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Combination Effects of 7β-Hydroxycholesterol and Radiation in Human Lung Cancer Cells

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Abstract – The present study was performed to evaluate combination effect of 7β-hydroxycholesterol (7β-OHC) and γ-radiation in NCI-H460 human lung cancer cells. 7β-OHC in combination with γ-irradiation has an enhanced effect in decreasing clonogenic survival and increasing cellular DNA damage. Pretreatment of cells with 7β-OHC enhanced radiation-induced apoptosis. Apoptosis of the cells by combined treatment of 7β-OHC and γ-irradiation was associated with reactive oxygen species generation and loss of mitochondrial membrane potential, resulting in the activation of caspase 9 and caspase 3. The combined treatment also resulted in an increased G_1 cell cycle distribution. These results indicate that 7β-OHC shows the additive effect of radiation sensitivity in human lung carcinoma cells *in vitro*.

Keywords □ 7β-hydroxycholesterol; radiation sensitivity; NCI-H460 human lung cancer cells

INTRODUCTION

Oxysterols are naturally occurring compounds present in vegetal and animal organisms. Several of these oxygenated sterols have been reported to be highly cytotoxic towards normal and tumor cells, according to their structure and the cell type (Schroepfer, 2000). Their cytotoxicity mainly results from the inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase, a key-enzyme in the endogenous cholesterol synthesis pathway. Thus, in diving cells exposed to active concentrations of oxysterols, membrane formation is impaired and thereby their growth is prevented or severely hindered (Chen, 1984; Schroepfer, 2000). 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. Also, oxysterols affected the membrane enzyme activities (Moog *et al.*, 1991), and their permeability to ions (Boissoneault *et al.*, 1985) and proteins (Boissoneault *et al.*, 1991). It was reported that 7β-hydroxycholesterol (7β-OHC), one of oxysterols, and its derivatives exhibited a pronounced cytotoxic activity against normal and tumor cells in culture and its cytotoxicity has been shown through apoptosis (Hietter *et al.*, 1986; Christ *et al.*, 1991 and 1993; Aupeix *et al.*, 1995; Lizard *et al.*, 1996; Hyun *et al.*, 1997; Lim *et al.*, 2003; Kang *et al.*, 2005). Recently, we reported that 7β-OHC enhanced the radiosensitivity in murine RDM4 thymoma cells (Hyun *et al.*, 2002). In this study, we undertook to assess whether 7β-OHC could enhance the susceptibility of human lung cancer cells to ionizing radiation, and to elucidate its mechanism.

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MATERIALS AND METHODS

Cell culture

NCI-H460 human lung carcinoma cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin and streptomycin.

Reagents

7β-OHC was obtained from Sigma chemical company (St. Louis, MO, USA), freshly dissolved in ethanol, and the final

concentration of 7β-OHC did not exceed 0.2%.

Clonogenic forming assay

NCI-H460 human lung carcinoma cells were seeded into 60 mm dishes at a density to produce approximately 300 colonies per dish after irradiation at various doses and/or treatment of 7β-OHC and were incubated for 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.

Single cell gel electrophoresis (Comet assay)

Comet assay was performed to determine the cellular DNA damage (Singh, 2000; Rajgopalan *et al.,* 2003). The cell suspension was mixed with 75 μl of 0.5% low melting agarose (LMA) at 39°C and spread on a fully frosted microscopic slide precoated with 200 ml of 1% normal melting agarose (NMA). After solidification of the agarose, slide was covered with another 75 μl of 0.5% LMA and then immersed in lysis solution (2.5 M NaCl, 100 mM Na-EDTA, 10 mM Tris, 1% Triton X-100, and 10% DMSO, pH 10) for 1 h at 4°C. The slides were next placed a gel-electrophoresis apparatus containing 300 mM NaOH and 10 mM Na-EDTA (pH 13) for 40 min to allow the unwinding of the DNA and the expression of the alkali labile damage. Then an electric field was applied (300 mA, 25 V) for 20 min at 4°C to draw negatively charged DNA toward anode. After electrophoresis, the slides were washed three times for 5 min at 4°C in a neutralizing buffer (0.4 M Tris, pH 7.5) and then stained with 75 ml of propidium iodide (20 μg/ml). The slides were observed by fluorescence microscope and image analysis (Kinetic Imaging. Komet 5.5, UK). The percent of total fluorescence in the tail and the tail length of the 50 cells per slide was recorded.

Flow cytometric analysis

Apoptosis and cell distribution at cell cycle phase were measured by flow cytometry with propidium iodide (PI) staining. Cells were treated with 7β-OHC at 15 μM alone, γ-irradiation at 4 Gy alone, or combination of 7β-OHC and γ-irradiation. After 48 h, the cells were washed with ice-cold PBS and fixed with 70% ice-cold ethanol overnight at 4°C. Fixed cells were washed twice with PBS and treated with 1 mg/ml RNaseA 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). From the analysis of DNA histograms, the percentage of cells in cell cycle phase was evaluated. Cells with DNA content less than the cells in the G_1 phase (sub-G_1) were taken as apoptotic cells (Nicoletti *et al.,* 1991).

Fluorescence microscopy

Cells were treated with 7β-OHC at 15 μM alone, γ-irradiation at 4 Gy alone, or combination of both 7β-OHC at 15 μM and γ-irradiation at 4 Gy. After 48 h, the cells were washed with ice-cold PBS and 1.5 μl of Hoechst 33342 (stock 10 mg/ml), a DNA specific fluorescent dye, was added to each well (1.5 ml) and incubated for 10 min at 37°C. Stained cells were then observed under a fluorescent microscope, equipped with a CoolSNAP-Pro color digital camera, to examine the degree of nuclear condensation.

Fluorescece measurement of intracellular reactive oxygen species (ROS)

The fluorescent probe 2'7'-dichlorofluorescin diacetate (DCFH-DA) was used for the assessment of intracellular ROS (Rosenkranz *et al.,* 1992). After cells were plated in 60 mm dishes (1×10^5 cells/dish) and were treated with 7β-OHC at 15 μM alone, γ-irradiation at 4 Gy alone, or combination of both 7β-OHC at 15 μM and γ-irradiation at 4 Gy. After 24 h, cells were loaded for 30 min at 37°C with 10 mM DCFH-DA and supernatant was removed by suction and after trypsin treatment, cells were washed with PBS. Fluorescence of DCFH-DA loaded cells was measured using a flow cytometer.

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential was determined as the retention of the mitochondria-specific dye $DiOC_6(3)$ (Mercie *et al.,* 2000). Cells were treated with 7β-OHC at 15 μM alone, γ-irradiation at 4 Gy alone, or combination of both 7β-OHC at 15 μM and γ-irradiation at 4 Gy. After 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.

Western blot analysis

Cells were treated with 7β-OHC at 15 μM alone, γ-irradiation at 4 Gy alone, or combination of both 7β-OHC at 15 μM and γ-irradiation at 4 Gy. After 48 h, the cells were harvested, and washed twice with PBS. The harvested cells were then lysed on ice for 30 min in 100 μl of a lysis buffer [120 mM NaCl, 40 μM Tris (pH 8), 0.1% NP 40] and centrifuged at

13,000 × g for 15 min. Supernatants were collected from the lysates and protein concentrations were determined. Aliquots of the lysates (40 μg of protein) were boiled for 5 min and electrophoresed in 10% SDS-polyacrylamide gel. Blots in the gels were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA), which were then incubated with primary rabbit monoclonal -caspase 9, 3 and -PARP antibodies. The membranes were further incubated with goat anti-rabbit immunoglobulin-G-horseradish peroxidase conjugates (Pierce, Rockford, IL, USA), and then exposed to X-ray film. Protein bands were detected using an enhanced chemiluminescence Western blotting detection kit (Amersham, Little Chalfont, USA).

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

Radiation sensitizing effect of 7β-OHC on NCI-H460 cells *in vitro*

Clonogenic survival assays were performed at 1, 2, 3, and 4 Gy of γ-irradiation alone and in combination with 7β-OHC at 7.5 μM and 15 μM. As shown in Fig. 1, clonogenic survival of NCI-H460 cells was inhibited when γ-irradiation treatment was combined with 7β-OHC. In addition, damage to cellular DNA *in vitro* was studied by comet assay. The cells treated with 7β-OHC at 15 μM and radiation at 4 Gy increased comet parameters like a tail length and percent of DNA in tail of cells (Fig. 2A). As shown in Fig. 2B, when the cells were exposed to γ-radiation at 4 Gy, 7β-OHC at 15 μM, and combination with 7β-OHC and radiation, the percent of DNA in the tail was increased 16.5 ± 1.2, 16.1 ± 0.3, and 20.4 ± 0.8, respectively, compared to control, 5.6 ± 0.5. These data indicate that 7β-OHC can sensitize human lung cancer cells to γ-irradiation *in vitro*.

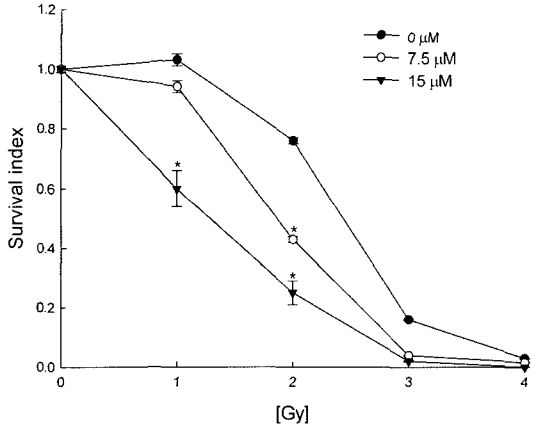

Fig. 1. Radiosensitizing effect of 7β-OHC on NCI-H460 cells *in vitro*. Cells were treated with indicated concentrations of 7β-OHC and irradiated with increasing doses of γ-radiation 1 h after addition of 7β-OHC. Cells were allowed to grow for 14 days and were stained with trypan blue and scored for colony formation. Results are given as means ± S.E. *significantly different from control ($p<0.05$).

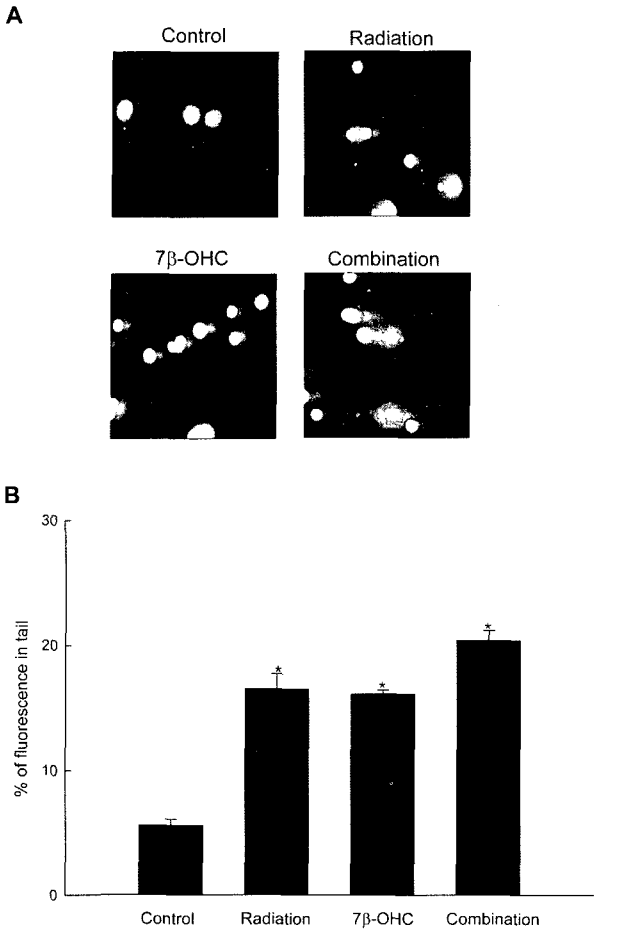

Fig. 2. Radiosensitizing effect of 7β-OHC on cellular DNA damage in NCI-H460 cells. Cells were treated with 7β-OHC at 15 μM alone, γ-irradiation at 4 Gy alone, or combination of 7β-OHC and γ-irradiation. Detection of the cellular DNA damage was determined by Comet assay. (A) Tail length of DNA in cells was observed under a fluorescent microscope after ethidium bromide staining. (B) The percent of total fluorescence in the tail was determined from the 50 cells per slide. Results are given as means ± S.E. *significantly different from control ($p< 0.05$).

7β-OHC enhances irradiation-mediated G_1 arrest

We next examined whether the reduced clonogenic survival by combined treatment of 7β-OHC and γ-irradiation was associated with alteration of cell cycle. As shown in Fig. 3, γ-irradiation alone induced accumulation of cells at G_1 phase on 48 h after radiation, 61 ± 1.1% compared to untreated cells, 56 ± 1.7 %. The 15 μM of 7β-OHC had accumulation of cells at G_1 phase (62 ± 0.9%), in combination with irradiation increased the cell population of arrested G_1 phase (76 ± 1.4%; significantly different from control, $p<0.05$). These data indicate that 7β-OHC delays cell cycle progression after irradiation.

Effect of 7β-OHC on radiation-induced apoptosis

In addition to cell cycle, we investigated whether the reduced clonogenic survival by combined treatment of 7β-OHC and γ-irradiation was associated with induction of apoptosis. Sub G_1-hypodiploid cell population and apoptotic bodies, indicators of apoptosis phenomenon, were performed to evaluate the extent of apoptosis. As shown in Fig. 4A, sub G_1-hypodiploid cell population was additively enhanced in combination with 7β-OHC at 15 μM and γ-irradiation at 4 Gy. The extent of apoptosis of NCI-H460 in response to this combined application was further confirmed by formation of apoptotic body (Fig. 4B). Taken together, these results demonstrate that 7β-OHC augments apoptotic cell death of human lung cancer cells in response to the γ-irradiation.

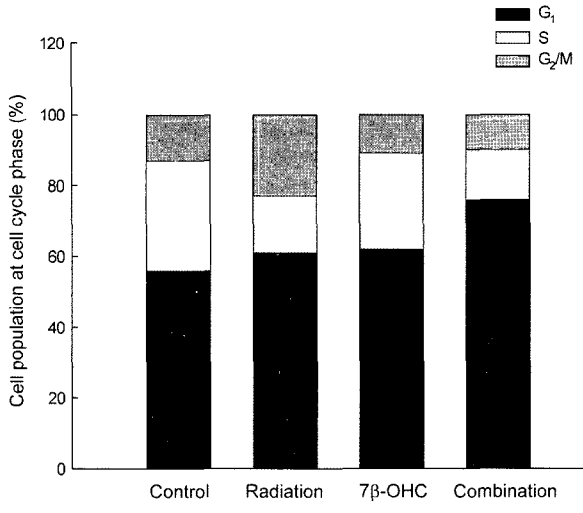

Fig. 3. Enhancing effect of 7β-OHC on irradiation-mediated G_1 arrest. Cells were treated with 7β-OHC at 15 μM alone, γ-irradiation at 4 Gy alone, or combination of 7β-OHC and γ-irradiation. After 48 h, the cells were harvested and subjected to flow cytometry to determine cell cycle distribution.

Loss of mitochondrial membrane potential and caspase activation are involved in 7β-OHC induced radiosensitization

To assess the induction of apoptosis at the biochemical level, changes in mitochondrial membrane potential, activation of caspase 9, the initiator caspase, and caspase-3, the major effecter caspase of the apoptotic process, were determined after treatment with 7β-OHC alone, γ-irradiation alone or in combination. Treatment with 15 μM 7β-OHC alone decreased $DiOC_6(3)$ retention, which reflects loss of mitochondrial membrane potential. The loss of mitochondrial membrane potential was further enhanced additively by the combined treatment with 7β-OHC and γ-irradiation (Fig. 5). We also observed enhanced activation of caspase 9, caspase 3, and PARP cleav-

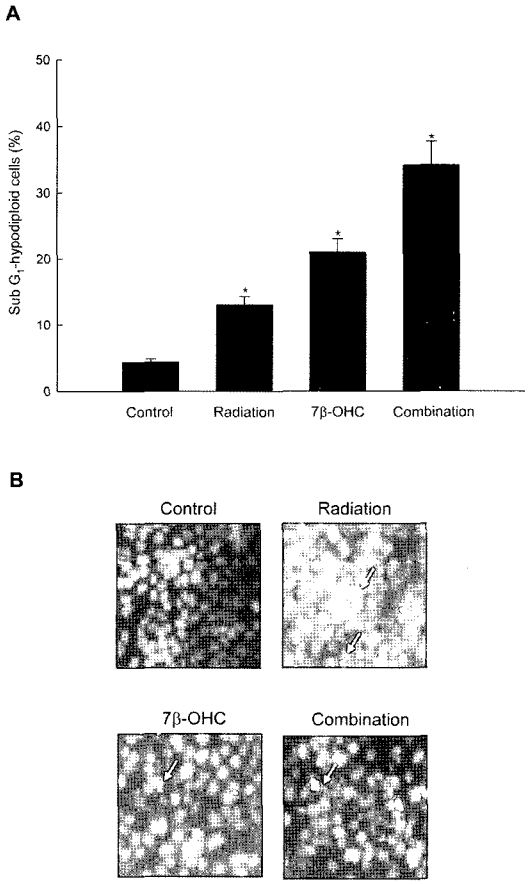

Fig. 4. Effect of 7β-OHC on radiation induced apoptosis. Cells were treated with 7β-OHC at 15 μM alone, γ-irradiation at 4 Gy alone, or combination of 7β-OHC and γ-irradiation. (A) Apoptotic sub-G_1 DNA content was detected by flow cytometry after propidium iodide staining. (B) Apoptotic body formation was observed under a fluorescent microscope after Hoechst 33342 staining and apoptotic bodies are indicated by arrows.

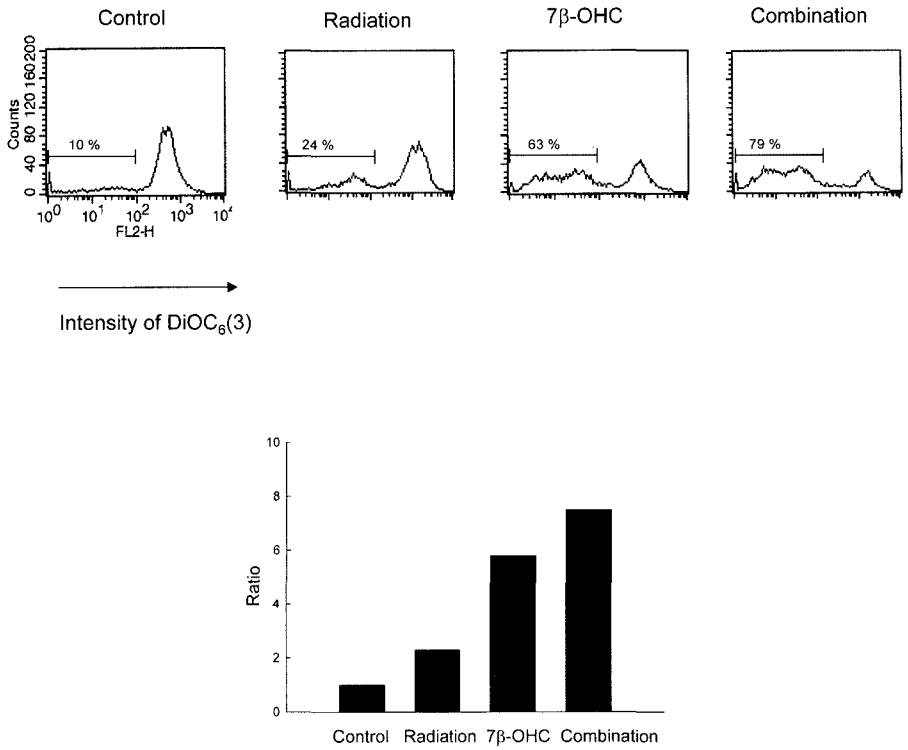

Fig. 5. Effect of 7β-OHC on radiation induced mitochondrial membrane potential. Cells were treated with 7β-OHC at 15 μM alone, γ-irradiation at 4 Gy alone, or combination of 7β-OHC and γ-irradiation. After 48 h, cells were loaded for 30 min at 37°C with 30 nM $DiO_6(3)$. The fluorescence of $DiOC_6(3)$ loaded cells was measured using a flow cytometer.

age in combination with 7β-OHC and γ-irradiation compared to 7β-OHC alone, or γ-irradiation alone (Fig. 6). These findings suggest that γ-irradiation in combination with 7β-OHC modulates caspase activity following the loss of mitochondrial membrane potential, thereby enhancing apoptotic cell death.

Combined effect of 7β-OHC and γ-irradiation on ROS generation

It has been reported that the generation of ROS regulates apoptosis (Chen *et al.*, 1998; Jing *et al.*, 1999; Dai *et al.*, 1999). To investigate the relationship between ROS production and enhancement of radiation-induced apoptosis by 7β-OHC, the ROS sensitive dye DCF-DA was used in the flow cytometric analysis to detect ROS generation (Fig. 7). The production of ROS was enhanced by the combined treatment of 7β-OHC and γ-irradiation. These results indicate that ROS generated by combined treatment of 7β-OHC and γ-irradiation regulates apoptotic process including intracellular caspase signaling.

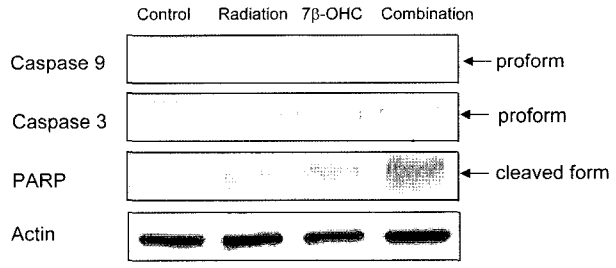

Fig. 6. Effect of 7β-OHC on caspases activities of irradiated NCI-H460 cells. Cells were treated with 7β-OHC at 15 μM alone, γ-irradiation at 4 Gy alone, or combination of 7β-OHC and γ-irradiation. After 48 h, cell lysates were prepared and Western blot analysis was performed using anti-caspase 9, caspase 3 and PARP antibodies.

DISCUSSION

The purpose of this study was to determine whether 7β-OHC could act as sensitizer to enhance the action of ionizing radiation. 7β-OHC affects the membrane formation and homeostasis by inhibiting the endogenous cholesterol synthesis, and by inserting into the phospholipid bilayers. This effect of 7β-OHC to cell membrane could account for their overall action on cell growth and cytotoxicity. Several studies have reported that 7β-OHC has an anti cancer effect in various cancer cells.

Our data suggest that 7β-OHC enhances the response of

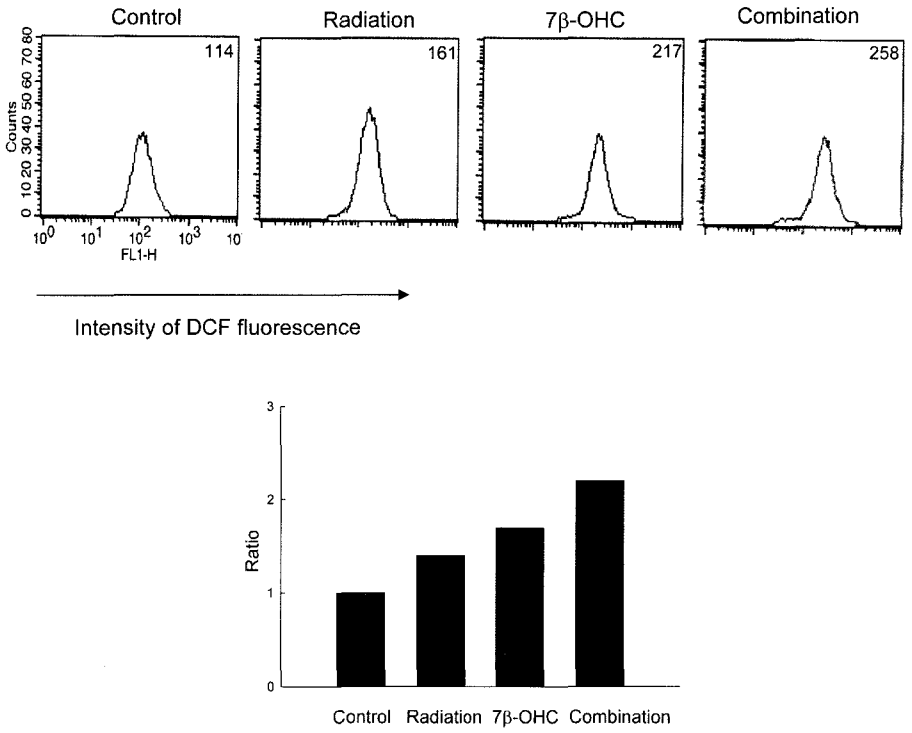

Fig. 7. Combination effect of 7β-OHC and radiation on production of ROS. Cells were treated with 7β-OHC at 15 μM alone, γ-irradiation at 4 Gy alone, or combination of 7β-OHC and γ-irradiation. After 24 h, cells were loaded for 30 min at 37°C with 10 μM DCFH-DA. The Fluorescence of DCFH-DA loaded cells was measured using a flow cytometer.

human lung cancer cells to radiation *in vitro*. 7β-OHC decreases survival of cancer cells via sensitizing cells to radiation-induced apoptosis. This apoptosis induced by the combined treatment in human lung cancer cells was associated with loss of mitochondrial membrane potential, enhanced generation of ROS, and activation of caspases. In particular, interaction of 7β-OHC with plasma membrane, a cell compartment, which participates in radiation induced injuries and apoptosis (Ojeda *et al.*, 19994), could contribute to the radiosensitizing effect. Here, 7β-OHC shows the addictive effect of radiation sensitivity in human lung carcinoma cells *in vitro* system.

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