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Metabolites of dietary carotenoids as potential cancer preventive agents

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Abstract: There is abundant epidemiological evidence that consumption of dietary carotenoids reduces the risk of cancer, but it is unclear which of the more than 24 carotenoids, including 8 metabolites, found in human plasma is active. Here we provide evidence that 3 major dietary carotenoids, beta-carotene, lutein and lycopene, can increase connexin 43 gene expression in 10T1/2 cells and in human keratinocytes in organotypic culture. This activity is shared with all-*trans* retinoic acid and is limited to suprabasal cells as is expression of this gene in intact human skin. Furthermore, (3*R*, 3′*R*)-zeaxanthin and 2,6-cyclolycopene-1,5 diol, metabolic derivatives found in human serum of lutein and lycopene respectively, exhibit greater activity than the parent compounds. We suggest that the conversion of dietary carotenoids to compounds which can increase gap junctional communication may play a role in the protective action of carotenoid-rich foods.

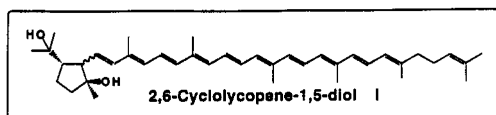
INTRODUCTION

With our increasing understanding of the causes of cancer at the environmental and genetic level there has grown an increasing realization that many cancers may be preventable. While primary prevention, i.e. the avoidance or elimination of carcinogenic agents such as tobacco, is clearly desirable, epidemiological research has indicated that consumption of many foods, principally green and yellow vegetables and fruits, results in lower risk of cancer at many sites. Among the constituents of such foods their content of carotenoids correlates most strongly with decreased risk (ref. 1). Because of the known importance of the pro-vitamin A carotenoids, principally beta-carotene (β-C), in human nutrition and the known role of the retinoids as cancer preventive agents (ref. 2), initial studies focused almost exclusively on this carotenoid. In response to the highly persuasive epidemiological evidence a number of clinical intervention trials were initiated utilizing purified, synthetic beta-carotene administered at doses approximately 10-fold higher than that found in a normal Western diet. The results of three of the largest trials became known recently (refs. 3-5). In all three trials beta-carotene failed to decrease cancer rates and, more disturbingly, in two of these trials both conducted in current or former smokers and/or asbestos-exposed workers, there was a suggestion that lung cancer rates were actually increased. During the conduct of these trials, additional quantitative information became available regarding the presence of other carotenoids in foods associated with lower cancer risk (ref. 6). Recent HPLC analysis has revealed a total of about 24 carotenoids in human serum (ref. 7). These include metabolites of lutein and of lycopene, a carotenoid associated with decreased risk of prostate cancer (ref. 8). These new studies presented the opportunity to determine if these oxidation products have biological activity which may explain some of the actions attributed to carotenoids. In previous research we had shown that carotenoids were capable of inhibiting neoplastic transformation in a model cell system regardless of their pro-vitamin A activity (ref. 9). Moreover we discovered that chemopreventive activity strongly correlated with their ability to increase expression of a gene,

connexin 43, coding for a gap junctional protein (ref. 10). Because gap junctional communication (GJC) has in many studies been linked to increased growth control (ref. 11), and because the chemopreventive retinoids also increase expression of this gene (ref. 12), these studies suggested a mechanism for the effects of carotenoids. In the present studies we utilize the expression of this gene as an intermediate marker of response in mouse and in human immortalized keratinocytes.

MATERIALS AND METHODS

Canthaxanthin (CTX) and β-C were gifts from Hoffmann-La Roche, Switzerland. Lycopene, lutein, zeaxanthin, ε,ε-carotene-3,3'-dione, 3-hydroxy-β,ε-caroten-3'-one and 2,6-cyclolycopene 1,5-diol (cyclolycopene) 1 were prepared and separated by HPLC as described (ref. 7).



Cell culture conditions and molecular techniques

Two immortalized cell lines were used in this study: the mouse embryonal fibroblast cell line 10T1/2 (ref. 13) and the human keratinocyte cell line HaCaT (refs. 14,15). All cultures were incubated at 37°C, 5% carbon dioxide and 95% humidity. 10T1/2 cells were cultured as monolayers as described previously (ref. 9) and treated when confluent for the indicated time periods. HaCaT cells were cultured in low-calcium (0.1 mmol/l) serum-free keratinocyte medium (Gibco-BRL, Grand Island, NY) supplemented with epidermal growth factor (5 μg/ml), bovine pituitary extract (35 μg/ml) and insulin (5 μg/ml) in monolayer culture until sub-confluent. They were then harvested and placed on Millicell-CM collagen-coated culture plate inserts (Millipore, Bedford, MA) for approximately 7 days when high-calcium medium (1.15 mmol/l), supplemented as described above, was placed below the filters while the surface of the filters was exposed to the atmosphere. During this time a multi-layered differentiating organotypic culture was produced (ref. 16). Cells were treated with carotenoids or retinoic acid dissolved in THF or acetone respectively using the precautions and procedures discussed previously (ref. 17) and added to culture medium concurrent with exposure to the air interface. Cells were re-fed and re-treated every two days. HaCaT cells and 10T1/2 cells were harvested and connexin 43 (Cx43) solubilized as previously described (ref. 18). Proteins were electrophoresed on 10% SDS-polyacrylamide gels, and subsequently incubated with a rabbit polyclonal antibody raised against a synthetic peptide corresponding to the 15 residues of the C-terminal domain of Cx43 (ref. 18). This sequence is 100% homologous in mouse, rat and humans. Bound antibody was visualized by means of a chemiluminescent detection system (Tropix, Bedford, MA) and recorded on X-ray film. Immunofluorescence was performed on 10T1/2 cells as previously described (ref. 18).

RESULTS

Lycopene and lutein induce connexin 43 in 10T1/2 murine embryo cells

To investigate if lycopene and lutein modulate Cx43 expression in confluent 10T1/2 cells, cultures were treated for 7 days then harvested and analysed by Western blotting. Lycopene induced Cx43 at 1×10^{-5} M (Fig. 1, lane 3). Although the lycopene oxidation product cyclolycopene was active, this activity did not exceed that of the parent compound (lane 9). At a concentration of 10^{-5}M, zeaxanthin was clearly the most potent of the carotenoids tested, producing a dramatic increase in Cx43 at 10^{-5}M (Lane 6). This increase greatly exceeded that produced by the parent compound lutein when tested at 1×10^{-5} M or 1×10^{-6} M (lanes 10-11 respectively). Two other known metabolites of lutein found in human serum, ε,ε-carotene-3,3'-dione, 3-hydroxy-β,ε-caroten-3'-one, were marginally active in inducing connexin 43 (lanes 7-8 respectively). While these data strongly suggest that zeaxanthin is a more potent molecule than other tested carotenoids in its ability to upregulate connexin 43, we must add the caveat that the stability of this and the other metabolites in cells and in culture medium has yet to be investigated. Because of limitations in the supply of zeaxanthin, its chemopreventive properties are at present unknown. However, if the correlation between induction of Cx43 and ability to inhibit transformation (ref. 10) is maintained with this molecule, we predict strong activity.

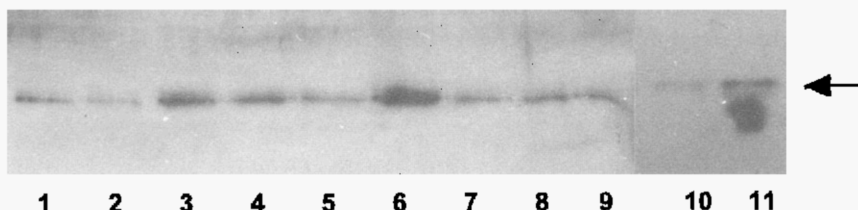


Figure 1. Induction of Cx43 by carotenoids and their metabolites.
Confluent 10T1/2 cells were treated with carotenoids for 7 days. Gel electrophoresis, Western blotting and detection of Cx43 were performed as described previously (ref. 18). Lane1, control no treatment ; lane 2, THF 0.5 % (solvent control); lane 3, lycopene 10^{-5}M; lane 4, CTX 10^{-5}M; lane 5, β-C 10^{-5}M; lane 6, zeaxanthin 10^{-5}M; lane 7, ε,ε-carotene-3,3'-dione 10^{-5}M; lane 8, 3-hydroxy-β,ε-caroten-3'-one 10^{-5}M; lane 9; 2,6-cyclolycopene 10^{-5}M; lanes 10 and 11, lutein 10^{-5} and 10^{-6}M respectively.

Immunofluorescence studies of connexin localization

To be functional, connexins must be integrated into the plasma membrane, assemble into hemichannels and dock with hemichannels on adjacent cells to form a connexon. Large numbers of these connexons aggregate together into junctional plaques which can be visualized by immunofluorescent techniques. As shown in Fig. 2, zeaxanthin-treated cells (panel B) demonstrated an increase in the number of immunofluorescent junctional plaques in regions of cell-cell contact as compared to the THF control (panel A). Previous studies of carotenoid-treated cells had shown that an increase in immunofluorescent plaques was associated with an increase in intercellular dye transfer following microinjection of Lucifer Yellow (ref. 10), indicating that gap junctional communication is also increased by carotenoids.

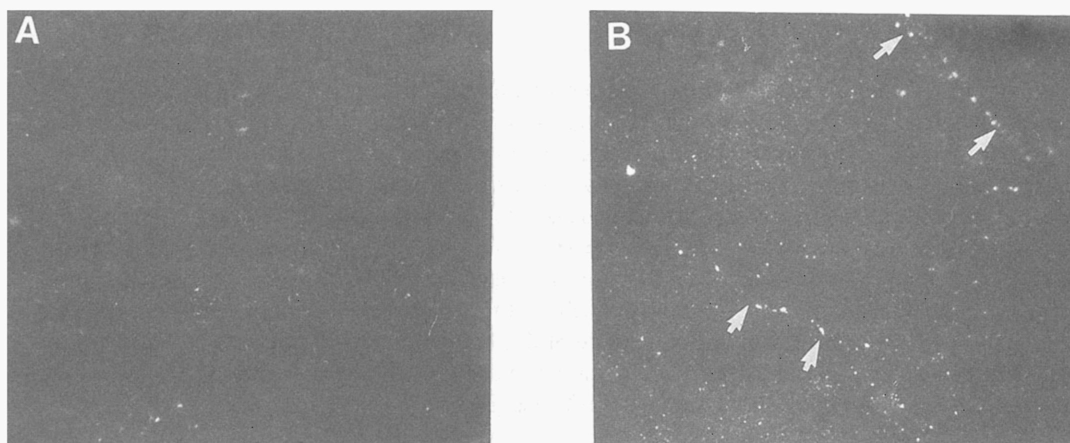


Figure 2. Carotenoid treatment of 10T1/2 cells increases number and size of immunofluorescent plaques in regions of cell-cell contact.
10T1/2 cells were seeded on Permanox plastic slides, grown to confluence and treated for 7 days with carotenoids in THF. Cultures were fixed and incubated with an anti-connexin 43 antibody as described (ref. 18). Panel A) THF 0.5% (solvent control) immunofluorescence; panel B) zeaxanthin 10^{-6}M. Gap junctions localized in the membrane in areas of cell-cell contact → were visualized as linear arrays of fluorescent plaques.

Carotenoids and their effects in HaCaT cells

Connexin 43 is expressed in differentiating human keratinocytes and we have demonstrated that treatment of intact human skin with all-*trans* retinoic acid causes induction of connexin 43 in suprabasal cells of the epidermis (ref. 19). Because of the requirement for differentiation for connexin expression and inducibility, keratinocytes were cultured as an organotypic culture in order to allow differentiation to proceed. For reasons of reproducibility, availability and ease of use, these new studies did not utilize primary keratinocytes as before (ref. 16); instead we used the immortalized

human keratinocyte cell line HaCaT. Studies by others had shown this line to resemble closely normal human keratinocytes in its differentiation pattern in organotypic culture (ref. 14).

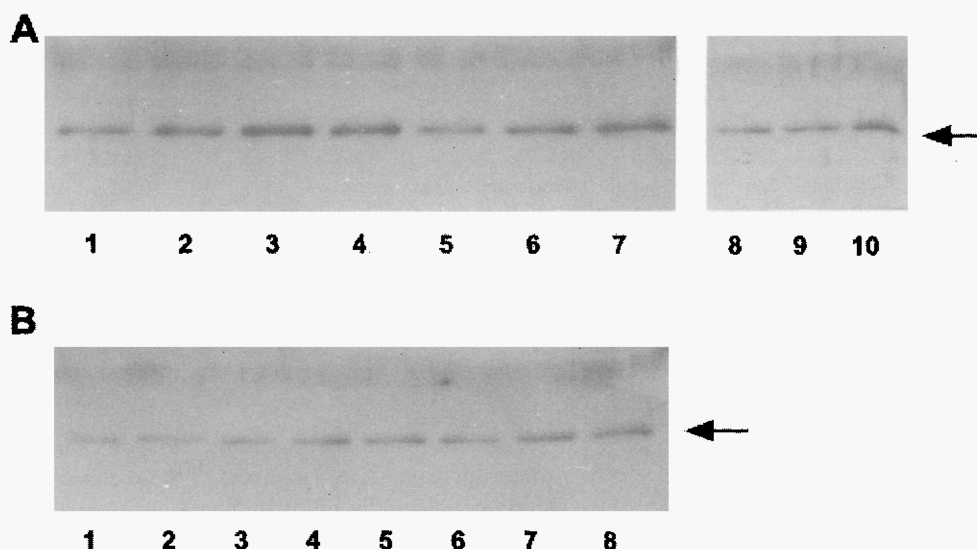


Figure 3. Effects of retinoic acid and carotenoids on Cx43 expression in organotypic HaCaT cell cultures.

HaCaT cells were grown in organotypic culture as previously described (ref. 16). Cells were treated with retinoic acid or carotenoids for 7 days. Gel electrophoresis, Western blotting and detection of Cx43 were performed as described previously (ref. 18). **Panel A**: Lane 1, control (no treatment); lanes 2, 3 and 4, retinoic acid 10^{-7}, 10^{-6} and 10^{-5}M respectively; lanes 5, 6 and 7, β-C 10^{-7}, 10^{-6} and 10^{-5}M respectively; lanes 8, 9 and 10 CTX 10^{-7}, 10^{-6} and 10^{-5}M respectively. **Panel B:** lane 1, control (no treatment); lane 2, THF 0.5 %; lanes 3, 4 and 5 lycopene 10^{-7}, 10^{-6} and 10^{-5}M respectively; lanes 6, 7 and 8, cyclolycopene 10^{-7}, 10^{-6} and 10^{-5}M respectively.

As shown in Fig. 3, panel A, lanes 3 and 4, treatments with retinoic acid, β-C and CTX increased the abundance of connexin 43 in organotypic cultures of HaCaT cells. This further underlines the similarity between the actions of carotenoids and retinoids in cultured keratinocytes. In similar experiments HaCaT cells were treated with lycopene and cyclolycopene (Fig. 4, panel B; lanes 13-20). As can be seen lycopene and its metabolite both increased the level of connexin 43 in a dose-dependent manner; of interest was the observation that the metabolite caused a marginally higher level of connexin 43 induction. We have not determined what proportion if any of added lycopene becomes converted to cyclolycopene under conditions of cell culture. If substantial conversion occurs, the apparent activity of lycopene may be a consequence of this conversion. With regard to the activity of zeaxanthin in this system, preliminary results (data not shown) show that it possesses a comparably high level of activity as indicated in studies in 10T1/2 cells (Fig. 1).

DISCUSSION

We have previously demonstrated that certain retinoids (ref. 12) and carotenoids, including β-carotene (ref. 9), can inhibit carcinogen-induced neoplastic transformation of 10T1/2 cells and that this inhibition correlates with increased gap junctional intercellular communication (GJIC) (refs. 10,20). In both cases the observed increase in GJIC is mediated through an increase in Cx43 at the mRNA and protein level (refs. 18,21). Because β-carotene is readily converted in mammals to retinoids, many of its effects have been considered to be mediated through its breakdown, either spontaneously or enzymatically to retinoids. However, our demonstration that several non-provitamin A carotenoids also mediate responses similar to retinoic acid (ref. 21) has led to a re-evaluation of this concept, particularly the question of whether conversion of non-provitamin A carotenoids to retinoid-like molecules can occur. Most of the actions of retinoic acid appear to be mediated by nuclear retinoic acid receptors RAR-α,β and γ and RXR-α,β and γ through interactions with their cognate responsive elements in retinoid-responsive genes (ref. 22). One such gene, RAR-β has been shown to be induced

in 10T1/2 cells at the transcriptional level by the synthetic retinoid, TTNPB (ref. 21). However, CTX, a non-provitamin A carotenoid, which had been shown to induce connexin 43 mRNA, did not induce RAR-β mRNA (ref. 21), indicating that an independent mechanism for mRNA induction by carotenoids may exist. Studies utilizing liarozole, an inhibitor of cytochrome-P450-mediated catabolism of retinoic acid, also failed to indicate conversion of CTX to the expected retinoid, 4-oxo-retinoic acid (ref. 23). Others however have demonstrated by chemical (ref. 24) and molecular techniques (ref. 25) the conversion of CTX to 4-oxo-retinoic acid. The reason for this discrepancy is not known.

While conversion of many dietary carotenoids to compounds shown to have retinoid-like properties is certainly feasible on a chemical basis, it is more difficult to imagine such conversion in the case of the straight-chain carotenoid lycopene, which exhibits chemopreventive activity and increases GJC in murine cells (ref. 9) and, as we demonstrate here, induces connexin 43 in 10T1/2 cells (Fig 1) and human keratinocytes (Fig. 3). The results presented here suggest a possible explanation for this surprising activity of lycopene; its conversion to the cyclic compound 2,6-cyclo-lycopene-1,5-diol as a consequence of oxidation and subsequent rearrangement. Because this conversion may occur under conditions of cell culture, the activity of lycopene in our systems may be entirely due to the activity of this oxidation product. Furthermore, the apparent protective effects of a lycopene-rich diet (ref. 8) may be due to the presence of this cyclocompound in the blood and presumably in tissues at risk (ref. 26).

Retinoids are known to be potent modifiers of differentiation in human keratinocytes in culture and in human skin (ref. 27). This is the basis of their extensive and successful use in dermatology. This effect can be observed as a decrease in expression of mature keratins such as K1 (ref. 28) and markers of terminal differentiation such as loricrin (ref. 29). This regulation is known to occur at the transcriptional level and to be mediated through retinoic acid receptors (RARs) and the retinoid X receptors (RXRs) (ref. 30). In the case of loricrin, functional interactions between these nuclear receptors and the promoter region appear to involve AP-1 sites (ref. 31). We have previously shown certain carotenoids to also alter differentiation in organotypic culture (ref. 16). HaCaT cells undergo pseudo-normal differentiation in organotypic culture and express connexin 43 in suprabasal cells in a manner similar to that observed *in vivo* (refs. 19,32). Under these conditions expression of this gene is increased when exposed to retinoic acid and to dietary carotenoids found in human serum (Fig. 3). This response to retinoic acid was predicted by our previous clinical studies in which we demonstrated that topical retinoic acid would induce connexin 43 in intact human skin (ref. 19). The present results with carotenoids predict that, in sufficient concentration, these molecules should produce dermatological effects; whether these effects would mirror those seen after retinoic acid treatment is uncertain given the known disparities between responses of intact human skin and keratinocytes in organotypic culture (reviewed in ref. 27). However, the ability of retinoic acid to elevate levels of connexin 43 under both situations (i.e. *in vivo* and in culture) suggests that carotenoids may also have this ability *in vivo*. If so, we would suggest that this action may be significant in terms of cancer chemoprevention and the proposed role of carotenoids as micronutrients which reduce cancer risk (ref. 33). However we are unaware of reports that dietary carotenoids, or β-carotene supplementation, have had retinoid-like effects on skin or other organs. On the contrary, β-carotene has not been reported to exert any clinical toxicity even after supplementation at levels up to 180mg/day (ref. 34). *In vivo*, a low concentration of retinoic acid causes increased thickening of the epidermis, *de novo* synthesis of collagen in the dermis and inhibition of UV-induced proteases, effects beneficial to photo-aged skin (ref. 27) and it is these effects we would predict to result from carotenoid exposure. Moreover, the carotenoids used in these studies were at concentrations (10^{-6}-10^{-7}M) that can be reached by dietary supplementation with lycopene- and lutein-rich foods (ref. 26).

The research presented here has two implications: first, that products of the oxidation of two major dietary carotenoids increase the expression of connexin 43 to levels marginally (cyclolycopene) and dramatically (zeaxanthin) higher than the parent compounds lycopene and lutein respectively. While zeaxanthin can be obtained directly from the diet as a component of maize, its conversion from lutein found in green leaves offers a quantitatively greater source. Second, these compounds are active in human keratinocytes and modulate differentiation and the expression of the gap junction gene connexin

43. The accumulation of connexin 43 into immunofluorescent junctional plaques and the observation on Western blots of multiple immunoreactive bands previously shown to be associated with functional junctions (refs. 18,35), suggests that competent channels are induced in treated keratinocytes. We have demonstrated that increased GJC is associated with increased growth control (refs. 20,36-38), and others have shown that transfection of connexin genes into a variety of tumor cell lines results in a partial loss of their neoplastic phenotype (refs. 39-44). Thus, if the *in vitro* models are predictive of *in vivo* behavior, increased connexin expression in epithelium as a consequence of exposure to dietary carotenoids, or therapeutic application of retinoids, could decrease the proliferation of carcinogen-initiated cells, thereby inhibiting their neoplastic conversion. Support for this hypothesis comes from our recent data conducted in head and neck cancer patients in which we demonstrated an association between loss of expression of connexin 43 oral mucosa and abnormal proliferation in dysplastic and malignant tissue (ref. 45).

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