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Carotenoids Induce Apoptosis in the T-lymphoblast Cell Line Jurkat E6.1

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Epidemiologically, a high-carotenoid intake via a fruit- and vegetable-rich diet is associated with a decreased risk of various forms of cancer. The mechanisms by which carotenoids exert this protective effect are controversial. In this study, we examined the potency of a range of carotenoids commonly found in human plasma to induce apoptosis in Jurkat E6.1 malignant T-lymphoblast cells. At a concentration of 20 μM, the order of potency to induce apoptosis after 24 h was: β-carotene > lycopene > lutein > β-cryptoxanthin = zeaxanthin. Canthaxanthin failed to induce apoptosis under these conditions. β-Carotene induced apoptosis in a time- and concentration-dependent manner with a lowest effective concentration of about 3 μM. Pre-conditioning of β-carotene for 72 h destroyed its pro-apoptotic activity almost completely, whereas degradation for 6 h or less did not, indicating that either β-carotene itself and/or an early degradation product of β-carotene are the death-inducing compounds. Apoptosis induced by β-carotene was characterized by chromatin condensation and nuclear fragmentation, DNA degradation, PARP cleavage and caspase-3 activation. The antioxidant BO-653 inhibited the degradation of β-carotene *in vitro* and significantly increased its cytotoxicity, indicating that a pro-oxidant effect of β-carotene is unlikely to cause its pro-apoptotic activity. The induction of apoptosis in transformed cells by carotenoids may explain their protective effect against cancer formation in humans. Possible pathways for induction of apoptosis by carotenoids are discussed.

Keywords: β-Carotene; Apoptosis; Jurkat cells; Carotenoids; Antioxidants

INTRODUCTION

Carotenoids are naturally occurring yellow to red pigments which function in photosynthesis and photoprotection in plants.[1] Over 600 carotenoids have been described and about 34 have been found in human plasma.[2] In humans, carotenoids are derived from the diet predominantly from fruit and green-leafy vegetables[3] and from carotenoids used as food additives.[4] Epidemiologically, a high-carotenoid intake via a fruit and vegetable-rich diet is associated with a decreased risk of various forms of cancer, of cardiovascular disease and some age-related degenerative diseases.[5] However, intervention trials have shown little, no or even detrimental effects of β-carotene supplementation on human health.[6,7] The benefit of carotenoid supplementation in humans is therefore still controversial.

Carotenoids have many biological effects. They can inhibit cell proliferation in various cell types,[8,9] they inhibit neoplastic transformation[10] and enhance gap junctional communication.[11] They have important roles as vitamin A precursors, for example during vertebrate development[12] and are thought to modulate the immune system.[13] The manifold effects of carotenoids could be mediated by various mechanisms. Provitamin A carotenoids, such as β-carotene and β-cryptoxanthin, could exert their effects by giving rise to vitamin A (retinol) and retinoic acid.[12,14] Alternatively, many of the

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protective effects of carotenoids on human health have been ascribed to their antioxidant properties.[15] However, carotenoids can have anti- or pro-oxidant properties in biological systems depending on conditions.[16–18] Elucidating the mechanism(s) by which carotenoids exert their effects on human health is of considerable interest.

Apoptosis is an active form of cell death, often described as cell suicide,[19] and is important in carcinogenesis,[20] atherogenesis[21] and a number of other diseases.[22] However, the pro-apoptotic effect of carotenoids has hardly been investigated. In this study, we examined the potency of a range of carotenoids commonly found in human plasma, β-carotene, lycopene, lutein, canthaxanthin, β-cryptoxanthin and zeaxanthin, to induce apoptosis in the human T-lymphoblast cell line Jurkat E6.1, a cell line frequently used for apoptosis studies. Subsequent experiments were performed with β-carotene as it was the most potent inducer of apoptosis and also is the only carotenoid readily available in large amounts. β-Carotene is quickly oxidized in solution[23,24] and some reports indicate that the oxidation products of β-carotene can also be bioactive.[25,26] For this reason we also examined the pro-apoptotic potency of pre-conditioned β-carotene in our study. The effect of various antioxidants on β-carotene-induced apoptosis was also investigated.

MATERIALS AND METHODS

Chemicals

All chemicals were from Sigma-Aldrich (Poole, Dorset, UK) unless stated otherwise and were of the highest grade of purity available. Zeaxanthin, lutein, lycopene, canthaxanthin and β-cryptoxanthin were gifts of Hoffmann-La Roche AG (Basel, Switzerland) and were stored in crystalline form at −70°C under argon. The antioxidant BO-653 was a gift of Chugai Pharmaceutical Company (Gotemba, Shizuoka, Japan).

Carotenoid and α-tocopherol stock solutions were prepared in tetrahydrofuran (THF) and ethanol (EtOH), respectively, and kept under argon at −70°C. Stock solutions of BO-653, probucol and etoposide were prepared in EtOH and were stored at −20°C. Carotenoid and antioxidant stock solution were used within 2 days of preparation. z-VAD-fmk (Calbiochem-Novabiochem, Nottingham, UK) was dissolved in DMSO and kept at −20°C. Polyclonal antibodies against PARP and caspase-3 were from Santa Cruz Biotechnology (Santa Cruz, California, USA) and Pharmingen (San Diego, California, USA), respectively.

Cell Culture

The human leukaemic T-lymphoblast cell line Jurkat E6.1, obtained from the European Collection of Cell Cultures (Salisbury, UK), was routinely cultured in RPMI 1640 medium containing 10% foetal calf serum, 2 mM L-glutamine, 100 μg/ml streptomycin and 63 μg/ml penicillin at 37°C in a humidified atmosphere at 5% CO_2 in air. This culture medium was also used for pre-conditioning of β-carotene and for the incubation of cells with carotenoids.

Pre-conditioning of β-carotene

β-Carotene was dissolved in culture medium at 25, 50 and 100 mg/l in sterile glass tubes loosely covered with foil and incubated at 37°C for 72 h in the dark in a humidified atmosphere at 5% CO_2 in air. The percentage loss of β-carotene after 72 h of pre-conditioning was more than 85%. Prior to the addition to cells at indicated concentrations, β-carotene solutions were filter sterilized (0.45 μm).

β-Carotene consumption in the absence of cells and the effect of antioxidants on this process were monitored by measuring β-carotene absorbance at 450 nm in a 96-well microplate reader. β-Carotene at 12.5 mg/l was dissolved in culture medium and incubated at 37°C in the dark in the absence of antioxidants, or in the presence of 200 μM α-tocopherol, 20 μM or 100 μM BO-653, 80 μM probucol, 40 μM BHT (butylated hydroxytoluene) or 100 μM ascorbic acid for up to 24 h. Degradation of β-carotene was expressed in % as the ratio of absorbance at 450 nm at a particular time point/absorbance at 450 nm at the 0h-time point.

Cell Treatments

Jurkat cells were seeded in 24-well tissue culture plates (Becton Dickinson, Franklin Lakes, New Jersey, USA) at 250,000 cells/well in 500 μl culture medium. Additions were diluted in culture medium at twice the concentration required and 500 μl were added to each well. Two wells were combined for each sample. Controls included a medium control with no additions (NA), vehicle controls (0.5% THF or 0.5% EtOH) and 5 μM etoposide as a positive control for the induction of apoptosis. Antioxidants were added simultaneously with β-carotene at indicated concentrations. An additional vehicle control (0.5% THF + 0.5% EtOH) was included as the appropriate vehicle control for the combined additions.

Flow Cytometry and Fluorescence Microscopy

DNA fragmentation was measured by flow cytometry after staining of cellular DNA with the

fluorescent dye propidium iodide (PI).[27] Following the various treatments, Jurkat cells were transferred into flow cytometry tubes and spun for 10 min at 300*g*. The cell pellet was resuspended in 200 µl PBS. 2 ml of cold EtOH (70% v/v in saline) was added to each sample and the cells were fixed for at least 30 min at 4°C. After centrifugation for 10 min at 600*g*, the fixative was removed and the cells were resuspended in 800 µl PBS. 100 µl ribonuclease A (from bovine pancreas, 1 mg/ml in PBS) and 100 µl PI (400 µg/ml in PBS) were added and the cells were incubated at 37°C for 30 min. DNA content was analysed by flow cytometry (FACScan, Becton Dickinson, San José, California, USA) using Cell Quest software. For each sample 10,000 cells were counted and results represent the percentage of cells in the hypodiploid region (sub-G_0/G_1).

Aliquots of samples stained as for flow cytometric analysis were centrifuged at 600*g* for 5 min at 4°C. Cells were resuspended in 20 µl of Vectashield (Vector Laboratories, Burlingame, CA, USA) and mounted on glass slides for microscopy. The percentage of cells with condensed chromatin and/or fragmented nuclei was assessed by fluorescence microscopy using a Leitz Axiophot fluorescence microscope at a magnification of ×400. 400–600 cells/sample were counted.

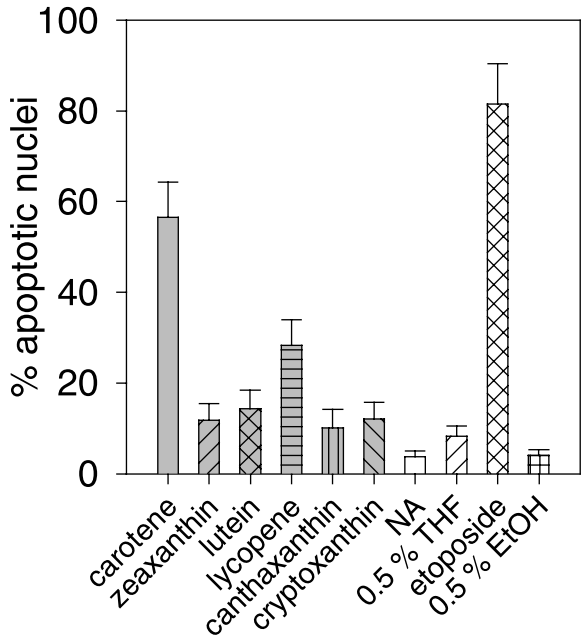

FIGURE 1 Induction of apoptosis by carotenoids. Jurkat cells were incubated for 24 h with 20 µM of the indicated carotenoid or with the indicated controls. Subsequently, the percentage of apoptotic nuclei was assessed by fluorescence microscopy after PI staining. Results represent the *mean* ± SD of three experiments performed in triplicate ($n = 9$). Apoptosis induced by β-carotene, lutein and lycopene ($p \leq 0.001$), and zeaxanthin and β-cryptoxanthin ($p \leq 0.05$) was significantly different from that induced by the vehicle (0.5% THF) control. Abbreviations: THF, tetrahydrofuran; NA, no additions; EtOH, ethanol.

Western Blotting and Enhanced Chemiluminescence (ECL)

Following treatments, Jurkat cells were pelleted by centrifugation at 4°C for 3 min at 1500*g*. After washing once with ice-cold PBS, cells were lysed in double concentrated electrophoresis sample buffer (100 mM Tris/HCl pH 6.8, 2% SDS, 20% glycerol, 0.002% bromphenol blue and 2% β-mercaptoethanol) and boiled for 10 min. Lysates were stored at −20°C until analysis by SDS-PAGE.

Lysate proteins were separated by SDS-PAGE according to Laemmli *et al.*[28] on 15% gels for analysis of caspase activation and on 7.5% gels for detection of PARP and β-actin. Proteins were transferred to PVDF membranes (Bio-Rad Laboratories, Hemel Hempstead, UK) by Western blotting at 400 mA (40 min to 1 h) using 25 mM Tris/192 mM glycine/20% methanol as transfer buffer. After blocking the membrane overnight with 5% non-fat dried milk in PBS, the primary antibody (in 5% milk/PBS) was applied for 1 h. The anti-PARP, anti-caspase and anti-actin antibodies were used at a dilution of 1:4000, 1:1000 and 1:2500, respectively. After washing four times in PBS containing 0.25% Tween 20, secondary antibodies (at 1:20,000 in 5% milk/PBS) were applied for 40 min. Blots were washed five times for 5 min in PBS/Tween and twice in PBS. The ECL was performed using the ECL Plus detection kit from Amersham (Little Chalfont, Bucks, UK) and BIOMAX ML films from Kodak (Rochester, New York, USA). Molecular weights were estimated using the Broad-Range Marker Kit from Bio-Rad Laboratories (Hemel Hempstead, UK).

Transmission Electron Microscopy (TEM)

Following incubation, cells were transferred to centrifuge tubes and spun for 2 min at 600*g*. Then, the cell pellet was washed once in ice-cold Tris-buffered saline (50 mM Tris, 150 mM NaCl, pH 7.4). Cells were fixed on ice in TEM fixative (3% glutaraldehyde, 0.2% formaldeyde, 2 mM calcium chloride in 0.1 M piperazine-1,4-bis[2-ethene-sulphonic acid] (PIPES) buffer, pH 7.2) and processed for TEM as described by Hardwick *et al.*[29]

Statistics

Significance of results was calculated by unpaired Student's *t*-test using Microsoft Excel. The level of significance was taken as $p = 0.05$.

RESULTS

Induction of Cell Death by Carotenoids

To compare a range of carotenoids for their pro-apoptotic potency, Jurkat cells were incubated with 20 μM β-carotene, zeaxanthin, lutein, lycopene, canthaxanthin or β-cryptoxanthin (Fig. 1). After 24 h, β-carotene induced the highest degree of apoptosis with 56.5% hypodiploid cells, which was significantly higher than all the other carotenoids tested. This was followed by lycopene, which induced 28.3% hypodiploid cells. Lutein, β-cryptoxanthin and zeaxanthin induced 14.4, 12.0 and 11.7% hypodiploid cells, respectively, which was significantly lower than the pro-apoptotic potencies of β-carotene or lycopene. Canthaxanthin was not significantly toxic compared to the THF vehicle (8.2%). In comparison, the positive control etoposide at 5 μM induced 81.5% hypodiploid cells after 24 h. As β-carotene was the most potent of the carotenoids tested, its cell death-inducing properties were examined in more detail.

Induction of Apoptosis by Fresh and Pre-conditioned β-carotene

Chromatin Condensation, Nuclear Fragmentation and DNA Degradation

Jurkat cells were incubated with 12.5, 25 or 50 mg/l (corresponding to 23.3, 46.6 or 93.1 μM) fresh or pre-conditioned β-carotene for 24 h (Fig. 2A). At all concentrations of fresh β-carotene tested 98–99% of Jurkat cells showed abnormal nuclei on fluorescence microscopy when compared to controls. At 12.5 mg/l chromatin condensation and nuclear fragmentation were predominant (as with etoposide), whereas at 25 and 50 mg/l nuclei mainly showed chromatin condensation. DNA degradation as measured by flow cytometry was significantly increased at 12.5 mg/l fresh β-carotene, whereas at 25 and 50 mg/l it was not significantly different from controls (not shown). Pre-conditioned β-carotene, which contained less than 15% of unchanged

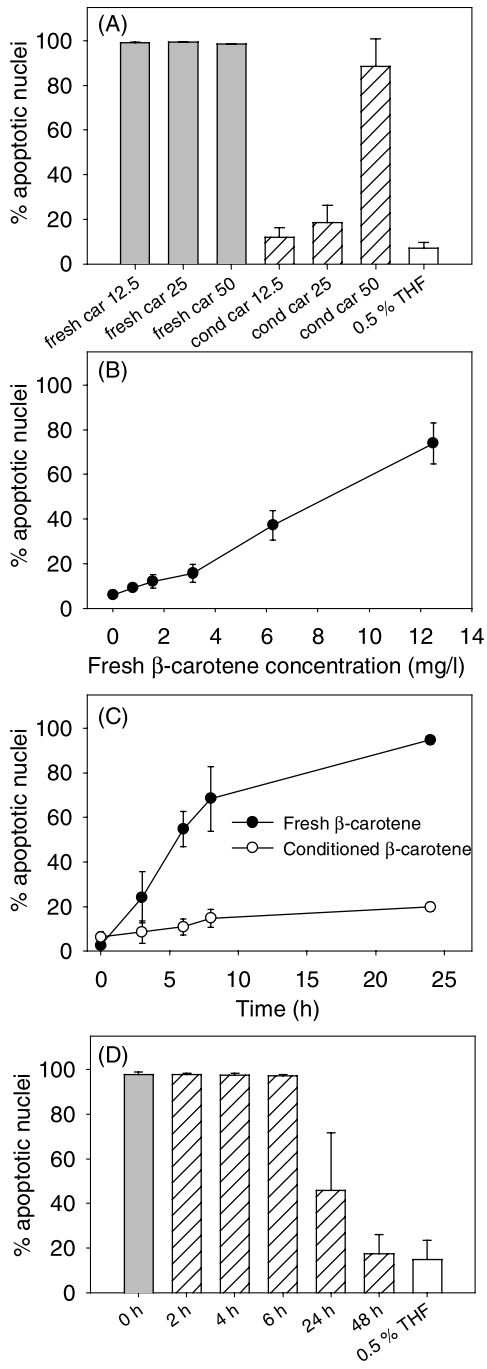

FIGURE 2 Induction of apoptosis in Jurkat cells by fresh and conditioned β-carotene. Following incubations, the percentage of condensed/fragmented nuclei was assessed by fluorescence microscopy. (A) Cells were incubated for 24 h with the indicated concentrations of fresh (solid grey bars) or 72 h-conditioned (open hatched bars) β-carotene. Apoptosis induced by fresh β-carotene at 12.5, 25 and 50 mg/l ($p \leq 0.001$) and by conditioned β-carotene at 50 mg/l ($p \leq 0.01$) was significantly different from that induced by the vehicle (0.5% THF) control. (B) Apoptosis induced by fresh β-carotene at 0.78, 1.56, 3.125, 6.25 and 12.5 mg/l for 24 h was significantly different ($p \leq 0.001$) from that induced by vehicle (0.5% THF) control (i.e. 0 mg/l β-carotene). (C) Time-course of induction of apoptosis by 12.5 mg/l fresh β-carotene (•) or 50 mg/l 72 h-conditioned (○) β-carotene, for up to 24 h incubation with cells. Apoptosis induced by fresh β-carotene at 24 h ($p \leq 0.001$), 8 h and 6 h ($p \leq 0.01$), and by 72 h-conditioned β-carotene at 24 h ($p \leq 0.001$) was significantly different from that induced by the vehicle (0.5% THF) control. (D) Fresh β-carotene (0 h conditioned, grey filled bar) or β-carotene which had been conditioned for the times stated (open hatched bars) was incubated with cells for 24 h. Apoptosis induced by fresh β-carotene (0 h-conditioned) and β-carotene conditioned for 2, 4, and 6 h ($p \leq 0.001$), and 24 h ($p \leq 0.05$) was significantly different from that induced by the vehicle (0.5% THF) control. In addition to the treatments shown in (A–D) all experiments included a positive control for the induction of apoptosis (5 μM etoposide), a vehicle control for etoposide (0.5% EtOH) and a NA control; all three of these controls gave results similar to those in Fig. 1. Results represent the mean ± SD of (A) two experiments with single estimations ($n = 2$), (B) and (D) two experiments in triplicate ($n = 6$) and (C) two experiments in duplicate ($n = 4$).

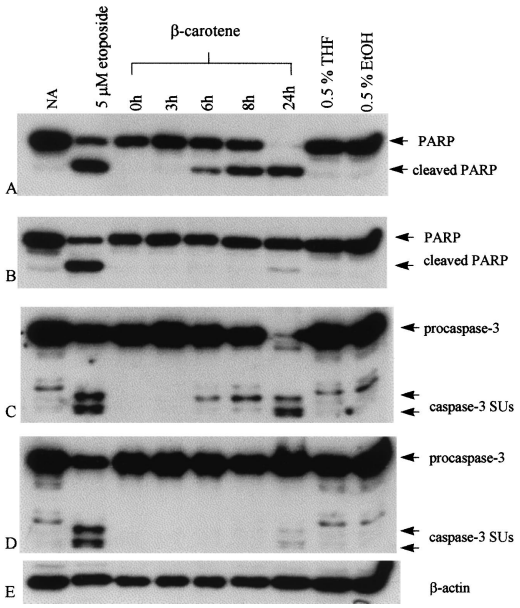

FIGURE 3 PARP cleavage and caspase-3 activation. Jurkat cells were incubated with (A + C) 12.5 mg/l fresh or (B + D) 50 mg/l conditioned β-carotene for up to 24 h. Controls included a medium control (NA), 5 μM etoposide, 0.5% THF or 0.5% EtOH (all for 24 h). Cell lysates were analysed using (A + B) an antibody against PARP, or (C + D) an antibody against caspase-3. (E) shows β-actin as a control for protein loading.

β-carotene, induced significant chromatin condensation, nuclear fragmentation and DNA degradation only at 50 mg/l.

Subsequently, Jurkat cells were incubated with a lower range of fresh β-carotene concentrations for 24 h (Fig. 2B). Significant nuclear fragmentation was induced down to a concentration of 0.78 mg/l (1.45 μM) as judged by microscopy. Significant DNA degradation was observed by flow cytometry down to a concentration of 1.56 mg/l (2.9 μM) fresh β-carotene (not shown).

To follow the time course of toxicity, Jurkat cells were incubated with either 12.5 mg/l fresh β-carotene or with 50 mg/l pre-conditioned β-carotene for 0, 3, 6, 8 and 24 h (Fig. 2C). Fresh β-carotene induced significant DNA degradation (not shown) and nuclear fragmentation after 6 h. Pre-conditioned β-carotene induced significant nuclear fragmentation only after 24 h.

To examine the relationship between β-carotene degradation and apoptosis induction, β-carotene was pre-conditioned for 0, 2, 4, 6, 24 and 48 h prior to addition to the cells for 24 h, (Fig. 2D). β-Carotene retained its full activity to induce nuclear fragmentation after 6 h of pre-conditioning, whereas after 24 and 48 h of pre-conditioning its activity was significantly reduced.

In all these experiments, 5 μM etoposide significantly increased nuclear fragmentation, whereas the EtOH vehicle control was innocuous. THF (0.5%) led to a slight increase in nuclear fragmentation above NA controls in most experiments, which was statistically significant in only one instance.

Caspase-3 Activation and PARP Cleavage

Two biochemical markers of apoptosis, PARP cleavage and caspase-3 activation, were assessed by Western Blotting (Fig. 3). After incubation with 12.5 mg/l fresh β-carotene, PARP cleavage was just detectable after 3 h and pronounced after 6–8 h. After 24 h hardly any uncleaved PARP remained, whereas a PARP cleavage product of about 92 kDa was prominent (Fig. 3A). Similarly, caspase-3 activation was pronounced after 6–8 h with 12.5 mg/l fresh β-carotene and after 24 h, the majority of procaspase-3 was cleaved into the subunits of active caspase-3 (Fig. 3C). Pre-conditioned β-carotene at 50 mg/l resulted in weak PARP cleavage and caspase-3 activation after 24 h, (Fig. 3B and 3D, respectively). No PARP cleavage and caspase-3 activation was detectable in the medium control (NA) and the vehicle controls (0.5% THF or EtOH), whereas extensive PARP cleavage and caspase-3 activation was present with 5 μM etoposide (Fig. 3A–D). Equal protein loading was monitored by staining for β-actin (Fig. 3E).

Transmission Electron Microscopy

Ultrastructural changes during β-carotene-induced cell death were further evaluated by TEM (Fig. 4). Jurkat cells were incubated with 12.5 mg/l fresh β-carotene for 6 h. Controls included medium only (NA) and a 0.5% THF vehicle control. In the medium control (Fig. 4A), Jurkat cells showed a normal cell morphology with abundant surface microvilli, cytoplasm with extensive mitochondria and a nucleus with finely granular heterochromatin. The majority of cells in the β-carotene-treated sample showed typical signs of apoptosis, including loss of surface microvilli, cell and nuclear shrinkage, compacted electron dense cytoplasm and condensed chromatin, with frequent nuclear fragmentation into apoptotic bodies (Fig. 4B).

Effect of Antioxidants on β-carotene Degradation *In Vitro*

Fresh β-carotene at 12.5 mg/l was incubated alone or with 200 μM α-tocopherol, 20 or 100 μM BO-653, 80 μM probucol, 40 μM BHT or 100 μM ascorbic acid in culture medium at 37°C for up to 24 h in the dark. The effect of these antioxidants on β-carotene degradation was examined by monitoring β-carotene levels by measuring absorbance at 450 nm (Fig. 5). In the absence of antioxidants, β-carotene absorbance exponentially decreased to 15.3% of the

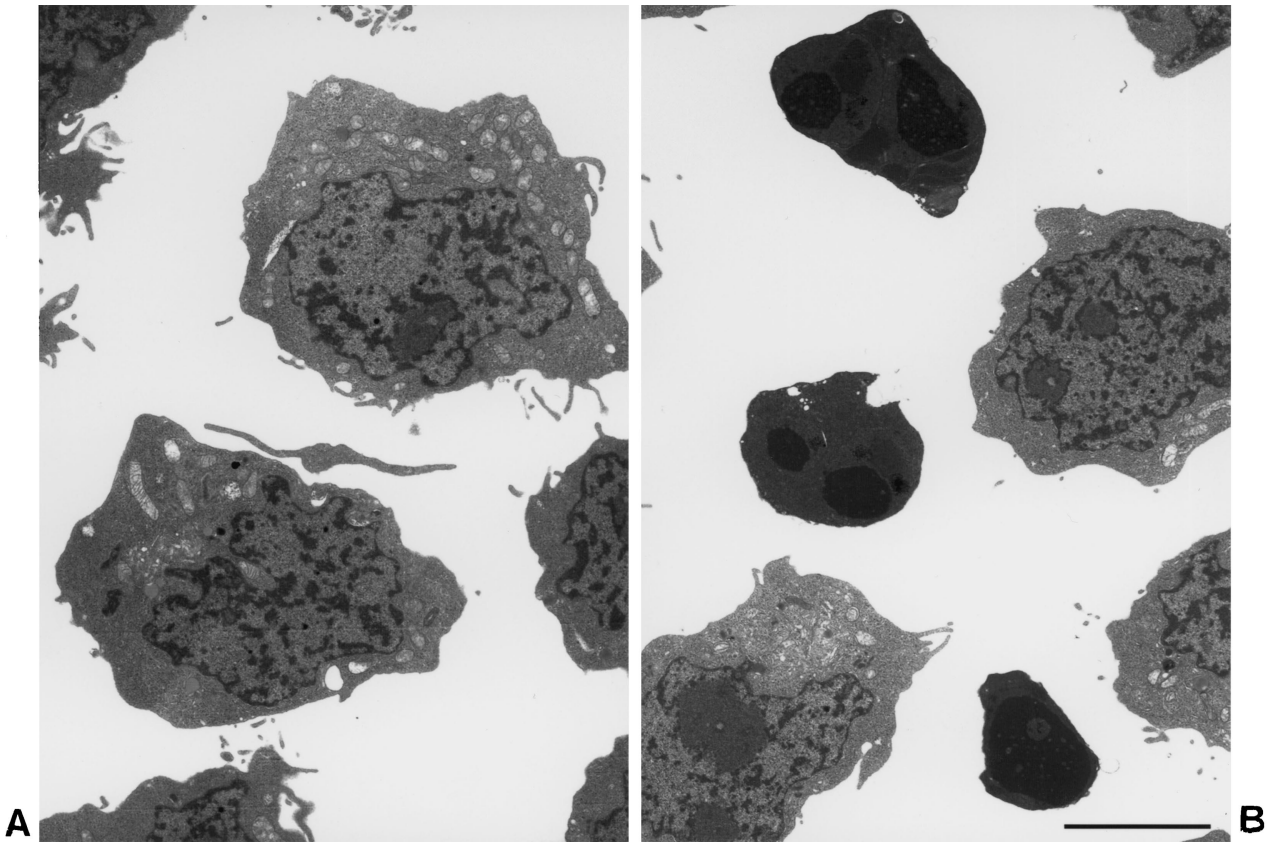

FIGURE 4 Transmission electron micrographs. Jurkat cells were incubated with (A) medium only, or with (B) 12.5 mg/l fresh β-carotene for 6 h. Cells in (A) show normal appearance, while three cells in (B) show increased electron density of the cytoplasm, condensed chromatin and nuclear fragmentation. Bars represent 5 μm.

0h-value after 24 h, which is probably due to the destruction of the β-carotene chromophore by an oxidative mechanism. BO-653 at 100 μM protected β-carotene best from degradation, with about 80% of the β-carotene still remaining at 24 h. Within the first 5 h of incubation, there was no substantial difference in β-carotene protection between 20 μM BO-653, α-tocopherol, probucol and BHT. After 24 h of incubation, the order of protection was: 100 μM BO-653 > 20 μM BO-653 > 80 μM probucol > 200 μM α-tocopherol > 40 μM BHT. No protection against degradation was conferred by ascorbic acid. Recent studies have shown that ascorbate can have pro-oxidant effects in cell culture media at least partly due to production of hydrogen peroxide.[30,31]

Effect of Antioxidants on β-carotene Induced Apoptosis

To evaluate the relationship between β-carotene degradation and its apoptosis-inducing potency further, Jurkat cells were incubated for 6 h with 12.5 mg/l fresh β-carotene in the absence or presence of antioxidants. Three antioxidant treatments—100 μM BO-653, 200 μM α-tocopherol and 80 μM probucol—were chosen from the *in vitro* experiment for their strong to intermediate ability to protect β-carotene from degradation.

α-Tocopherol and probucol alone were innocuous, whereas BO-653 led to a slight but statistically significant increase in hypodiploid cells (Fig. 6). In the absence of antioxidants, 12.5 mg/l β-carotene increased the proportion of hypodiploid cells to 24.65% as compared to 8.7% in the THF control. In combination with α-tocopherol or probucol a slight increase in hypodiploid cells was observed, which

TABLE I Inhibition of β-carotene-induced apoptosis by caspase inhibitor z-VAD-fmk*

	% sub-G_0/G_1	% Apoptotic nuclei
NA	6.07 ± 0.37	4.93 ± 2.08
z-VAD	5.16 ± 0.68	5.01 ± 1.3
Car	14.63 ± 1.15	31.61 ± 8.24
Car + z-VAD	6.74 ± 0.78**	45.51 ± 5.71*

*Cells were incubated with 12.5 mg/l fresh β-carotene for 6 h in the absence or presence of 20 μM z-VAD-fmk. The percentages of hypodiploid cells and cells with condensed/fragmented nuclei were estimated by flow cytometry and fluorescence microscopy, respectively. Results represent the mean ± SD of two experiments in triplicate ($n = 6$). z-VAD-fmk significantly inhibited the percentage of hypo-diploid cells (** $p \leq 0.0001$), but increased the percentage of apoptotic nuclei (* $p \leq 0.001$).

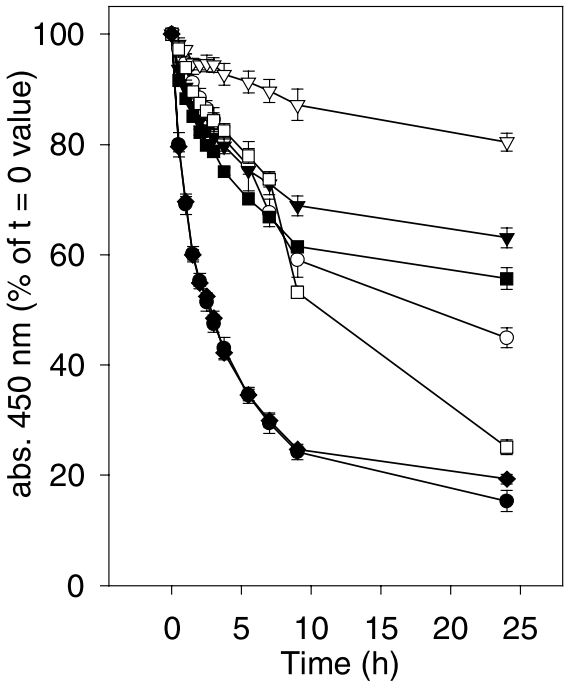

FIGURE 5 Degradation of β-carotene *in vitro*. Fresh β-carotene (12.5 mg/l) was incubated in the absence of antioxidants (●) or in the presence of 200 μM α-tocopherol (○), 20 μM (▼) or 100 μM (σ) BO-653, 80 μM probucol (▽), 40 μM BHT (□) or 100 μM ascorbic acid (◆) at 37°C for up to 24 h in the dark, in the absence of cells. Results are expressed in % as the ratio of absorbance at 450 nm at the indicated time point/absorbance at 450 nm at the 0h-time point and each data point represents the mean ± SD of eight replicate wells.

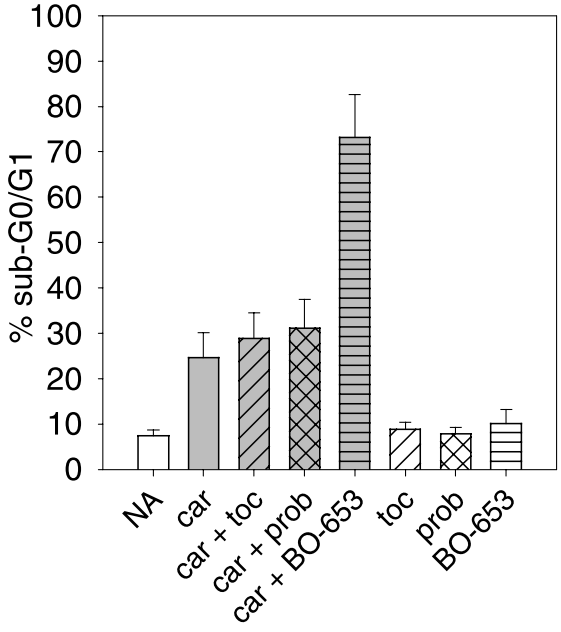

FIGURE 6 Effect of antioxidants on β-carotene-induced apoptosis in Jurkat cells. The proportion of sub-G_0/G_1 cells was assessed by flow cytometry. Cells were incubated for 6 h with 12.5 mg/l fresh β-carotene (car), in the absence or presence of 200 μM α-tocopherol (toc), 80 μM probucol (prob) or 100 μM BO-653. Controls shown were no additions (NA), and individual antioxidants (toc, prob, BO-653) in the absence of β-carotene. Additional controls were 0.5% THF (8.66 ± 1.76% sub-G_0/G_1), 5 μM etoposide (24.53 ± 5.4% sub-G_0/G_1), 0.5% EtOH (7.54 ± 1.46% sub-G_0/G_1) and 0.5% THF + 0.5% EtOH (14.4 ± 6.11% sub-G_0/G_1). Results represent the mean ± SD of three experiments performed in triplicate ($n = 9$). Apoptosis induced by β-carotene + BO-653 ($p = 0.001$) and by β-carotene + probucol ($p = 0.05$) was significantly different from that induced by β-carotene alone.

was non-significant for the former and significant for the latter. A dramatic increase to 73.1% hypodiploid cells was observed for BO-653 in combination with β-carotene. The effect of the combined treatment was considerably higher than a simple additive effect of the two compounds alone.

We also examined the effect of 20 μM z-VAD-fmk, a general caspase inhibitor, on β-carotene-induced apoptosis (Table I). After 6 h with 12.5 mg/l β-carotene, z-VAD-fmk significantly reduced the proportion of hypodiploid cells from 14.63 to 6.74%, which was near background levels seen with the vehicle control. In contrast, the percentage of nuclei with condensed chromatin induced by 12.5 mg/l β-carotene was increased by z-VAD-fmk (Table I).

DISCUSSION

This study shows that five out of six carotenoids tested induce apoptosis in the human leukaemic T-lymphoblast cell line Jurkat E6.1. At a concentration of 20 μM, the order of potency to induce apoptosis after 24 h was: β-carotene > lycopene > lutein > β-cryptoxanthin = zeaxanthin. Canthaxanthin did not induce apoptosis under these conditions. β-Carotene induced apoptosis in a time- and concentration-dependent manner. Some variation in the degree of β-carotene-induced apoptosis was observed between different sets of experiments, which was probably due to variations in cell growth and density of Jurkat stock cultures prior to plating. After 24 h, the lowest effective concentration of β-carotene to induce apoptosis, as judged by both microscopy and flow cytometry, was 1.56 mg/l (~3 μM). Apoptosis estimates obtained by flow cytometry were always lower than those obtained by fluorescence microscopy, but both methods showed similar trends except at extremely high concentrations of β-carotene. In Jurkat cells, degradation of genomic DNA and nuclear morphological changes during apoptosis are controlled independently.[32] The lack of DNA degradation in the presence of chromatin condensation at 50 mg/l β-carotene may indicate that Jurkat cells were ultimately undergoing necrosis at these extreme concentrations. The mode of cell death at lower concentrations of β-carotene was confirmed as apoptosis as the cells showed chromatin condensation and nuclear fragmentation by light and electron microscopy, DNA degradation by flow

cytometry and PARP cleavage and caspase-3 activation by Western blot analysis. DNA degradation was inhibited by the general caspase inhibitor z-VAD-fmk, whereas chromatin condensation was not, indicating the action of a caspase-independent mediator of chromatin condensation, such as apoptosis-inducing factor (AIF).[33] Extensive degradation of β-carotene for 48–72 h destroyed its pro-apoptotic potency almost completely, whereas mild degradation for 6 h or less did not, indicating that either β-carotene itself or an early degradation product of β-carotene, or both, are the active compounds. The antioxidant BO-653 at a concentration of 100 μM inhibited the degradation of β-carotene *in vitro* and significantly increased its cytotoxicity, again pointing to β-carotene itself and/or to an early degradation product as the death-inducing molecules.

Concentrations of Carotenoids *In Vivo*

The first question which arises is, whether these carotenoid concentrations reflect those observed *in vivo*. Several studies have measured the plasma concentrations of carotenoids in normal healthy populations,[34–36] in subjects on a fruit- and vegetable-rich diet[37] and after β-carotene supplementation.[38,39] In unsupplemented subjects, the mean plasma concentrations for β-carotene, lycopene, lutein, β-cryptoxanthin and zeaxanthin were 0.87, 0.66, 0.4, 0.23 and 0.09 μM, respectively, and β-carotene at the top of its range could reach concentrations of 2.2 μM.[36] After supplementation, β-carotene in plasma can reach concentrations of up to 6.45 μM.[39] Therefore, the top concentrations used in our study exceed those seen *in vivo* even after supplementation, whereas the lowest effective concentrations used (~3 μM) may be reached *in vivo* at least in supplemented subjects. However, higher carotenoid concentrations should not be considered as irrelevant for the *in vivo* situation for several reasons. Firstly, carotenoids may act synergistically and total carotenoid concentrations in plasma or tissue may be more relevant than the concentration of a single carotenoid. Secondly, plasma concentrations of carotenoids may not necessarily reflect tissue concentrations, as cells and tissues can accumulate carotenoids to various degrees.[40] In human liver for instance total carotenoids can reach concentrations of up to 77 nmol/g,[41] which corresponds to approximately 77 μM. Also, carotenoid concentrations in the lumen of the gastrointestinal tract prior to absorption may be much higher than in plasma.[42] Thirdly, cells and tissues *in vivo* may be exposed to carotenoids for a long time, whereas *in vitro* experiments are often performed over short time periods.

Induction of Apoptosis by Carotenoids

The cytotoxic effect of carotenoids has been reported before. β-Carotene induces apoptosis in several human cancer cell lines,[43] in human adenocarcinoma cells[44] and in human cervical dysplasia-derived cell lines.[45] Canthaxanthin induced apoptosis in human colon adenocarcinoma and melanoma cells[46] and lutein induced apoptosis in transformed but not normal mammary epithelium.[47] This study shows that lycopene and, to a lesser extent, zeaxanthin and β-cryptoxanthin can induce apoptosis in Jurkat cells. The mechanism(s) by which carotenoids induce apoptosis are only starting to be elucidated.

Induction of Apoptosis by Pro-oxidant Activity

Carotenoids may induce apoptosis by their pro-oxidant activity, especially at high carotenoid concentrations and at high oxygen partial pressures.[16–18] In human colon adenocarcinoma cells, 50 μM β-carotene inhibited cell growth, induced apoptosis and down-regulated Bcl-2 expression, associated with an increased production in intracellular reactive oxygen species (ROS). Addition of 10 μM α-tocopherol inhibited the rise in intracellular ROS and also apoptosis.[44] In contrast, β-carotene-induced apoptosis observed in our study is probably not caused by a pro-oxidant effect, as an increase in apoptosis was observed in the presence of the antioxidants probucol and BO-653. Furthermore, we used carotenoids at a concentration of 20 μM, a concentration at which β-carotene did not increase intracellular ROS.[44] β-Carotene in our study was still pro-apoptotic at 3 μM, a concentration at which pro-oxidant activity appears even less likely. A high concentration of α-tocopherol failed to protect colon cancer cells from β-carotene-induced apoptosis,[43] which is thought to be due to a pro-oxidant activity of α-tocopherol itself at this concentration.[44] Recently, ascorbate was shown to have a pro-oxidant effect in cell culture media, partly by the production of hydrogen peroxide, which could cause toxicity to cells in culture.[30,31] The exacerbation of β-carotene-induced apoptosis by 100 μM BO-653 observed in our study is unlikely to be caused by a pro-oxidant effect, as BO-653 effectively inhibits cell-mediated oxidation of low density lipoprotein at this concentration,[48] but rather by the inhibition of β-carotene degradation, which would otherwise destroy its pro-apoptotic activity.

Induction of Apoptosis Via RAR/RXR Receptors

A second pathway by which carotenoids could induce apoptosis is via the formation of retinoic acid. Pro-vitamin A carotenoids, such as β-carotene and

β-cryptoxanthin, can be metabolized by cells to retinol (vitamin A) and subsequently to retinoic acid.[49] Retinoic acid can induce or suppress apoptosis depending on the cell type and the apoptotic stimulus via the retinoid receptors RXR and RAR, and also by nuclear receptor-independent mechanisms.[50,51] Furthermore, retinoids are effective against many types of cancer in humans and may constitute new compounds for chemoprevention.[52,53] On the other hand, non-provitamin A carotenoids, e.g. canthaxanthin and lutein, can also induce apoptosis of cells in culture.[46,47] The non-provitamin A carotenoids lycopene, lutein and zeaxanthin also induced apoptosis in our study in Jurkat cells. Furthermore, non-provitamin A carotenoids are also protective against cancer *in vivo*.[54,55] However, "retinoic acid-like" substances can be formed from non-provitamin A carotenoids via oxidation. Oxidation of canthaxanthin can produce 4-oxo-retinoic acid, which promotes differentiation of embryonal carcinoma cells and enhances gap junctional communication via RAR-β.[56,57] Auto-oxidation of lycopene produced a range of compounds, including acycloretinal and apo-lycopenals.[58] Acycloretinal was converted by microsomal enzymes to acycloretinoic acid,[58] which also enhances gap junctional communication and activates the RAR-β2 promoter.[59] Carotenoids might therefore induce apoptosis via the formation of retinoic acid or retinoic acid analogues independent of their provitamin A status.

Induction of Apoptosis by Modulating Cyclo-oxygenase-2 Activity

Another possibility of apoptosis induction by carotenoids is by modulating the activity of the inducible cyclo-oxygenase enzyme, COX-2. COX-2 is overexpressed in a number of tumour types and may be a target for chemoprevention.[60–62] Exactly how COX-2 overexpression promotes tumourigenesis is unclear, but suppression of apoptosis is one option.[63,64] Provitamin A carotenoids can decrease COX-2 activity via formation of retinoids.[65,66] A preliminary report indicates that lycopene can down-regulate COX-2 mRNA expression accompanied by an increase in apoptosis.[67] The apoptosis-inducing effect of carotenoids in our study could be partially mediated by an inhibition of COX-2 as Jurkat cells express this enzyme.[68]

Induction of Apoptosis by Other Mechanisms

Other mechanisms by which carotenoids could induce apoptosis include DNA damage. *In vitro*, β-carotene and lycopene protected various cell types against oxidative DNA damage at low concentrations (1–3 μM).[69,70] However, at higher concentrations (4–10 μM), the carotenoids lost their protective activity and actually led to an increase in oxidative DNA damage,[70] which could then signal apoptosis. Lycopene and β-carotene, pre-oxidized by heat treatment in air, led to oxidative DNA damage both in calf thymus DNA and in human foreskin fibroblasts at concentrations of 20–40 μM.[71] Concomitantly, fibroblast viability was significantly reduced in response to the pre-oxidized carotenoids, and cell death was probably by apoptosis.[71] In contrast to our study in Jurkat cells, unoxidized lycopene and β-carotene were not toxic to foreskin fibroblasts.[71] This variation in toxicity may be due to differences in cell types or different pre-conditioning treatments.

In human cervical dysplasia-derived cell lines β-carotene induced apoptosis via the down-regulation of the epidermal growth factor (EGF) receptor.[45] Both lutein and all-trans retinoic acid induced apoptosis in transformed but not normal mammary epithelium.[47] Furthermore, both compounds protected normal but not transformed cells from the apoptosis-inducing effect of etoposide and cisplatin. This effect was associated with an increase in the Bcl-xL: Bax ratio in normal but not transformed cells.[47] Recently, carotenoids have also been shown to interact with key proteins controlling cell cycle progression. In normal human fibroblasts, β-carotene induced a cell cycle delay in the G_1 phase independent of its provitamin A activity. The G_1 arrest was mediated by an increase in the cyclin-dependent kinase inhibitor $p21^{waf1/cip1}$[72] In breast and endometrial carcinoma cells, lycopene at a concentration of 2–3 μM inhibited G_1/S transition by down-regulation of cyclin D and retention of p27 in cyclin E-cdk2 complexes, leading to inhibition of G_1 cyclin-dependent kinase activities.[73] Neither of these studies investigated apoptosis. However, in some circumstances cell cycle arrest may trigger apoptosis in malignant cells.[74]

Pro-carcinogenic Activity of Carotenoids

The selective induction of apoptosis in transformed cells by carotenoids *in vivo* could explain the protective effect of fruit- and vegetable-rich diets against cancer observed in epidemiological studies. However, how can the pro-carcinogenic effect of β-carotene supplementation be explained in this context? Since carcinogenesis is a multi-step process, carotenoids may only induce apoptosis at early stages of neoplastic transformation, when cells are still able to undergo apoptosis. At later stages, when apoptotic pathways in transformed cells may be abrogated, carotenoids may be unable to induce apoptosis. Any pro-carcinogenic properties of carotenoids may then accelerate carcinogenesis of pre-existing transformed cells. However, the increased

incidence of lung cancer and mortality in heavy smokers and asbestos workers after β-carotene supplementation has also been explained in other ways. High oxygen partial pressures may reduce or abrogate the antioxidant efficacy of β-carotene in the lung[18] and may lead to increased formation of β-carotene oxidation products. Oxidation products of β-carotene may have pro-carcinogenic activity,[26] may cause direct DNA damage,[71] and can also increase the binding of benzo[a]pyrene, one of the major carcinogens in tobacco smoke, to DNA *in vitro*.[75] This pro-carcinogenic activity of β-carotene oxidation products may be exacerbated by lower vitamin C and E levels in plasma of smokers, which are thought to restore unoxidized β-carotene levels *in vivo*.[26] In animal models, β-carotene supplementation led to an upregulation of carcinogen-metabolizing enzymes (P450 isoforms), which in humans would increase the cancer risk from bioactivated tobacco-smoke carcinogens.[76] The pro-oxidant environment in the lungs of smokers may also destroy the pro-apoptotic effect of carotenoids in favour of the pro-carcinogenic properties of its oxidation products.

CONCLUSIONS

Irrespective of the signalling pathway, the selective induction of apoptosis in transformed cells may be a mechanism by which carotenoids could exert their anti-carcinogenic effect in humans, in addition to their antioxidant and growth-inhibitory properties. This study also highlights the need for further study to elucidate the various effects of carotenoids in human cells and tissues. Carotenoid metabolism and accumulation in certain tissues, their conversion to retinoic acid or retinoic acid analogues and the interaction of carotenoids, their metabolites and oxidation products with RAR/RXR receptors, COX-2 and key control elements of the cell cycle would be of particular interest.

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