Induction of cell cycle arrest and apoptosis in human colon adenocarcinoma cell lines by β-carotene through down-regulation of cyclin A and Bcl-2 family proteins

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Although the pharmacological role of β-carotene in the prevention and treatment of colon cancer has received increasing attention, little is known about the molecular mechanisms of action of this carotenoid. The present study demonstrates that β-carotene, a natural pigment widely present in fruit and vegetables, inhibits the growth of several human colon adenocarcinoma cell lines (COLO 320 HSR, LS-174, HT-29 and WiDr) by inducing cell cycle arrest in G2/M phase and apoptosis. These effects were dose and time dependent and strictly related to cell ability to accumulate the carotenoid. COLO 320 HSR cells incorporated β-carotene to a greater extent than LS-174, HT-29 and WiDr cells and, concomitantly, they exhibited a higher sensitivity to the growth inhibitory effects of the carotenoid. At inhibitory concentrations β-carotene reduced the expression of cyclin A, a key regulator of G2/M progression. Neither p21 nor p27, two cyclin kinase inhibitors, were significantly modified by carotenoid treatment. With respect to apoptosis induction, decreased levels of the apoptosis blocking proteins Bcl-2 and Bcl-xL were also observed. On the other hand, no changes in expression of the apoptosis promoter protein Bax were detected. This study represents a novel aspect of the biological profile of β-carotene and a new step in elucidating the underlying molecular mechanisms of its antitumor action. In addition, since cell growth inhibitory effects were reached at β-carotene concentrations achievable in vivo following its supplementation, this study provides a rational approach for the use of β-carotene in colon cancer.

Introduction

Colon cancer is one of the commonest causes of cancer mortality among Western populations (1) and its occurrence is commonly ascribed to the transformation of normal colon epithelium to adenomatous polyps and ultimately invasive cancer. According to the model proposed by Fearon and Vogelstein (2), cancer develops as a consequence of genetic alterations which accumulate over one or two decades. Experimental and epidemiological data have linked dietary composition with colorectal carcinogenesis. In particular, evidence from recent epidemiological studies has shown that a high dietary intake of fruit and vegetables, rich in β-carotene and other carotenoids, is associated with a low risk for colon neoplasia (3–6). Although some human intervention trials

failed to demonstrate prevention of colon cancer by β-carotene supplements (7–9), an extensive intervention study in China showed a significant protective effect of β-carotene in combination with vitamin E and selenium on gastrointestinal cancer in a population at high risk (10). Moreover, β-carotene supplementation reduced the rate of colon cell proliferation in patients with adenomatous polyps (11). Interestingly, the carotenoid has been reported to accumulate in colonic neoplastic tissues in humans (12,13) and several experimental studies suggest that it can be used to enhance cytotoxicity of chemotherapeutics (for a review see 14). Concomitantly, several reports have shown protective effects of β-carotene against colon carcinogenesis in animal models (15–18) and growth inhibitory effects by carotenoids were also observed in human colon cancer cell lines (19–21).

The mechanism by which β-carotene may protect from colon cancer is poorly understood. We recently reported that β-carotene was able to inhibit the growth of a human colon adenocarcinoma cell line through a mechanism involving apoptosis induction (22). In the same cell line we also demonstrated that this effect was independent of pro-vitamin A activity of the carotenoid, since the 4,4’-diketo-β-carotene canthaxanthin, which is not a precursor of vitamin A, also acted as a potent growth inhibitory agent (20). However, such experiments were conducted in a single cell line and at carotenoid concentrations which exceed normal dietary intake. On the other hand, it has been suggested that carotenoids may reduce cell growth by affecting cell cycle progression in some cell lines, including GOTO (23), HL-60 (24), SCC-25 (25) and mammary cancer cells (25,26). However, no data are available on the effects of β-carotene on cell cycle distribution of colon cancer cells.

In this study four different lines of human colon adenocarcinoma cells, growing in suspension (COLO 320 HSR) or as a monolayer (LS-174, HT-29 and WiDr), were exposed to varying β-carotene concentrations. We investigated whether: (i) the growth inhibitory effects of the carotenoid are due to changes in cell cycle progression and/or apoptosis; (ii) β-carotene modifies molecular pathways involved in the regulation of cell growth. In particular, we evaluated the expression of cyclin kinase inhibitors (p21 and p27), cyclins (cyclin A) and apoptosis-related proteins (Bcl-2, Bcl-xL and Bax).

In this study we have demonstrated that the mechanism of β-carotene inhibition of colon cancer cell growth involves both interference with cell cycle progression and apoptosis. Moreover, we show for the first time that the carotenoid, at concentrations achievable in vivo, may modulate expression of cell cycle and apoptosis regulators.

Materials and methods

Cell culture

WiDr (kindly provided by Dr S.D.Showalter, NCI, Frederick, MD), LS-174 (CCL 220.1; American Type Culture Collection, Rockville, MD) and COLO

Abbreviations: PBS, phosphate-buffered saline; PI, propidium iodide.
320 HSR (CL 188; American Type Culture Collection) human adenocarcinoma cancer cell lines were cultured in RPMI 1640 medium (Gibco Biocult, Paisley, UK) without antibiotics, supplemented with 10% fetal calf serum (Flow Laboratories, Irvine, UK) and 2 mM glutamine. The HT-29 adenocarcinoma cell line (HTB 38; American Type Culture Collection) was cultured in MEM medium (Gibco) without antibiotics, supplemented with 10% heat-inactivated fetal calf serum (Gibco) and 2 mM glutamine. WiDr, LS-174 and HT-29 cells grew as a monolayer, while COLO 320 HSR cells grew in suspension. They were maintained in log phase by seeding twice a week at a density of 3 × 10^5 cells/ml. All cell lines grew at 37°C under 5% CO2, 95% air at 99% humidity. β-Carotene (Fluka Chemika-Biochemika, Buchs, Switzerland) was delivered to the cells using THF as solvent, containing 0.025% butylated hydroxyltoluene to avoid formation of peroxides. The stock solutions (2 and 20 mM) were prepared immediately before each experiment. From the stock solutions, aliquots of β-carotene were rapidly added to the culture medium to give the final concentrations indicated. Control cultures received an amount of THF equal to that present in β-carotene-treated ones. The amount of THF added to the cells was not greater than 0.5% (v/v). The medium was not replaced throughout the experiments. At the times indicated, cells were harvested by trypsinization and quadruplicate hemocytometer counts of triplicate cultures were performed. The trypan blue dye exclusion method was used to evaluate the percentage of necrotic cells.

**Cell growth inhibition assay**

Curves of cell growth inhibition were determined for all four tumor cell lines. Serial dilutions of the carotenoid were used at 0, 1, 5, 10, 25 and 50 µM. Tumor cells were seeded in 24-well plates with 2 × 10^4 cells/well and divided into control and treatment groups. The control groups consisted of controls treated with the same volume of solvent and controls without any treatment. After 24 h, cells were removed from each well, stained with trypan blue and counted under a microscope for viable and dead cells. The results were then calculated by plotting cell growth inhibition as a function of carotenoid concentration.

**Extraction and analysis of β-carotene**

The carotenoid was extracted with 1 vol methanol and 3 vol hexane:diethyl ether (1:1) from 4 × 10^6 cells after 24 h treatment with 5 µM β-carotene and visualized by HPLC as described earlier (21).

**Cell cycle analysis**

Cell cycle stage was analyzed by flow cytometry. Aliquots of 10^6 cells were harvested by centrifugation, washed in phosphate-buffered saline (PBS), fixed with ice-cold 70% ethanol and treated with 1 mg/ml RNase for 30 min. Propidium iodide (PI) was added to a final concentration of 50 µg/ml. Data were collected, stored and analyzed using Flowcytometry Software (Minitab Inc., State College, PA).

**Apoptosis detection**

Apoptosis was detected with an annexin V–FITC kit (Oncoregene Research, Cambridge, MA) according to the manufacturer's instructions. Briefly, at the times indicated cells were collected, washed with ice-cold PBS and centrifuged; cells growing as a monolayer (LS-174, HT-29 and WiDr) included both those harvested by trypsinization and those attached to the culture plate. The cell pellet was resuspended in ice-cold binding buffer. After that, annexin V–FITC (1.25 µg/ml) and PI (10 µg/ml) solutions were added. The tube was incubated for 15 min in the dark before being analyzed by flow cytometry (Coulter Epics XL-MCL, 620 nm filter).

To demonstrate morphological features of apoptosis, we also stained cells with acridine orange and analyzed them by fluorescence microscopy.

**Immunohistochemical analysis of p21WAF-1/CIP-1, p27KIP-1, cyclins, Bcl-2, Bcl-xL and Bax expression**

Cytospins of colon cancer cells (50 × 10^3/slide) prepared with Cytospin 3 (Shandon, Cheshire, UK) were fixed with 4% paraformaldehyde in PBS for 10 min and permeabilized with cold (−20°C) methanol for 10 min. Cells were then washed with PBS and incubated for 1 h at room temperature with PBS containing one of the following monoclonal antibodies: anti-p21WAF-1/CIP-1 (clone F-5, catalog no. SC 6246; Santa Cruz Biotechnology, Santa Cruz, CA); anti-p27KIP-1 (clone F-8, catalog no. SC 1641; Santa Cruz Biotechnology); anti-cyclin A (clone 6E6; YLEM, Rome, Italy); anti-cyclin D1 (clone D1; Santa Cruz Biotechnology); and anti-Bax (clone B-19, catalog no. SC-526; Santa Cruz Biotechnology). Mouse IgG matched isotypes were used as negative controls. Endogenous biotin sites were blocked by sequential incubations with avidin–biotin solutions (Blocking Kit; Vector Laboratories, Burlingame, CA). Hydrogen peroxide; normal goat blocking serum; biotinylated Ig avidin–biotin complexes and DAB substrate solutions (ABC ELITE detection system; Vector Laboratories) were used according to the manufacturer’s instructions. For each slide, four randomly selected microscopic fields were observed and at least 100 cells per field were evaluated. The results were quantified as the percentage of positive cells with respect to total cells.

**Results**

**Effect of β-carotene on cell growth**

Figure 1 shows the effect of varying β-carotene concentrations on cell growth of COLO 320 HSR colon adenocarcinoma cells. The cells were incubated with the carotenoid for 72 h. The values are the means ± SEM of five different experiments.

![Graph showing cell growth inhibition induced by varying β-carotene concentrations.](image-url)

**Fig. 1. Effect of varying β-carotene concentration on cell growth of human COLO 320 HSR colon adenocarcinoma cells. The cells were incubated with the carotenoid for 72 h. The values are the means ± SEM of five different experiments.**

Three separate cultures per treatment were used for analysis in each experiment. Data were analyzed using one-way analysis of variance (ANOVA). When significant values were found (P < 0.05), post hoc comparisons of means were made using Fisher’s test. Differences were analyzed using Minitab Software (Minitab Inc., State College, PA).

![Graph showing cell growth inhibition induced by varying β-carotene concentrations.](image-url)

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**Results**

**Effect of β-carotene on cell growth**

Figure 1 shows the effect of varying β-carotene concentrations on cell growth of COLO 320 HSR cells. The cells were incubated with the carotenoid for 72 h. β-Carotene addition caused growth inhibition when compared with controls. This inhibition was dose and time dependent. It was significant (P < 0.05) after 24 h incubation at a concentration of 1 µM carotenoid. On the other hand, no significant differences in cell number were found between untreated and vehicle controls (data not shown), suggesting that THF at a concentration up to 0.5% did not modify cell growth. Among the various colon cancer cells, COLO 320 HSR cells seemed to be the most susceptible to β-carotene treatment, followed by LS-174, HT-29 and WiDr cells in that order (Figure 2). The calculated ID_{25} and ID_{50} values for COLO 320 HSR cells were 2 and 8 µM, respectively. The ID_{25} values for LS-174, HT-29 and WiDr cells were 5, 11 and 36 µM, respectively. To verify whether the different sensitivities to β-carotene of the various colon cancer cells is due to changes in cell ability to incorporate the carotenoid, we measured β-carotene accumulation in the cells. Figure 3 shows accumulation of the carotenoid in the various cell lines treated with 5 µM β-carotene for 24 h. Such a time was the minimum required to obtain maximum accumulation of the carotenoid in the four cell lines (data not shown). In accord with the effects on cell growth, COLO 320 HSR cells exhibited the highest β-carotene accumulation, followed by LS-174, HT-29 and WiDr cells. Such different cell abilities to incorporate the carotenoid was observed at all concentrations tested (<50 µM). Interestingly, β-carotene up to 50 µM did not induce cell necrosis, measured as trypan blue dye exclusion (data not shown).
Effects of β-carotene on cell cycle

To elucidate the mechanism(s) responsible for the reduction in cell number by β-carotene in colon cancer cells, we first examined whether such a reduction was associated with a cytostatic effect due to changes in cell cycle progression. Figure 4 shows the cell cycle distribution of COLO 320 HSR cells incubated in the absence (Figure 4A) or presence of 5 (Figure 4B) and 25 μM (Figure 4C) β-carotene for 24 h. In addition, Figure 4D summarizes the relative percentages of COLO 320 HSR cells in each phase of the cell cycle, following a 24 h treatment with varying β-carotene concentrations (0, 1, 5, 10 and 25 μM). In the absence of β-carotene, most COLO 320 HSR cells (~52%) were in S phase, due to the high proliferative state. However, in the presence of β-carotene we observed a net dose-dependent increase in the percentage of cells in G2/M, which was maintained throughout the treatment (72 h) (data not shown). The G2/M phase accumulation was accompanied by a corresponding reduction in the percentages of cells in S phase. In addition, the presence of a distinct sub-G1 peak (subdiploid DNA content), suggestive of the presence of apoptotic cells, was found following β-carotene treatment. Such a peak increased in a dose-dependent manner. The various colon cancer cell lines treated with the carotenoid at concentrations corresponding to their respective ID25 values accumulated in G2/M phase of the cell cycle (Table I).

Effect of β-carotene on apoptosis

Apoptosis induction by the carotenoid was further studied by a double staining method using FITC-conjugated annexin V and PI. Annexin V, a calcium- and phospholipid-binding protein, binds preferentially to a negatively charged inner membrane phospholipid, phosphatidylserine, on exposure to the cell surface and detects early cellular apoptotic changes, whereas the normally impermeable vital dye PI detects cells undergoing necrotic changes. Figure 5 shows representative cytograms relative to apoptosis of COLO 320 HSR cells incubated in the absence (Figure 5A) or presence of 5 (Figure 5B) and 25 μM (Figure 5C) β-carotene for 24 h, as measured by this method. β-Carotene treatment increased the number of early and late apoptotic cells (annexin-positive cells) in quadrants 4 and 2, respectively, but it did not affect the percentage of necrotic cells (PI-positive cells, quadrant 1), which was ~2% in both untreated and β-carotene-treated cells. Figure 5D summarizes the percentage of apoptotic cells following a 24 h treatment with varying β-carotene concentrations (0, 1, 5, 10 and 25 μM). Evidence for apoptosis induction by β-carotene in the other cell lines is reported in Table II. The carotenoid was added to the cells at the respective ID25 values for 24 h and apoptosis was evaluated by the annexin V−FITC method.

Moreover, β-carotene also induced morphological changes associated with apoptosis, such as apoptotic bodies, cell shrinkage, condensation of cytoplasm and/or nuclear chromatin, as shown by acridine orange staining.

Effect of β-carotene on proteins involved in cell cycle progression and apoptosis

Since COLO 320 HSR cells exhibited the highest sensitivity to the effects of β-carotene on cell cycle progression and apoptosis, we investigated possible mechanisms by which the carotenoid would interfere with these processes in this cell line. We evaluated the cellular content of G2/M phase-related cyclin A and of the cyclin kinase inhibitors p21 and p27. Figure 6 shows the effects of varying concentrations of β-carotene (0, 1, 5, 10 and 25 μM) on the levels of this cyclin in COLO 320 HSR cells incubated for 24 h. The carotenoid induced a dose-dependent reduction in cyclin A-positive cells, as compared with the respective untreated controls. This finding was consistent with blockage of the cell cycle in G2/M phase. In contrast, expression of p21 and p27 was not modified by the carotenoid up to 25 μM for 24 h (data not shown).

In an attempt to explore the effects of β-carotene on apoptosis regulating proteins, we examined expression of Bcl-2 and Bcl-xL, which suppress programmed cell death, and that of Bax, which appears to promote it. Figure 7 shows the results of immunohistochemical analysis of Bcl-2 (Figure 7A) and Bcl-xL (Figure 7B) expression in COLO 320 HSR cells.
Fig. 4. Distribution of cell cycle phases of COLO 320 HSR cells after treatment with β-carotene. Representative histograms of DNA content in cells incubated for 24 h in the absence (A) or presence of 5 (B) and 25 µM (C) β-carotene. (D) Percentage of cells in the G0/G1, S and G2/M phases after a 24 h incubation with varying β-carotene concentrations (0, 1, 5, 10 and 25 µM). The values are the means ± SEM of three different experiments. Within the same phase, the values with different letters are significantly different from each other (P < 0.05).

Table I. Effect of a 24 h β-carotene treatment on cell cycle distribution of different human colon adenocarcinoma cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>G0/G1 (%)</th>
<th>β-Carotene&lt;sup&gt;a&lt;/sup&gt;</th>
<th>S (%)</th>
<th>β-Carotene&lt;sup&gt;a&lt;/sup&gt;</th>
<th>G2/M (%)</th>
<th>β-Carotene&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td>COLO 320</td>
<td>47.9 ± 4.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>48.5 ± 4.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>51.6 ± 3.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>40.0 ± 4.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.5 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.5 ± 1.4&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>LS-174</td>
<td>65.1 ± 6.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>64.0 ± 7.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>28.0 ± 2.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18.0 ± 1.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.9 ± 1.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18.0 ± 2.0&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>HT-29</td>
<td>68.0 ± 6.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>65.0 ± 6.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>30.0 ± 3.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23.0 ± 2.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.0 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.0 ± 1.4&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>WiDr</td>
<td>67.2 ± 6.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>66.5 ± 6.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>32.2 ± 3.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>22.5 ± 2.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.6 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.0 ± 1.1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>β-Carotene was added at the respective ID<sub>25</sub> (see text).
The values are the means ± SEM of three experiments. Within the same cell cycle phase, values not sharing the same superscript were significantly different from their respective controls at P < 0.05.

incubated with varying β-carotene concentrations (0, 1, 5, 10 and 25 µM) for 24 h. Treatment with the carotenoid significantly reduced the percentage of Bcl-2- and Bcl-xL-positive cells in a dose-dependent manner. Interestingly, cells with apoptotic bodies were always Bcl-2-negative. The reduction in the percentage of both Bcl-2- and Bcl-xL-positive cells was consistent with the pro-apoptotic effect of β-carotene observed in these cells. In contrast, no significant changes in the percentage of Bax-positive cells were found following a 24 h β-carotene treatment. Figure 8 shows micrographs from the
β-Carotene and growth of colon cancer cells

Fig. 5. Flow cytometric analysis of apoptosis induction by β-carotene in COLO 320 HSR cells, measured by the annexin V–FITC and PI methods. Representative cytograms of apoptosis in COLO 320 HSR cells incubated for 24 h in the absence (A) or presence of 5 (B) and 25 µM (C) β-carotene. Within a cytogram, quadrants 2 and 4 represent late and early apoptotic cells, respectively; quadrant 1, necrotic cells; quadrant 3, viable cells. (D) Percentage of apoptotic cells after a 24 h incubation with varying β-carotene concentrations (0, 1, 5, 10 and 25 µM). The values are the means ± SEM of three different experiments. The values with different letters are significantly different from each other (P < 0.05).

Table II. Effect of a 24 h β-carotene treatment on apoptosis induction in different human colon adenocarcinoma cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Apoptosis (%)</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>COLO 320</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>LS-174</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>HT-29</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>WiDr</td>
<td>3.0 ± 0.2</td>
</tr>
</tbody>
</table>

The values are the means ± SEM of three experiments.

aβ-Carotene was added at the respective ID25 (see text).
bValues significantly different from their respective controls at P < 0.05.

Discussion

The present study provides evidence that β-carotene may act as a potent growth inhibitory compound in human colon adenocarcinoma cells and supports the possibility of a chemopreventive or chemotherapeutic potential for this carotenoid in colon cancer. The effectiveness of β-carotene in inhibiting cell growth has been established in several tumor cells, including colon (19), melanoma (27), prostate (28), oral, lung and breast (29) cancer cells. However, our study clearly shows that colon adenocarcinoma cells differed significantly in their sensitivity to β-carotene. COLO 320 HSR cells exhibited an ID25 much lower than those calculated for LS-174, HT-29 and WiDr cells. The different sensitivities of these cells to β-carotene is presumably due to their different abilities to incorporate the carotenoid. According to this hypothesis, a strict relationship between cell β-carotene accumulation and arrest of cell growth was found. COLO 320 HSR cells accumulated β-carotene to a greater extent and were more sensitive to the growth inhibitory effects of the carotenoid as compared with LS-174, HT-29 and WiDr cells. Differences in β-carotene uptake among cell lines have been previously

immunohistochemical analysis of cyclin A (Figure 8A and B), Bcl-2 (Figure 8C and D) and Bcl-xL (Figure 8E and F) in the absence (Figure 8A, C and E) or presence of 5 µM β-carotene (Figure 8B, D and F) in COLO 320 HSR cells treated with the carotenoid for 24 h.
Fig. 6. Percentage of cyclin A-positive COLO 320 HSR cells after a 24 h incubation with varying β-carotene concentrations (0, 1, 5, 10 and 25 µM). The values are the means ± SEM of three different experiments. The values with different letters are significantly different from each other (P < 0.05).

reported (21). It is possible that cell growth modality (as a monolayer or in suspension) as well as cell metabolic status and membrane composition influence β-carotene accumulation. Recently, it has been suggested that β-carotene uptake into cells may be dependent on two different mechanisms: one involving passive transport of the carotenoid across the cell membrane and the other requiring metabolically driven endocytosis (30). In this study, it is noteworthy that cell growth inhibitory effects occurred at concentrations of β-carotene which were in the range or were lower than those found in serum of human subjects supplemented with the carotenoid. Nierenberg and co-workers reported that supplementation with 50 mg/day β-carotene in humans resulted in plasma β-carotene concentrations of up to 16.1 µM (31). In another study, Prince and Frisoli demonstrated that human subjects ingesting various doses of β-carotene as supplements (51 mg/day to 102 mg/three times a day) could average serum steady-state concentrations of the carotenoid in the range ~2 to ~13.2 µM (32). The ID_{25} and ID_{50} values reported here for COLO 320 HSR cells were in this range. Therefore, this study provides a rational approach for the in vivo use of β-carotene.

We also report that the mechanism of β-carotene inhibition of colon cancer cell growth involves interference in both cell cycle progression and apoptosis. The carotenoid was able to induce cell cycle arrest in G2/M phase and apoptotic death in a dose- and time-dependent manner in the human colon cancer cell lines tested. Necrotic or lytic effects were excluded by the lack of plasma membrane permeability as assessed by the absence of PI uptake.

Apoptosis is a crucial element in the behavior of mammalian cells in many different situations (33). Programmed cell death is characterized by particular morphological features (34–37). In cells treated with effective concentrations of β-carotene we have clearly observed many of the typical structural modifications that happen during apoptosis, which include translocation of phosphatidylserine to the outer layer of the plasma membrane, apoptotic bodies, cell shrinkage and condensation of the cytoplasm and/or nuclear chromatin. It is interesting that the induction of apoptosis by β-carotene in our model can be independent of the presence of wild-type p53, the tumor suppressor gene product which acts as a transcription factor. In this study, β-carotene was shown to induce apoptosis in COLO 320 HSR, HT-29 and WiDr cells expressing a mutant p53, as well as in LS-174 cells containing wild-type p53 (38,39). The observation that the growth inhibitory effects of β-carotene are not apparently related to p53 expression is extremely important from a therapeutic point of view as many anticancer agents, such as doxorubicin, etoposide and 5-fluorouracil, induce apoptosis through a
of cells in G2/M phase. This is also shown by the decreased expression of cyclin A, a protein known to regulate cdc2
kinase activity in G2/M phase (43). On the other hand, in our
model cdk inhibitors (p21 and p27) are not implicated in
negative regulation of cell cycle progression by β-carotene.
In this study we have demonstrated that the mechanism of
β-carotene inhibition of colon cancer cell growth involves
interference with both cell cycle progression and apoptosis.
Moreover, we report for the first time that the carotenoid, at
concentrations achievable in vivo, may modulate expression
of regulators of the cell cycle and apoptosis.

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