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REGULATION OF CELL CYCLE PROGRESSION AND APOPTOSIS BY β-CAROTENE IN UNDIFFERENTIATED AND DIFFERENTIATED HL-60 LEUKEMIA CELLS: POSSIBLE INVOLVEMENT OF A REDOX MECHANISM

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Although epidemiologic studies have demonstrated that a high intake of vegetables containing β-carotene lowers the risk of cancer, recent intervention studies have revealed that β-carotene supplementation to smokers resulted in a high incidence of lung cancer. We hypothesized that β-carotene may act as a pro- or anticancerogenic agent by modulating pathways involved in cell growth and that such a modulation may involve a redox mechanism. To test this hypothesis, cell proliferation, apoptosis and redox status were evaluated in undifferentiated and dimethylsulfoxide-differentiated HL-60 cells exposed to β-carotene. The carotenoid modified cell cycle progression and induced apoptosis in a dose-dependent manner. These effects were more remarkable in undifferentiated cells than in differentiated cells. In accord with these findings, in undifferentiated cells, β-carotene was more effective in decreasing cyclin A and Bcl-2 expression and in increasing p21 and p27 expression. Neither Bcl-xL nor Bax expression were significantly modified by the carotenoid. From a mechanistic point of view, the delay in cell growth by β-carotene was highly coincident with the increased intracellular reactive oxygen species production and oxidized glutathione content induced by the carotenoid. Moreover, α-tocopherol minimized the effects of β-carotene on cell growth. These data provide evidence that β-carotene modulates molecular pathways involved in cell cycle progression and apoptosis and support the hypothesis that a redox mechanism may be implicated. They also suggest that differentiated cells may be less susceptible to the carotenoid than highly neoplastic undifferentiated cells.
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Key words: *β-carotene; cell cycle progression; apoptosis; intracellular redox potential; HL-60 cells*

Considerable attention is currently being given to carotenoids as potential chemopreventive agents. Epidemiologic studies have demonstrated that individuals who eat more fruits and vegetables rich in carotenoids and/or who have higher levels of serum β-carotene show a lower risk of cancer.[1] On the other hand, there is some contradictory evidence from recent human intervention studies using β-carotene supplements. An increase in the risk of lung cancer among smokers who took β-carotene supplements was reported in the Alpha-Tocopherol, β-Carotene Cancer Prevention Study[2] and among smokers and asbestos workers in the Beta-Carotene and Retinol Efficacy Trial,[3] but not among male physicians in the United States in the Physicians' Health Study.[4]

These contradictory findings have focused interest on elucidating the mechanisms by which β-carotene may modify tumor incidence and development.[5] It is known that β-carotene is converted to retinoids (vitamin A and its analogs) in humans, and the hormone-like effects of these compounds may exert potent influences on cell differentiation, proliferation and development.[6] β-Carotene also acts as an immunomodulatory agent, increasing immune surveillance in carcinogenesis.[7] Moreover, it may enhance gap junction communication, restricting clonal expansion of initiated cells. It may also influence carcinogenesis by interfering with the metabolic pathways involved in the metabolization of chemical carcinogens.[8]

On the other hand, it has recently been reported that β-carotene may act as a promoter of carcinogenesis through its metabolization to biologically active products, such as apocarotenoids.[9] In ferrets exposed to tobacco smoke, these metabolites can interfere with the expression of transcription factors (RAR and AP-1) regulating cell growth. Another possibility is that β-carotene may modulate molecular pathways involved in cell growth and/or programmed cell death by acting as a redox agent. It could alternatively behave as an antioxidant or as a prooxidant molecule, depending on its redox potential and on the cellular environment.[10–13] In accord with this hypothesis, we recently reported that β-carotene at high concentrations effectively acted as a proapoptotic agent in an adenocarcinoma cell line and that this effect was accompanied by an increased production of intracellular reactive oxygen species (ROS).[14] However, the involvement of a redox mechanism in the growth-inhibitory effects of β-carotene remains to be evaluated in other tumor cell lines at different degrees of differentiation and in the respective normal counterparts.

HL-60 leukemia cells have been extensively used as a model system because of their ability to differentiate by DMSO or by retinoic acid into morphologically mature myeloid cells, possessing properties similar to those found in normal neutrophils.[15,16] This differentiation concomitantly inhibits cell proliferation and is usually followed by cell death via apoptosis. Therefore, HL-60 cells and their respective differentiated cells, having a different rate of growth and apoptosis, seem to be an appropriate model system to study the effect of β-carotene on cell redox status and its influence on molecular pathways involved in the regulation of cell growth and death.

In our study, undifferentiated and DMSO-differentiated HL-60 cells were exposed to various concentrations of β-carotene for 24 hr. Such a time was required to achieve the maximum β-carotene incorporation and to avoid possible carotenoid-induced differentiative effects. We investigated (*i*) whether molecular pathways regulating cell growth and/or apoptosis may be affected by the carotenoid; (*ii*) whether there is a relationship between modifications of intracellular redox status and changes in cell growth and/or death by β-carotene; and (*iii*) whether the differentiation process modifies the sensitivity to β-carotene.

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MATERIAL AND METHODS

Cell culture

The human promyelocytic leukemia HL-60 cell line was maintained in log phase by seeding twice a week at a density of 3×10^5 cells/ml. Cells were routinely cultured in RPMI-1640 medium (Sigma Italia, Milan, Italy) supplemented with 10% heat-inactivated FBS (Life Technologies Italia, Milan, Italy) at 37°C in 5% CO_2/air atmosphere. HL-60 cells were induced to differentiate to neutrophyl granulocytes by culturing the cells in a medium containing 1.3% dimethyl sulfoxide (DMSO; Sigma Italia) for 5 days.[15] The agent was subsequently removed and cell cultures utilized after 24 hr. The differentiated status was verified by morphologic inspection (smaller nucleus-to-cytoplasm ratio and a more irregular shape) and by a respiratory-burst assay performed by measuring superoxide production after stimulation with phorbol 12-myristate 13-acetate (PMA; results not shown).[17] In our preparations, about 95% of the cells were considered to be fully differentiated. In contrast, no evidence of spontaneous terminal differentiation was observed in DMSO-untreated HL-60 cells, in agreement with the original description of Collins *et al.*[18]

β-carotene (Fluka Chemika-bioChemika, Buchs, Switzerland) and DL-α-tocopherol (Fluka Chemika-bioChemika) were delivered to the cells (10^6 cells/ml) using tetrahydrofuran (THF) as a solvent, containing 0.025% butylated hydroxytoluene (BHT) to avoid the formation of peroxides.[19] The purity of β-carotene and that of α-tocopherol was verified to be 97% and 98%, respectively. The stock solutions of β-carotene (2 and 20 mM) and those of α-tocopherol (10, 25 and 50 mM) were prepared immediately before each experiment. From the stock solutions, aliquots of β-carotene and/or α-tocopherol were rapidly added to the culture medium to give the final concentrations indicated. In both β-carotene and α-tocopherol experiments, the amount of THF added to the cells was not greater than 0.5% (v/v). Control cultures received an amount of THF equal to that present in β-carotene- and/or α-tocopherol-treated cultures. Since no differences were found between cells treated with different amounts of THF containing BHT and untreated cells in terms of cell number, viability, cell cycle progression, apoptosis or reactive oxygen species (ROS) production, "controls" refers to untreated cells. After the addition of β-carotene and/or α-tocopherol, the medium was not further replaced throughout the experiments.

Experiments were routinely carried out on triplicate cultures. At the times indicated, cells were harvested, and quadruplicate hemocytometer counts were performed. The trypan blue dye exclusion method was used to evaluate the percentage of viable cells.

Cell cycle analysis

The cell cycle was analyzed by flow cytometry, as described in ref. 20. Aliquots of 10^6 cells were harvested by centrifugation, washed in PBS, fixed with ice-cold 70% ethanol and treated with 1 mg/ml RNase for 30 min in the dark at room temperature. Propidium iodide was added to a final concentration of 50 μg/ml. The stained nuclei were analyzed on an Epics Profile flow cytometer (Coulter, Hialeah, FL) with an argon laser (Omnicrome 500, 15 mW, and excitation wavelength of 488 nm). From each sample, 20,000 cells were counted. Data were collected, stored and analyzed using the Multicycle software (Phoenix).

Apoptosis detection

Apoptosis was quantified by scoring the morphologic features of nuclear pyknosis and chromatin condensation on acridine orange-stained cells.[21] At least 300 cells for each condition were counted. Double blind examination was performed.

Immunohistochemical analysis of $p21^{WAF\text{-}1/CIP\text{-}2}$, $p27^{KIP\text{-}1}$, cyclin A, Bcl-2, Bcl-xL and Bax expression

Cytospins of undifferentiated and DMSO-differentiated HL-60 cells (50×10^3/slide) prepared with Shandon (Cheshire, UK) Cytospin 3 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 hr at room temperature with PBS containing anti-$p21^{WAF\text{-}1/CIP\text{-}2}$ [clone: (F-5) cat. no. SC 6246, Santa Cruz Biotechnology, Santa Cruz, CA], anti-p27 [clone: (F-8) cat. no. SC 1641, Santa Cruz Biotechnology], anti-cyclin A (clone: 6E6, YLEM, Rome, Italy), anti-Bcl-2 (clone: Bcl-2/100/D5, YLEM), anti-Bcl-xL S/L [(L-19): cat. no. SC-1041, Santa Cruz Biotechnology] or anti-Bax [(P-19): cat. no. SC-526, Santa Cruz Biotechnology] monoclonal antibodies. Mouse preimmune Igs were used as negative control. Endogenous biotin sites were blocked by sequential incubations with avidin-biotin solutions (Blocking Kit; Vector, Burlingame, CA). Hydrogen peroxide, normal goat blocking serum, biotinylated Igs, avidin-biotin complexes and diaminobenzidine (DAB) substrate solutions (ABC ELITE detection system; Vector) were used according to the manufacturer's instructions. For each slide, 4 randomly selected microscopic fields were observed and at least 100 cells/field were evaluated.

Extraction and analysis of β-carotene

The carotenoid was extracted from 4×10^6 cells and analyzed by high-performance liquid chromatography (HPLC), as described earlier.[21]

Measurement of ROS

Cells (10^6 cells/ml) treated with varying β-carotene concentrations for 24 hr were harvested to evaluate cellular peroxides and hydroxyl radical levels using the di(acetoxymethyl ester) analog (C-2938) of 6-carboxy-2′,7′-CF or DHR (D-632) (Molecular Probes, Eugene, OR) as described.[23] Before the addition of the fluorescent probes, media were removed to eliminate the amount of β-carotene not cell-associated. Fluorescent units were measured in each well after a 30-min incubation with DCF (10 μM) or DHR (5 μM) by use of a Cytofluor 2300/2350 Fluorescence Measurement System (Millipore, Bedford, MA). β-Carotene alone did not alter the basal fluorescence of DHR or DCF.

Glutathione assay

For the analysis of total and oxidized glutathione, 10×10^6 cells were homogenized in ice with $HClO_4$ (1 M)/EDTA (2 mM) and centrifuged at 1,000*g* for 10 min at 4°C. The supernatant, neutralized with NaOH, was extracted and analyzed by HPLC as indicated.[24] Chromatography was carried out with an LC-18 Supelcosil column, 25 × 0.46 cm, 5 μm particle size (Supelco, Bellefonte, PA). A C-18 Supelcosil precolumn, 2 × 0.46 cm, 5 μm packing, was used. The mobil phase was 7.5% methanol/92.5% acetate buffer (0.15 M, pH 7.00). The flow rate was 1.5 ml/min. Peaks were detected by fluorescence measurement at 420 nm after excitation at 340 nm. Reduced glutathione (GSH) and oxidized glutatione (GSSG) concentrations in samples were derived comparing the derivative peak area with a standard curve generated by derivatizing known amounts of GSH.

Statistical analysis

Three separate cultures per treatment were utilized for analysis in each experiment. Data were analyzed using 1-way analysis of variance (ANOVA). When significant values were found ($p < 0.05$), post hoc comparisons of means were made using the Fisher's test. Differences were analyzed using Minitab Software (Minitab, State College, PA).

RESULTS

Effect of β-carotene on cell number and viability

β-Carotene, administered at micromolar concentrations, reduced cell number of both undifferentiated and DMSO-differentiated HL-60 in a dose-dependent manner, as measured by the percentage of growth with respect to control (Fig. 1*a*). This inhibition was exhibited after 24 hr of incubation, although it was already observed after 12 hr, and was maintained for at least 48 hr (data not shown). Within this time, β-carotene treatment did not induce differentiation of normal HL-60 cells, analyzed by mor-

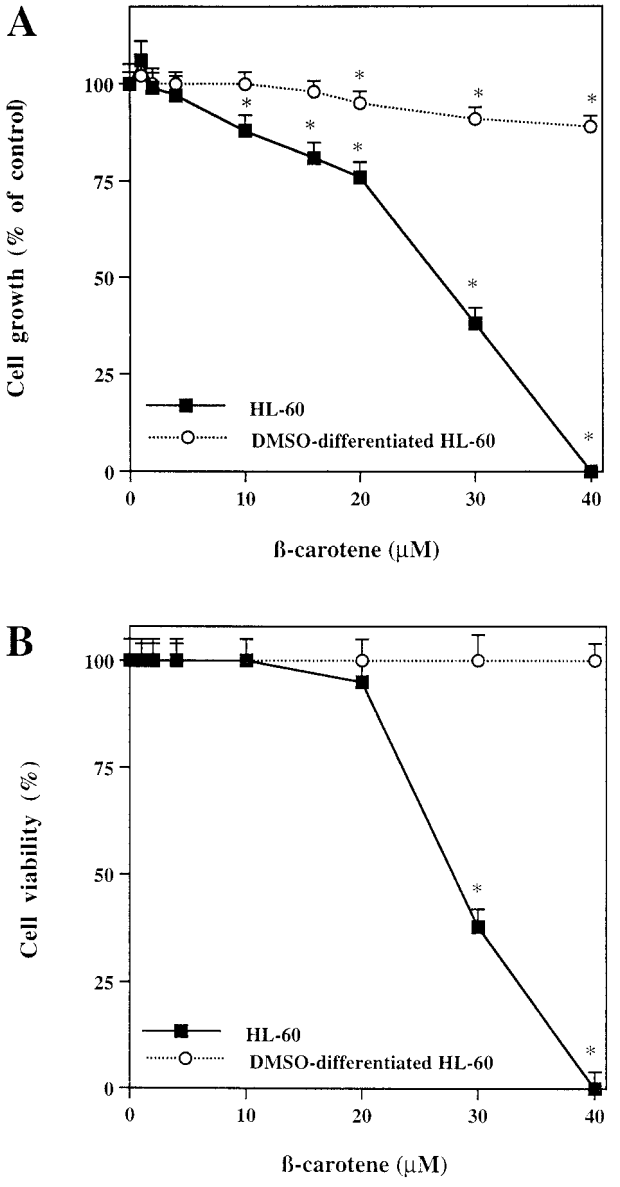

FIGURE 1 – Effect of varying β-carotene concentrations on cell growth (*a*) and viability (*b*) of undifferentiated and DMSO-differentiated HL-60 cells. The cells were incubated with the carotenoid for 24 h. After this time, in the absence of the carotenoid, the number of undifferentiated cells was $(1.455 \pm 0.050) \times 10^6$ and that of DMSO-differentiated cells was $(0.990 \pm 0.030) \times 10^6$. Values are the means ± SEM of 3 different experiments. The values with asterisks were significantly different from the respective controls at $p < 0.05$.

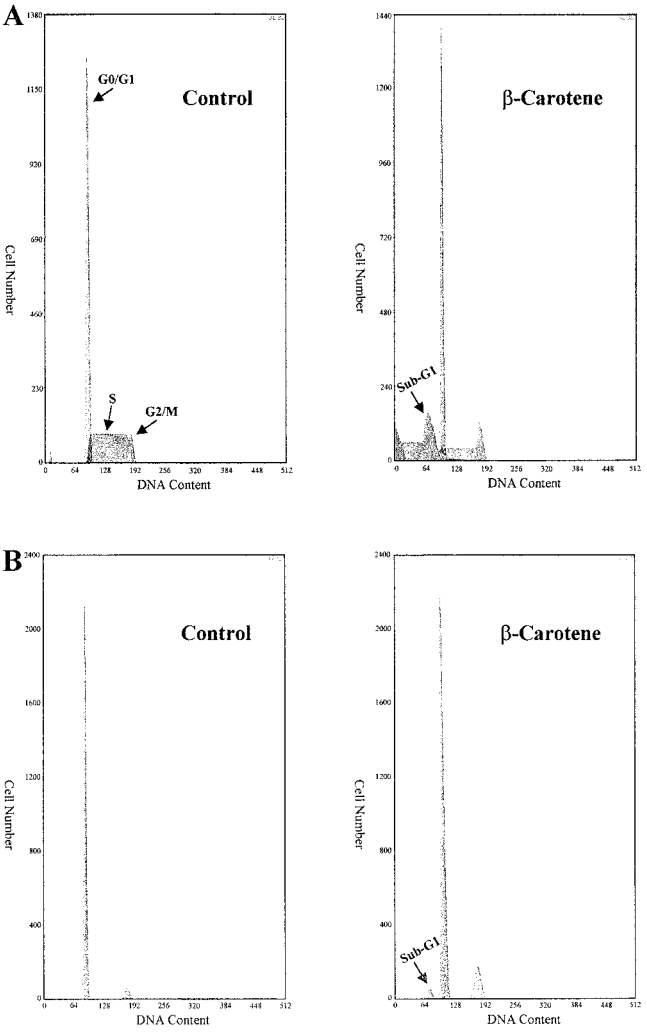

FIGURE 2 – Distribution of cell cycle phases after a 24 hr treatment with 20 μM β-carotene in undifferentiated (*a*) and DMSO-differentiated (*b*) HL-60 cells.

phologic inspection and by a respiratory-burst assay (data not shown). Normal HL-60 cells were extremely sensitive to β-carotene [inhibitory dose at 50% (ID_{50}) = 27 μM], and their growth was significantly inhibited at the concentration of 10 μM. On the other hand, DMSO-treated HL-60 cells were more resistant to the carotenoid ($ID_{50} > 40$ μM) and a significant reduction in cell number was observed at 20 μM of β-carotene.

The different cell sensitivity to β-carotene after DMSO differentiation was also observed by measuring cell viability using the trypan blue dye exclusion method (Fig. 1*b*). The carotenoid profoundly reduced the viability of undifferentiated HL-60 cells at concentrations higher than 20 μM. Such an effect was not observed in DMSO-differentiated cells, which were resistant even at very high concentrations of the carotenoid (40 μM). It is noteworthy that in undifferentiated HL-60 cells, β-carotene reduced the cell number at concentrations lower than those responsible for the loss of cell viability. This finding suggests that the carotenoid induced cytostatic effects at concentrations up to 20 μM and cytotoxic effects at higher concentrations. On the other hand, in differentiated HL-60 cells, β-carotene exerted only cytostatic effects at concentrations up to 40 μM. Moreover, it should be noted that the greater resistance of differentiated HL-60 cells to β-carotene was not due to DMSO itself, since comparable effects of the carotenoid on the reduction of cell number and cell viability were also observed when the differentiation of HL-60 cells was induced by a 3-day treatment with retinoic acid. After such treatment, a significant decrease in cell number was observed starting from 20 μM β-carotene (data not shown).

Effect of β-carotene on the cell cycle

To elucidate the mechanism(s) responsible for the reduction of cell number by β-carotene in differentiated and undifferentiated HL-60 cells, we first examined whether such a reduction was associated with changes in cell cycle progression. Figure 2 shows the distribution of cells in the cell cycle phases after 24 hr of β-carotene treatment with 20 μM β-carotene in undifferentiated (*a*) and DMSO-differentiated (*b*) HL-60 cells, and Table I shows

TABLE I – EFFECT OF VARYING β-CAROTENE CONCENTRATIONS ON CELL CYCLE PROGRESSION IN UNDIFFERENTIATED AND DMSO-DIFFERENTIATED HL-60 CELLS[1]

β-carotene (μM)	Phase (%)		
	G0/G1	S	G2/M
Undifferentiated HL-60 cells			
0	47.3 ± 4.0^2	46.2 ± 4.2^2	6.5 ± 0.4^2
2	47.0 ± 4.2^2	46.5 ± 4.4^2	6.5 ± 0.4^2
4	48.3 ± 4.3^2	45.3 ± 4.0^2	6.2 ± 0.5^2
10	53.8 ± 4.2^2	39.4 ± 4.0^2	6.8 ± 0.5^2
20	57.3 ± 4.4^3	24.3 ± 2.0^3	18.4 ± 0.9^3
DMSO-differentiated HL-60 cells			
0	94.4 ± 5.5^2	0.7 ± 0.1^2	4.9 ± 0.4^2
4	95.3 ± 4.8^2	0.7 ± 0.1^2	4.1 ± 0.4^2
10	95.0 ± 4.3^2	0.6 ± 0.1^2	4.4 ± 1.0^2
20	86.8 ± 4.2^3	1.6 ± 0.1^3	11.6 ± 1.0^3
40	86.3 ± 4.4^3	1.3 ± 0.1^3	12.4 ± 1.1^3

[1]The values were obtained by multicycle analysis of cells (without pre-G1 apoptotic cell peak) and are the means ± SEM of 3 different experiments. Within a column, the values with different superscripts (2, 3) were significantly different from their respective controls (0) ($p < 0.05$).

TABLE II – EFFECT OF VARYING β-CAROTENE CONCENTRATIONS ON APOPTOSIS INDUCTION IN UNDIFFERENTIATED AND DMSO-DIFFERENTIATED HL-60 CELLS

β-Carotene (μM)	Apoptosis (%)[1]	
	Undifferentiated	DMSO-differentiated
0	7.0 ± 0.9^2	3.0 ± 0.4^2
2	7.5 ± 0.8^2	2.0 ± 0.3^2
4	9.0 ± 0.9^2	3.0 ± 0.3^2
10	22.0 ± 2.3^3	3.5 ± 0.3^2
20	65.0 ± 6.0^4	10.0 ± 1.9^3
40	n.d.	28.0 ± 2.5^4

[1]A minimum of 300 cells/data point were counted. The values are the means ± SEM of 3 different experiments. Within a column, the values with different superscripts (2, 3, 4) were significantly different from their respective controls (0) ($p < 0.05$).

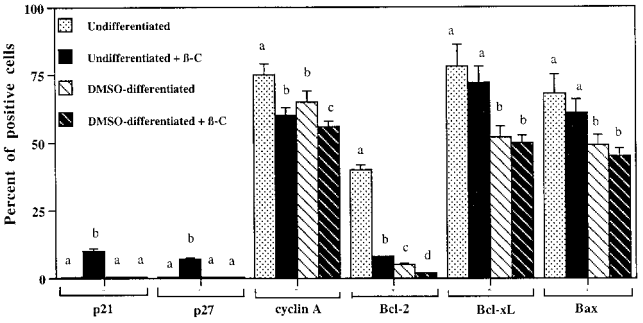

FIGURE 3 – Expression of $p21^{WAF-1/CIP-1}$, $p27^{KIP-1}$, cyclin A, Bcl-2, Bcl-xL and Bax in undifferentiated and DMSO-differentiated HL-60 cells treated with β-carotene (20 μM) for 24 hr. Protein expression was measured as the percentage of positive cells. Values are the means ± SEM of 3 different experiments. Within the same kind of analysis, the values with different letters were significantly different from each other ($p < 0.05$).

the percentages of cells in each phase of the cell cycle, after treatment with varying concentrations of the carotenoid. β-Carotene was added to cells in the range of concentrations that did not induce cell trypan blue staining during the first 24 hr of treatment. In the absence of β-carotene, most undifferentiated cells (about 46%) were in S phase, due to the high proliferative state, whereas most differentiated cells (about 94%) accumulated in G0/G1 phase, indicating that cell differentiation induced a drastic reduction in cell proliferation.

In the presence of β-carotene up to 10 μM, a progressive but not significant increase in the percentages of undifferentiated cells in the G0/G1 phase was observed. Such an increase became significant at the concentration of 20 μM β-carotene, and it was accompanied by a concomitant accumulation of undifferentiated cells in the G2/M phase and by a decreased percentage of cells in the S phase. On the other hand, in differentiated HL-60 cells, an increase in the S phase followed by an accumulation in the G2/M phase was observed at concentrations starting from 20 μM β-carotene. In addition to these changes in cell cycle distribution, the presence of a distinct sub-G1 peak, (subdiploid DNA content), characteristic of apoptotic cells, was also found. However, such a peak became apparent after exposure to 10 μM β-carotene in undifferentiated cells and exposure to 20 μM in differentiated cells.

Effect of β-carotene on cell apoptosis

Confirmation of the induction of apoptosis by β-carotene under the above-described conditions was based on acridine orange assay. Cell morphology showed that both undifferentiated and differentiated HL-60 cells treated with β-carotene had condensed and structureless chromatin, hyperchromatic DNA, diminished size and blebbing of the plasma membrane and nuclear fragmentation. Table II summarizes the respective percentages of undifferentiated and differentiated apoptotic cells after 24 hr of treatment with varying β-carotene concentrations. β-Carotene induced apoptosis in both undifferentiated and differentiated HL-60 cells at the same concentrations responsible for the arrest in cell cycle. These data further point out that undifferentiated HL-60 cells are much more sensitive to the carotenoid than differentiated cells.

Effect of β-carotene on expression of proteins involved in regulation of the cell cycle ($p21^{WAF-1/CIP-2}$p3, $p27^{KIP-1}$ and cyclin A) and apoptosis (Bcl-2, Bcl-xL, Bax)

To investigate possible mechanisms by which β-carotene would interfere with cell cycle progression, we evaluated the cellular content of the cyclin kinase inhibitors $p21^{WAF-1/CIP-2}$ and $p27^{KIP-1}$ (Fig. 3). In the absence of β-carotene, both undifferentiated and DMSO-differentiated HL-60 cells did not express detectable $p21^{WAF-1/CIP-2}$. However, after 24 hr of treatment with 20 μM β-carotene, induction of expression of both these cyclin kinase inhibitors was observed in undifferentiated cells. In contrast, no changes in the expression of these inhibitors were observed in differentiated cells. We also evaluated the cellular content of the G2/M phase-related cyclin A. Figure 3 shows that, according to the blockage in the G2/M phase, both undifferentiated and DMSO-differentiated cells treated with 20 μM β-carotene expressed a lower number of cyclin A-positive cells compared with the respective untreated controls. Such a reduction was higher in undifferentiated (20%) than in differentiated cells (14%).

In an effort to investigate the molecular pathways involved in apoptosis induction by β-carotene, we also examined the effect of the carotenoid on the expression of Bcl-2 and Bcl-xL, 2 apoptosis-blocking proteins, and Bax, an apoptosis promoter protein (Fig. 3). In the absence of β-carotene, differentiated cells expressed lower levels of Bcl-2, Bcl-xL and Bax than undifferentiated cells. Treatment with β-carotene significantly decreased the percentage of Bcl-2-positive cells. Such a decrease was more remarkable in undifferentiated (80%) than in differentiated cells (40%). Moreover, this effect was also evidenced at 10 μM β-carotene in undifferentiated cells (data not shown). In contrast, neither Bcl-xL nor Bax expression was significantly modified by β-carotene.

Similar results were obtained by measuring the above-mentioned cell cycle- and apoptosis-related proteins by Western blot (data not shown).

Cell incorporation of β-carotene

To verify that the differences in β-carotene sensitivity between undifferentiated and differentiated cells were not due to changes in the ability of the cell to incorporate the carotenoid, we measured β-carotene accumulation in undifferentiated and differentiated HL-60 cells after 24 hr. The 24 hr treatment represents the minimum time required to obtain the maximum cell accumulation of the carotenoid (data not shown). During this time, β-carotene was incorporated in and/or associated with both undifferentiated and differentiated HL-60 cells in a dose-dependent manner (data not shown). The amount of β-carotene found in undifferentiated and differentiated HL-60 cells after 24 hr of treatment was 0.79 ± 0.07 nmol/10^6 cells and 0.70 ± 0.07 nmol/10^6 cells, respectively, upon incubation with 20 μM β-carotene. Thus, the amount of β-carotene associated with both undifferentiated and differentiated HL-60 cells was about 4% of the total amount present in the medium. These data show that cell differentiation did not modify carotenoid incorporation.

Effect of β-carotene on intracellular ROS production

Figure 4 shows the spontaneous intracellular ROS production induced by 24 hr of β-carotene treatment in both undifferentiated and differentiated HL-60 cells, measured by both DCF (*a*) and DHR (*b*) as fluorescent probes. In the absence of β-carotene, similar basal levels of ROS were found in normal and DMSO-treated cells. β-Carotene addition deeply modified intracellular ROS levels using both DCF and DHR. The carotenoid acted as an antioxidant at low concentrations and as a prooxidant at high concentrations in both differentiated and undifferentiated cells, inhibiting and increasing ROS production, respectively. These effects were specific for β-carotene since cells treated with THF alone did not significantly differ from untreated cells and appeared after only 4 hr of incubation with the carotenoid (data not shown). However, β-carotene antioxidant activity shifted into a prooxidant activity at much higher concentrations in differentiated than in undifferentiated cells. Prooxidant effects of the carotenoid were observed at 10 μM in untreated HL-60 cells and at 20 μM in DMSO-treated cells.

Effect of β-carotene on glutathione content

To explore the importance of glutathione in regulating stress response and in controlling cell growth and apoptosis, we measured the effects of 24 hr of β-carotene treatment on the endogenous levels of both GSH and GSSG in undifferentiated and DMSO-differentiated HL-60 cells (Fig. 5). Differentiated cells exhibited higher basal levels of glutathione compared with the respective undifferentiated cells. Carotenoid treatment significantly decreased GSH content and increased GSSG content in both undifferentiated and differentiated cells. However, the decrease in GSH and the increase in GSSG were observed at 10 μM β-carotene in undifferentiated cells and at 20 μM in DMSO-differentiated cells.

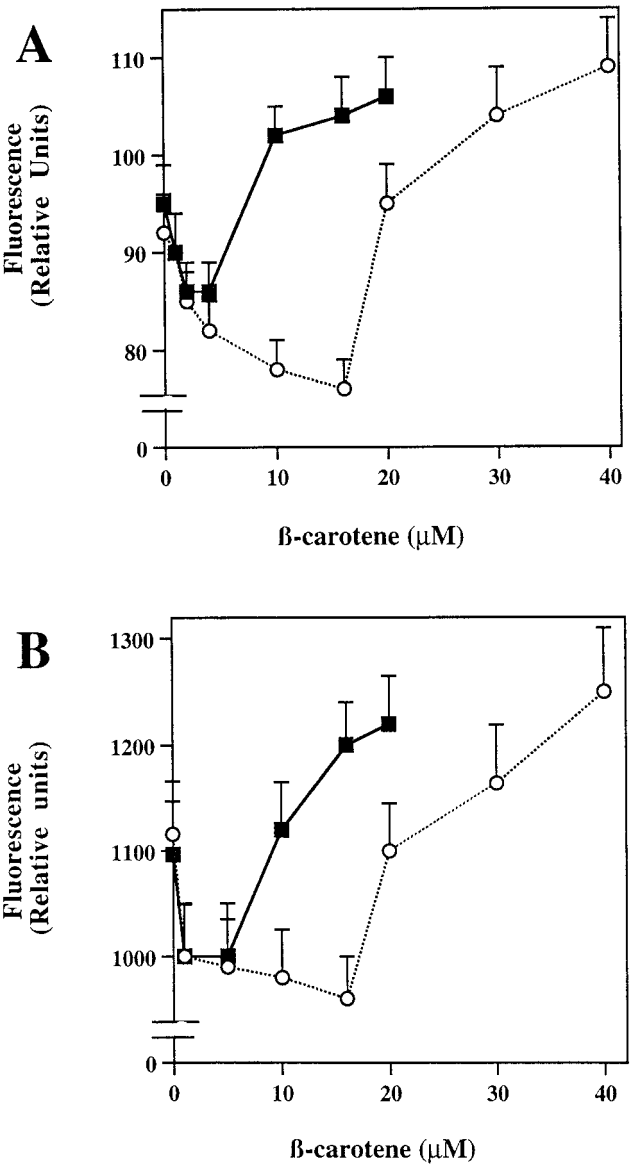

FIGURE 4 – Effect of varying β-carotene concentrations on production of intracellular reactive oxygen species (ROS) in undifferentiated and DMSO-differentiated HL-60 cells, using the di(acetoxymethyl ester) analog of 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCF;*a*) and dihydrorhodamine (DHR; *b*) as fluorescent probes. The cells (10^6 cells/ml) were incubated with the carotenoid for 24 hr. Values are the means ± SEM of 3 different experiments.

Effect of α-tocopherol on the growth-inhibitory and proapoptotic effects of β-carotene

Table III shows that the combined addition of α-tocopherol and β-carotene to undifferentiated HL-60 cells for 24 hr reduced the growth-inhibitory and proapoptotic effects of β-carotene. These effects were dependent on the concentration of α-tocopherol used.

DISCUSSION

In our study, we demonstrated that β-carotene can act as a potent antiproliferative and apoptosis-inducing agent. Our results show that the carotenoid induced a dose-dependent inhibition of cell growth in HL-60 cells. The potential for this compound as an antineoplastic agent has been evidenced in other cell lines, includ-

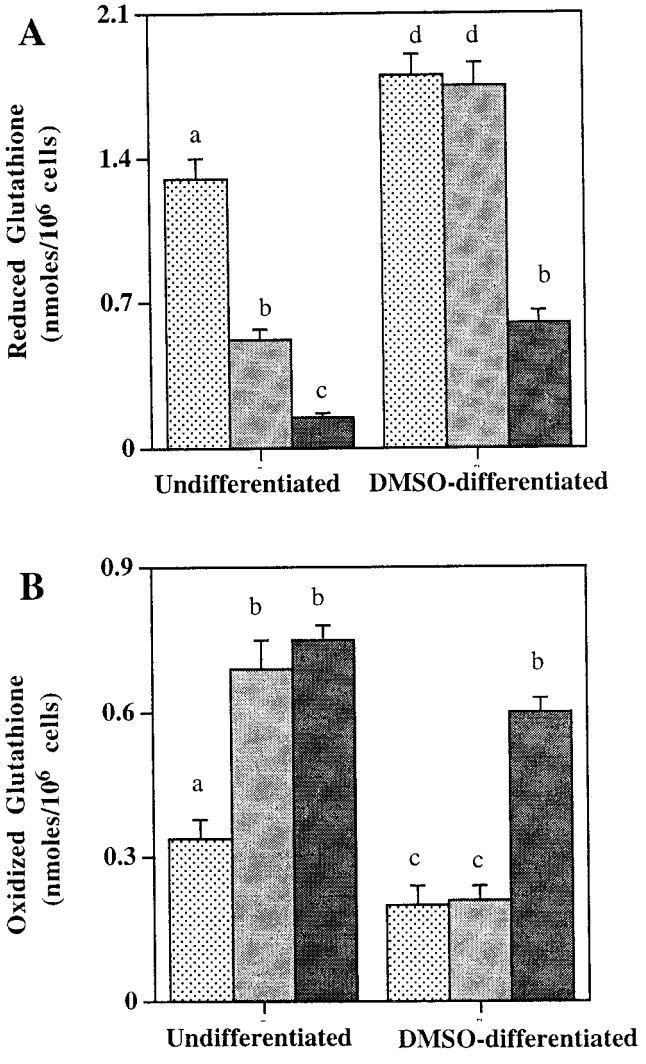

FIGURE 5 – Effect of 24 hr treatment with β-carotene (10 and 20 μM) on the content of reduced (*a*) and oxidized (*b*) glutathione in undifferentiated and DMSO-differentiated HL-60 cells. Values are the means ± SEM of 3 different experiments. The values with different letters were significantly different from each other ($p < 0.05$).

TABLE III – EFFECT OF VARYING α-TOCOPHEROL (α-T) CONCENTRATIONS ON CELL NUMBER AND APOPTOSIS INDUCTION IN UNDIFFERENTIATED HL-60 CELLS TREATED WITH β-CAROTENE (β-C)

Treatment	No. of cells ($\times 10^{-3}$)	Apoptosis (%)
None	$1,455 \pm 70^2$	7.0 ± 0.9^2
β-C		
10 μM	$1,100 \pm 40^3$	22.0 ± 2.3^3
α-T		
10 μM	$1,459 \pm 65^2$	7.2 ± 0.8^2
25 μM	$1,450 \pm 64^2$	7.1 ± 0.7^2
50 μM	$1,465 \pm 60^2$	7.5 ± 0.8^2
β-C + α-T		
10 + 10 μM	$1,217 \pm 55^3$	18.0 ± 2.3^3
10 + 25 μM	$1,250 \pm 60^3$	15.0 ± 2.3^3
10 + 50 μM	$1,380 \pm 60^2$	10.0 ± 1.0^2

α-Tocopherol was added alone or in combination with β-carotene for 24 hr. The values are the means ± SEM of 3 different experiments. Within a column, the values not sharing the same superscript (2, 3) were significantly different at $p < 0.05$.

ing colon,[25] melanoma,[26] prostate,[27] oral, lung and breast[28] cancer cells. Our finding that β-carotene has growth-inhibitory effects on tumor cells at 10 μM is particularly relevant, since this concentration is within the range of the concentrations achieved *in vivo* in sera from human subjects supplemented with various doses of the carotenoid. An average serum steady-state concentration of β-carotene of up to 7.7 μM was found in human subjects ingesting 51–102 mg/day β-carotene, and a serum β-carotene concentration of 13.2 μM was reached at a higher doses.[29] In another study by Nierenberg and co-workers,[30] supplementation with 50 mg/day of β-carotene in humans resulted in plasma β-carotene concentrations of up to 16.1 μM. Differentiated HL-60 cells were much more resistant to β-carotene than undifferentiated cells, since a reduction in their cell number was observed only at 20 μM. In addition, they did not show the loss of viability found in undifferentiated cells at higher β-carotene concentrations (30–40 μM).

The growth-inhibitory effect of β-carotene in HL-60 cells was accompanied by an arrest in cell cycle progression, which was apparently influenced by the concentration of the carotenoid as well as by cell differentiative status. β-Carotene at higher concentrations (20 μM) induced an accumulation of both undifferentiated and differentiated cells in G2/M phase, whereas at lower concentrations (10 μM) it also induced an arrest in G0/G1 phase. However, such an effect was evidenced only in undifferentiated cells. The changes in cell cycle distribution were accompanied by modifications in the expression of molecules involved in its regulation. Namely, β-carotene increased the expression of the cyclin-dependent kinase inhibitors $p21^{WAF-1/CIP-2}$ and $p27^{KIP-1}$.[31] Moreover, it decreased the expression of cyclin A, a protein known to regulate cdc2 kinase activity at the G2/M phase.[32] There are some indications showing that carotenoids are able to modify cell cycle progression. In particular, lycopene (6 μM)[33] and α-carotene (5 μM)[34] were able to accumulate HL-60 and GOTO cells, respectively, in the G0/G1 phase of the cycle. On the other hand, β-carotene (70 μM) tended to accumulate in the human oral cancer SCC-25 cell line at the G2/M phase of the cell cycle.[35] It has recently been reported that, in mammary cancer cells synchronized by mimosine treatment, lycopene delayed cell cycle progression through the G1 and S phases, after removal of mimosine block.[36]

The arrest of cell cycle progression was also accompanied by apoptosis induction. Similar proapoptotic effects by β-carotene and other carotenoids have been reported in other cell lines, including cervical dysplasia-derived cells[37] melanoma and adenocarcinoma cells.[38] Our experiments regarding the proapoptotic effects of β-carotene in HL-60 cells highlight several important points: (*i*) undifferentiated cells were more susceptible to β-carotene than differentiated cells; (*ii*) β-carotene profoundly decreased the expression of *bcl-2*, a protooncogene product involved in protecting cells from apoptosis; and (*iii*) apoptosis was induced through *p53*-independent mechanisms, since this gene is completely deleted in HL-60 cells.[39] It has recently been reported that β-carotene was able to enhance H_2O_2-induced DNA damage in human hepatocellular HepG2 cells. In view of this observation, DNA damage might represent a contributory mechanism to the induction of apoptosis observed in our model.[40]

Modulation of intracellular redox status is an important event in the regulation of cellular processes controlling cell growth and apoptosis.[41] It has been proposed that β-carotene can act as an intracellular redox agent, protecting against free radicals in some circumstances and promoting free radical formation in others.[10–12] In accord with this hypothesis, in our study, we demonstrated that β-carotene acted as an antioxidant, inhibiting intracellular ROS production at low concentrations, and as a prooxidant, increasing ROS at high concentrations. Other authors have reported modulatory effects of varying β-carotene concentrations on intracellular ROS production. Using the DCF test, β-carotene, at doses ranging

from 1 to 100 μM, was able to trap oxygen species generated by H_2O_2 in Chinese hamster ovary cells. However, in these cells, the carotenoid, particularly at the highest dose, increased H_2O_2-induced chromosomal aberrations.[42] Moreover, whereas β-carotene did not alter susceptibility to H_2O_2 in Caco-2 cells cultured between 3 and 8 days at low doses (0.1–0.5 μM), it profoundly enhanced such a toxicity at persistent high doses (5–50 μM).[43]

It has recently been reported that cytochrome c is a potent catalyst of DCF oxidation.[44] Therefore, the increase in DCF fluorescence found in our model at high β-carotene concentrations might actually reflect a release of cytochrome c into the cytoplasm, as would be expected for cells undergoing apoptosis, rather than an increase in ROS production. However, the results obtained with DCF were confirmed by the use of DHR, another ROS detector, suggesting a specific role for β-carotene in modulating intracellular ROS production. From a mechanistic point of view, the delay in cell cycle progression and the induction of apoptosis by β-carotene were highly coincident with the increase in intracellular ROS production caused by the carotenoid in both undifferentiated and differentiated HL-60 cells. These data are also in agreement with the fact that Bcl-2 is suggested to interfere with signal transduction events caused by oxidative stress.[23] In fact, the antiapoptotic effect of Bcl-2 has been at least partially explained by its antioxidant properties.[45] On the other hand, it has been demonstrated that an increase in ROS production was able to induce an accumulation of inhibitors of cyclin-dependent kinases, such as $p21^{WAF-1}$.[46] It should be noted that the increase in ROS production by β-carotene occurred at concentrations lower in undifferentiated cells than in differentiated cells. It has been reported that during differentiation, HL-60 cells acquired more resistance to oxidative stress, increasing the content and/or the activity of antioxidants and modifying their cell distribution.[47–49] In accord with these findings, we reported that DMSO-differentiated HL-60 cells exhibited higher levels of reduced GSH than undifferentiated HL-60 cells. In this context, the prooxidant character of the carotenoid may be minimized and evidenced only at higher concentrations in differentiated HL-60 cells. This hypothesis is supported by our finding that, concomitantly with the increase in ROS production, β-carotene decreased GSH and increased GSSG at lower concentrations in undifferentiated than in differentiated cells.

At this time we cannot prove a direct functional association between the proapoptotic and the prooxidant effects of β-carotene. However, the finding that a well-known antioxidant such as α-tocopherol protected in a dose-dependent manner the effects of β-carotene on cell growth and apoptosis strongly suggests a correlation between the proapoptotic activity of the carotenoid and its redox properties. It also remains unclear whether the growth-inhibitory effects induced by β-carotene in our model are due to β-carotene molecule *per se* or to the formation of its oxidative metabolites. It has been reported that β-apocarotenals, excentric cleavage products of β-carotene, may act as strong inducers of cytochrome P450 enzymes[8] and may affect retinoid signaling.[9]

On the other hand, it is also possible that some of the effects induced by the carotenoid are due to its conversion to retinoic acid. β-Carotene cleavage to retinoic acid was demonstrated in some cultured cancer cells, such as small cell lung cancer cells[50] and colon adenocarcinoma cells.[51] However, it has been reported that several cancer cell lines, such as human breast, oral carcinoma and malignant melanoma, were not able to convert β-carotene to retinoic acid.[35] It has also been suggested recently that antiproliferative effects of carotenoids may occur in cancer cells without their conversion to retinoic acid.[52] In agreement with this observation, preliminary data from our laboratory seem to exclude a role of this metabolite in the growth-inhibitory effects of β-carotene in our study, since no significant differences were found in the levels of retinoic acid in HL-60 cells after β-carotene treatment (data not shown).

Although the significance of these findings in a broader context must be proved by further studies *in vitro* and *in vivo*, the data presented here provide evidence for the antileukemic activity of β-carotene and demonstrate its role as an intracellular redox agent. Moreover, they provide evidence that differentiated cells, morphologically and biochemically similar to normal neutrophils, are more resistant to the proapoptotic and prooxidant effects of β-carotene than undifferentiated neoplastic cells. Our findings also offer a possible mechanistic understanding of what was observed in the human trials in which β-carotene increased cancer incidence and/or mortality,[2,3] as supplementation with high doses of the carotenoid may induce prooxidant effects that can favor neoplastic transformation of normal cells.

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