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Effect of β-carotene on Cell Growth Inhibition of KB Human Oral Cancer Cells

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β-carotene is present in carrots, pumpkins, and sweet potatoes. It suppresses many types of cancers by regulating cellular proliferation and apoptosis through a variety of mechanisms. However, the effects of β-carotene on oral cancer cells have not been clearly established. The main goal of this study was to investigate the effects of β-carotene on cell growth and apoptosis in oral cancer cells. Our results demonstrate that treatment with β-carotene induced inhibition of cell growth, and that the effect was dependent on β-carotene treatment time and concentration in KB cells. Furthermore, treatment with β-carotene induced nuclear condensation and fragmentation in KB cells. β-carotene promoted proteolytic cleavage of procaspase-3, -7, -8 and –9 with associated increases in the concentration of cleaved caspase-3, -7, -8 and –9. In addition, the level of cleaved PARP was increased by β-carotene treatment in KB cells. These results suggest that β-carotene can suppress cell growth and induce apoptosis in KB human oral cancer cells, and that it may have potential usefulness in anti-cancer drug discovery efforts.

Key words: β-carotene, cell death, apoptosis, oral cancer cells

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Introduction

Carotenoids are organic pigments that are found in the chloroplasts and chromoplasts of plants and some other photosynthetic organisms [1,2]. Carotenoids are divided into two classes, xanthophylls which contain oxygen and carotenes which are purely hydrocarbons and contain no oxygen [1,2]. In humans, three carotenoids, α-carotene, β-carotene and β-cryptoxanthin have vitamin A activity [2].

β-Carotene is one of the major dietary cartenoids, the most important precursor of retinol and other retinoids [3]. β-carotene is present in carrots, pumpkins and sweet potatoes and has been used to treat various disorders such as erythropoietic protoporphyria and age-related macular degeneration [3-10]. Of particular interest is that β-carotene acts as a chemopreventive agent and induces apoptotic cell death in various types of cancer cells such as pancreatic cancer, colorectal cancer, prostate cancer, breast cancer, skin cancer, lung cancer, melanoma and leukemia [3-10].

However, β-carotene effects on oral cancer are not clearly established.

It has been known that oral cancer is the sixth most common cancer globally [11]. Despite the introduction of novel therapeutic modalities into the treatment of oral cancer, improvements in long-term survival rates have only been modest [12]. Advances in the underlying mechanisms of oral cancer are likely to be necessary to improve survival rates, which, despite the better early detection of oral cancer, have plateaued over the past two decades and remain among the worst of all cancer sites [12].

In this study, therefore, the effect of β-carotene on cell growth and the mechanism of cell death elicited by β-carotene were examined in KB human oral cancer cells.

Materials and Methods

Materials

β-Carotene and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were supplied by Sigma (St Louis, MO, USA). Anti-cleaved caspase-3, -7, -8, -9 and anti-cleaved PARP antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Other analytical reagents were purchased based on the analytical grade.

Cell line and cell cultures

The human oral cancer cell line, KB, was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured as according to the cell culture instructions provided by ATCC. Briefly, the KB cells were grown in MEM medium (Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA). The cells were maintained as monolayers in plastic culture plate at 37°C in a humidified atmosphere containing 5% CO_2 [13].

Cell proliferation assay

The cell viability test was performed according to the previously described method with minor modifications [14,15]. The cells were seeded at a concentration of 5 X 10^3 cells/well in 24-well plates. After 24 hours growth, the cells were treated with β-carotene at various concentrations and incubation times. The cell viability was assessed using MTT assay. Four separate experiments were performed for each concentration/exposure time combination.

Nuclear staining with DAPI

Nuclear staining with DAPI (40,60-diamidino-2-phenylindole) was performed to examine the level of apoptosis. The KB cells were cultured in 24-well plates at a seeding density of 5 X 10^3 cells/well. After 24 hours growth, the cells were treated with 0, 50 or 100 µM β-carotene for 72 hours. The treated KB cells were fixed with 1% paraformaldehyde for 30 min at room temperature and washed twice with PBS. The cells were permeated with ice-cold ethanol for 5 min at room temperature and washed twice with PBS. The fixed cells were stained with DAPI (300 nM) for 5 min at room temperature in the dark, washed twice with PBS and examined by fluorescent inverted microscopy (IX71, Olympus, Japan).

DNA fragmentation analysis

Following treatment with 0, 50 and 100 µM β-carotene for 72 hours, approximately 5 X 10^6 cells were collected and transferred to lysis buffer containing 100 mM NaCl, 10 mM EDTA, 300 mM Tris-HCl, pH 7.5, 200 mM sucrose, 0.5% SDS and 0.5 mg/ml proteinase K and incubated at 65°C. DNA was extracted with an equal volume of phenol/chloroform/ isoamylalcohol (25:24:1, v/v) and precipitated with ethanol. The DNA was resuspended in Tris-EDTA buffer, pH 8.0 containing 5 µg/ml DNase-free RNase and incubated at 37°C for 1 hour. The DNA was visualized on 2% agarose gel in the presence of 0.5 µg/ml ethidium bromide.

Immunoblotting

The KB cells were treated with 0, 50 or 100 µM β-carotene for 72 hours. Immunoblotting was performed according to the previously described method with minor modifications [16,17]. The anti-cleaved caspase-3, -7, -8, -9 or anti-cleaved PARP antibody (1:1000 dilution, Cell Signaling Technology, Inc., Danvers, MA, USA) was used as the primary antibody.

Data analysis

All experiments were performed at least three times. The results were presented as mean ± SEM. The statistical significance was analyzed by using Student's t-test for two groups and one way analysis of variance for multi-group comparisons. All statistical analyses were performed using SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). A p value <0.05 was considered statistically significant.

Results

Cytotoxic effect of β−carotene in KB cells

To analyze the effect of β-carotene on the viability of KB cells, the cells were treated with β-carotene at various concentrations for 72 hours, and then the MTT assay was performed. When the KB cells were treated with β-carotene for 72 hours, β-carotene inhibited the proliferation of KB cells in a dose-dependent manner (Fig. 1), suggesting that β-carotene induces KB cell death. From 3 to 500 μM treatment of β-carotene, the inhibition of KB cell growth depended on the β-carotene treatment time (Fig. 1). The IC_{50} values of β-carotene on the cell viability are shown in Table 1.

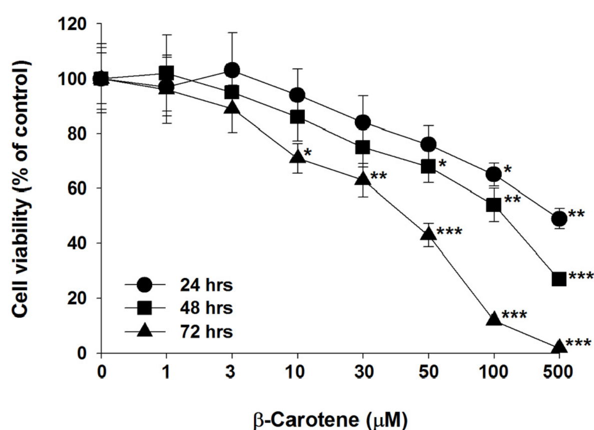

Fig. 1. Concentration- and time-dependent effects of β-carotene on the cell viability in KB human oral cancer cells. (A) Concentration-dependent effect of β-carotene on the cell viability in KB cells. The KB cells were treated with various concentrations of β-carotene or without β-carotene for 24 (circle), 48 (square) and 72 hours (triangle). (B) Time- dependent effect of β-carotene on the cell viability in KB cells. The KB cells were treated with 3 (circle), 10 (square), 30 (triangle), 50 (diamond), 100 (hexagon) and 500 μM (inverted triangle) β-carotene for 0 - 72 hours. The cell viabilities were determined by the MTT assays. The percentage of cell viability was calculated as a ratio of $A570_{nms}$ of β-carotene treated cells and untreated control cells. Each data point represents the mean ± SEM of four experiments. $^{*}P<0.05$ vs. control, $^{**}P<0.01$ vs. control and $^{***}P<0.001$ vs. control (the control cells measured in the absence of β-carotene).

Table 1. Anti-proliferative effect of β-carotene in KB human oral cancer cells

Time	IC_{50} (μM)
24 hours	467.3 ± 50.4
48 hours	158.2 ± 12.9
72 hours	42.9 ± 5.8

The IC_{50} values represent the mean ± SEM for four experiments.

Changes in the nuclear morphology by β−carotene

The nuclear morphological changes were assessed by DAPI staining. As shown in Fig. 2A, the nuclei of the control KB cells (Control) had a normal regular and oval shape. Treatment with 50 or 100 μM β-carotene for 72 hours resulted in nuclear condensation and fragmentation, which are the characteristics of apoptosis (Fig. 2A). As quantified in Fig. 2B, 50 and 100 μM β-carotene increased the apoptotic rate of KB cells significantly to 38.8 ± 5.8% and 86.7 ± 4.3%, respectively.

DNA fragmentation

Increased cellular apoptosis is only one among several possible mechanisms involved in reduced cell proliferation [18-21]. To determine if apoptosis is indeed the underlying mechanism for the reduced cell proliferation observed, the KB cells treated with β-carotene were subjected to DNA fragmentation. As shown in Fig. 3, the formation of DNA ladder in the KB cells treated with 50 and 100 μM β-carotene was observed.

Activation of caspases by β−carotene

The levels of cleaved caspase-3, -7, -8 and −9 were

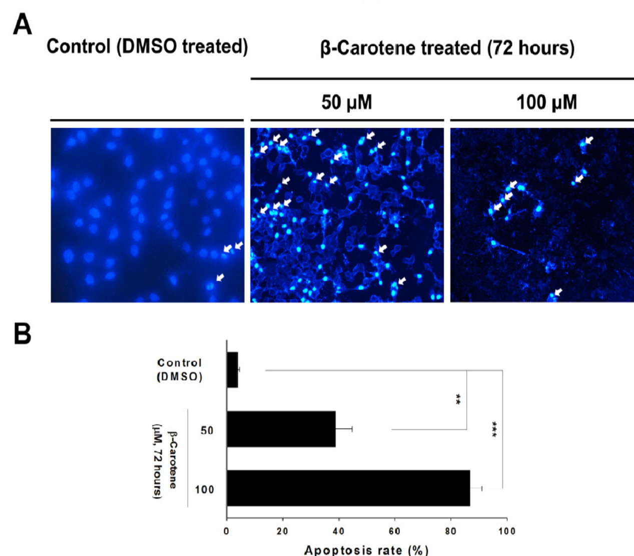

Fig. 2. Induction of apoptosis by β-carotene in KB cells. (A) Changes in nuclear morphology by β-carotene. The cells were treated with 0, 50 or 100 μM β-carotene for 72 hours. Representative fluorescence photomicrographs show the nuclei morphology of KB cells. The arrows indicate chromatin condensation, reduced nuclear size and nuclear fragmentation typically observed in apoptotic cells. (B) The percentage of apoptotic cells was calculated as the ratio of apoptotic cells to living KB cells. $^{**}P<0.01$ vs. control and $^{***}P<0.001$ vs. control (the control cells measured in the absence of β-carotene).

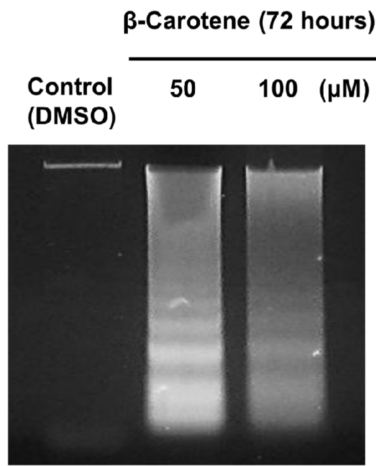

Fig. 3. Fragmentation of internucleosomal DNA by β-carotene in KB cells. The cells were treated with 0, 50 or 100 μM β-carotene for 72 hours and nuclear DNA was subjected to agarose gel electrophoresis.

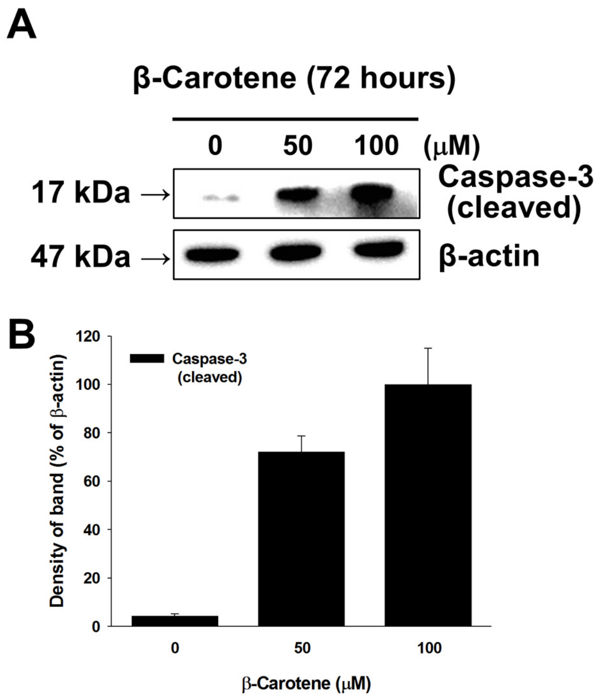

Fig. 4. Proteolytic cleavage of caspase-3 by β-carotene treatment in KB cells. (A) Expression of cleaved caspase-3 by β-carotene was measured in KB cells. The cells were treated with 0, 50 or 100 μM β-carotene for 72 hours. The cell lysate was prepared and analyzed by immunoblotting as described in "Materials and Methods". (B) Quantitative data for (A) were analyzed by using Imagegauge 3.12 software after β-actin normalization. The deviations in the results represent four separate experiments.

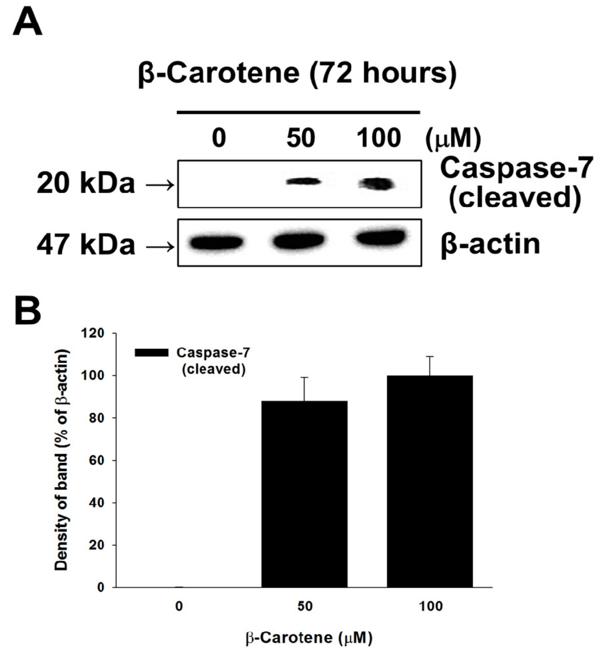

Fig. 5. Proteolytic cleavage of caspase-7 by β-carotene treatment in KB cells. (A) Expression of cleaved caspase-7 by β-carotene was measured in KB cells. (B) Quantitative data for (A) were analyzed by using Imagegauge 3.12 software after β-actin normalization. The deviations in the results represent four separate experiments.

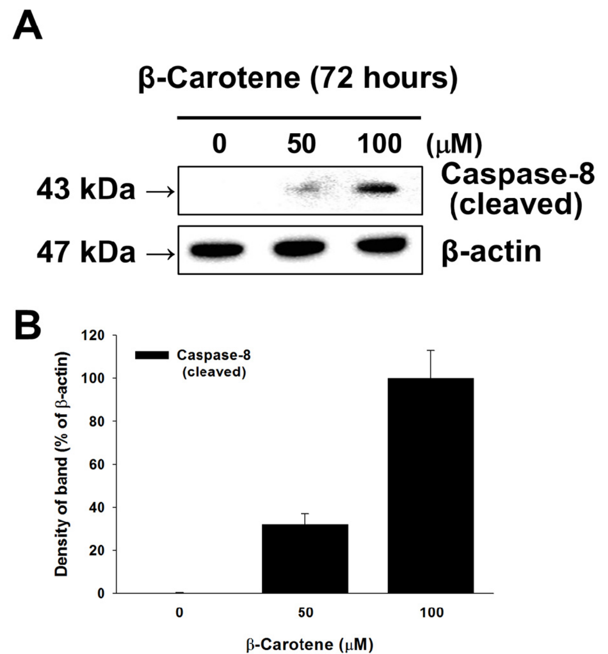

Fig. 6. Proteolytic cleavage of caspase-8 by β-carotene treatment in KB cells. (A) Expression of cleaved caspase-8 by β-carotene was measured in KB cells. (B) Quantitative data for (A) were analyzed by using Imagegauge 3.12 software after β-actin normalization. The deviations in the results represent four separate experiments.

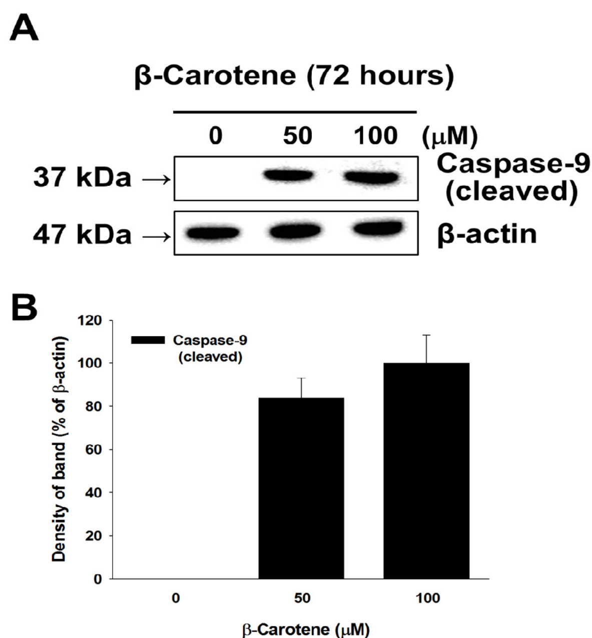

Fig. 7. Proteolytic cleavage of caspase-9 by β-carotene treatment in KB cells. (A) Expression of cleaved caspase-9 by β-carotene was measured in KB cells. (B) Quantitative data for (A) were analyzed by using Imagegauge 3.12 software after β-actin normalization. The deviations in the results represent four separate experiments.

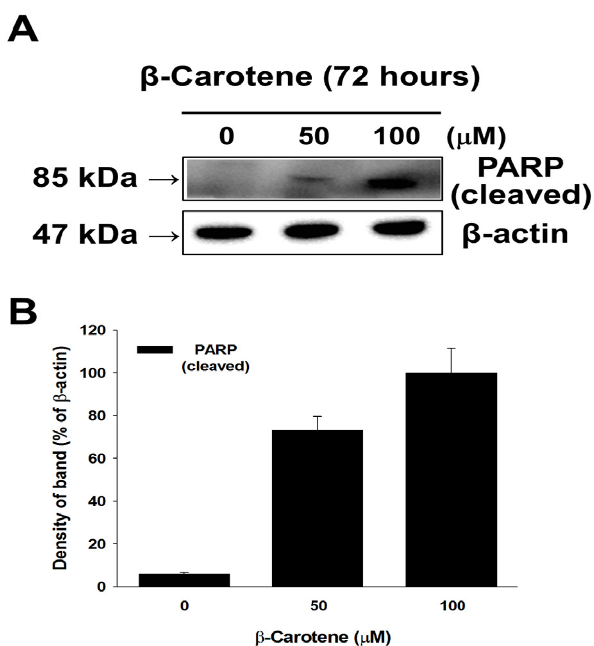

Fig. 8. Activation of cleaved PARP by β-carotene treatment in KB cells. (A) Expression of cleaved PARP by β-carotene was measured in KB cells. The KB cells were stimulated with 0, 50 or 100 μM β-carotene for 72 hours, harvested and lyzed using a cell lysate buffer. (B) Quantitative data for (A) were analyzed by using Imagegauge 3.12 software after β-actin normalization. The deviations in the results represent four separate experiments.

examined by immunoblotting since caspase-3, -7, -8 and -9 are effector caspases of apoptotic cell death [21-24]. The expression levels of cleaved caspase-3 (Fig. 4) and −7 (Fig. 5) were significantly increased in the KB cells treated with 50 or 100 μM β-carotene for 72 hours. β-carotene (50 and 100 μM) also promoted the expression levels of cleaved caspase-8 (Fig. 6) and −9 (Fig. 7) in the KB cells.

Apoptosis mediated via PARP by β−carotene

To determine how β-carotene induce the apoptosis of KB cells, immunoblotting was performed to measure the expression of the PARP at the protein level. As shown in Fig. 8, cleaved-PARP was increased by β-carotene (50 and 100 μM) compared to the control.

Discussion

β-Carotene is one of the major solvent soluble natural colorants widely present in majority of fruits and vegetables and used as chemopreventive agents [3-10]. However, the β-carotene effects on oral cancer cells are not clearly established. In this study, therefore, the cytotoxic activity of β-carotene and the mechanism of cell death exhibited by β-carotene were examined in KB human oral cancer cells. The present study demonstrated that the β-carotene can act as apoptotic inducer in human oral cancer cells.

In MTT assay, β-carotene inhibited growth of KB cells in a concentration- and a time-dependent manner (Fig. 1). This corresponded with the results of β-carotene that has anti-cancer effects via the suppression of cancer cell growth in various types of cancer cells [3-10]. These results speculated that β-carotene has cytotoxicity for oral cancer cells and potential value for anti-cancer drug discovery.

Apoptosis is an important way to maintain cellular homeostasis between cell division and cell death [18-20]. The induction of apoptosis in cancer cells is one of useful strategies for anti-cancer drug development [20]. So, many studies were performed for screening of apoptosis from natural materials. In this study, treatment with β-carotene induced the nuclear condensation and fragmentation in KB cells (Fig. 2 and 3), suggesting apoptotic cell death. These results indicated that β-carotene inhibits the growth of KB cells by activating cell apoptosis.

The activation of a family of intracellular cysteine

proteases, called caspases, is known to play an important role in the initiation and execution of apoptosis induced by various stimuli [22,23]. Among the caspases identified in mammalian cells, caspase-3, -7, -8 and -9 can serve as effector caspases of apoptotic cell death [22-24]. Caspase-3, -7, -8 and -9 are synthesized as inactive proenzymes, which require proteolytic activation to cleaved enzymes (of sizes 17 kDa, 20 kDa, 43 kDa and 37 kDa, respectively) [22-24]. Our results show that low levels of cleaved capase-3, -7, -8 and -9 were present in β-carotene-untreated KB cells, and the amount of cleaved enzymes was increased after β -carotene treatment in KB cells (Fig. 4, 5, 6 and 7). Also, the cleaved PARP, which is an important regulatory factor of death receptor-mediated extrinsic apoptotic pathway [25], was increased by β-carotene in KB cells (Fig. 8). These results suggest that β-carotene induces apoptotic cell death through both the mitochondria-mediated intrinsic pathway and the death receptor-mediated extrinsic pathway by the activation of caspases-3, -7, -8, −9 and PARP in KB cells. However, the mechanisms of apoptosis induced by β -carotene are not yet completely understood. Further studies will reveal the precise cellular and molecular mechanisms of apoptosis induced by β-carotene.

In conclusion, these *in vitro* results suggest that the β -carotene inhibits cell proliferation and induces apoptotic cell death in KB human oral cancer cells through both the mitochondria-mediated intrinsic pathway and the death receptor-mediated extrinsic pathway. Moreover, these results suggest that the β-carotene may provide a novel strategy for preventing and treating oral cancer and more research is needed to explore the molecular mechanisms.

Acknowledgements

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Conflict of interest

The authors declare that they have no conflicting interest.

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