Dietary Lutein But Not Astaxanthin or β-Carotene Increases pim-1 Gene Expression in Murine Lymphocytes

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Abstract: This study investigates the effect of dietary carotenoids on pim-1 gene expression in mouse splenocytes. Female BALB/c mice were fed 0%, 0.02%, or 0.4% astaxanthin, β-carotene, and lutein for two weeks. Plasma and liver were obtained for the analysis of carotenoids. Splenocytes were isolated and cultured in the presence of concanavalin A, and the level of pim-1 mRNA was determined by Northern blot analysis. None of the carotenoids were detectable in the plasma and liver of unsupplemented mice. In plasma, the concentration of astaxanthin (4.9–54.7 μmol/l) was dramatically higher than that of lutein (1.4–2.0 μmol/l) and β-carotene (0.1–0.7 μmol/l). Carotenoid uptake by the spleen but not the liver reflected that observed in plasma. In mice fed 0.4% of each carotenoid, the absolute concentration of the carotenoid in the liver was highest for astaxanthin (24 nmol/g) followed by β-carotene (7.5 nmol/g) and lutein (1.58 nmol/g). Mice fed lutein showed a dose-related increase in pim-1 mRNA expression. The steady-state level of pim-1 mRNA in mice fed 0.4% lutein was sixfold higher than in mice fed 0.02% lutein. In contrast, dietary astaxanthin and β-carotene did not affect pim-1 expression. Therefore, an increase in pim-1 mRNA was observed in splenocytes stimulated with concanavalin A in lutein-fed mice. This appears to be a unique effect of lutein and may be associated with its antitumor activity observed in vivo.

Introduction

Numerous epidemiological studies have demonstrated a negative correlation between high intakes of carotenoid-rich foods and the occurrence of major clinical diseases such as cancer, cardiovascular disease, and macular degeneration (1–3). However, recent studies on the anticancer activity of β-carotene have contradicted these observations. For instance, two epidemiological studies (4,5) failed to show an inverse relationship between carotenoid intake and breast cancer. β-Carotene supplementation was found to have no effect on the incidence of cancer and heart disease (6). Furthermore, β-carotene supplementation to a high-risk population of heavy smokers in southwestern Finland did not reduce the incidence of prostate, bladder, colorectal, or stomach cancers; instead, β-carotene supplementation increased lung cancer incidence by 18% (7). In recent years, encouraging results on the anticancer activities of carotenoids other than β-carotene have begun to emerge. For instance, carotenoids such as astaxanthin (8), canthaxanthin (8,9), lycopene (10,11), and lutein (12,13) have been shown to possess anticancer activity.

The mechanism by which carotenoids inhibit cancer is not clear. However, some proposed mechanisms include their role as antioxidants (14), in modulating immune function (8,15–17) and in increasing gap-junctional communication (18–20). Also, carotenoids have been shown to regulate gene expression, for example, in modulating mutagenesis in Salmonella typhimurium (21,22) and in increasing connexin 43 gene expression in C3H10T1/2 cells (18–20). That carotenoids may regulate immunity and carcinogenesis by modulating gene expression has recently gained greater acceptance. No data are available to demonstrate a possible direct effect of carotenoids in modulating the protein and mRNA expression of certain genes in immune cells.

The pim-1 gene was first identified in induced T cell lymphomas as a proviral integration site of Moloney’s murine leukemia virus (23) and thus was designated pim-1. It is involved in T cell transformation and is expressed as an immediate early gene in normal lymphocytes activated by mitogens, certain cytokines, and antigens (24–27). The expressed pim-1 gene codes for a highly conserved cytoplasmic serine/threonine kinase. The pim-1 kinase may play a role in the downstream signal transduction pathways associated with hematopoietic cells' activation, such as proliferation, differentiation, and apoptosis (28–33). The pim-1 gene may cooperate with the c-myc gene in regulating apoptotic cell death (28); however, the pim-1 protein also is implicated in lymphoid tumorigenesis with other protooncogene protein products.

Overall, carotenoids may be involved in gene activation and DNA repair in lymphocytes and, thereby, modulate immunity. Certain carotenoids are taken up in substantial quantities into the nucleus of blood lymphocytes, where they
may augment lymphocyte activation (15,34). Therefore, the pim-1 gene, being involved in early activation of T cells and other lineage cells, may be a good model for exploring carotenoid actions on gene expression by lymphocytes. Our objective is to determine whether astaxanthin, β-carotene, and lutein when fed to BALB/c mice can influence pim-1 gene expression in concanavalin A-stimulated splenocytes.

Methods

Animals and Diet

Eight-week-old female BALB/c mice obtained from our mouse breeding colony were fed a semipurified diet (35) containing 0%, 0.02%, or 0.4% astaxanthin (Hoffmann-La Roche, Nutley, NJ), β-carotene (BASF, Ludwigshafen, Germany), or lutein (INEXA, Industria Extractora, Quito, Ecuador). Carotenoids were mixed with the oil portion (preheated to 60°C) of the diet before being incorporated into the rest of the dietary ingredients. The prepared diets were sealed and stored at 4°C until used. At weekly intervals, the diets were solidified with 4% agar solution to reduce food wastage and improve intake measurements (35). Mice were housed in a light- and temperature-controlled room. Animals had free access to water and food.

Sampling

Mice were fed their respective diets for 14 days and killed using CO2 asphyxiation. Blood was collected into heparinized tubes by heart puncture. Liver and spleen also were obtained. Plasma and liver were immediately frozen at -80°C under a layer of nitrogen until analyzed for carotenoid concentrations by high-performance liquid chromatography (HPLC).

Splenocyte Culture

Splenic lymphocytes were obtained by flushing the spleen with RPMI 1640 medium containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 5% fetal bovine serum. Contaminating erythrocytes were lysed using 0.84% ammonium chloride. After they were washed, the cells were resuspended in the culture medium, and cells were enumerated using a particle counter (Coulter Electronics, Hialeah, FL). Cells were resuspended to 4 x 10⁶ cells/ml in the culture medium and cultured for four hours at 37°C in a 95% air-5% CO2 atmosphere in the presence of 10 μg/ml of concanavalin A (27). Thereafter, cells were collected and washed twice, and one aliquot of 1 x 10⁷ cells was placed in a ribonuclease-free tube. The supernatant was removed, and the remaining cell pellet was stored at -80°C until used for RNA isolation.

RNA Isolation and Northern Blot Analysis

Total RNA was isolated by a modified guanidium isothiocyanate-cesium chloride method (27) using a commercial kit (RNeasy Kit, Qiagen, Hilden, Germany). The amount of RNA was determined by spectrophotometry (model DU 640B, Beckman, Fullerton, CA) by measuring the optical density at 260 and 280 nm. Exactly 25 μg of RNA were denatured in a loading buffer (36) and electrophoresed on a 1.1% agarose gel containing 80 ml of H2O, 10 ml 10x 3-(N-morpholino)propanesulfonic acid, and 10 ml of 37% formaldehyde (27). The gel was transferred to a nylon membrane (MagnaGraph nylon membranes, MSI, Westborough, MA), and the nucleic acid was immobilized by ultraviolet cross-linking. The nylon membrane was prehybridized in a buffer containing 5x saline-sodium phosphate-EDTA buffer (SSPE), 5x Denhard’s solution, 0.1% sodium dodecyl sulfate (SDS), 50% formamide (47%), and 100 μg/ml of denatured salmon sperm DNA for two hours at 42°C. The membrane was subsequently hybridized in a buffer containing 5x SSPE, 5x Denhard’s solution, 0.2% SDS, 50% formamide (47%), and 100 μg/ml of denatured salmon sperm DNA with 32P-labeled 743-bp Xho/Hind III pim-1 DNA or human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA (37). The 32P-labeled pim-1 DNA or GAPDH cDNA was prepared by the Nicking translation method (Nicking Translation System, Life Technologies, Gaithersburg, MD). After overnight hybridization at 42°C, the membrane was washed twice with 5x saline-sodium citrate (SSC) and 0.5% SDS for 15 minutes at room temperature, twice with 1x SSC and 0.75% SDS for 15 minutes at 37°C, and finally once with 0.1x SSC and 1% SDS for 15 minutes at 37°C. The washed membrane was wrapped in plastic wrap and subjected to autoradiography overnight at -80°C in a cassette with an intensifying screen. Equal RNA loading on the gel was determined by ethidium bromide staining of ribosomal RNA and by detection of GAPDH mRNA with use of Northern blot analysis. The K-562 cell line was used as a positive control, because it expresses high levels of pim-1 mRNA (37). Quantification of specific transcripts was accomplished by densitometry (Alpha-Imager 2000, Innotech, San Leandro, CA).

Lutein Extraction and HPLC

Plasma carotenoids were measured by HPLC analysis (35). Plasma (300 μl) was pipetted, and the protein was precipitated by adding an equal volume of ethanol containing 0.1% butylated hydroxytoluene. After it was vortexed briefly, the mixture was extracted with a 1:1 (vol/vol) anhydrous diethyl ether-petroleum ether with the aid of a multitube vortexer. After centrifugation for 10 minutes at 1,000 g, the ether layer was removed and dried under a stream of nitrogen in a 40°C water bath.

The liver and spleen were washed with phosphate-buffered saline and finely diced. Approximately 0.3 g of the
tissue was weighed, and 1 ml of ethanol containing 0.1% butylated hydroxytoluene was added. The tissue was homogenized for 20 seconds (Kinematica, Luzern, Switzerland), and 2 ml of 10 N KOH were added. The mixture was saponified at 60°C for 45 minutes. After addition of 2 ml of cold deionized H₂O, the mixture was allowed to cool and extracted twice with 10 ml of ether mixture, as described for plasma.

Residues from the plasma, liver, and spleen extracts were redissolved in mobile phase and analyzed for carotenoid content by reverse-phase HPLC (Waters, Milford, MA) using a 5-μm spherical, C₁₈ column (Resolve, Waters; 3.9 × 150 mm). The mobile phase was 47:47:6 (vol/vol/vol) acetonitrile-methanol-chloroform delivered at a flow rate of 1 ml/min. Astaxanthin, β-carotene, and lutein were detected at 450 nm. All procedures were conducted under yellow light.

Table 1. Concentrations of Astaxanthin, β-Carotene, and Lutein in Plasma, Liver, and Spleen of Mice Fed 0% (Control), 0.02%, or 0.4% Astaxanthin, β-Carotene, or Lutein for 14 Days

<table>
<thead>
<tr>
<th></th>
<th>Plasma, µmol/l</th>
<th>Liver, nmol/g</th>
<th>Spleen, nmol/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Astaxanthin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.02%</td>
<td>4.9 ± 0.53 †</td>
<td>0.53 ± 0.04 †</td>
<td>ND</td>
</tr>
<tr>
<td>0.4%</td>
<td>54.7 ± 4.28 †</td>
<td>24.08 ± 1.51 †</td>
<td>1.763 ± 0.35 †</td>
</tr>
<tr>
<td>β-Carotene</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.02%</td>
<td>0.1 ± 0.07 †</td>
<td>0.78 ± 0.06 †</td>
<td>ND</td>
</tr>
<tr>
<td>0.4%</td>
<td>0.7 ± 0.09 †</td>
<td>7.56 ± 1.84 †</td>
<td>0.034 ± 0.01 †</td>
</tr>
<tr>
<td>Lutein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.02%</td>
<td>1.4 ± 0.09 †</td>
<td>0.88 ± 0.09 †</td>
<td>ND</td>
</tr>
<tr>
<td>0.4%</td>
<td>2.0 ± 0.17 †</td>
<td>1.58 ± 0.24 †</td>
<td>0.140 ± 0.03 †</td>
</tr>
</tbody>
</table>

a: Values are means ± SEM (n = 42). Carotenoids were not detectable (ND) in unsupplemented mice.

Results

Plasma, Liver, and Spleen Carotenoids

After 14 days of dietary carotenoid supplementation, concentrations of astaxanthin, β-carotene, and lutein in plasma increased dose dependently (Table 1). Plasma carotenoid concentrations in mice fed 0.4% astaxanthin, β-carotene, and lutein were 11, 7, and 1.4 times higher (p < 0.05), respectively, than those observed in mice fed 0.02% of the same carotenoid. The absolute concentration of the carotenoids in plasma was highest with astaxanthin and lowest with β-carotene. Concentrations of plasma astaxanthin averaged approximately 50- to 80-fold higher than concentrations of β-carotene and 3- to 28-fold higher than concentrations of lutein. Plasma lutein concentrations were 3-14 times higher than concentrations of β-carotene. In contrast, carotenoids were not detectable in the plasma of unsupplemented mice.

Concentrations of liver carotenoids in mice fed diets containing the carotenoids for 14 days increased (p < 0.05) in a dose-dependent manner (Table 1). There was no significant difference in the concentration of the three carotenoids in mice fed 0.02% of the respective carotenoids. Mice fed 0.4% astaxanthin, β-carotene, or lutein had approximately 46-, 11-, and 2-fold higher liver carotenoid concentrations than those fed 0.02% of the respective carotenoid. Also, large differences were observed in the absolute concentrations of each carotenoid in the liver of mice fed 0.4% of the carotenoid. Concentrations of astaxanthin in the liver of mice fed 0.4% astaxanthin averaged >3- and 15-fold higher than those fed the same amount of β-carotene and lutein, respectively. In contrast to plasma, the concentration of β-carotene in the liver was higher than the concentration of lutein in mice fed 0.4% of these two carotenoids. None of the carotenoids were detectable in the liver of unsupplemented mice.

The absolute concentration of astaxanthin (1.763 nmol/g) in the spleen of mice fed 0.4% of the carotenoid was 52 and 13 times (p < 0.05) higher than in the spleen of mice fed β-carotene (0.034 nmol/g) and lutein (0.140 nmol/g), respectively. As in plasma, spleen lutein concentration was higher (4 times, p < 0.05) than β-carotene concentration.

Expression of pim-1 Gene

Figure 1 illustrates the effects of dietary astaxanthin, β-carotene, and lutein on pim-1 mRNA expression by concanavalin A-stimulated splenocytes. The splenocytes from mice fed lutein showed a dose-dependent increase in steady-state levels of pim-1 mRNA (Figure 2). The steady-state level of pim-1 mRNA was approximately sixfold higher in mice fed 0.4% lutein than that in mice fed 0.02% lutein. However, astaxanthin and β-carotene when fed at 0.02% or 0.4% did not significantly increase the steady-state levels of pim-1 compared with unsupplemented mice. Equal loading of mRNA was determined and confirmed by probing the blot for GAPDH transcripts (Figure 1). The analysis was repeated six times, and results were highly repeatable.

Discussion

The possible mechanisms of gene regulation induced by carotenoids have not been studied and certainly merit future research. In this study we demonstrated for the first time that dietary lutein, but not astaxanthin or β-carotene, activates pim-1 gene mRNA expression in concanavalin A-stimulated mouse splenocytes in a dose-dependent manner in vivo. Lutein and astaxanthin do not possess provitamin
A activity in mice, whereas β-carotene has the highest provitamin A activity. Even though astaxanthin and β-carotene have higher antioxidant activities than lutein (8), they did not affect pim-1 gene expression. We are unable to explain why lutein but not β-carotene or astaxanthin can upregulate pim-1 gene expression in this study. In previous studies on other genes, all three carotenoids upregulated connexin 43 gene expression in CH310T1/2 cells (19–20) and in human and mouse cells in vitro (18). Also, all-trans-β-carotene suppressed unc C gene expression in S. typhimurium (39). It is possible that different carotenoids may have different target genes. Like retinoic acid (40), lutein may bind to specific nuclear binding regions or response elements in the lymphocyte to accelerate pim-1 gene transcription.

It has been long speculated that pim-1 is involved in signal transduction, but only recently has a clearer understanding of its role in such signaling events become apparent. In cross-breeding experiments with pim-1 transgenic mice and mice genetically deficient in apoptotic signals (lpr/lpr), dramatically enlarged lymph nodes resulted, suggesting that pim-1 contributes to lymphoproliferation or prevention of apoptosis (29). Treatment of the Nb2 prolactin-dependent T cell line with sodium butyrate arrested the cells in the G1 phase of the cell cycle, an effect similar to that produced by prolactin.
deprivation (41). Mitogenic stimulation of these cells with prolactin reinitiated cell cycle progression with biphasic expression of pim-1 (41). Apoptosis can be induced in this prolactin-dependent cell line with dexamethasone. However, apoptosis can also be inhibited if prolactin is given along with dexamethasone. Because prolactin induces pim-1 expression but not the antia apoptotic gene bcl-2, it appears that pim-1 is responsible for cell survival (24,42). Overexpression of pim-1 in interleukin-3-dependent FDC-P1 cells also causes a decrease in apoptotic cell death and increases the number of G0/G1 phase cells that remain viable when interleukin-3 is withdrawn from the cells (43). Also, the antia apoptotic agent aurantricarboxylic acid sustained growth in the prolactin-deprived, prolactin-dependent Nb2 cells by activating the Janus kinase 2 (JAK2)-STAT5a pathway. This pathway induces the expression of pim-1 (44). Finally, pim-1 phosphorylates p100, a transcriptional coactivator that interacts with the c-Myb transcription factor. c-Myb has been linked to the regulation of cell proliferation, differentiation, and apoptosis in a variety of cell types, including immature hematopoietic cells (45). Taken together, these data suggest that pim-1 expression is a survival factor and is associated with differentiation and not necessarily proliferation, as originally thought. These findings are important, because they suggest that pim-1 may be involved in survival and differentiation signals, both of which are important for augmenting the immune response. Only when other genes are aberrantly expressed does pim-1 become an accomplice in cancer.

The enhancement of pim-1 gene expression is by no means the only mechanism by which carotenoids modulate immune function to inhibit tumor development. For instance, astaxanthin did not enhance pim-1 gene expression in this study but is a potent inhibitor of mammalian tumor growth (46). Splenocytes from mice injected with lutein and astaxanthin but not with β-carotene showed enhanced antibody response to T cell-dependent antigens (16).

Mice and rats are considered very poor carotenoid absorbers compared with humans, cattle, or ferrets. For example, 14C-labeled β-carotene was barely detectable in the plasma of rats fed β-carotene for eight weeks before being given a single dose of the radiolabel (47). In the present study it is interesting to observe that the concentration of astaxanthin in plasma is much higher than the concentration of β-carotene and lutein. This could reflect dramatic differences in intestinal absorption and/or blood clearance. The physical form of the carotenoid could explain the difference in plasma concentrations of the carotenoids. It has been suggested that the intestinal absorption of carotenoids may be related to the presence of hydroxyl or keto groups (35,48). Mathews-Roth and co-workers (48) found that the absorption of dietary canthaxanthin was greater than the absorption of β-carotene. On the other hand, lycopene is readily absorbed, even though it is not an oxycarotenoid (48). Blood clearance of carotenoids may depend on their location in the lipoprotein structure. The more polar carotenoids tend to be located on the outside of the lipoprotein structure. At this location, these polar carotenoids will more likely be taken up or exchanged with other lipoproteins and tissues, thereby increasing their clearance from the blood. Results from this study failed to support the latter as the sole explanation, because the most polar carotenoid (astaxanthin) was present in the highest concentration in the blood.

At the levels fed in this study, none of the dietary carotenoids produced external signs of toxicity in the mice, which is in agreement with other longer-term studies (12,35). Concentrations of plasma and spleen astaxanthin averaged about 50 times higher than concentrations of β-carotene and about 3 (plasma) to 13 (spleen) times higher than concentrations of lutein. Similarly, liver uptake of astaxanthin in mice fed 0.4% astaxanthin was at least three times higher than that in mice fed the same level of β-carotene and lutein. Even though lutein concentrations in plasma and spleen were higher than β-carotene concentrations, the reverse was true with liver carotenoids, thus demonstrating that plasma carotenoid concentrations may not necessarily reflect tissue (liver) uptake. These results are in agreement with our previous studies (8,35). Therefore, the absolute concentration of the carotenoid did not seem to be a factor in pim-1 gene regulation. Astaxanthin concentrations in the plasma, liver, and spleen and β-carotene concentrations in the liver were many times higher than concentrations of lutein, yet lutein but not astaxanthin or β-carotene stimulated pim-1 mRNA expression.

In summary, astaxanthin, β-carotene, and lutein are rapidly absorbed into plasma and liver of mice but with different efficiencies. Dietary lutein increased the expression of pim-1 mRNA in mouse splenocytes. This upregulation of pim-1 mRNA is unique to lutein, rather than a general effect of all carotenoids, because astaxanthin and β-carotene did not elicit the same response.

Acknowledgments and Notes

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