Subcellular Accumulation of β-Carotene and Retinoids in Growth-Inhibited NCI-H69 Small Cell Lung Cancer Cells

Pankaj Prakash, Cynthia L. Jackson, and Leonard E. Gerber

Abstract: Delivery of β-carotene in tetrahydrofuran slowed the growth of NCI-H69 small cell lung cancer cells. Analysis of cells and cellular fractions revealed that β-carotene-treated cells accumulated β-carotene as well as some polar metabolites, primarily in the crude nuclei. Cells were grown at $1 \times 10^6$ cells/ml and treated with 20 μM β-carotene. Growth monitoring up to 15 days indicated an inverse relationship between the duration of β-carotene treatment and the rate of cell growth. Reverse-phase high-performance liquid chromatography analysis of treated cells showed the presence of β-carotene, retinoic acid, retinol, and retinal, with β-carotene accounting for the major material recovered. When cellular fractions were analyzed for β-carotene, it was found to be located primarily in the crude nuclei. These results demonstrate that treatment of small cell lung cancer cells with β-carotene results in a reduced growth of the cells. Further investigation is required to show a direct effect of β-carotene or its intracellular polar metabolites on these cells. Accumulation of β-carotene in the nucleus suggests a need for evaluating the nuclear role for β-carotene.

Introduction

Lung cancer is the major cancer-related cause of death among men and women in the United States (1). The types of lung cancer that afflict the human population with the corresponding percentage of the total are as follows: squamous cell carcinoma (45%), adenocarcinoma (25%), small cell carcinoma (20%), and large cell carcinoma (10%) (2). People with small cell lung carcinoma (SCLC) have a very poor prognosis, since long-term survival is <15% and only 3–5% are cured (3). To further hinder treatment, the current screening technologies for early diagnosis of SCLC are poor compared with other types of lung cancer and other cancers in general (3). Clearly, SCLC is a high priority for preventive strategies because of its poor detection and prognosis.

Several authors have reviewed the epidemiological evidence suggesting a protective role for carotenoids and fruits and vegetables in cancer incidence (4). The most consistent cancer for which this relationship occurs is lung cancer (5). An inverse association between the dietary consumption of β-carotene and the risk of developing lung cancer has been observed in a number of studies (6–8). The consumption of β-carotene-containing fruits and vegetables or β-carotene blood levels have been inversely correlated with lung cancer incidence, particularly for small cell and squamous cell carcinoma (9–11). Serum β-carotene (but not retinol) was significantly lower in lung cancer cases than in controls in a number of studies (12,13).

In animal model experiments, a suppression in the progression of spontaneous mammary tumors in virgin mice by a β-carotene-rich algae (Dunaliella bardawil)-supplemented diet was demonstrated (14). In cell culture studies, using beadlet and crystalline forms of β-carotene, Bertram and co-workers (15–18) found β-carotene to cause a dose-dependent decrease in the growth rate of C5H/10T1/2 murine fibroblast cells.

On the basis of the evidence that β-carotene might reduce the risk of lung cancer, recent studies have been designed to measure the impact of supplemental β-carotene on lung premalignant end points or on lung cancer. The Tyler Chemoprevention Trial (19), in which β-carotene and vitamin A were used, did not result in reduction of atypical cells in sputum in asbestos workers; however, van Poppel (20) did see reduced levels of cellular micronuclei in sputum from heavy smokers.

Three recent trials in which lung cancer was used as a primary end point have now been completed. In the α-Tocopherol β-Carotene Trial (21), heavy cigarette smokers received supplemental α-tocopherol and/or β-carotene or placebo for up to eight years. Participants receiving β-carotene had a significantly higher incidence of lung cancer than those receiving placebo. Within the placebo group, higher dietary β-carotene intake was associated with lower lung cancer incidence, consistent with the dietary intake and lung cancer incidence observed in numerous other studies. In another study, the Carotene and Retinol Efficacy Trial, as-
bestos workers and smokers received supplemental β-carotene and vitamin A or placebo (22). This study was terminated earlier than intended, because lung cancer incidence was increased in those subjects consuming β-carotene and vitamin A. In the US Physician’s Health Study (23), male physicians consuming supplemental β-carotene for 12 years demonstrated no change in their risk for all types of cancer.

The inconclusive results obtained with human and animal studies demonstrate the need to establish cell culture models to determine the effect of β-carotene on lung and other cancers. This includes effects on cell growth, the determination of the cellular accumulation of β-carotene, its metabolism to retinoids, and intracellular distribution of β-carotene and its bioactive metabolites. The present study was intended to obtain some answers to these questions. Specifically, β-carotene in crystalline form was delivered to NCI-H69 SCLC cells, and its effect on the growth of these cells was studied along with the high-performance liquid chromatography (HPLC) analysis of cells and cellular fractions to reveal any intracellular conversion of β-carotene to β-apocarotenals and/or retinoids and the localization site(s) of carotenoid and its metabolites (if any) in the cells.

Materials and Methods

Materials

The human SCLC line NCI-H69 was obtained from the American Type Culture Collection (Rockville, MD). RPMI 1640 growth medium and other growth regulators were purchased from GIBCO (Gaithersburg, MD). Crystalline β-carotene was kindly provided by Hoffmann-La Roche (Nutley, NJ). Tetrahydrofuran (THF) containing butylated hydroxytoluene was purchased from Aldrich (Allentown, PA). All reagents for chromatography work were of HPLC grade and were purchased from Fisher Scientific (Pittsburgh, PA). The reagents used for the fractionation work were purchased from Sigma Chemical (St. Louis, MO) and Fisher Scientific.

Cell Culture

NCI-H69 cells were grown in RPMI 1640 medium, supplemented with 10% calf serum, 1% penicillin-streptomycin, and 1% glutamine in a humidified atmosphere of 5% CO₂ in air at 37°C. Cells were grown and maintained at a concentration of 1 × 10⁵/ml in the medium for the experiments. At the range of cell concentrations used in our studies, typical doubling time of cells was approximately two days. The viability of the cells was determined using the trypan blue exclusion method before and during carotenoid treatment of the cells in all experimental groups. A 0.4% trypan blue solution was routinely used for this purpose.

Treatment of Cells With β-Carotene

β-Carotene was used at a concentration of 20 μM in the medium. THF containing butylated hydroxytoluene was used as a delivery vehicle of crystalline β-carotene to the cells. On the basis of the molecular weight of β-carotene, the appropriate amount of β-carotene was dissolved in THF to make a stock solution of 20 mM. One microliter of this stock β-carotene solution was added to each milliliter of the medium to achieve a final concentration of 20 μM β-carotene in the medium, as described previously (18). An equivalent amount of THF was added to the medium for control cells. In this way, the concentration of THF in the control and experimental medium was 0.1%. Cells were diluted from stock cultures to approximately 1 × 10⁵ cells/ml in 10 ml of treatment or control medium. In one group, THF was omitted to determine whether THF had any toxic effect on the growth of the cells. Three replications were used in each group. The cells were exposed to treatment to monitor growth for 5, 11, or 15 days; for metabolite quantitation experiments, treatment exposure was 6 days. Red light was used while working with β-carotene solution to prevent photodamage to the carotenoid. Solutions of β-carotene were made in the hood equipped with ultraviolet light to maintain sterility. A new bottle of THF was used for each experiment, purged, and filled with nitrogen after each use.

Growth-Monitoring Experiments

Flasks of cells were grown in the presence or absence of 20 μM β-carotene, as described above, for 5, 11, or 15 days at approximately 1 × 10⁵ cells/ml. The medium was changed at Days 5 and 11. For the cultures allowed to grow for 15 days, culture flasks were diluted with medium appropriately at Day 11 to bring the concentrations back to approximately 1 × 10⁵ cells/ml. This was accomplished to prevent the effects of high cell density on the reduction of cell growth.

Growth of the cells in control and treatment groups was monitored by cell counts with a model ZM Coulter counter. Before they were counted, the cells were passed through a syringe-and-needle combination to bring them down to a single cell suspension and also suspended several times in the isotonic buffer before they were counted. The Coulter counter window for counting was adjusted to prevent counting of particles smaller than the cells and to prevent counting of possible clumped cells that may have remained. During the course of the experiment, cells in each group were also critically viewed under a phase-contrast microscope to monitor the general growth pattern.

 Extraction and HPLC Quantitation of β-Carotene and Retinoids

Cells grown at 1 × 10⁵/ml and treated with 20 μM β-carotene for six days were used for this determination. Cells were harvested by washing four times with phosphate-buff-
ered saline (PBS). Cell samples were then pelleted at 2,000 rpm for five minutes and stored at -70°C until ready to use. For extraction of the carotenoids, cell pellets containing approximately 5 x 10⁶ cells were suspended in 4 ml of PBS to which 4 ml of acetone were added and vortexed for about two minutes. This mixture was extracted by adding 4 ml of hexane and 4 ml of distilled water and vortexed for two minutes. The mixture was centrifuged, the upper hexane layer was removed, and the hexane extraction was repeated. The extracts were combined and evaporated under nitrogen. The residue was reconstituted in 100 µl of acetone. For retinoid extraction, cell pellets containing approximately 5 x 10⁶ cells were suspended in 4 ml of PBS, and 1 ml of 4N KOH in ethanol was added. This solution was saponified at 60°C in water for 40 minutes, then 2 ml of distilled water were added and the mixture was extracted twice by addition of 2 ml of hexane. It was vortexed for two minutes and centrifuged. The upper hexane layers were combined and evaporated under nitrogen. The residue was reconstituted in 100 µl of ethanol. These procedures were modified from published procedures (24).

A 20-µl aliquot of the reconstituted carotenoid or retinoid extract was injected onto a 5-µm C₁₈ (4.6 mm x 25 cm) column (Vydac 201TP54) and eluted with methanol-THF (95:5, vol/vol) at 0.8 ml/min (25) for the carotenoids and methanol-water (95:5, vol/vol) at 0.8 ml/min (26) for the retinoids. Measurement of the carotenoids was determined by absorbance at 450 nm and retinoids at 325 nm. Echinonene and retinyl acetate were used as internal standards for the quantification of carotenoids and retinoids, respectively. Recovery of the internal standards was always >90% for all experiments.

Cell Fractionation

Approximately 1.7 x 10⁷ cells, which had been cultured at a concentration of 1 x 10⁶/ml in 20 µM β-carotene-supplemented medium or in control medium for six days, were suspended in 5 ml of lysis buffer [10 mM tris(hydroxymethyl)aminomethane-Cl, 10 mM NaCl, 1.5 mM MgCl₂, pH 7.4] and permitted to swell for 10 minutes at 4°C. Cells were then broken in a dounce homogenizer with seven to eight strokes of a B pestle. The extent of cell disruption was monitored using light microscopy to ensure that there were no unbroken cells in the samples. The homogenate was centrifuged at 200 g for 10 minutes, yielding a pellet of crude nuclei and a postnuclear supernatant. The supernatant fraction was separated and further centrifuged at 30,000 g for 30 minutes to obtain a pellet of crude membranes. These two cellular fractions were again viewed under a microscope and washed with PBS before extraction. This fractionation scheme was followed as described previously (24) to determine carotenoid and retinoid distribution in cell fractions. Crude nuclear, crude membrane, and cytoplasmic fractions were extracted and subjected to reverse-phase HPLC quantitation of carotenoids and retinoids by use of appropriate internal standards, as described above.

Statistical Analysis

The results of growth experiments, quantification of carotenoids and retinoids in the cell, and cellular fractions were analyzed using Student's t-test for each individual test, and the significance of differences between control and treatment was determined at p < 0.05 (27).

Results

Effect of β-Carotene on Cell Growth

Figure 1 shows the effect of 20 µM crystalline β-carotene, dissolved in THF, on the growth of NCI-H69 SCLC cells grown at 1 x 10⁶/ml in the medium. For all growth experiments, three separate flasks were used for each control and treatment. Figure 1 is representative of the multiple experiments conducted in this manner. β-Carotene inhibited the growth of the cells in a time-dependent manner. Significant reduction in the growth of cells grown in the presence of β-carotene compared with control cell flasks was observed on Days 11 and 15 of treatment (p < 0.05). At time points beyond 15 days, cell growth in the presence of β-carotene remained suppressed compared with control cell flasks (data not shown). No toxicity of THF was noticed in the control cells when these were compared with a separate set of cells that was grown in a manner identical to this group but was

![Figure 1](image-url)

**Figure 1.** Effect of 20 µM β-carotene (BC) on growth of NCI-H69 small cell lung cancer cells grown at a concentration of 1 x 10⁶ cells/ml. Values are expressed as total number of cells. β-Carotene was dissolved in tetrahydrofuran, and control cells received tetrahydrofuran only. Growth medium was changed on Days 5 and 11. Each bar is mean of 3 replications. Growth of cells was reduced on Days 11 and 15 of β-carotene treatment. *, p < 0.05.
not treated with carotenoid or THF (data not shown). Viability determination using 0.4% trypan blue revealed >90% of the cells in all groups to be viable during the entire course of the experiment. As is typical for this suspension cell line, phase-contrast microscopy revealed control cells growing in clumps (not shown). Cells treated with β-carotene were found to be growing in a scattered fashion, indicating a reduction in proliferation under β-carotene treatment.

Accumulation of β-Carotene and Its Metabolites in Cells

Figure 2 shows the HPLC detection of retinoic acid (Peak 1) in the cells treated with β-carotene. The cells were grown at 1 × 10^5 cells/ml medium and given 20 μM β-carotene for six days. An unknown retinoid (Peak 2) was detected at the retention time of 6.90 minutes and could be one of the retinoic acid cis-isomers, although it could not be compared because of the lack of standards. Retinal (Peak 3) was also detected in this analysis of treatment cells.

The accumulation of β-carotene and retinoids in NCI-H69 cells treated with 20 μM β-carotene for six days as quantified by HPLC is shown in Table 1. The results are the averages of three determinations. Echinone and retinyl acetate were used as internal standards for the quantification of the carotenoids and retinoids, respectively. Treated cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control Cells</th>
<th>Experimental Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Carotene</td>
<td>ND</td>
<td>101.75</td>
</tr>
<tr>
<td>Retinoic acid</td>
<td>11.51</td>
<td>58.08*</td>
</tr>
<tr>
<td>Retinol</td>
<td>4.35</td>
<td>10.39</td>
</tr>
<tr>
<td>Retinal</td>
<td>ND</td>
<td>3.22</td>
</tr>
<tr>
<td>Total retinoids</td>
<td>15.86</td>
<td>71.69*</td>
</tr>
</tbody>
</table>

*a: Values are averages of 3 determinations expressed as ng/10^6 cells. ND, not detectable.
b: Statistical significance is as follows: *, significantly different from control, p < 0.05.

were found to accumulate 101.75 ng of β-carotene. Retinoic acid was found to be the major retinoid accumulated (58.08 ng). Retinol was found to be the second major retinoid (10.39 ng), followed by retinal (3.22 ng). A significantly increased accumulation was observed in treatment cells when retinoic acid and total retinoids (retinoic acid, retinol, and retinal) were compared between control and treatment cells (p < 0.05).

Accumulation of β-Carotene in Cellular Fractions

Table 2 shows the quantitative accumulation of β-carotene on Day 6 in the cellular fractions of NCI-H69 cells treated with 20 μM β-carotene. Echinone was used as an internal standard to quantify the carotenoid in different fractions. Results are the averages of three determinations. β-Carotene was found to be localized primarily in the crude nuclear fraction of the cells at an average value of 40.61 ± 2.4 ng. Crude membrane was also the site of β-carotene localization in the cells, with an average accumulation of 4.34 ± 0.5 ng. No β-carotene was detected in the cytoplasmic fraction.

<table>
<thead>
<tr>
<th>Cell Fractions</th>
<th>Control Cells</th>
<th>Experimental Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude nuclei</td>
<td>ND</td>
<td>40.6 ± 2.4</td>
</tr>
<tr>
<td>Crude membranes</td>
<td>ND</td>
<td>4.3 ± 0.5</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*a: Values are averages ± SD of 3 determinations expressed as ng/10^6 cells.

Discussion

The antiproliferative effects of carotenoids, including β-carotene, have been observed on various types of cancers by use of cell culture models, namely, the human lung cancer 801 cell line (28), squamous cell lung carcinoma cells (29), NCI-H69 SCLC cells (30), neuroblastoma cells (31), neo-
plastic transformation of C3H/10T1/2 murine fibroblast cells (15), F9 embryonal carcinoma (32), X-ray-induced transformation of C3H/10T1/2 cells (33), and human colon adenocarcinoma (34). Investigators have used different methods of carotenoid delivery to the cells in culture and have used a variety of carotenoid concentrations and molecules. In the present study, THF was used to dissolve β-carotene and also as the delivery vehicle of the carotenoid to NCI-H69 SCLC cells, as detailed elsewhere (18). THF was used at 0.1% in the medium, and consequently no toxicity to the cells was observed when the cell viability, by use of the trypan blue exclusion method, was compared with control cells not exposed to THF (data not shown).

As stated earlier, the level of β-carotene used and the delivery system vary widely in published in vitro studies. In the present study, 20 and 40 μM β-carotene dissolved in THF were used and found to be effective in decreasing the proliferation of the cells, with 20 μM showing greater effect in terms of percent reduction (data not shown). This concentration was selected on the basis of the previous observations in our laboratory (30), where 20 μM β-carotene consistently reduced the proliferation of NCI-H69 cells in dose-response experiments in which 2–20 μM β-carotene was used. β-Carotene at 20 μM is slightly above the level that can be achieved in the serum of human subjects taking oral β-carotene supplementation. Previously, serum β-carotene levels up to 16.1 μM in response to a dose of 50 mg/day (35) have been reported.

β-Carotene is a highly lipophilic compound that is poorly absorbed by most animals (36). Determining the cellular uptake and accumulation of β-carotene and its distribution in target tissues are important to clinical applications. In addition, the compound(s) resulting from β-carotene metabolism is another consideration. The results of this study indicate the metabolism of β-carotene to retinoic acid, retinol, and retinal in NCI-H69 cells. An accumulation of β-apo-12'-carotenal and β-apo-8'-carotenal was also noticed, but their low levels precluded quantification (data not shown). It is possible that because radioactively labeled β-carotene was not used and the sensitivity of the HPLC system was limited, certain potent retinoid metabolites may have been undetected. Caco-2 cells derived from an adenocarcinoma of the human colon were shown to metabolize β-carotene to retinoids (34). Similarly, β-carotene conversion to retinol was shown in BALB/c 3T3 cells of nonintestinal origin (24). Additionally, cleavage of β-carotene to retinol and retinoic acid was also demonstrated in small intestinal cells in culture (37). In contrast to these studies, C3H/10T1/2 mouse fibroblast cells were not found to convert β-carotene to retinoids (17). Conversion of carotenoid to retinoid was also not demonstrated in β-carotene-treated cells in human breast, oral carcinoma, and malignant melanoma (38).

The in vitro studies available to date indicate that several cellular compartments or molecular targets can be protected by β-carotene. It has been reported that β-carotene can protect cell membranes from oxidative damage (39). Furthermore, in vitro studies have shown that membrane-associated events such as gap junctional communication (39) can be modulated by β-carotene. Because β-carotene can reduce damage to membrane and nuclear components, knowledge of β-carotene's subcellular distribution may help establish the relationship between localization site and the mechanism of action involved. In this study, β-carotene was found to be localized primarily in the crude nuclear fraction (90%) and the rest in the crude membranes, suggesting a need to further evaluate a nuclear role for β-carotene. Recovered β-carotene was not found to accumulate in the cytoplasm. Different subcellular sites of β-carotene accumulation have been found in other cell types. In BALB/c 3T3 fibroblast cells, β-carotene was found to be largely associated with the membranes (24). Studies done on bovine corpus luteum showed recovery of β-carotene predominantly in lipid and crude nuclear fractions, with smaller amounts in mitochondria (40). In contrast, liver cells of chicken showed the highest accumulation of β-carotene in mitochondria, followed by lysosomes, microsomes, and nuclei (41). However, rat liver cells accumulated β-carotene primarily in cytosol, with smaller amounts in nuclei, mitochondria, and lysosomes (42).

The cellular accumulation of β-carotene in NCI-H69 cells was found to be 0.19 nmol/10⁶ cells over a period of six days. These cellular concentrations are within the range reported in similar studies using different cell lines. An accumulation of 0.004 nmol/10⁶cells was found when 7 μM β-carotene was added to MCF10A human mammary epithelial cells for five days (43). The highest cellular levels were found to be 0.008 nmol/10⁶ in Chinese hamster ovary cells exposed to 0.07 μM β-carotene for 48 hours (44). An accumulation of 0.011 nmol/10⁶ was reported in human buccal mucosal cells (45). In BALB/c 3T3 cells treated with 0.3 μM β-carotene in crystalline and beadlet forms, an accumulation of 0.006 and 0.07 nmol/10⁶ cells, respectively, was reported (24). In C3H/10T1/2 cells, cellular β-carotene concentrations were 1.0–2.0 nmol/10⁶ cells when 10 μM β-carotene solution was given for one week (17). On the basis of these observations, the accumulation of β-carotene seems to vary with cell type, carotenoid type and concentration, and duration of treatment.

In the present study, HPLC analysis of the cells treated with β-carotene showed metabolism of the carotenoid to retinoids and apocarotenoids. These results in isolation do not allow a conclusion as to whether β-carotene or its metabolites are responsible for the antiproliferative effect. In conjunction, however, with the previous work in our laboratory which demonstrated that neither retinol nor retinoic acid suppressed the growth of these cells (30), these results suggest that metabolism to retinoids is not required for the growth-reducing effect of β-carotene.

In conclusion, our studies demonstrate that when optimal physiological levels of β-carotene are taken up by SCLC cells, the cells metabolize β-carotene to some retinoic acid, retinol, and retinal, but most of the β-carotene remains intact. Most of the intact β-carotene associates with the nucleus.
Concomitant with these metabolic events, the growth of the SCLC cells is slowed. Recent studies from our laboratory also indicate that gene expression is altered in the β-carotene-treated cells through decreased N-myc mRNA levels and other mRNA species that are being identified. It is possible that β-carotene directly modifies gene expression in lung cancer cells, reducing their growth. Further investigation of the nature of the gene expression changes should provide clues as to the mechanism(s) involved in β-carotene’s antiproliferative effects.

Acknowledgments and Notes

The authors thank Dr. Norman I. Krinsky (Dept. of Biochemistry, Tufts University School of Medicine, Boston, MA) for the kind review of the manuscript. This work was supported by grants from the Rhode Island Agricultural Experiment Station and American Cancer Society Grant IN-45-37. This manuscript is Contribution 3635 of the College of the Environment and Life Sciences, University of Rhode Island. The entire work was accomplished at the University of Rhode Island and Rhode Island Hospital. Address reprint requests to Dr. Leonard E. Gerber, 15 Woodward Hall, Dept. of Food Science and Nutrition, University of Rhode Island, Kingston, RI 02881.

Submitted 2 June 1998; accepted in final form 18 March 1999.

References

16. Pung, AO, Rundhaug, JE, Yoshizawa, CN, and Bertram, JS: β-Caro
tene and canthaxanthin inhibit chemically- and physically-induced neo
18. Cooney, RV, Kappock, TJ, IV, Pung, A, and Bertram, JS: Solubili
29. Schwartz, J, and Shidler, G: The selective cytotoxic effect of caro
34. Quick, TC, and Ong, DE: Vitamin A metabolism in the human intestinal Caco-2 cell line. Biochemistry 29, 11116–11123, 1990.