Effect of β-carotene on cell cycle progression of human fibroblasts

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The uptake of β-carotene (BC) and its effect on the cell cycle progression of normal human fibroblasts in primary culture were investigated by using two different delivery methods: exposure to BC solubilized in the organic solvent tetrahydrofuran (THF) or to BC incorporated into dipalmitoylphosphatidylcholine (DPPC) liposomes. Cell cycle progression was evaluated by immunofluorescence detection and flow cytometric analysis of the proliferating cell nuclear antigen (PCNA). In contrast to THF, which induced a marked reduction in the number of cells in S phase and in the extent of PCNA immunolabelling, DPPC liposomes proved to be an effective delivery system that does not interfere with cell proliferation. Cellular uptake of 0.23 nmol/106 cells was found after 24 h incubation in BC-containing DPPC liposomes. This value increased to 1.2 nmol/106 cells after 72 h. After the first day of incubation, the number of cells in S phase was reduced by ~50%, with a consequent accumulation of cells in G1 phase. This effect was maintained up to 3 days incubation, with no detectable effects on cell viability. This cell cycle delay was found to be reversible, returning the percentage of cells in S phase to the control value 24 h after removal of BC from the medium. In order to determine whether the activity of BC could be attributed to the molecule itself or to its conversion into retinoids, the production of BC metabolites was assessed. Analysis of cellular levels of retinoids failed to demonstrate the presence of retinal, retinoic acid or retinyl esters during an incubation period of 6 days. These results suggest that in normal human fibroblasts, BC induces a cell cycle delay in the G1 phase and that this effect is independent of conversion to known retinoids.

Introduction

β-Carotene (BC*), a carotenoid distributed mostly in green and yellow vegetables, has been reported to protect cells against cytotoxicity and genotoxicity in both in vitro and in vivo experimental model systems; BC can inhibit cell transformation in vitro and carcinogenesis induced by chemical and physical agents in animal experimental models (for a review see 1). Moreover, epidemiological studies have suggested an association between an increased dietary intake of carotenoids and a reduced incidence of cancer (2), even if recent results of clinical trials indicated that BC supplements do not protect against cancer and might actually increase smokers’ risk of lung tumors (3). Although several anticarcinogenic mechanisms of carotenoids have been proposed, there are still conflicting opinions and little is known regarding their mechanisms at the cellular and molecular levels. The protection afforded by carotenoids can in part be attributed to the quenching of singlet oxygen (4,5). In addition to this mechanism, carotenoids has been reported to quench not only singlet oxygen, but also to scavenge a variety of free radical species (6). However, BC is considered an ‘unusual antioxidant’, because its activity depends, in a chemical system, on the partial pressure of oxygen (7) and, in a cellular system, on its concentration (8). Although these observations account for some of the biological effects of BC, they are not sufficient to explain its antitumor properties. Thus, possible additional mechanisms have been suggested, such as: conversion to vitamin A, which is a potent modulator of cell differentiation and proliferation (9); an enhancement of immunological response (10,11); an increase in synthesis of the cytoskeletal proteins (12); an increase in gap junctional communication (13); a decrease in expression of proto-oncogenes (14). The last two mechanisms are related to the antiproliferative effects of BC observed in tumor cell lines (for a review see 12). On the other hand, little and contrasting evidence concerning the inhibition of proliferation of normal cells is available (12,15).

BC can be metabolized to retinoids, which are known to be strong inhibitors of cell proliferation. However, in normal tissues the enzyme 15,15'-dioxygenase, responsible for the conversion of BC to retinoids, has been detected up to now only in the small intestine, the liver and corpus luteum (16,17). The ability of the BC molecule itself to inhibit cell growth has yet to be elucidated.

The aim of this study was to determine the effect of BC on cell cycle progression as such or in relation to the production of BC metabolites in primary cultures of normal human fibroblasts.

Because BC is a highly lipophilic compound poorly taken up by most animals (18) and cells in culture (19), two delivery methods were investigated: (i) exposure of cells to BC solubilized in tetrahydrofuran (THF); (ii) exposure of cells to BC incorporated into dipalmitoylphosphatidylcholine (DPPC) liposomes. Cellular uptake of BC was determined by HPLC.

Cell cycle progression was evaluated by immunostaining of proliferating cell nuclear antigen (PCNA) tightly bound to DNA replication sites (20), which allows the determination of cells in S phase (21). In addition, the effect of BC on the incorporation of bromodeoxyuridine (BrdU) was assessed by flow cytometry.

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Materials and methods

Chemicals
A stock solution of 5 mM crystalline BC (synthetic all-trans, type I; Sigma CAS no. 7235-40-7) was prepared in THF (BDH). Fresh solutions of BC/THF were prepared on each day of the experiments as needed. The absence of THF peroxide was monitored by an iodometric method.

For lipidosome preparation, 5 mM crystalline BC was dissolved in chloroform (Sigma) and an equal volume of a lipid solution (1 mg/ml) was used. The liposomes were prepared from 100 mg DPPC (Sigma) dissolved in chloroform in the presence or absence of BC. The lipid mixtures with or without 500 μl BC solution were dried with nitrogen, resuspended in 10 ml phosphate-buffered saline (PBS) and ultracentrifuged after 15 min sonication or, alternatively by filter (1 μm pore size; Nucleopore) filtration with an extruder device (Lipex Biomembranes Inc., Canada). The amount of BC incorporated into liposomes was determined by extraction of the BC from the liposomes and by subsequent detection by HPLC. Starting from an initial solution of 250 μM, the concentration of BC retained by liposomes was determined to be 210 (85% of initial concentration), while in the liposomes prepared by filtration it was 40 μM (16% of initial concentration).

Cell culture and treatments
Human embryonic lung fibroblasts (PEU) were grown in E-MEM medium (Bio-Whittaker) supplemented with 10% fetal calf serum (Gibco, UK), 100 IU/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml fungizone. Cells were used between the fifth and twentieth passages. The estimated doubling time for these cells was 30.3 ± 2.5 h. Cell treatment was performed by diluting BC-containing liposomes 1:10 in culture medium. The medium containing BC was replaced daily in order to avoid formation of oxidation products. Cell viability, determined by the release of lactate dehydrogenase (LDH) into the culture medium (22), was shown to be unaffected by the treatment. All experiments were performed at least in triplicate.

Extraction of BC and HPLC determination
After treatment, 1 ml aliquots of cell suspensions (1x10^6 cells) were washed three times with distilled water and subsequently lysed with Triton X-100 (1%). BC was extracted twice in ethanol/hexane containing 0.025% butylated hydroxytoluene as an antioxidant. All solvents (HPLC grade) were provided by BDH (UK). The hexane phase was collected, evaporated under nitrogen and the extract was finally resuspended in HPLC mobile phase (methanol/hexane 85:15) and assayed immediately. Aliquots of 50 μl were injected into the HPLC system (Hewlett Packard 1090) equipped with a diode array detector (DAD).

The analytical procedure to analyze the intracellular concentrations of the BC was an isotropic reverse phase HPLC analysis carried out with a mobile phase flux of 1 ml/min at constant temperature (30°C). The column used for separation was a Spherisorb ODS 2, 250 mm in length and with a diameter of 4 mm ( Particle Technology Packard). The DAD was programmed to acquire signals at 450 (BC absorption maximum) and 280 nm (tocopherol acetate absorption maximum, an internal standard periodically used for extraction and analysis quality control purposes). The entire analytical procedure (treatment, extraction and chromatographic run) was carried out in dim light to avoid carotenoid photo-autooxidation. For analytic quantitation, a calibration curve was constructed by using pure standard BC and tocopherol acetate (Sigma).

Extraction of BC metabolites and HPLC determination
Pellets containing 1x10^6 cells were used for quantitation of retinoids. The extraction method and the analytical procedure to determine the intracellular concentration of BC metabolites were the same as used for BC, as reported above. For better chromatographic resolution of retinoid peaks, a HPLC gradient method modified from the above isotropic one was also used. The DAD was programmed to acquire signals at 325 (retinol absorption maximum), 382 (retinal absorption maximum) and 335 nm (retinoic acid absorption maximum). For analytic quantitation, calibration curves were periodically built with pure standard retinol, retinal and retinoic acid (Sigma).

For detection of the presence of retinyl esters, extracts from BC-supplemented cells, dried with nitrogen, underwent saponification for 6 h in the dark by adding 1 ml of a solution containing 10 mg/ml KOH/CH3OH and 25 μl twice distilled H2O. Cell extracts without BC and extracts with added retinyl palmitate were used as negative and positive controls respectively. The saponification procedure proved to be effective since, after 6 h, in the positive control group no retinyl palmitate was present, while an almost complete transformation into retinol was detected.

Cell cycle analysis
At the end of incubation, cells were detached using a standard trypsinization procedure, counted by hemocytometer, transferred to plastic tubes and washed in PBS. Fixation was performed according to the following protocol (23). Cells were lysed at 4°C for 8 min in a hypotonic solution containing 10 mM Tris–HCl pH 7.4, 2.5 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride (BH2) and 0.5% Nonidet P-40 (Sigma). After lysis was complete, cells were resuspended in saline and cold ethanol was added to a final 70% concentration. Fixed cells were stored at -20°C.

Cells were removed from the fixative, washed in PBS containing 1% BSA (Sigma) and 0.2% Tween 20 (PBT) (Sigma) and incubated for 30 min in 100 μl mouse anti-PCNA monoclonal antibody (PC10 clone, Dako, Denmark) diluted 1:100 in PBT. At the end of incubation, cells were washed twice and incubated for 30 min in 100 μl fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (Sigma) diluted 1:100. The last antibody incubation, cells were again washed twice in PBT and resuspended in PBS containing 5 μg/ml propidium iodide (PI) (Sigma) and 100 μg/ml RNase A (Sigma). Staining occurred for at least 30 min at room temperature.

Cell cycle distribution was also assessed by determining BrdU incorporation versus DNA content. Cells were incubated with 30 μM BrdU (Sigma) during the last hour of culture, harvested and fixed in cold 70% ethanol. Fixed cells were washed in PBS, resuspended in 2 N HCl for 30 min at room temperature, centrifuged at 200 g for 3 min and then resuspended in 0.1 N sodium tetraborate for 15 min. The samples were then washed in PBS, incubated for 15 min in PBT and for 30 min in 100 μl anti-BrdU monoclonal antibody (Becton Dickinson) diluted 1:10 in PBT. After two washes with PBT, cells were incubated for 30 min in FITC-conjugated anti-mouse antibody (Sigma) diluted 1:50 in PBT, then washed twice and resuspended in PBS containing 5 μg/ml PI and 100 μg/ml RNase A.

Flow cytometric measurements
Cells were analyzed with a FACScan (Becton-Dickinson) or with a Coulter XL (Coulter Corp.) flow cytometer. Both instruments were equipped with an argon laser tuned at 488 nm for fluorescence excitation. FITC fluorescence was measured with a band-pass filter at 530/30 nm; PI fluorescence was measured with a 585/44 or a 620/30 nm band-pass filter. Ten thousand cells were measured for each sample. Computer statistical analysis of mean fluorescence intensity (MFI) and graphic representation were performed with Lysis II software (Becton-Dickinson) or with XL software (Coulter). Statistical significance analysis was obtained by Student’s t-test.

Results
Since BC is poorly water soluble, preliminary studies were performed to optimize its solubilization with organic solvents whose concentrations will not interfere with cell cycle progression. Thus, solubilization in dimethylsulfoxide (DMSO), ethanol and THF were assessed. An alternative delivering system with DPPC liposomes was also investigated. To reach a useful concentration of 5 mM in the stock solution, BC could be solubilized only in THF.

In order to analyze the effect of THF or of DPPC liposomes on the cell cycle, the distribution of PCNA bound to DNA replication sites was determined. PCNA bound to DNA synthesis sites is found only in S phase cells, thus allowing discrimination of these cells from those present in the other phases of the cell cycle, G1 and G2+M (Figure 1, control).

THF was consistently found to remarkably influence cell cycle progression. In fact, this solvent strongly reduced the percentage of cells in S phase even at the minimal concentration (0.2%) necessary to dissolve BC. In contrast, DPPC liposomes alone were not found to affect cell proliferation (Figure 1).

Therefore, subsequent experiments were all performed by delivering BC dissolved in DPPC liposomes to reach a final extracellular concentration of 21 μM. Figure 2 shows the cellular uptake of BC delivered from liposomes prepared by sonication, after treatment for 24, 48 and 72 h. BC is taken up by exponentially growing human fibroblasts, achieving cellular levels of 0.12 μg/10^6 cells (0.23 mmol/10^8 cells) after...
Control

DPPC liposomes

THF

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Effect of DPPC liposomes and THF on cell cycle progression of human fibroblasts. Two-parameter dot plots of PCNA immunofluorescence versus DNA content are shown; the regions relative to each compartment of the cell cycle are also indicated. Untreated cells (control) and samples incubated for 24 h with DPPC liposomes or with 0.2% THF.

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Time course of cellular uptake of BC delivered from DPPC liposomes in exponentially growing human fibroblasts. BC-containing liposomes were prepared by sonication to reach a final extracellular concentration of 21 μM. Mean values ± SD of three independent experiments are shown.

24 h of incubation and reaching a value of 0.94 μg/10^6 cells (1.2 nmol/10^6 cells) at 72 h. Comparable results were obtained when BC was administered with liposomes prepared by extrusion (data not shown), even if the BC concentration in liposomes was lower (40 μM) than that obtained in liposomes prepared by sonication (210 μM), indicating that BC cellular uptake reached a saturation level at the lower concentration.

Figure 3A shows the chromatogram of a cell extract after 72 h BC treatment. The BC chromatographic peak acquired at a wavelength of 450 nm (BC absorption λ_max) is well resolved at a retention time (RT) of 10.71 min (the same as BC standards previously acquired); the peak spectrum is superimposable on that of the BC standard. Peaks acquired at 325 nm (i.e. the retinol absorption maximum) neither match in RT nor show the spectral properties of retinol or retinoids, thus excluding a metabolic intracellular transformation of BC into vitamin A or retinoids. Figure 3B shows the chromatogram of a cellular extract obtained from human fibroblasts after addition of retinyl palmitate, before (1) and after (2) saponification. No evidence of retinoids was observed during a period of 6 days treatment with liposomes containing BC. Observations were confirmed using the modified HPLC gradient method.

Figure 4 shows a dot plot of PCNA immunofluorescence versus DNA content in control cells and in fibroblasts daily supplemented with BC-containing liposomes. BC induced a reduction in the number of cells in S phase and a consequent accumulation of cells in G1 phase. The data obtained by PCNA immunostaining were also confirmed by cytometric evaluation of BrdU incorporation. In fact, the number of cells incorporating the nucleotide was reduced in BC-treated samples to an extent similar to that determined by PCNA immunostaining. However, those cells that were in S phase exhibited levels of BrdU labeling comparable with those measured in control cells (Figure 4).

After the first day of incubation, the number of cells in S phase was reduced by ~50%. On days 2 and 3, this inhibition was maintained at the same value (Figure 5). This effect was found to be reversible, since 24 h following the end of BC treatment, the percentage of cells in S phase returned to the control value (Table I).

The inhibitory effect of BC on cell proliferation was also assessed by determining the number of viable cells after treatment. As shown in Figure 6, a reduction in cell number of ~35% was observed only after 3 days of incubation in the presence of BC.

**Discussion**

Before investigating the biological effect of BC on the cell cycle, preliminary studies were performed to optimize the solubilization of this lipophilic substance. It was previously reported that THF is a highly effective and non-toxic solubilizing agent (24), even if one of its peroxide derivatives was detected (25). It has recently been shown that this solvent causes a reduction in cell proliferation over a 7 day period (15). In our experimental model, THF induced a marked reduction in the number of cells in S phase after 24 h, thus confirming the effect of this molecule on cell cycle progression. In contrast, DPPC liposomes have been found to be a reliable delivery system that does not interfere with cell proliferation.

Previous investigations have reported on BC uptake in different cell lines. The intracellular concentration attained in murine C3H/10T1/2 fibroblasts treated with a water-dispersible BC beadlet (10 μM) ranged between 1 and 2 nmol/10^6 cells over a 1 week incubation period (26). On the other hand, in
BALB/c 3T3 cells, BC achieved an intracellular concentration of 0.07 and 0.58 nmol/10^6 cells after 36 h treatment with beadlet BC preparations of 0.3 and 3 μM respectively (27). In the present study, the intracellular concentration of BC between 0.23 and 1.2 nmol/10^6 cells, quantified through HPLC, was within the range reported by the above studies. In human lung fibroblasts, an intracellular concentration of ~0.09 nmol/10^6 cells was obtained after 24 h treatment with BC (2 nM) dissolved in THF/DMSO (1:1) (28). Recently, an uptake of 0.004 nmol/10^6 cells after 5 days treatment was found in human mammary epithelial cells treated with BC (7 μM) dissolved in THF (15).

In vivo BC concentrations in cells, tissues and biological fluids were found to be extremely variable and dependent on diet, smoking and other parameters (1). However, it is of interest that levels of BC comparable with those reported here were found in exfoliated cells of the oral mucosa after dietary supplementation (29).

On the basis of both epidemiological data and experimental studies, it has been proposed that BC inhibits in vivo neoplastic cell proliferation and subsequent tumor formation (1,30). Several reports have dealt with the effect of BC on cell proliferation of tumor cells line: BC was found to suppress the proliferation of various human malignant tumor cell lines such as PANC-1 (pancreatic cancer), A-172 (glioblastoma), HGC-27 (gastric cancer), A-375 (malignant melanoma), SCC-25 (squamous cell carcinoma), SQ-38 (oral carcinoma), GOTO (neuroblastoma) (14), MCF-7 (breast carcinoma), SK-MS and NCI-H226 (lung carcinoma) and Ishikawa (endometrial cancer) (33). Only a few studies address the interest about cell growth inhibition by BC in normal cells. In normal human keratinocytes (31) and in human mammary epithelial cells (15), no inhibitory effect on cell growth was found over a period of time not exceeding 1 week. However, in the latter cell line, a dramatic effect on cell proliferation was observed after 10 days BC treatment (15). In all these studies the effect on cell proliferation was assessed by measuring total [3H]thymidine incorporation or by cell counting. In the present investigation, it has been found that BC does not affect the rate of DNA synthesis, given that the levels of PCNA bound to DNA replication sites per cell as well as the amount of BrdU incorporated were similar to those of control (12,31,32).

In the present study, the accumulation of cells in G1 phase, as assessed by BrdU incorporation or by cell counting, was found over a period of time not exceeding 1 week. However, in the latter cell line, a dramatic effect on cell proliferation was observed after 10 days BC treatment (15). In all these studies the effect on cell proliferation was assessed by measuring total [3H]thymidine incorporation or by cell counting. In the present investigation, it has been found that BC does not affect the rate of DNA synthesis, given that the levels of PCNA bound to DNA replication sites per cell as well as the amount of BrdU incorporated were similar to those of control cells. Thus, the reduction in the number of cells in S phase of human fibroblasts appears to be a consequence of the accumulation of cells in G1 phase. This evidence is in agreement with the results obtained by Schwartz, who reported a similar accumulation in G1 phase of malignant tumor cells line (MCF-7 or A375) after BC treatment. In contrast, in SCC-25 tumor cells an accumulation in G2+M phase was shown (12).

In our experiments, the accumulation of cells in G1 phase persisted throughout the incubation period, suggesting that a slowing down of growth rate was responsible for the reduction
Fig. 4. Effect of BC (21 μM final extracellular concentration) delivered from DPPC liposomes on the cell cycle progression of exponentially growing human fibroblasts after 72 h BC exposure. Two parameter dot plots of PCNA immunofluorescence (upper panels) or BrdU incorporation (lower panels) versus DNA content are shown.

Table I. Cell cycle analysis of human fibroblasts incubated for up to 72 h with BC-containing DPPC liposomes and after BC removal

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Percent cells in S phase (mean ± SD)</th>
<th>P (t-test)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>17.44 ± 2.4</td>
<td>8.76 ± 3.3</td>
</tr>
<tr>
<td>48</td>
<td>17.72 ± 4.9</td>
<td>9.96 ± 2.6</td>
</tr>
<tr>
<td>72</td>
<td>16.64 ± 4.1</td>
<td>7.35 ± 3.7</td>
</tr>
<tr>
<td>After BC removal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>10.2 ± 2.5</td>
<td>14.2 ± 3.1</td>
</tr>
<tr>
<td>24</td>
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Mean values ± SD of the percentage of cells in S phase of the cell cycle were obtained from three independent experiments. Statistical analyses were evaluated by the Student’s t-test.

In the number of cells observed after 3 days treatment. Since an effective G_{0} arrest was never observed, these results suggest that BC induced a growth delay in G_{1} phase. Interestingly, this delay was reversible, since cell cycle progression was restored at 24 h following the removal of BC from the medium.

While the antitumor activity of retinoids is unequivocal, there is considerable debate as to whether BC has an intrinsic activity or if it requires conversion to vitamin A. In our cell system, analysis of cellular levels of retinoids by HPLC failed to demonstrate the presence of retinal, retinol, retinoic acid or retinyl esters during a period of 6 days incubation. These negative results demonstrate that the delay induced by BC on cell cycle progression of human fibroblasts is independent of its conversion to known retinoids.

Contrasting results were previously reported on the biotransformation of BC to retinoids in cell culture. No metabolites were found in C3H10T1/2 mouse fibroblasts up to 7 days incubation (26); in contrast, Quick and Ong (34) showed that Caco-2 cells derived from an adenocarcinoma of the human colon were capable of metabolizing BC to retinoids. Furthermore, biotransformation of BC to retinol was also observed in BALB/c 3T3 cells after 3 days BC treatment (27) and in human fibroblasts (WI-38 and embryonic human lung fibroblasts HLF).
which BC influences the cell cycle of human fibroblasts is available. Further studies on the cellular levels of cyclins and the activity of cyclin-dependent kinases will be useful to elucidate the molecular effect of BC on the cell cycle.

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References


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