RESEARCH ARTICLE

Inhibition of Proliferation and Induction of Apoptosis by the Combination of β-carotene and 1,25-dihydroxyvitamin D3 in Human Esophageal Cancer EC9706 Cells

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Abstract

Esophageal cancer is a common malignant tumor occurring in human esophageal epithelial tissue. The primary purpose of this paper was to define the effects of β-carotene and 1,25-dihydroxyvitamin D_3, alone and in combination, on cell proliferation, cell cycle and apoptosis of human esophageal cancer EC9706 cells. Treatment with different concentrations of β-carotene and/or 1,25-dihydroxyvitamin D_3. MTT assay showed that β-carotene and 1,25-dihydroxyvitamin D_3 significantly inhibited proliferation of EC9706 cells in a dose- and time-dependent manner. Further studies also demonstrated that β-carotene alone or 1,25-dihydroxyvitamin D_3 alone caused a marked increase on the induction of apoptosis in EC9706 cells. The percentage of G0/G1-phase cells significantly increased on addition of 1,25-dihydroxyvitamin D_3 alone, but there were no significant changes with β-carotene alone. These two agents in combination synergistically inhibited cell growth and induced apoptosis. Therefore, our results indicate that β-carotene and 1,25-dihydroxyvitamin D_3 in combination may provide a novel strategy for preventing and treating esophageal cancer.

Keywords: β-carotene - 1,25-dihydroxyvitamin D_3 - proliferation - apoptosis - esophageal cancer cells

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Introduction

Esophageal cancer is the eighth most common cancer and the sixth most common cause of death from cancer worldwide. In the world, the age-standardized (World) incidence rate of esophageal cancer in 2008 was 7.0/100,000, which represented 3.8% of all new cancer cases, and the age-standardized (World) mortality rate was 5.8/100,000, which represented 5.4% of all cancer deaths (Ferlay et al., 2010). Over 80% of esophageal cancers occur in developing countries, where nearly all cases are esophageal squamous cell carcinoma (Wang et al., 2012).

Incidence of esophageal cancer varies considerably according to geographic location. It is more common in northern China, northern Iran, and southern republics of the former Soviet Union, and is less common in Japan, Great Britain, Europe and Canada. In China, an estimated 258,000 new esophageal cancer cases and 210,000 deaths occurred globally during 2008, accounting for 53.6% of the cases and 51.7% of the deaths worldwide respectively (Guo et al., 2012). In the United States, incidence is highest in urban areas and overall incidence is about 5.8/100,000 (Ferlay et al., 2010). Esophageal cancer is a serious threat to people's health and even life, and only 10% of esophageal cancera patients can live to more than 5 years (Kollarova et al., 2007).

There is a certain relationship between β-carotene, 1,25-dihydroxyvitamin D_3 or other fat-soluble vitamin with the occurrence of esophageal cancer (Grant et al., 2002; Grant et al., 2007; Jessri et al., 2011). Since Peto proposed β-carotene may reduce cancer risk in early 80s of 20th century (Peto et al., 1981), people have conducted a large number of studies about anti-cancer mechanism of β-carotene. People thought β-carotene play anti-cancer role through vitamin A as a precursor of it in the past, but some studies have observed that a high dietary intake of β-carotene was negatively correlated with lung cancer, while high retinol intake couldn't reduce the risk of lung cancer (Kvale et al., 1983). Therefore, β-carotene may also be an independent protective factors of cancer, its anti-cancer mechanism was enhanced immunity, inhibiting cancer gene expression, such as bcl-2, inhibiting tumor cell proliferation, interfering with DNA metabolism in cancer cells, interfering with the cell cycle, etc (Woods et al., 1999; Palozza et al., 2002).

Recent studies about anti-cancer mechanism of β-carotene in vitro are mostly focused on lung cancer, liver cancer, leukemia and other cancer cells (Luo et al., 2001; Al-Wadei et al., 2009; Sacha et al., 2011), the study in vitro about the effect on esophageal cancer cells of

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β-carotene has rarely been reported.

1,25-dihydroxyvitamin D_3 is a fat-soluble vitamins, which mainly involved in calcium and phosphorus metabolism in vivo. In recent years, along with the progress of science and research, 1,25-dihydroxyvitamin D_3 was shown some atypical biological functions such as inhibiting cell proliferation, inducing apoptosis and differentiation, regulating immune function, protecting the central nervous system and genes (van den Bemd et al., 2000; Rohan et al., 2009). Eisman reported 1,25-dihydroxyvitamin D_3 receptor existed in human breast cancer cells firstly, which inspired people to explore the anti-cancer effect of vitamin D (Eisman et al., 1986). But the excessive β-carotene may lead the body to a high oxidation state (Marques et al., 2004), which harmful to health, and while 1,25-dihydroxyvitamin D_3 is a promising material with anti-tumor activity, excessive 1,25-dihydroxyvitamin D_3 could also easily induce hypercalcemia (Iqbal et al., 2003). β-carotene and 1,25-dihydroxyvitamin D_3 in combination could not only reduce the amount of β-carotene, but also reduce the amount of 1,25-dihydroxyvitamin D_3, thus reducing their pharmaceutical concentration, their toxic effects and the resistance of tumor cells, so the combination of these two agents is a new research direction of prevention and treatment of cancer.

This study explored the mechanism of action of β-carotene and 1,25-dihydroxyvitamin D_3, alone and in combination, on a esophageal cancer cell line so that providing a theoretical basis for the prevention and treatment of esophageal cancer.

Materials and Methods

Materials

β-carotene, 1,25-dihydroxyvitamin D_3, Dimethylsufoxide (DMSO), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI) were purchased from Sigma (St Louis, MO, USA). RPMI 1640, fetal bovine serum (FBS), phosphate buffered saline (PBS), trypsin and ethylene-diaminetetra-acetic acid (EDTA) were purchased from Gibco (Rockville, MD, USA). Stock solutions of β-carotene and/or 1,25-dihydroxyvitamin D_3 for cellular assays was prepared in ethanol and then diluted in the optimal medium. The final concentration of ethanol in all cultures was 0.1% (v/v), a concentration which was non-toxic to the cells (Ellis et al., 2009).

Cell culture

A human esophageal cancer EC9706 cells line was purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China). The cells were grown in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated FBS, 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C with 5% CO_2 in a humidified incubator. The growth of cultured cell was observed by inverted microscope every day, and then RPMI 1640 medium was changed every other day. For subculturing, cells were rinsed once with PBS and incubated in 0.25% trypsin containing 0.02% EDTA in PBS for 2 min. The medium was always changed on the day before the experiment. In all experiments, cells were always incubated at 37°C with 5% CO_2 in a humidified incubator.

Cell viability

The effect of β-carotene, 1,25-dihydroxyvitamin D_3, alone and the combination of these agents on cell viability was determined by an MTT assay. The MTT assay was performed as described by Li et al.(Li et al., 2009). Briefly, EC9706 cells were seeded in 96-well microtiter plates (Becton Dickson Labware, Franklin Lakes, NJ, USA) at 5×10^3 per well with 100 μL culture medium, and incubated for 24 hr to allow attachment to the bottom of the well. The medium was removed and the cells were treated with various concentrations (20, 40, 80, and 160μM) of β-carotene and/or 1,25-dihydroxyvitamin D_3 for 12, 24 and 48 hr. Control cells were supplemented with 0.1% ethanol vehicle. After 100 μL MTT (5 mg/ml in PBS) was added in the culture medium, cells were incubated for 4 hr at 37 °C. The medium was aspirated and cells were suspended in 150 μL DMSO. The absorption was measured at 490 nm with a Mithras LB 940 multimode microplate reader (Berthold Technologoies, Germany). The cell proliferation was calculated as follows: (OD of the experimental samples/OD of the control) ×100%. The experiment and assay were repeated at least three times.

Cell cycle and apoptosis analysis by flow cytometry

Flow cytometry was employed to evaluate cell cycle distribution and inducing-apoptosis activity. Exponentially growing EC9706 cells at 1×10^5 cells/mL were seeded at a density of 1×10^5 cells/mL in 50 cm^2 culture flasks and allowed to grow in 4 ml of culture medium. After cells attachment, culture medium was poured away. Then cells were treated with β-carotene (80μM) and/or 1,25-dihydroxyvitamin D_3 (80μM) for 48 hr. Approximately 1×10^6 cells were trysinized, collected and washed in cold PBS. The cells were subsequently fixed with 1 ml ice-cold 70% ethanol for 30 min. After fixing, cells were pelleted by centrifugation to remove the fixatives, washed three times with PBS at 4°C, resuspended in 100 μL PBS, and treated with Vindelov's reagent (40 mM Tris, pH 7.6; 100 mM NaCl; 10 mg Rnase A/ml; 7.5% PI and 0.1% Nonidet p40) at 4°C for 30 min. The stained cells were stored at 4°C in the dark and analyzed within 2 hr. The stained samples were measured on a Becton Dickinson flow cytometer. Data from 10,000 cells were collected for each data file and analyzed using CellQuest software to assess cell-cycle distribution patterns (apoptotic, G0/G1, S, and G2/M phases). The experiment and assay were repeated three times.

Statistical analysis

The data are expressed as means ± S.D. Statistical analysis of the data was performed using the SPSS package(version13.0). Differences between the experimental groups and the negative control group were evaluated by Student's t-test. $P<0.05$ was considered as statistically significant.

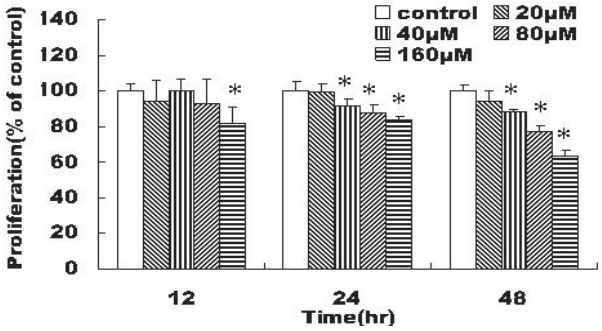

Figure 1. Effect of β-carotene on the Growth of EC9706 Cells. Cells were incubated in presence of the indicated doses of β-carotene for 12, 24 and 48 hr, respectively. And cell proliferation was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Results are presented as percentages of control. Data are shown in the mean±S.D. (n = 3). * $p < 0.05$, versus the control group

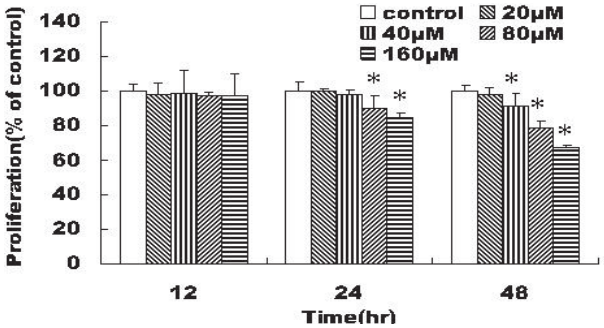

Figure 2. Effect of 1,25-dihydroxyvitamin D3 on the Growth of EC9706 Cells. Cells were incubated in presence of the indicated doses of 1,25-dihydroxyvitamin D3 for 12, 24 and 48 hr, respectively. And cell proliferation was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Results are presented as percentages of control. Data are shown in the mean±S.D. (n = 3). * $p < 0.05$, versus the control group

Results

Effects of β-carotene and 1,25-dihydroxyvitamin D_3 alone on proliferation of EC9706 cells

Cells were treated with different concentrations (20, 40, 80, 160 μM) of β-carotene for different periods of time (12, 24 and 48 hr). The results illustrated that β-carotene inhibited significantly proliferation of EC9706 cells in a concentration- and time-dependent manner. Treated for 12 hr, 160 μM β-carotene inhibited significantly proliferation of EC9706 cells (Figure 1). And treated for 24 and 48 hr, 40, 80, 160 μM β-carotene inhibited significantly proliferation of EC9706 cells, whereas EC9706 cells showed no significant change in proliferation after 48-hr culture with 20 μM β-carotene (Figure 1).

Cells were treated with different concentrations (20, 40, 80, 160 μM) of 1,25-dihydroxyvitamin D_3 for different periods of time (12, 24 and 48 hr), too. The results illustrated that 1,25-dihydroxyvitamin D_3 inhibited significantly proliferation of EC9706 cells in a concentration- and time-dependent manner. Treated for 12 hr, 20, 40, 80, 160 μM 1,25-dihydroxyvitamin D_3 didn't inhibit significantly proliferation of EC9706 cells (Figure 2). And treated for 24 and 48 hr, 80, 160 μM 1,25-dihydroxyvitamin D_3 inhibited significantly proliferation of EC9706 cells, whereas EC9706 cells showed no significant change in proliferation after 48-hr culture with 20 μM 1,25-dihydroxyvitamin D_3 (Figure 2).

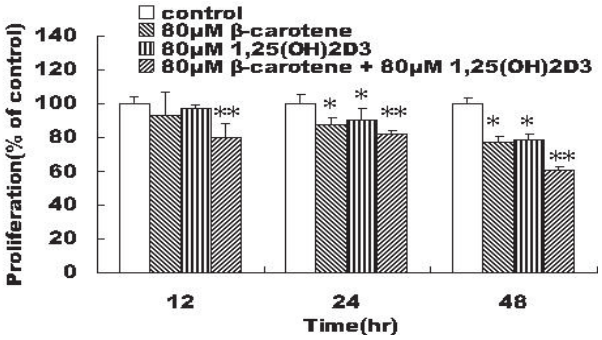

Figure 3. Effect of the Combination of β-carotene and 1,25-dihydroxyvitamin D3 on the Growth of EC9706 Cells. Cells were incubated in presence of the indicated doses of β-carotene and/or 1,25-dihydroxyvitamin D3 for 12, 24 and 48 hr, respectively. And cell proliferation was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Results are presented as percentages of control. Data are shown in the mean±S.D. (n = 3). * $p < 0.05$, versus the control group. ** $p < 0.05$, versus both 80 μM β-carotene treatment group and 80 μM 1,25-dihydroxyvitamin D3 treatment group

The effect of the combination of β-carotene and 1,25-dihydroxyvitamin D_3 on proliferation of EC9706 cells

To examine the combination effect on proliferation, EC9706 cells were treated with 80 μM β-carotene and/or 1,25-dihydroxyvitamin D_3 for 12, 24 and 48 hr. The results illustrated that β-carotene plus 1,25-dihydroxyvitamin D_3 group inhibition of EC9706 cells was significantly higher than the same concentration drug alone group (Figure 3).

The effect of β-carotene plus 1,25-dihydroxyvitamin D_3 acted on cell cycle distributions and apoptosis of EC9706 cells

To determine whether the decrease in cell proliferation was associated with cell cycle arrest or apoptosis, cells were treated with 80 μM β-carotene and/or 80 μM 1,25-dihydroxyvitamin D_3 for 48 hr. then cell cycle and apoptosis were analysed by using a flow cytometer. As indicated in Figure 4B, There was an increase in the number of cells at G0/G1 phase after 80 μM of 1,25-dihydroxyvitamin D_3 treatment (71.71% ± 2.25% versus control 61.59% ± 2.05%, $p < 0.05$), an increase in the number of cells at G0/G1 phase after 80 μM of β-carotene plus 1,25-dihydroxyvitamin D_3 combination treatment (77.30% ± 1.35% versus control 61.59% ± 2.05%, $p < 0.05$) (Figure 4B).

At the same time, we examined whether β-carotene and 1,25-dihydroxyvitamin D_3 caused apoptosis in EC9706 cells. β-carotene or 1,25-dihydroxyvitamin D_3 at a concentration of 80 μM caused minor apoptotic cell death in EC9706 cells (3.33% ± 0.20% and 1.57% ± 0.49%). The combination of 80 μM β-carotene plus 80 μM 1,25-dihydroxyvitamin D_3 further enhanced the induction of apoptosis to 5.11% ± 0.86% (Figure 4C).

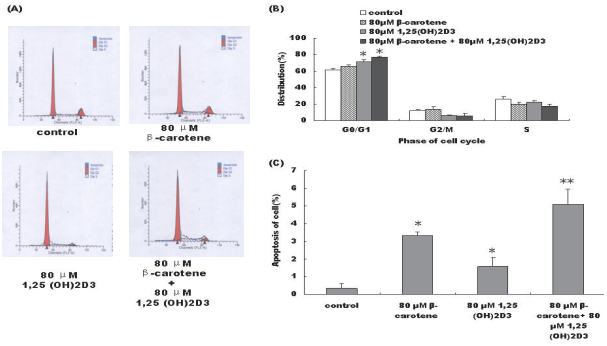

Figure 4. Effect of β-carotene and 1,25-dihydroxyvitamin D3 in Combination Acted on Cell Cycle Distributions and Apoptosis in EC9706 Cells. EC9706 cells were treated with β-carotene (80 μM) and/or 1,25-dihydroxyvitamin D3 (80 μM) for 48 hr. Then, cells were analysed by flow cytometry after PI staining and the relative percentage of cells in different cell cycle phases and apoptosis was reported. (B) The results are expressed as the percentage of the cell cycle distribution. (C) The results are expressed as the percentage of the cell apoptosis. * $p < 0.05$, versus the control group. ** $p < 0.05$, versus both 80 μM β-carotene treatment group and 80 μM 1,25-dihydroxyvitamin D3 treatment group

Discussion

The EC9706 tumor cells are a useful model to study cell growth inhibition of esophageal squamous cells by chemical, physical and physiological agents. Studies about the effect of β-carotene, 1,25-dihydroxyvitamin D_3 alone and in combination on esophageal cancer cells in vitro are rare. The aim of the present study was to investigate the effect of β-carotene, 1,25-dihydroxyvitamin D_3 alone and in combination on EC9706 cell growth.

Epidemiological investigations exploring the relationship between diet and esophageal cancer suggest an inverse correlation between consumption of fresh fruits, vegetables, seafood, and milk rich in carotenoids, flavonoids and vitamin D with esophageal cancer incidence rate (Hung et al., 2004; Fan et al., 2008; Lucenteforte et al., 2008).In our recent work, it was showed that luteolin, a kind of plant flavonoid, inhibited proliferation of human esophageal carcinoma cell line Eca109 by arresting cell cycle and inducing apoptosis in vitro (Wang et al., 2013). The results of an epidemiological study and a hospital-based case-control study demonstrate that β-carotene provides a protective effect on the incidence of esophageal cancer (Gao et al., 1994; Jessri et al., 2011). However, no protective effect of supplemental β-carotene was observed in a randomized primary esophageal and gastric cancer prevention trial to determine its effect on the incidence of esophageal cancer (Qiao et al., 2009).

In this study, β-carotene inhibited significantly proliferation and efficiently induced apoptosis of human esophageal cancer EC9706 cells, but there was not significantly increase in the number of cells at G0/G1 phase after β-carotene treatment. Several studies performed using other cancer cells line have reported that β-carotene may reduce cell proliferation rate by mechanisms that involve cellular differentiation or cell death by apoptosis. Inhibition of growth of endometrial (Ishikawa), mammary (MCF-7), and lung (NCI-H226) human cancer cells by β-carotene has been reported (Levy et al., 1995). In another study, the result shows that β-carotene does not affect the proliferation and differentiation process of the three human acute leukemia cell lines: U-937, HL-60 and TF-1, but can influence and enhance the apoptosis by modulating the expression of the regulatory genes (Sacha et al., 2005). β-carotene also have been observed to induce morphologic changes consistent with apoptosis in YTMLC-90 lung cancer cells, including cellular shrinkage, chromatin condensation and nuclear fragmentation (Lu et al., 2003). Microscopical observation of β-carotene treated cells showed a distinct pattern of morphological abnormalities with inclusion of apoptotic bodies in U937 and HL-60 leukemia cells (Upadhyaya et al., 2007). The different effects of β-carotene on cell proliferation, cell cycle and apoptosis are dependent on the dose of the carotenoids and the types of cancer cells, and so on.

Several studies have reported that enhancing vitamin D status could improve survival or response to therapy in patients living with cancer (Ng et al., 2009; Gugatschka et al., 2011; Reichrath et al., 2012). The epidemiologic evidence for cancer prevention by vitamin D is strongest for rectal, breast and colon cancer, and cancer of the esophagus may be vitamin D sensitive (Welsh et al., 2012).

Our experiments indicated 1,25-dihydroxyvitamin D_3 inhibited significantly proliferation, blocked the cell cycle progression in the G0/G1 phase and efficiently induced apoptosis of human esophageal cancer EC9706 cells.In recent years, a large number experiments find 1,25-dihydroxyvitamin D_3 could inhibit tumor cell proliferation on breast cancer, prostate cancer, colon cancer, leukemia, skin cancer(Beer et al., 2004; Rohan et al., 2009), and its mechanism of inhibiting tumor cell is inhibition of tumor cell cycle progression, inducing apoptosis and differentiation of tumor cells (Liu et al., 1996; Narvaez et al., 2001; Zinser et al., 2003).

In the present study, we treated EC9706 cells with β-carotene plus 1,25-dihydroxyvitamin D_3 too. Our data implied inhibition of cell growth by the combination of these agents in EC9706 cells was significantly higher than β-carotene or 1,25-dihydroxyvitamin D_3 alone, there was an increase in the number of cells at G0/G1 phase after β-carotene plus 1,25-dihydroxyvitamin D_3 combination treatment (77.30% ± 1.35% versus control 61.59% ± 2.05%, $p < 0.05$), and the combination of β-carotene and 1,25-dihydroxyvitamin D_3 further enhanced the induction of apoptosis to 5.11% ± 0.86% in these cells. These results strongly suggest that β-carotene plus 1,25-dihydroxyvitamin D_3 has a synergistic effect on the cell growth and apoptosis in EC9706 cells. Too much of β-carotene or 1,25-dihydroxyvitamin D_3 for long-term administration can be extremely harmful. Recent studies have revealed that oxidized beta-carotene is cytotoxic and that mitochondrial function is decreased in both human K562 erythroleukaemic and 28 SV4 retinal pigment epithelium cells (Hurst et al., 2005). And it has been reported that mice receiving 1,25-dihydroxyvitamin D_3 supplementation had significantly lower Lung tumor incidence and tumor multiplicity, but experienced significant body weight loss, kidney calcium deposition,

and decreased fasting plasma 1,25-dihydroxyvitamin D_3 levels (Mernitz et al., 2007).Therefore, the doses of β-carotene and 1,25-dihydroxyvitamin D_3 can be reduced by use in combination of these agents.

In conclusion, our study demonstrated that treatment with β-carotene plus 1,25-dihydroxyvitamin D_3 is more effective and safe than β-carotene or 1,25-dihydroxyvitamin D_3 alone. The synergistic effects of β-carotene and 1,25-dihydroxyvitamin D_3 on EC9706 cells shown in this study will provide a novel strategy for treating esophageal cancer, and more research is needed to explore the molecular mechanisms.

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