Environmental Mutagens & Carcinogens 25-2 : 76-81 (2005)

Combined Effect of Radiation and 25-Hydroxycholesterol on Human Cervix and Lung Cancer Cells *in vitro*

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(Received May 31, 2005 / Accepted June 21, 2005)

***ABSTRACT* : 25-Hydroxycholesterol (cholest-5-ene-3, 25-diol, 25-OHC) showed the cytotoxicity on HeLa human cervix and NCI-H460 human lung cancer cells, 0.5 μM of 50% inhibitory concentration. We studied 25-OHC as the possibility of radiation sensitizer. The combination effect of 25-OHC and γ-irradiation measured using flow cytometer with propidium iodide stained cells. The combined treatment of 25-OHC and γ-irradiation did not show significant enhancing effects on Hela and NCI-H460 cells.**

Key words :

Introduction

Oxysterols, or oxygenated derivatives of cholesterol, 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). Cytotoxicity has been identified to involve apoptosis in certain cell lines (Hyun, *et al.*, 1997; Hietter, *et al.*, 1986; Christ, *et al.*, 1993; 1991; Aupeix, *et al.*, 1995; Ayala-Torres, 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 (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) in rat hepatocytes, which, in turn, means oxidative stress generation (Cantwell and Devery, 1998).

Their cytotoxicity 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 therby 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 (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 treatment can alter cellular functions and in this paper, we investigate whether 25-OHC, a potent oxysterol, could enhance the susceptibility to ionizing radiation HeLa and NCI-H460 cells.

Materials and Methods

Cell culture

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

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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 g-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

25-OHC was obtained from Sigma, freshly dissolved in ethanol, and the final concentration of 25-OHC did not exceed 0.2%.

Clonogenic forming assay

HeLa human cervix and NCI-H460 human lung carcinoma cells were seeded into 60 mm dishes at a density to produce approximately 300 colonies per dish and it was treated at various doses of 25-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 25-OHC at 10 μM alone, γ-irradiation at 10 Gy alone, or combination of both 25-OHC at 10 μM and γ-irradiation at 10 Gy. After 24, 48, and 72 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). From the analysis of DNA histograms.

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 0.5, 1, 2, and 4 μM of 25-OHC on NCI-H460 and HeLa cells. As shown in Fig. 1 and 2, clonogenic survival of Hela and NCI-H460 cells was inhibited at dose dependent pattern in 25-OHC treated cells. The 50% of inhibitory concentration of 25-OHC on Hela and NCI-H460 cells was about 0.5 μM. These data indicate that 25-OHC can inhibit the cell growth of Hela and NCI-H460 cells *in vitro*. We next investigated whether combined treatment of 25-OHC and γ-irradiation showed the radiation sensitivity. The extent of radiation sensitivity was performed using flow cytometry with propidium iodide staining at 24, 48, and 72 h. As shown in Fig. 3,

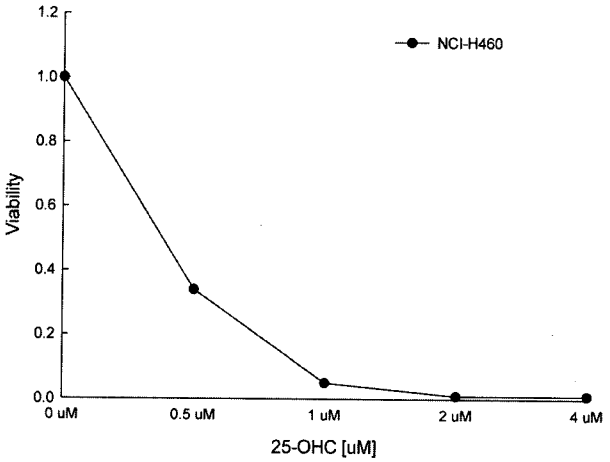

Fig. 1. Effect of 25-OHC on NCI-H460 cells *in vitro*. Cells were treated with various concentrations of 25-OHC. Cells were allowed to grow for 10-14 days and were stained with trypan blue and scored for colony formation. Results are given as means ± S.D.

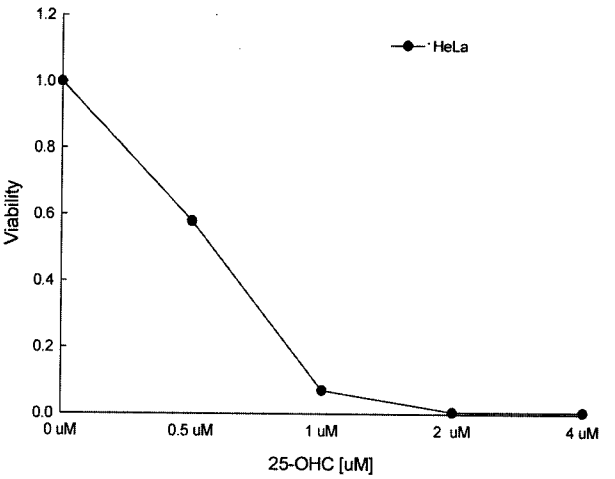

Fig. 2. Effect of 25-OHC on HeLa cells *in vitro*. Cells were treated with various concentrations of 25-OHC. Cells were allowed to grow for 10-14 days and were stained with trypan blue and scored for colony formation. Results are given as means ± S.D.

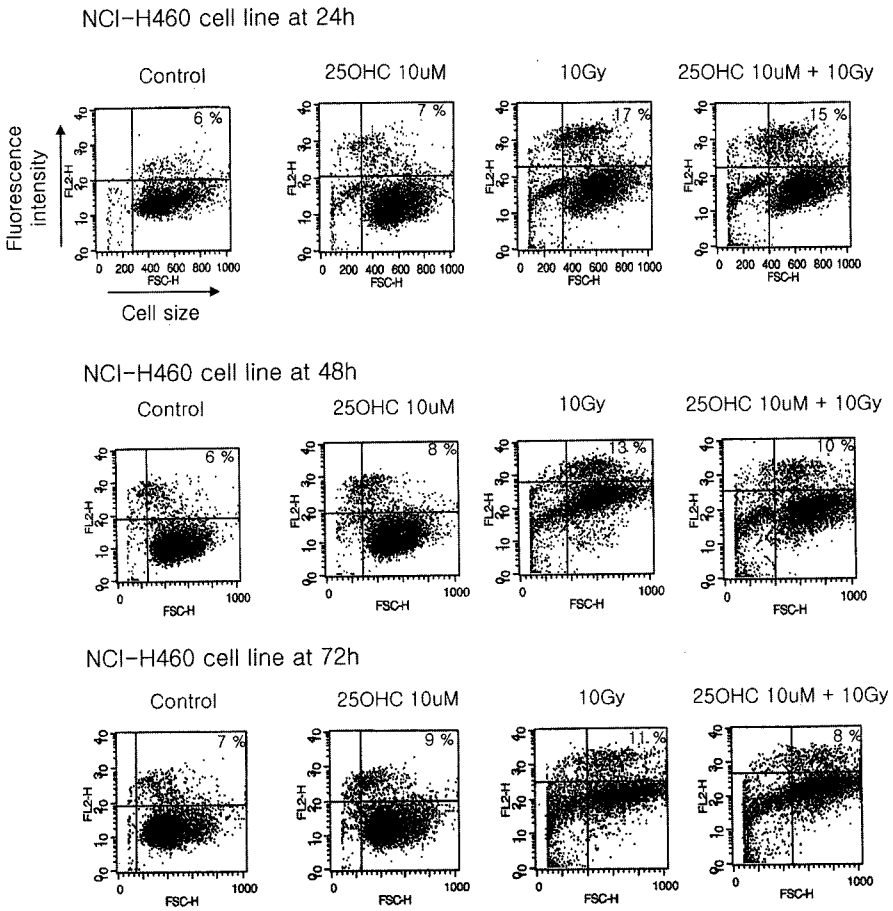

Fig. 3. Combined effect of 25-OHC on NCI-H460 cells *in vitro*. The cell death was measured by flow cytometry with propidium iodide (PI) staining.

percentage of cell death in NCI-H460 cells was 7% in 25-OHC, 17% in 10 Gy radiation, 15% in combined treatment at 24 h, 8% in 25-OHC, 13% in 10 Gy radiation, 10% in combined treatment at 48 h, and 9% in 25-OHC, 11% in 10 Gy radiation, 8% in combined treatment at 72 h. As shown in Fig. 4, percentage of cell death in HeLa cells was 3% in 25-OHC, 2% in 10 Gy radiation, 3% in combined treatment at 24 h, 7 % in 25-OHC, 4% in 10 Gy radiation, 6% in combined treatment at 48 h, and 26% in 25-OHC, 13% in 10 Gy radiation, 20% in combined treatment at 72 h. Taken together, 25-OHC showed the cytotoxicity on NCI-H460 and HeLa cells, however, did not enhance the radiation sensitivity.

Discussion

The purpose of this study was to determine whether 25-OHC could act as sensitizer to enhance the action of ionizing radiation (IR). It was reported that 25-OHC induced apoptosis in human lymphoblastic leukemic CEM cells via c-*myc* gene regulation (Ayala-Torres, *et al.,* 1999) and in THP-1 cells through the accumulation of cell cycle at G_2/M (Lim, *et al.,* 2003), also 25-OHC treated cells arrest at the G_1 phase of the cell cycle in various cell line (Ayala-Torres, *et al.,* 1997). Previous study have shown that the inhibition of caspase-3 protects against 25-OHC induced apoptosis (Nishio and Watanabe, 1996), and in the treatment of 7β-OHC, the increase of caspase 3 was observed in U 937 cells (O'Callaghan, 2002). Also, there are experiments with oxystrols showing 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

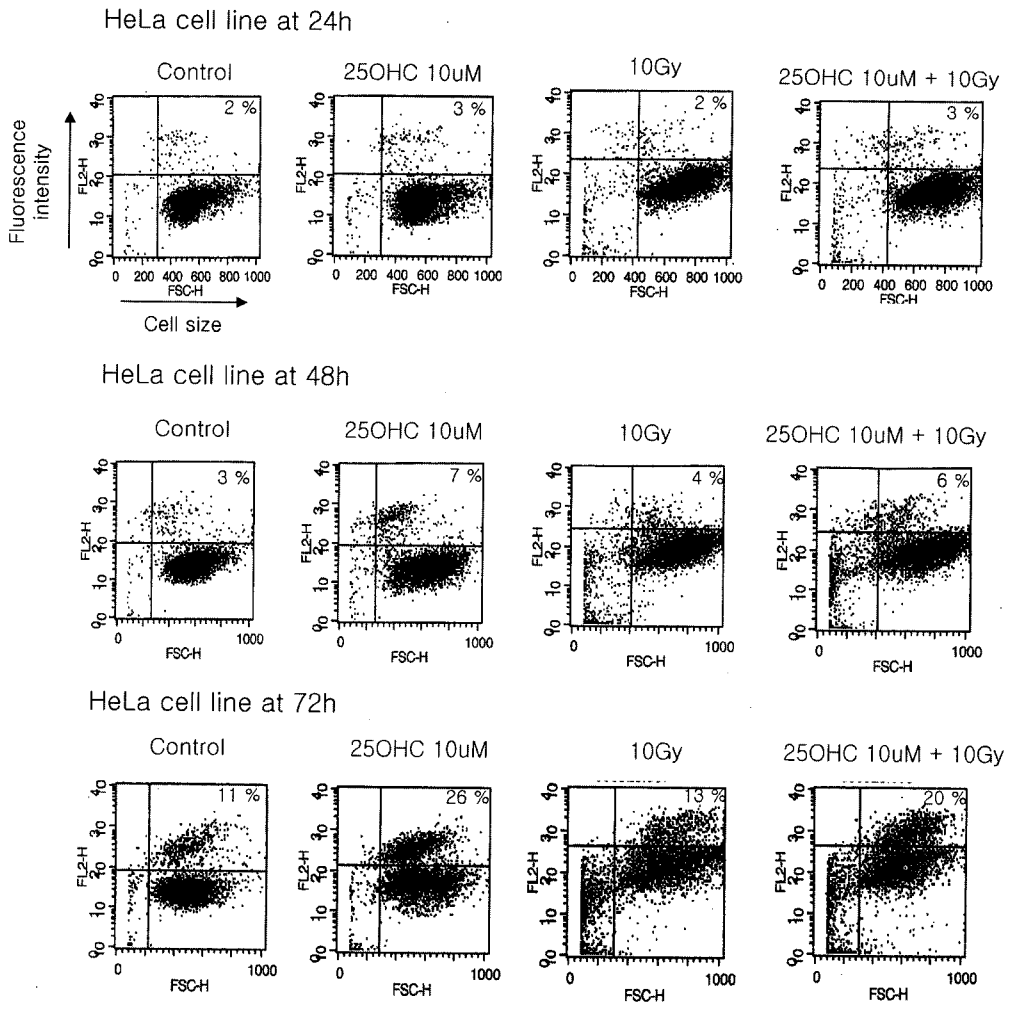

Fig. 4. Combined effect of 25-OHC on HeLa cells *in vitro*. The cell death was measured by flow cytometry with propidium iodide (PI) staining.

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). 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 chemoradiation in the medical literature (Kvols, 2005). The purpose of combined treatment for cancer, or chemoradiation, is essential part to improve

cancer patient survival not only to enhance the therapeutic effect but also to use the advantages of each single treatment, decreasing 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, a series of many trials for finding out a new candidate compound, whether it is naturally occurring or synthetically made, which have a closeness to ideal chemo-radiation should be recognized as important future alternatives for clinical treatment. In this paper, we would like to make an attempt to investigate whether 25-OHC have properties of radiation sensitizing effect or not. Taken together, 25-OHC showed the cytotoxicity on NCI-H460 and HeLa cells, however, did not enhance the radiation sensitivity.

Acknowledgement

This study was supported by Korea Science & Engineering Foundation (KOSEF) and Ministry of Science & Technology (MOST), Korean government.

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