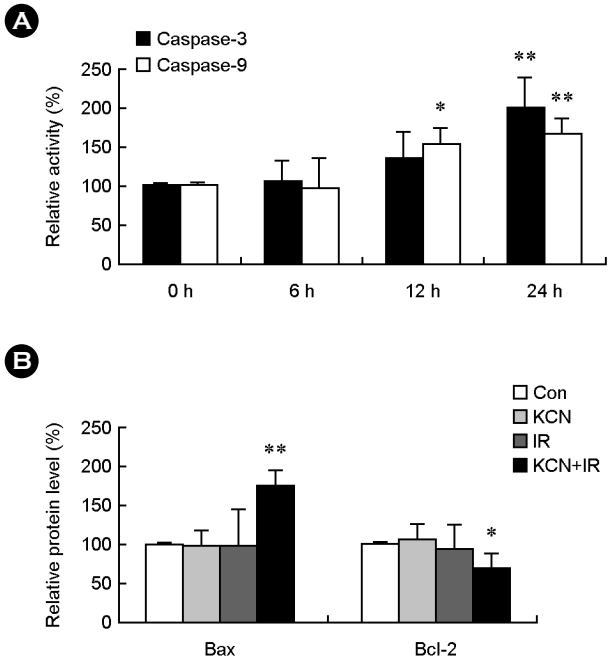

Fig. 5. The effect of KCN, irradiation and the combined treatment on the apoptosis-associated proteins in HCT 116 cells. (A) HCT 116 cells were treated with KCN (5 mM) and irradiation (4 Gy) for the indicated time. Harvested cells were lysed and cell lysates were used for activities of caspase 3 and caspase 9 using luciferase assay. $*P < 0.05$ and $**P < 0.01$ were considered as significant difference between the control group (0 h) and KCN + IR treated group. (B) HCT 116 cells were treated either with KCN (KCN), irradiation of 4 Gy (IR) or combinations (KCN + IR). After incubation for 24 h, harvested cells were lysed and cell lysates were performed ELISA for determination of Bax and Bcl-2 levels in HCT 116 cells. The data are expressed as the relative ratio to the absorbance of the untreated cells (Con), which was set at 100%. $*P < 0.05$ and $**P < 0.01$ were considered as significant difference between the control group and treatment group. All Data are expressed as the means ± SD in three individual experiments.

activity assay of both caspase 3 and caspase 9 were performed. Radiation is known to induce the intrinsic caspase pathway that involves caspase 9 (Johnson and Jarvis, 2004). Although the levels of caspase 3 and caspase 9 activation were slightly rose to that observed in control levels when cell were incubated until 12 hours after treatment with KCN and irradiation, the activities of caspase 3 and caspase 9 were markedly increased at 24 hours after irradiation with KCN (Fig. 5A).

I then investigated whether the effect of KCN on radiosensitization is associated with changes in the levels of apoptosis-associated proteins such as Bax and Bcl-2. ELISA was performed to estimate the levels of Bax and Bcl-2. Up-regulation of Bax and down-regulation of Bcl-2 induce the pro-apoptotic response in the cell leading to the release of cytochrome *c* via mitochondrial pathway and

promoting cell death. When HCT 116 cells pretreated with KCN were irradiated, the level of Bax, a pro-apoptotic signal protein, increased until 24 hours incubation (Fig. 5B). In contrast, KCN continuously suppressed the level of Bcl-2, a anti-apoptotic signal protein (Fig. 5C). These data indicate that the cell death of HCT 116 cells mediated by combination treatments is induced by the regulation of Bax/Bcl-2 expression levels and the activation of caspase 3/9. And the enhanced radioresponsiveness of HCT 116 cells was associated with promoting apoptosis.

DISCUSSION

In the colorectal cancer therapy, surgical resection is important for tumor clearance and radiation is an adjunctive process before or after surgery. However, tumor cells in colorectal cancers are known to be relatively radio-resistant cells than other tumor cells (Itani et al, 2007). In intracellular signaling pathway of colorectal cancer cells, the signaling activities of survival-associated proteins strongly increase, however, other pro-apoptotic proteins decrease (Markowitz and Bertagnolli, 2009). Therefore, there is a need for the development of potent drugs for the enhanced radio-responsiveness of colorectal cancer cells.

Among the intracellular process for the regulation of biological activity of cells, carbamylation can change the structure of proteins, and can modify the activity of proteins (Kuckel et al., 1993; Inoue et al, 2001). Recently, the carbamylated proteins induce cell death in various diseases, including chronic kidney diseases, atherosclerosis and coronary artery disease (OK et al., 2005; Apostolov et al., 2011). This carbamylation is induced by potassium cyanate (KCN). KCN is an inorganic compound and is the conjugate based of cyanic acid. In the process of carbamylation, the cyanic acid reacts irreversibly with the N-terminal groups of amino acid, peptides within proteins (Kraus and Kraus, 2001). In the healthy control, cyanic acid is derived from urea and normally present in human blood plasma and is not harmful to normal cells (OK et al., 2005; Apostolov et al., 2011). So, in this study, I used KCN to reduced the radio-resistance of HCT 116 cells, the colorectal cancer cell line.

I first demonstrated that both KCN and irradiation had no effects on the proliferation, apoptosis and cell cycle of HCT 116 cells, respectively (Fig. 1 and Fig. 2A). However, the cell death of HCT 116 cells was significantly increased by combined treatment with 5 mM KCN and 4 Gy irradiation (Fig. 4). These cell death occurred via apoptotic pathway. Apoptosis occurs by extracellular factors and induces the intrinsic death pathways. In early stage of apoptosis, mitochondria membrane potential (MMP, $\Delta\Psi m$) is altered by opening of permeability transition pore (PTP), and it continuously mediates pro-apoptotic signals. KCN-induced apoptosis in irradiated HCT 116 cells causes by alteration of apoptosis-associated protein, including the suppression of Bcl-2 expression and the increase of Bax expression (Fig. 5B and 5C). The anti-apoptotic Bcl-2 protein has been involved in resistance to conventional cancer treatment. Bcl-2 has been reported to exert its inhibitory effects on apoptosis by blocking the release of cytochrome c and the loss of MMP (Gross et al., 1999). In contrast, Bax is a pro-apoptotic protein and integrates to the outer mitochondrial membrane and causes cytochrome *c* release. These process also can induce mitochondria hyperpolarization and then can generate ROS. The excess ROS generation into cell has been associated with cell death, such as apoptosis, and mediate the activation of the caspase pathway (Terasaka et al., 2005; Ishihama et al., 2008). This increase of ROS generation in response to both KCN and irradiation may be related to the induction of apoptosis and cell cycle arrest. As shown in Fig. 2B, sub G_0/G_1 phase of irradiated HCT 116 cells increased by pretreatment of KCN. The increased in sub G_0/G_1 phase was considered to arrest of cell cycle.

In conclusion, KCN enhances the sensitivity for radiation in radio-resistant cancer cells and its process occurred by increased apoptosis via pro-apoptotic signaling pathway and cell cycle arrest. These data indicate that KCN could be used as a drug of radiosensitizer to involved in radiation enhancement.

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REFERENCES

Apostolov EO, Ray Debarti, Alovuia WM, Mikhailova MV, Wang X, Basnakian AG, Shah SV. Endonuclease G mediates endothelial cell death induced by carbamylated LDL. Am J Physiol Heart Circ Physiol. 2011. 300: H1997-H2004.

Blumberg D, Ramanathan RK. Treatment of colon and rectal cancer. J Clin Gastro Enterol. 2002. 34: 15-26.

Bouzourene H, Bosman FT, Seelentag M, Matter M, Coucke P. Importance of tumor regression assessment in predicting the outcome in patients with locally advanced rectal carcinoma who are treated with preoperative radiotherapy. Cancer. 2002. 94: 1121-1130.

Fluckiger R, Harmon W, Meier W, Gabbay KH. Hemoglobin carbamylation in uremia. N Engl J Med. 1981. 304: 823-827.

Gross A, McDonnell JM, Korsmeyer SJ. Bcl-2 family members and the mitochondria in apoptosis. Genes Dev. 1999. 13: 1899-1911.

Inoue EN, Nagano I, Ichinohasama R, Asato N, Kondo Y, Iinuma K. Bimodal effects of platelet-derived growth factor on rat mesangial cell proliferation and death, and the role of lysophosphatidic acid in cell survival. Clin Sci (Lond). 2001. 101: 11-19.

Itani W, Geara F, Haykal J, Haddadin M, Gali-Muhtasib H. Radiosensitization by 2-benzoyl-3-phenyl-6,7-dichloroquinoxaline 1,4-dioxide under oxia and hypoxia in human colon cancer cells. Radiation Oncology. 2007. 2: 1.

Johnson CR, Jarvis WD. Caspase-9 regulation: an update. Apoptosis. 2004. 9: 423-427.

Kraus LM, Kraus AP. Carbamylation of amino acids and proteins in uremia. Kidney Int. 2001. 78: S102-S107.

Kuckel CL, Lubit BW, Lambooy PK, Farnsworth PN. Methylisocyanate and actin polymerization: The in vitro effects of carbamylation. Biochim Biophys Acta. 1993. 1162: 143-148.

Markowitz SD, Bertagnolli MM. Molecular basis of colorectal cancer. N Engl J Med. 2009. 361: 2449-2460.

Mehta VK, Poen J, Ford J, Edelstein PS, Vierra M, Bastidas AJ, Young H, Fisher G. Radiotherapy, concomitant protracted-venous infusion 5-fluoro-uracil, and surgery for ultrasound-staged T3 or T4 rectal cancer. Dis Colon Rectum. 2001. 44: 52-58.

OK E, Basnakian AG, Apostolov EO, Barri YM, Shah SV. Carbamylated low-density lipoprotein induces death of endothelial cells: A link to atherosclerosis in patients with kidney disease. Kidney Int. 2005. 68: 173-178.

Read TE, McNevin EKM, Gross HM, Lewis JL, Ratkin G, Picus J, Birnbaum EH, Fleshman JW, Kodner IJ, Myerson RJ. Neoadjuvant therapy for adenocarcinoma of the rectum: tumor response and acute toxicity. Dis Colon Rectum. 2001. 44: 513-522.

Terasaka H, Kadoma Y, Sakagami H, Fujisawa S. Cytotoxicity and apoptosis-inducing activity of bisphenol A and hydroquinonein HL-60 cells. Anticancer Res. 2005. 3B: 2241-2247.