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Combined Effect of Radiation and 7β-Hydroxycholesterol on Human Cervical Cancer Cells *in vitro*

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ABSTRACT : 7β-Hydroxycholesterol (cholest-5-ene-3, 7-diol, 7β-OHC) showed the cytotoxicity on human cervical carcinoma cells (HeLa), 10 μM of 50% inhibitory concentration. We evaluated 7β-OHC as the possibility of radiation sensitizer. The combination effect of 7β-OHC and γ-irradiation was measured using colony forming assay and flow cytometer with propidium iodide and $DiOC_6$ stained cells, respectively. The combined treatment of 7β-OHC and γ-irradiation did not show significant enhancing effects on HeLa cells.
Key words : 7β-OHC, radiation, HeLa cells

Introduction

Oxysterols, or the products of cholesterol oxidation, are naturally occurring compounds present in vegetal and animal organisms. Oxysterols can be formed either from lanosterol, the precursor of cholesterol, or from diversion of squalene 2 : 2-epoxide to squalene 2 : 3, 22 : 23-dioxide and subsequent cyclization of epoxysterols, or from controlled enzymic oxidation of endogenous cholesterol or exogenous cholesterol derived from low-density lipoproteins, LDL (Gibbons, 1983; Gupta *et al.*, 1986).

Several of these oxygenated cholesterols have been reported to be highly cytotoxic towards normal and tumor cells, according to their structure and the cell type (Schroepfer, 2000). The mode of cytotoxicity has been identified to involve apoptosis in certain cell lines (Hyun *et al.*, 1997; Hietter *et al.*, 1986; Christ *et al.*, 1991 and 1993; Aupeix *et al.*, 1995; Ayala-Torres *et al.*, 1999). Though not fully elucidated, the exact mechanism through which oxysterol induces cell death is related with the generation of an oxidative stress (Ryan *et al.*, 2004). 7-keto-cholesterol and cholestane-triol have been shown to increase the antioxidant enzyme activities, such as catalase, superoxide dismutase and glutathione peroxidase in rat hepatocytes, which is indicative of oxidative stress generation (Cantwell and Devery, 1998). During oxidative stress, the intracellular antioxidant enzyme, glutathione (GSH) becomes oxidized and exits the cell, leading to glutathione depletion (Reed, 1990). Thus, it cause decrease in the glutathione concentration of the cells and may be involved in the opening of a pore in the mitochondrial membrane known as the mitochondrial permeability transition pore (Chernyak and Bernardi, 1996), which results in loss of mitochondrial transmembrane potential and organelle swelling (Hengartner, 2000), indicating that glutathione depletion may be necessary process for apoptosis. In addition, glutathione metabolic pathways contribute to protect against oxidative stress in the brain, due to its effective reduction of peroxides in a non-enzymatic reaction (Meister and Anderson, 1983). And the recent studies showed that intracellular ROS was produced via the lowering of the glutathione level in aluminum-mediated cell death (Satoh *et al.*, 2005) and attenuation of glutathione depletion by antioxidant (Sohn *et al.*, 2005).

The cytotoxicity of oxysterols mainly results from the inhibition of HMG-CoA reductase, a key-enzyme in the endogenous cholesterol synthesis pathway. Thus, in dividing cells treated with oxysterols, membrane formation is impaired and thereby their growth is prevented or severely hindered (Schroepfer, 2000; Luu, 1988; Chen, 1984). Moreover, the interaction of these substances with plasma membranes following their insertion into the phospholipid bilayers could lead to structural distortion, which may also participate in their cytotoxicity. Oxysterols affected the membrane enzyme activities (Moog *et al.*, 1991), and their permeability to ions (Boissoneault and Heiniger, 1985) and proteins

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(Boissoneault *et al.*, 1991). Also, it was reported that cholesterol oxides can inhibit DNA synthesis in replicating cells (Astruc *et al.*, 1983; Defay *et al.*, 1982) and that they can potentially disturb normal cellular metabolism and homeostasis by inducing excess lipid accumulation in cells (Higley and Taylor, 1984).

Thus, these cholesterol oxides treatments can alter cellular functions and the objective of the present study was to investigate whether 7β-OHC, a potent oxysterol, could enhance the susceptibility to irradiated HeLa cells.

Materials and methods

Cell culture

HeLa human cervix cancer cells were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin and streptomycin.

Irradiation

Cells were plated in 6-cm dishes and incubated at 37°C under humidified 5% CO_2, 95% air in culture medium until 70% to 80% confluent. Cells were then exposed to γ-rays from a ^{137}Cs γ-ray source (Atomic Energy of Canada, Canada, located in Korea Institute of Radiological and Medical Sciences, Seoul, Korea) at a dose rate of 3.81 Gy/minute.

Reagents

7β-OHC was obtained from Sigma, freshly dissolved in ethanol, and the final concentration of 7β-OHC did not exceed 0.2%.

Clonogenic forming assay

HeLa human cervix cancer cells were seeded into 60 mm dishes at a density to produce approximately 300 colonies per dish. And they were treated at various doses of 7β-OHC and were incubated for 10-14 days. The formed colonies were fixed with 75% ethanol and 25% acetic acid, and stained with trypan blue. The number of colonies consisting of more than 50 cells was counted.

Flow cytometric analysis

The cell death was measured by flow cytometry with propidium iodide (PI) staining. Cells were treated with 7β-OHC at 10 μM alone, γ-irradiation at 10 Gy alone, and combination of both 7β-OHC at 10 μM and γ-irradiation at 10 Gy. After 24 and 48 h, the cells were washed with ice-cold PBS and treated with 1 mg/ml RNase for 30 min at 37°C. Cellular DNA was stained with 50 μg/ml PI and in 2 mM EDTA-PBS. Cells were then analyzed by FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential was determined as the retention of the mitochondria-specific dye $DiOC_6(3)$. Cells were treated with 7β-OHC at 10 μM alone, γ-irradiation at 10 Gy alone, and combination of both 7β-OHC at 10 μM and γ-irradiation at 10 Gy. After 24 and 48 h, cells were loaded for 30 min at 37°C with 30 nM $DiO_6(3)$ and supernatant was removed by suction and after trypsin treatment, cells were washed with PBS. Fluorescence of $DiOC_6(3)$ loaded cells was measured using a flow cytometer.

Statistical analysis

All the measurements were made in triplicate. The results were subjected to an analysis of the variance (ANOVA) using the Turkey test to analyze the difference. $p < 0.05$ were considered significantly.

Results

Clonogenic survival assays were performed at 5, 10, 15 and 20 μM of 7β-OHC on HeLa cells. As shown in Fig. 1, clonogenic survival of HeLa cells was inhibited

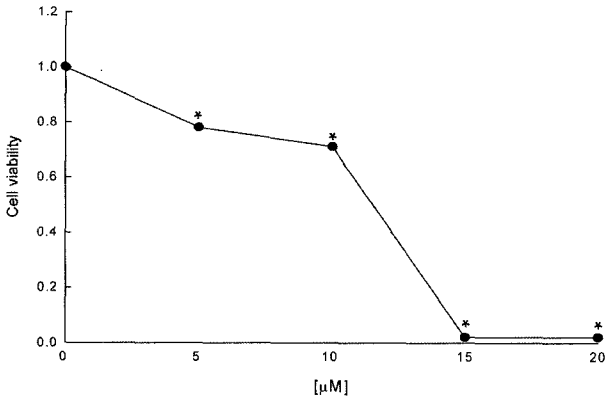

Fig. 1. Effect of 7β-OHC on HeLa cells *in vitro*. Cells were treated with various concentrations of 7β-OHC. Cells were allowed to grow for 10-14 days and were stained with trypan blue and scored for colony formation. Results are given as means ± S.D.

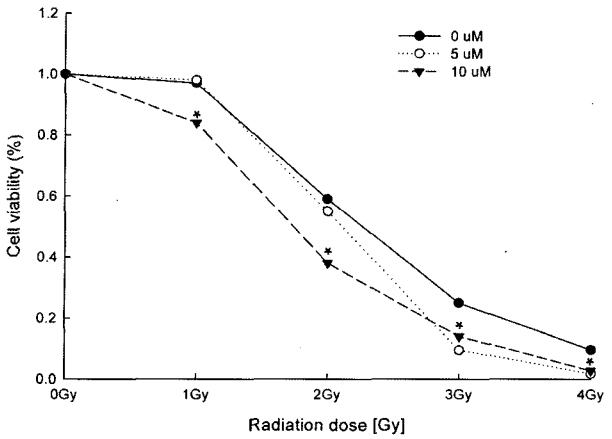

Fig. 2. Combined effect of 7β-OHC and γ-irradiation on HeLa cells *in vitro*. Cells were treated with γ-irradiation at various doses alone, and combination of both 7β-OHC at 5 and 10 μM and γ-irradiation at various doses. After 14 days, the cell death was measured by colony forming assay.

at dose dependent pattern in 7β-OHC treated cells. The 50% of inhibitory concentration of 7β-OHC on HeLa cells was about 10 μM. These data indicate that 7β-OHC can inhibit the cell growth of HeLa cells *in vitro*.

We next investigated whether combined treatment of 7β-OHC and γ-irradiation showed the radiation sensitivity or not. The extent of radiation sensitivity was performed using clonogenic assay. As shown in Fig. 2, combined treatment of 7β-OHC and γ-irradiation at various doses showed the radiation sensitivity in HeLa cells. However, γ-irradiation at various doses did not show the radiation sensitivity in HeLa cells. In addition, flow cytometry with propidium iodide staining at 24 and 48 h. As shown in Fig. 3, percentage of cell death in HeLa cells was 7% in 10 μM 7β-OHC, 11% in 10 Gy radiation, 10 % in combined treatment at 24 h. Changes in mitochondrial membrane potential were determined after treatment with 7β-OHC alone, γ-irradiation alone and in combination. Treatment with 10 μM 7β-OHC and 10 Gy radiation decreased $DiOC_6(3)$ retention, which reflects loss of mitochondrial membrane potential (Fig. 4), with 84% of $DiOC_6(3)$ retention in 10 μM 7β-OHC and 92% of $DiOC_6(3)$ retention in 10 Gy radiation when compared to 94% of $DiOC_6(3)$ retention in control cells at 24 h. The loss of mitochondrial membrane potential was not enhanced by the combined treatment

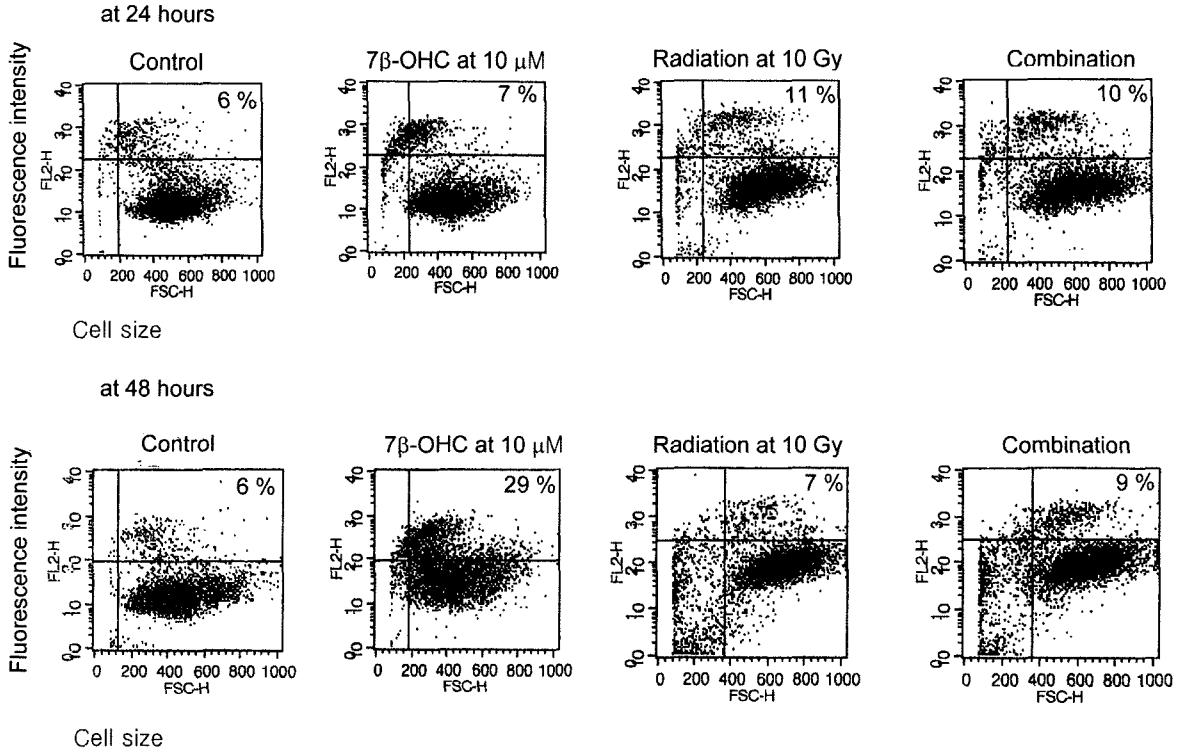

Fig. 3. Combined effect of 7β-OHC and γ-irradiation on induction of apoptosis in HeLa cells *in vitro*. Cells were treated with 7β-OHC at 10 μM alone, γ-irradiation at 10 Gy, and combination of both 7β-OHC at 10 μM and γ-irradiation at 10 Gy. After 24 and 48 h, the cell death was measured by flow cytometry with propidium iodide (PI) staining.

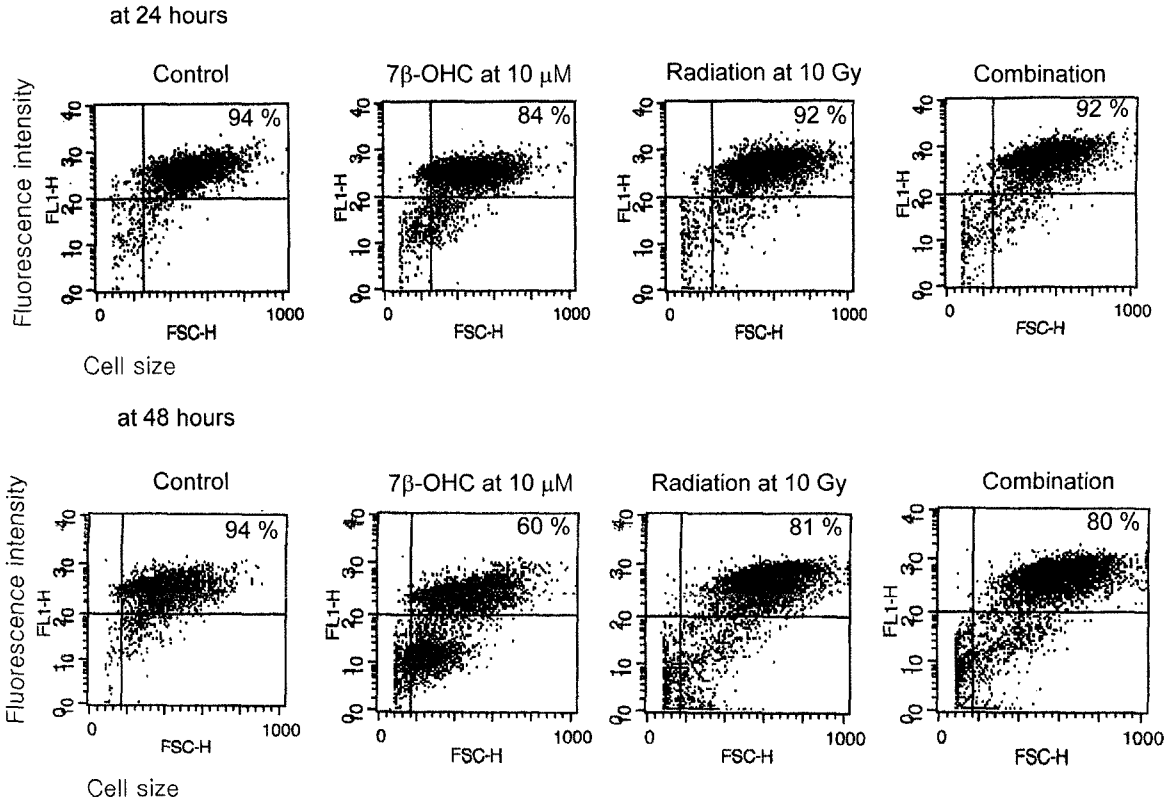

Fig. 4. Loss of mitochondrial membrane potential by 7β-OHC. Cells were treated with 7β-OHC at 10 μM alone, γ-irradiation at 10 Gy alone, and combination of both 7β-OHC at 10 μM and γ-irradiation at 10 Gy. After 24 and 48 h, cells were loaded for 30 min at 37°C with 30 nM $DiO_6(3)$. The fluorescence of $DiOC_6(3)$ loaded cells was measured using a flow cytometer.

with 7β-OHC and γ-irradiation at 24 h (Fig. 4). These findings suggest that γ-irradiation in combination with 7β-OHC dose not modulate the loss of mitochondrial membrane potential. Taken together, 7β-OHC showed the cytotoxicity on HeLa cells, however, did not enhance the radiation sensitivity.

Discussion

The purpose of this study was to assess possibility of 7β-OHC as sensitizer to enhance the action of ionizing radiation (IR). 7β-OHC affects the membrane formation and homeostasis by inhibiting the endogenous cholesterol synthesis, and by inserting into the phospholipid bilayers. 7β-OHC effects cell membrane, accounting for their overall action on cell growth and cytotoxicity. Several studies have reported that 7β-OHC has an anti cancer activity in various cancer cell lines. Also, oxysterols show various biological effect such as, inhibition of DNA synthesis and cell proliferation and alteration of intercellular communication throughout gap junctions (Marinovich *et al.*, 1995). On the other hand, exposure to ionizing radiation is believed to cause cell damage via the production of reactive oxygen species to induce oxidative stress (Lin *et al.*, 2003), and apoptotic signaling via mitochondrial pathway involving caspase-9 and -3 activation (Verheij and Bartelink, 2000; Fei and El-Deiry, 2003). Before occurring caspase cascade to induce apoptosis, mitochodrial permeability transition (MPT), one of major pathways involved in stress-inducing apoptosis, is causative event (Kim *et al.*, 2003). Mitochondrial membrane permeabilization is considered to be one of the initial events of the apoptotic process induced by chemotherapeutic drugs (Green and Reed, 1998; Crompton, 1999; Gottlieb, 2000). Also, glutathione depletion which has been shown to be one of the earliest events in oxysterol-induced apoptosis (O'Callaghan *et al.*, 2002) might be related with loss of the mitochondrial transmembrane potential. For the results of the loss of mitochondrial

membrane potential we obtained, the cell death was considered to be associated with the extrusion of glutathione. And the intracellular concentration of glutathione remained to be measured, whether it is decreased or not, for further research. Therefore, combined treatment of oxysterol and IR for cancer is expected to be an essential aspect of attempts to remove tumor cells, in a synergistic manner.

Radiosensitizer can enhance radiation-induced cell death through by perturbing various physiological phenomena such as, inhibition of angiogenesis, arrest or disruption of the cell cycle, induction of apoptosis, and blockade of cell survival signaling pathways (Chernikova *et al.*, 1999; Edwards *et al.*, 2002; Eshleman *et al.*, 2002; Sarkaria *et al.*, 1998; Wang *et al.*, 2001). Only a single treatment often causes therapeutic resistance and side effects in all types of cancers. Chemotherapy-radiotherapy combination is based on the theory that two types of cancer treatment act via different mechanisms (Park *et al.*, 2004). The use of a combination of radiation and chemotherapy is often called chemo-radiation in the medical literature (Kvols, 2005). Combined treatment for cancer, or chemoradiation, is important part for offering the hope of improved cancer patient survival not only to enhance the therapeutic effect but also to use the advantages of each single treatment, minimizing treatment doses and side effects of the patient, simultaneously. An ideal radiation sensitizer should have properties of more effective in increasing the apoptotic cell death of tumor cells and less toxic to normal cells. However, the ideal radiation sensitizer does not exist today (Kvols, 2005). Therefore, many attemps to pursuing a new promising candidate compound are being developed (Stevens *et al.*, 2000), whether it is naturally occurring or synthetically made, which is close to ideal chemoradiation should be recognized as important future alternatives for chemo-therapy. In this study, we evaluated whether 7β-OHC have properties of radiation sensitizing effect or not. Taken together, 7β-OHC showed the cytotoxicity on HeLa cells, however, did not sensitize HeLa cells to radiation.

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