

Carotenoids Normally Present in Serum Inhibit Proliferation and Induce Differentiation of a Human Monocyte/Macrophage Cell Line (U937)¹

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ABSTRACT Carotenoids, plant pigments with potent antioxidant activity, are implicated in chronic disease protection. They are absorbed from the diet and transported by plasma lipoproteins. Monocytes, as circulating blood cells, are exposed to carotenoid-rich lipoproteins. Such exposure may lead to enrichment with carotenoids and may affect the functions of monocyte-derived macrophages. This study explored the effect of cellular enrichment *in vitro* with β -carotene, lycopene, or lutein on monocyte/macrophage function, using U937 cells as a model. Cell proliferation, production of reactive oxygen species, and cell-substrate adhesion were examined. Maximal carotenoid levels in medium supplemented with preenriched human serum were 2–8 $\mu\text{mol/L}$; incubation for 1–6 d resulted in 0.2–1.1 nmol carotenoid/mg cell protein (0.25–1 nmol/ 10^6 cells), \sim 10-fold more than that reported in normal tissue *in vivo* but within the range that might be anticipated with dietary supplementation. β -Carotene, lycopene, and lutein markedly inhibited the proliferation of U937 cells, to an extent similar to or greater than that due to phorbol myristic acetate, a known differentiation/activation agent. Lycopene, but not β -carotene or lutein, caused a significant increase in reactive oxygen species, indicating the induction of cell differentiation. Adhesion and LDL oxidation were unaffected. Thus, cellular carotenoids inhibit proliferation, and for lycopene at least, this may involve cell differentiation. The effectiveness of lycopene, a nonprovitamin A carotenoid, is consistent with a vitamin A-independent pathway modulating cell function. *J. Nutr.* 135: 160–164, 2005.

KEY WORDS: • human • carotenoids • monocytes/macrophages • U937 cells • differentiation

Carotenoids are plant pigments responsible for the color observed in many fruits and vegetables as well as the natural colors seen in selected animal species (1). Humans, incapable of synthesizing carotenoids, obtain them by eating foods rich in these compounds (1–3). The predominant carotenoids found in human plasma are β -carotene, lycopene, and lutein, and their concentrations vary from 0 to 8 $\mu\text{mol/L}$ depending upon dietary intake (4). They are transported in plasma exclusively by lipoproteins; the nonpolar carotenoids, β -carotene and lycopene, are associated primarily with LDL, whereas the xanthophylls, including lutein, are more equally distributed between LDL and HDL (5–8).

Carotenoids, collectively or individually, have been studied with respect to chronic diseases such as cancer and atherosclerosis because of the association between increased intake of fruits and vegetables rich in carotenoids and decreased incidence of disease (9–12). For example, in a large clinical study in Linxian, China, supplementation with β -carotene and vitamin E decreased the incidence of cancer by 13%; however, the precise role of β -carotene was unclear because of the cotreatment with vitamin E (10). Other studies implicated lycopene in the prevention of prostate cancer (11,12) and lutein and zeaxanthin in protection from age-related macular degeneration (13–15).

Antioxidant activity is one of the mechanisms proposed to explain the putative protective role of carotenoids observed in chronic disease. With their highly conjugated structures, carotenoids can quench reactive oxygen species, scavenge free radicals or terminate lipid peroxidation reactions effectively (16,17). Because LDL oxidation is thought to play a critical role in the development of atherosclerosis, antioxidant activity may slow or prevent this disease (18,19). Several studies, including our own, suggested that LDL oxidation can be limited by the bulk antioxidant action of carotenoids (20–22). Cellular enrichment with carotenoids, however, did not alter cell-mediated LDL oxidation (23) although it does modulate the expression of a number of cellular proteins including those involved in cholesterol metabolism (24), gap junctions (25), and cell adhesion (26,27).

Inflammatory responses are thought to play an important role in chronic diseases. Monocytes/macrophages are an integral and essential part of the immune responses in inflammation, acting as antigen-presenting cells to cytotoxic T lymphocytes (28,29) as well as providing phagocytic and cytotoxic activity for removal of infective organisms or toxic substances (30–32). Supplementation studies have suggested that carotenoids (including β -carotene, lycopene, and lutein) can enhance immune function *in vivo* (26,29).

Circulating monocytes are precursors of tissue macrophages. Differentiation involves loss of proliferative activity and the development of specific attributes; for monocyte-

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derived macrophages, these include recognition of tumor cells, direct killing by release of lysosomal enzymes, or release of cytokines (28,31,32). Thus, agents that induce monocyte-macrophage differentiation could be considered cancer preventative.

Given the exposure of circulating monocytes to potentially high plasma concentrations of carotenoids and the putative involvement of monocytes/macrophages in aspects of chronic disease, a monocytic cell line is a reasonable model in which to examine potential carotenoid-induced modulation of cellular responses. U937 cells, a human promonocytic leukemic line, were used because, like monocytes, they are nonadherent until differentiated, and they are capable of generating reactive oxygen and oxidizing LDL upon challenge (33). Three aspects of cell function related to differentiation were examined: proliferation, production of reactive oxygen species as measured by nitroblue tetrazolium (NBT)³ reduction and LDL oxidation, and cell-substrate adhesion.

MATERIALS AND METHODS

Materials. β -Carotene, lycopene, and lutein were all purchased from Sigma, with purity verified by UV-visible (UV-VIS) spectroscopy and HPLC. Phorbol 12-myristate 13-acetate (PMA), NBT, zymosan A, dimethyl sulfoxide (DMSO), human serum, and assay chemicals were also obtained from Sigma. The U937 cell line was purchased from American Tissue Type Collection. RPMI 1640 and HBSS were obtained from Mediatech, fetal bovine serum (FBS) from Atlanta Biological, and tetrahydrofuran (THF; 0.025% BHT) from Fisher. The CyQUANT-GR[®] assay kit was purchased from Molecular Probes. LDL was purchased from Sigma or isolated from fresh plasma (Children's Hospital of Philadelphia) and used as described previously (20,21).

Cell culture and enrichment with carotenoids. U937 cells were maintained under standard conditions (33) and routinely subcultured (1:6) 2 times/wk.

Heat-inactivated human serum, preenriched with specific carotenoids, was used as a biological source of carotenoids for cellular enrichment. Preenrichment of serum was achieved by the addition of stock solutions (1 mmol/L, stored at -70°C in the dark and under nitrogen to prevent degradation) in either THF or absolute ethanol to human serum (1% v:v) with mixing, then incubated in the dark at 37°C for 24 h. Control incubations were done with solvent alone. Any aggregates were removed by filtration through a $0.45\ \mu\text{m}$ filter before mixing serum with medium and incubation with cells. In preliminary toxicity studies, no significant reduction in cell viability, as measured by trypan blue exclusion, was observed for up to 50% (v:v) serum and solvent (THF:ethanol) concentrations up to 0.5% (v:v) (data not shown). Thus, all experiments used a 1:1 (v:v) mix of serum with tissue culture media for enrichment of cells. Final carotenoid levels achieved in enrichment media ranged from 2 to $6\ \mu\text{mol/L}$, and were comparable from experiment to experiment for each specific carotenoid. Endogenous carotenoid concentrations (in 1:1 control medium) ranged from 0.08–0.10 $\mu\text{mol/L}$.

The carotenoid content in cells and media was measured by UV-VIS spectroscopy (model U-3110, double beam, Hitachi) and/or HPLC according to Barua et al. (34,35). The following extinction coefficients ($E_{1\ \text{cm}}^{1\%}$) for individual carotenoids were used to calculate concentrations using spectroscopy: 2592 for β -carotene at 452 nm, 3450 for lycopene 472 nm, and 2550 for lutein at 445 nm (35). In control studies both methods yielded comparable results.

Experimental protocols and measures. For adhesion and LDL oxidation studies, cells were preincubated with carotenoids for 24 h. For studies examining proliferation and NBT reduction, cells were continuously incubated with carotenoids for up to 144 h, based on

preliminary growth curve studies. Carotenoid concentrations in media remained stable throughout (data not shown). Cell carotenoid levels after incubation as well as cell protein concentrations were measured in samples pooled from 1 to 6 wells of a 24-well plate incubated in parallel.

Cell number was determined by nucleic acid dye binding (CyQUANT-GR cell proliferation assay kit, Molecular Probes). U937 cells seeded in 96-well plates (450 or 4500 cells/well) were incubated in the dark at 37°C with or without carotenoids, or PMA (5 nmol/L) as a positive control (36). Cell number was analyzed at selected time points (0, 48, 96, 144 h) using a plate reader (model HTS 7000 series, Perkin Elmer), according to kit instructions, from a standard curve run in each assay.

NBT reduction was assayed colorimetrically (37,38) in U937 cells cultured in 24-well plates ($1-2 \times 10^5$ cells/well) for 5–6 d with or without carotenoids, then challenged with PMA (200 $\mu\text{g/L}$) in the presence of NBT (5 g/L) in RPMI without phenol red for 30 min at 37°C . Cellular formazan deposits were solubilized in DMSO and quantitated at 570 nm.

For LDL oxidation, U937 cells in 24-well plates at (5.0×10^5 cells/well) were preincubated at 37°C overnight with or without carotenoids, then incubated with LDL (0.3 mg protein/mL in serum-free RPMI, 1 mL/well) and opsonized zymosan (3.0 g/L, after opsonization with human serum for 30 min at 37°C in the dark). Cell-free incubations with or without CuSO_4 (10 $\mu\text{mol/L}$) served as positive and background controls, respectively. Lipid hydroperoxides were assayed with ferrous oxidation:xylenol orange (38) as in our previous studies (20,21).

Cell-substrate adhesion was measured by plating cells preincubated for 24 h with or without carotenoids in 96-well plates (10,000 cells/well), incubating for 10 min before inverting and washing (4 times with RPMI), and enumeration of attached cells by CyQuant. PMA (120 nmol/L) addition served as a positive control, and background adhesion (0–2.0% in all groups) was determined by immediate washing.

Statistical analysis. Data were analyzed by 1-way ANOVA to determine whether there were any significant differences among the groups in each experiment, followed by the Tukey-Kramer pairwise multiple comparison test to determine the significance of differences among groups. Data are expressed as means \pm SEM from multiple independent experiments, each performed in triplicate or quadruplicate wells. Differences were considered significant at $P < 0.05$.

RESULTS

Cellular enrichment with carotenoids. In vitro preenrichment of human serum with a specific carotenoid effectively increased serum concentration of that carotenoid (Table 1). Attempts to achieve greater enrichments by the addition of a higher stock concentration yielded large variations in serum carotenoid concentrations achieved after incubation and filtration (data not shown). Given the greater consistency in

TABLE 1

Preenrichment of human serum with a specific carotenoid by addition of carotenoid in solvent (1% v:v) to serum

Carotenoid (solvent)	Stock concentration	Serum concentration ¹
	mmol/L	$\mu\text{mol/L}$
β -Carotene (THF)	1.0	17.5 ± 1.9
	4.0	17.0 ± 12.1
Lycopene (THF)	1.0	13.4 ± 1.3
	4.0	7.2 ± 5.6
Lutein (ethanol)	1.0	12.4 ± 1.6
	3.0	19.0 ± 17.6

¹ Values are means \pm SEM, $n = 3$ independent experiments.

³ Abbreviations used: DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; NBT, nitroblue tetrazolium; PMA, phorbol 12-myristate 13-acetate; THF, tetrahydrofuran; UV-VIS, UV-visible.

media enrichment using 1 mmol/L stock solutions, all cell enrichments were done in this manner. Incubation of U937 with media containing the preenriched serum for 24 h resulted in cellular enrichment (Table 2). No further cellular enrichment with carotenoids was achieved after 24 h (data not shown). Cell viability, assessed both pre- and postexposure to carotenoids, was $\geq 90\%$.

Cell proliferation. Control cells, plated at low density, proliferated 2000% over the 6-d period (Fig. 1, upper panel). PMA, a known inducer of monocyte differentiation, limited this proliferation to 400%. Each of the 3 carotenoids, β -carotene, lycopene and lutein, also limited cell proliferation, with lycopene being the most effective. That this was inhibition of proliferation, not toxicity, was evidenced by the $>95\%$ cell viability at all time points and the absence of a decline in cell number relative to the start. Because cell proliferation depends in part on plating density, we also examined the effect of increasing cell density on the ability of carotenoids to inhibit cell proliferation. At a high plating density, control cells proliferated to a lesser extent (700%) over 6 d (Fig. 1, lower panel). Proliferation of PMA-treated cells was inhibited to $\sim 500\%$ (a 30% inhibition of proliferation). Cell proliferation under these conditions was again inhibited by each of the carotenoids, and this difference was detectable earlier in the time course (after 2 d) than when cells were plated at low density. Under these conditions, however, the inhibition of proliferation in the presence of lutein was significant only at d 2.

Effect of carotenoids on NBT reduction. To explore the link between inhibition of proliferation and differentiation, the ability of cells to reduce NBT, a commonly used index of cell differentiation reflecting intracellular reactive oxygen production, was measured. As expected, the ability of cells to reduce NBT was markedly increased (120%) in the presence of PMA compared with the control (Fig. 2). Moreover, lycopene enrichment of cells significantly increased NBT reduction compared with control (90%). β -Carotene and lutein tended to increase NBT reduction by $\sim 30\%$ ($P = 0.10$) and 10% ($P > 0.10$), respectively.

LDL oxidation and cell substrate adhesion. The ability of U937 cells to mediate LDL oxidation in response to an activator (opsonized zymosan) was measured as an index of released reactive oxygen species. Cells (5.0×10^8 cells/L) in 24-well plates, incubated with or without carotenoids and then activated, oxidized the LDL in the media over 24 h, as seen by increased lipid hydroperoxide content (to ~ 160 nmol/mg protein) but preenrichment of cells with carotenoids had no effect (data not shown).

Because differentiation of U937 cells can enhance cell adhesion, we also examined the effect of preenrichment of

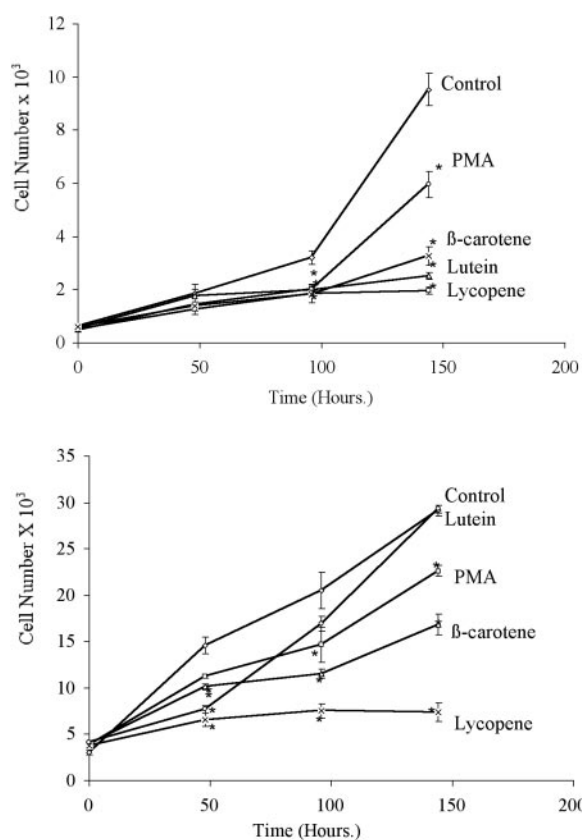


FIGURE 1 U937 cell proliferation at low (upper panel) and high plating density (lower panel) when incubated with PMA (5 nmol/L; positive control), β -carotene (3.8 μ mol/L), lycopene (2.5 μ mol/L), lutein (5.8 μ mol/L), or vehicle alone (control). Values are means \pm SEM, $n = 3$ independent experiments. *Different from control, $P < 0.05$.

U937 cells with carotenoids on adherence to tissue culture plastic as an index of any changes in U937 cell "stickiness." PMA, a differentiation and activation agent, increased the adherence of cells to plastic to $25 \pm 2.5\%$, whereas carotenoid-enriched cell-substrate adherence remained at $\sim 3\%$, basically at background levels.

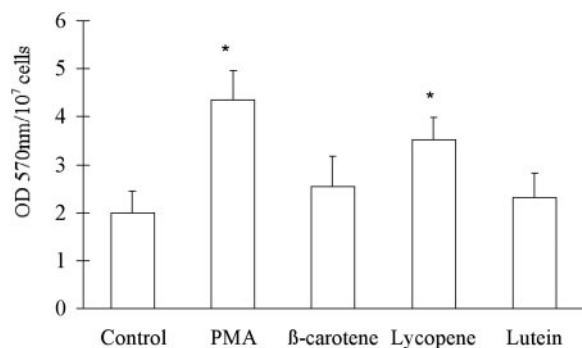


FIGURE 2 NBT reduction in U937 cells incubated with PMA (5 nmol/L; positive control), β -carotene (3.8 μ mol/L), lycopene (2.5 μ mol/L), lutein (5.8 μ mol/L), or vehicle alone (control). Values are means \pm SEM, $n = 3$ independent experiments. *Different from control, $P < 0.05$.

TABLE 2

Carotenoid levels in U937 cells after 24 h exposure to media preenriched with specific carotenoids¹

Carotenoid	Media concentration	Cell-associated carotenoid
	μ mol/L	nmol/mg cell protein
β -Carotene	8.7 \pm 0.95	0.75 \pm 0.20
Lycopene	6.7 \pm 0.65	0.77 \pm 0.11
Lutein	6.2 \pm 0.82	0.59 \pm 0.15
None (control)	0.09 \pm 0.05	Not detectable

¹ Values are means \pm SEM, $n = 4$ independent experiments.

DISCUSSION

The observation that exposure to serum enriched with a single carotenoid, β -carotene, lycopene, or lutein, was sufficient to inhibit U937 cell proliferation, and in the case of lycopene, to enhance generation of reactive oxygen species, suggests that carotenoids can influence monocyte-macrophage differentiation. Moreover, given the effectiveness of lycopene, a nonprovitamin A carotenoid, in both inhibiting proliferation and enhancing reactive oxygen production, this influence is independent of vitamin A or retinoic acid-mediated mechanisms.

The cellular enrichment with specific carotenoids achieved here (i.e., 0.19–1.1 nmol/mg cell protein or 0.25–1 nmol/10⁶ cells) is comparable to that found in limited reports in the literature. For example, the addition of carotenoids in THF to FBS-containing culture media reportedly resulted in cellular contents of 0.2–0.9 nmol/10⁶ cells for a mouse embryonic cell line (39), 0.004 nmol/10⁶ cells for a human mammary epithelial cell line (40), and 0.4–3.6 nmol/mg cell protein for J774 macrophages (24). However, this pre-enrichment of serum followed by dilution into media may be somewhat more “physiologic” than solvent addition to media in that cells are exposed only to those carotenoids associated with serum lipoproteins.

A direct comparison of these levels of enrichment to in vivo tissue levels is problematic because of a scarcity of information and differences in how concentrations are normalized in vitro and in vivo. It is of interest to note that tissue levels of 0.98 and 0.45 nmol β -carotene/g wet tissue for normal breast and adipose tissue, respectively, were reported (41). These concentrations are 5–20% of what was achieved by in vitro cell enrichment even assuming that cell protein constitutes ~1% of total wet weight. Levy et al. (23) demonstrated that in vivo enrichment of monocytic cells is plausible because β -carotene supplementation produced an ~2-fold enrichment of human peripheral blood monocyte-derived macrophages for ~3-fold enrichment of plasma LDL. Because dietary supplementation with carotenoids can increase LDL content up to 12-fold (20), it is possible that the in vitro enrichments achieved here serve as a model of the cellular levels that might be expected with supplementation.

The inhibition of cellular proliferation by specific carotenoids was reported previously. For example, lycopene effectively inhibited proliferation in several cancer cell lines including human endometrial, mammary, and lung cancers (42). Both β -carotene and lutein inhibited proliferation of HL-60 cells (43) and human aortic smooth muscle cells (44). In general, these reflect inhibition of proliferation at somewhat higher media concentrations than those used here and without other changes in cell characteristics.

In these studies, we observed a difference in timing and the degree of inhibition of cell proliferation induced by the carotenoids based on initial cell plating density. The earlier and more marked detection of carotenoid-mediated inhibition of proliferation for cells plated at high density most likely reflects a greater proportion of cells entering log phase growth simultaneously in the presence of carotenoid and may warrant further investigation.

The distinction in action between PMA, a known differentiation agent and activator, and that of the carotenoids, especially in the adhesion assay, is worthy of consideration. It is unclear whether there is a difference in potency, or stage of interaction, or response measurement that allows PMA to markedly decrease proliferation, increase reactive oxygen production, and increase cell-substrate adhesion whereas carot-

enoids affect only proliferation and reactive oxygen production. This difference may warrant further study.

In summary, these studies suggest that serum carotenoids can effectively enrich monocyte/macrophage-like cells and, in doing so, inhibit their proliferation. Lycopene appears to be most effective, with β -carotene and lutein exhibiting somewhat lesser effects. The effectiveness of lycopene suggests that the changes in cell function seen here are independent of the vitamin A-retinoic acid pathway. Further elaboration of the sequence of events involved in U937 cell proliferation and differentiation and the effect of carotenoids during crucial elements of that sequence is required to understand fully the potential effect of carotenoids on monocyte differentiation.

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