

Role of gene regulation in the anticancer activity of carotenoids*

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Abstract: There is extensive evidence that high intake of fruits and vegetables is associated with decreased risk of many types of cancers. Thus, it is widely accepted that diet changes are a powerful means to prevent cancer. Although there is a growing interest in the role of the tomato carotenoid lycopene in cancer prevention and treatment, we hypothesize that a single micronutrient cannot replace the power of the concerted action of multiple agents derived from a diet rich in fruits and vegetables. Indeed, we found that lycopene can synergize with other phytonutrients in the inhibition of cancer cell growth. The mechanism underlying the inhibitory effects of lycopene and other carotenoids involves interference in several pathways related to cancer cell proliferation and includes changes in the expression of many proteins participating in these processes, such as connexins, cyclins, cyclin-dependent kinases, and their inhibitors. These changes in protein expression suggest that the initial effect involves modulation of transcription by ligand-activated nuclear receptors or by other transcription factors. It is feasible to suggest that carotenoids and their oxidized derivatives interact with a network of transcription systems that are activated by different ligands at low affinity and specificity and that this activation leads to the synergistic inhibition of cell growth.

CAROTENOIDS AND CANCER

Fruits and vegetables play an important role in the prevention of degenerative diseases including cancer [1]. Among plant constituents, carotenoids have been implicated as cancer-preventive agents [2]. Most earlier studies were related to β -carotene, but in recent years, additional carotenoids found in the human diet have also begun to receive attention. Our research focuses primarily on tomato carotenoids, particularly lycopene, thus, in this paper, we will relate mainly to those studies that deal with this carotenoid.

Epidemiological studies

The biological activity of lycopene has been recently reviewed [3–7]. A comprehensive analysis of the epidemiological literature on the relation of tomato consumption and cancer has been published by Giovannucci [8]. He found that most of the published studies show an inverse association between tomato intake or blood lycopene level and cancer risk, suggesting that lycopene may contribute to the

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beneficial effects of foods containing tomato, but this still remains conjecture. Alternatively, the anti-cancer activity of tomatoes can be explained by interactions among their multiple components. More recent epidemiological studies also support this impression [9–15].

Laboratory studies

The epidemiological data described above are reinforced by studies showing the inhibitory effect of lycopene on tumor growth in animal models *in vivo* [16–21]. Additional support for a lycopene effect was found with diverse cancer cells *in vitro* [22–25]. Particularly, we have demonstrated that lycopene inhibits mammary, endometrial, lung, and leukemic cancer cell growth in a dose-dependent manner ($IC_{50} \sim 2 \mu\text{M}$) [23,25].

Human intervention studies with carotenoids

The epidemiological and laboratory studies reviewed above suggest a cancer-preventive activity for lycopene. Similar earlier studies on β -carotene led to collaborative large intervention studies with synthetic β -carotene. However, the use of a single plant-derived compound in human prevention studies has not been successful, revealing either no beneficial effect [26] or even a negative effect [27,28]. These results led to the hypothesis that a single micronutrient cannot replace the power of the concerted action of multiple compounds derived from a diet rich in fruits and vegetables. So, it is not surprising that more recent studies used a natural mixture of phytonutrients. Two small-scale, preliminary intervention studies in prostate cancer patients were carried out with natural tomato preparations.* In one, Bowen et al. [29] showed that after dietary intervention, serum and prostate lycopene concentrations were increased, whereas oxidative DNA damage both in leukocytes and in prostate tissue was significantly lower. Furthermore, serum levels of prostate-specific antigen (PSA) decreased after the intervention ($p < .001$). In the other study, Kucuk et al. [30] reported that supplementation with tomato extract in men with prostate cancer positively modulates the grade and volume of prostate intraepithelial neoplasia and tumors and the levels of both serum PSA and biomarkers of cell growth and differentiation.

Synergy between various phytonutrients

To support the hypothesis that a concerted action of several micronutrients is responsible for the anti-cancer activity of a diet enriched with fruits and vegetables, it has to be shown that plant-derived constituents, such as carotenoids, have the ability to synergize with other phytonutrients. Therefore, we have been studying the effects of combinations of various micronutrients or their metabolites on cancer cell proliferation and differentiation. These micronutrients include carotenoids (β -carotene, lycopene, phytoene, phytofluene, and astaxanthin); a polyphenolic antioxidant (carnosic acid from rosemary); an organosulfur compound (allicin from garlic); the active metabolite of vitamin D (1,25-dihydroxyvitamin D_3); the metabolite of β -carotene and vitamin A (retinoic acid); and a synthetic derivative of lycopene (acyclo-retinoic acid). We have found that various combinations of these compounds produce a synergistic or additive inhibition of cancer cell proliferation. For example, the combination of low concentrations of lycopene with 1,25-dihydroxyvitamin D_3 [$1,25(\text{OH})_2D_3$] synergistically inhibited proliferation and induced differentiation in HL-60 leukemic cells [25]. In addition, Pastori and colleagues have found that the simultaneous addition of lycopene and another vitamin, α -tocopherol, at physiological concentrations, resulted in a strong synergistic inhibition of prostate carcinoma cell proliferation [24].

* These two studies were presented at the 13th International Symposium on Carotenoids in Hawaii.

MECHANISM OF CANCER CELL GROWTH INHIBITION AT THE PROTEIN EXPRESSION LEVEL

The mechanisms underlying the anticancer activity of lycopene and other carotenoids may involve changes in pathways leading to cell growth or cell death. These include hormone and growth factor signaling, regulatory mechanisms of cell cycle progression, cell differentiation, and apoptosis. Examples of carotenoid effects on some of these pathways will be described below, with emphasis being placed on the changes in protein expression associated with these effects.

Gap junctional communication

One of the earliest discoveries related to carotenoids and modulation of protein level was made by Bertram and colleagues who found that carotenoids increase gap junctional communication between cells and induce the synthesis of connexin43, a component of the gap junction structure [31,32]. This effect was achieved independently of provitamin-A or the antioxidant properties of the carotenoids. Loss of gap junctional communication may be important for malignant transformation, and its restoration may reverse the malignant process.

Growth factor signaling

Growth factors, either in the blood or as part of autocrine or paracrine loops, are important for cancer cell growth. Recently, IGF-I has been implicated as a major cancer risk factor. It was reported that high blood levels of IGF-I existing years before malignancy detection, predict an increase in risk for breast, prostate, colorectal, and lung cancer [33–36]. Accordingly, two possible strategies might be used to reduce IGF-related cancer risk: reduction in IGF-I blood levels, and interference with IGF-I activity in the cancer cell. Preliminary results of our studies on the former strategy suggest that tomato phytonutrients lower IGF-I blood levels. In addition, we have recently shown that lycopene inhibits the mitogenic action of IGF-I in human cancer cells. In mammary cancer cells, lycopene treatment markedly reduced IGF-I stimulation of both tyrosine phosphorylation of insulin receptor substrate-1 and DNA binding capacity of the AP-1 transcription factor [37]. These effects were not associated with changes in the number or affinity of IGF-I receptors, but rather with an increase in membrane-associated IGF-binding proteins (IGFBPs). This finding can explain the suppression of IGF-I-signaling by lycopene, as based on our previous studies [38] showing that membrane-associated IGFBP-3 inhibits IGF-I receptor signaling in an IGF-dependent manner.

Cell cycle progression

Growth factors have a major effect in promoting cell cycle progression, primarily during G1 phase. We have shown that lycopene treatment of MCF-7 mammary cancer cells slowed down IGF-I-stimulated cell cycle progression [37], which was not accompanied by either apoptotic or necrotic cell death. Lycopene-induced delay in progression through G1 and S phases was also observed in other cancer cell lines (leukemic, endometrial, lung, and prostate) tested in our laboratory ([25] and an unpublished work). A similar effect of another carotenoid, α -carotene, was demonstrated in GOTO human neuroblastoma cells [39]. Likewise, β -carotene induced a cell-cycle delay in G1 phase in normal human fibroblasts [40]. Cell cycle transition through a late G1 checkpoint is governed by a mechanism known as the “pRb pathway” [41]. The central element in this pathway, retinoblastoma protein (pRb), is a tumor suppressor that prevents premature G1/S transition via physical interaction with transcription factors of the E2F family. The activity of pRb is regulated by an assembly of cyclins, cyclin-dependent kinases (Cdks), and Cdk inhibitors. Phosphorylation of pRb by Cdks results in the release of E2F, which leads to the synthesis of various cell growth-related proteins. Cdk activity is modulated in both a posi-

tive and a negative manner by cyclins and Cdk inhibitors, respectively. It is well documented that growth factors affect the cell cycle apparatus primarily during G1 phase, and that the D-type cyclins are the main elements acting as growth factor sensors [42]. Moreover, cyclin D1 is known as an oncogene and is found to be overexpressed in many breast cancer cell lines as well as in primary tumors [43]. In a recent study [44], we have demonstrated that cancer cells arrested by serum deprivation in the presence of lycopene are incapable of returning to the cell cycle after serum re-addition. This inhibition correlated with reduction in cyclin D1 protein levels that resulted in inhibition of both cdk4 and cdk2 kinase activity and in hypophosphorylation of pRb. Abundance of the Cdk inhibitor p21Cip1/Waf1 was reduced while p27Kip1 levels were unchanged. Inhibition of cdk4 was directly related to a lower amount of cyclin D1-cdk4 complexes while inhibition of cdk2 action was related to a shift of the inhibitor p27Kip1 molecules from cdk4 complexes to cyclin E-cdk2 complexes.

Differentiation-related proteins

Induction of differentiation to mature cells with distinct functions similar to nonmalignant cells has been proposed as an alternative to cytotoxic chemotherapy and may be useful for chronic chemoprevention. Differentiation therapy has been quite effective in treating acute promyelocytic leukemia and is currently being investigated for treatment of solid tumors. Differentiation inducers that are presently under laboratory and clinical investigation include vitamin D and its analogs, retinoids, polyamine inhibitors, and others. We have shown that lycopene alone induces differentiation of HL-60 promyelocytic leukemia cells [25]. A similar effect was described also for other carotenoids such as β -carotene, lutein, and the saffron carotenoids [25,45,46]. The differentiation effect of lycopene was associated with elevated expression of several differentiation-related proteins, such as cell surface antigen (CD14), oxygen burst oxidase (as measured by phorbol ester-stimulated reduction of nitroblue tetrazolium) [25], and chemotactic peptide receptors (unpublished work).

The mechanism of the differentiating activity of lycopene and its ability to synergize with $1,25(\text{OH})_2\text{D}_3$ in this effect [25] is largely unclear. However, in a similar study, we have recently shown that the differentiation-enhancing effect of another phytonutrient, carnosic acid from rosemary, is associated with induction of multiple differentiation-related proteins, such as Cdk inhibitor, p21Cip1, early growth response gene-1 (EGR-1), and Cdk5 and its activator protein, p35Nck5a [47,48]. Most importantly, carnosic acid and its combinations with $1,25(\text{OH})_2\text{D}_3$ and retinoic acid transcriptionally activated the expression of nuclear hormone receptors, such as vitamin D₃ receptor (VDR), retinoic acid receptor (RAR α), and retinoid X receptor (RXR α) [47]. This may represent a molecular basis for synergy between phytonutrients and differentiation inducers. The possibility that lycopene as well as other carotenoids and/or their derivatives may affect nuclear signaling pathways is an attractive suggestion, but requires experimental proof.

MODULATION OF TRANSCRIPTION BY CAROTENOIDS

Our main question is, by what mechanism do carotenoids affect so many and diverse cellular pathways as described above? The changes in the levels of many proteins suggest that the initial effect involves modulation of transcription. As illustrated below, such modulation can occur at the level of ligand-activated nuclear receptors or other transcription factors.

Retinoic acid receptor (RAR)

Initially, the structure similarity between lycopene and β -carotene suggested that lycopene or some of its oxidized derivatives may activate retinoid-like receptors. To test this assumption, Stahl and colleagues [49] synthesized a hypothetical oxidation product of lycopene, acyclo-retinoic acid, the open chain analog of retinoic acid. However, we found that although acyclo-retinoic acid was able to trans-

activate RAR α , the growth-inhibitory effect of lycopene was not mediated directly via this classical retinoid receptor [50]. In addition, Stahl [49] concluded that acyclo-retinoic acid does not have a role in gap junctional communication. Muto and colleagues [51] synthesized acyclo-retinoic acid and tested its biological activity as part of a series of acyclic retinoids, but did not observe transactivation by this compound in the RAR or RXR reporter gene systems [52]. However, they did find that other acyclic retinoids, lacking one or two double bonds (geranyl geranoic acid and 4,5-didehydro geranyl geranoic acid), caused transactivation of the reporter gene comparable to that achieved by retinoic acid. It is interesting to note that these acyclic retinoids may be potential derivatives of phytoene and phytofluene (two carotenoids that are present in tomatoes).

These studies suggest that carotenoids, their oxidized derivatives, and other phytonutrients interact with a network of transcription factors that are activated by different ligands at low affinity and specificity (Fig. 1). The activation of several transcription factor systems by different compounds may lead to the synergistic inhibition of cell growth. In addition to the retinoid receptors, other candidate transcription systems that may participate in this network are the peroxisome proliferator-activated receptors (PPARs) [53–55], the antioxidant response element (ARE) [56,57], AP-1 [58], the xenobiotic receptors [59,60] and yet unidentified orphan receptors.

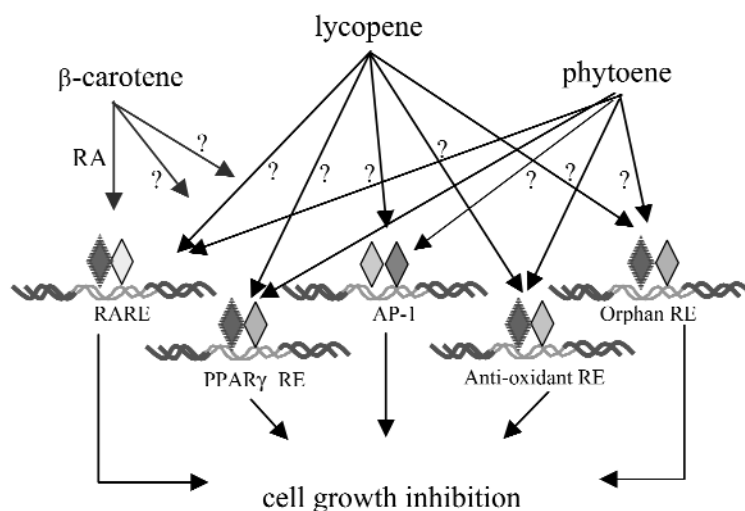


Fig. 1 Hypothetical network of transcription factors that may be activated by various carotenoids or their derivatives. RA—retinoic acid.

Peroxisome proliferator-activated receptor (PPAR)

These nuclear receptors have a key role in the differentiation of adipocytes, but recently their role in cancer cell growth inhibition and differentiation has also been demonstrated. PPAR γ is expressed at significant levels in human primary and metastatic breast adenocarcinomas, colon cancer cells, and liposarcomas [61–63]. Colon cancer in humans was found to be associated with loss-of-function mutations in PPAR γ [63]. Ligand activation of PPAR γ in cultured breast cancer cells [61] and in liposarcomas in vivo [62] causes changes in gene expression associated with a more differentiated, less malignant state. Human prostate cancer cells expressed PPAR γ at prominent levels while normal prostate tissues had a very low expression [54,55]. Activation of this receptor with specific ligands, such as troglitazone, exerts an inhibitory effect on the growth of prostate cancer cells and favorable changes in PSA dynamics in prostate cancer patients [55]. The presence of PPAR γ receptors in various cancer cells, their activation by fatty acids, prostaglandins, and related hydrophobic agents in the μ M range makes

this liganded transcription factor an interesting target for carotenoid derivatives. To support this hypothesis, we recently compared the relative efficacy of several carotenoids found in tomatoes in transactivation of the PPAR response element. Preliminary results suggest that lycopene, phytoene, phytofluene, and β -carotene transactivate PPAR γ in mammary cancer cells.

Xenobiotic and other orphan nuclear receptors

Orphan receptors include gene products that are structurally related to nuclear hormone receptors, but lack known physiological ligands. Thus, like all the recognized nuclear receptors, they should have multiple regulatory roles, some of which may be related to diet-derived compounds. Mammals encounter numerous foreign chemicals (xenobiotics), such as ingested food, environmental pollutants, carcinogens, and drugs, which are metabolized and eliminated mainly by cytochrome P450 (CYP) enzymes [60]. CYP enzymes are induced by various xenobiotic substrates, including phytonutrients, through response element of several orphan nuclear receptors, such as steroid and xenobiotic receptor/pregnane X receptor (SXR/PXR) and constitutive androstane receptor (CAR) [59,60]. St. John's wort, the herbal remedy used widely for the treatment of depression, illustrates the possible role of phytonutrients in this system. It has recently been found that its active compound, hyperforin, is a potent ligand for PXR, which promotes expression of CYP 3A4 [64].

Antioxidant response element (ARE)

Induction of phase 2 enzymes that neutralize reactive electrophiles and act as indirect antioxidants appears to be an effective means for achieving protection against a variety of carcinogens in animals and humans. Transcriptional control of the expression of these enzymes is mediated, at least in part, through ARE found in the regulatory regions of their genes. The transcription factor Nrf2, which binds to ARE, appears to be essential for the induction of prototypical phase 2 enzymes such as glutathione S-transferases (GSTs), NAD(P)H:quinone oxidoreductase (NQO1) [65], and thioredoxin [66]. Constitutive hepatic and gastric activities of GST and NQO1 were reduced by 50–80 % in Nrf2-deficient mice compared with wild-type mice [65]. Under basal conditions, Nrf1 and Nrf2 are located in the cytoplasm and are bound to the inhibitory protein, Keap1. Upon challenge with inducing agents, they are released from Keap1 and translocate to the nucleus [67,68]. Within the nucleus, these basic region leucine zipper transcription factors are recruited to ARE as heterodimers with either small Maf proteins, FosB, c-Jun, or JunD. Several studies have shown that dietary antioxidants, such as terpenoids [69]; phenolic flavonoids (e.g., green tea polyphenols and epigallocatechin-3-gallate [70]); and isothiocyanates, may work as anticancer agents by activating this transcription system. To illustrate, an isothiocyanate compound from Japanese horseradish extract induced both nuclear localization of Nrf2 (which binds to ARE) and expression of phase 2 enzyme genes. These effects were completely abrogated in Nrf2-deficient mice [71].

Activator protein-1 (AP-1)

As mentioned above, the activation of the AP-1 transcriptional complex is a middle-term event (1–2 h) in the mitogenic signaling pathway of IGF-I and other growth factors [72]. The AP-1 complex consists of protein from the Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, FosB, Fra-1, and Fra-2) families which associate as homo- (Jun/Jun) or heterodimers (Jun/Fos). These proteins are often induced by mitogenic stimuli and tumor-promoting agents and bind to an activator protein-1 (AP-1) site, known also as the TPA response element (TRE), on the promoter of many genes that are related to cell proliferation, such as cyclin-D [73]. Interestingly, as just discussed, some of these proteins participate in the ARE transcription complex as well. It has recently been shown that this transcriptional system is modulated by carotenoids. The AP-1 family members can form different complexes that bind to AP-1 sites. These

proteins differ considerably in their ability to activate transcription of target genes. A complex that is composed of weak transactivators will induce less-transcriptional activity than one containing more potent transactivators. Preliminary results from our studies suggest that lycopene and retinoic acid reduce growth factor-induced stimulation of AP-1 transcriptional activity by altering the composition of AP-1 complexes that bind to DNA. Wang et al. [58], reported that the expression of c-Jun and c-Fos genes in lungs of ferrets supplemented with high-dose β -carotene and exposed to tobacco smoke was elevated by three- to four-fold. In addition,* they observed a strong proliferative response in lung tissue and squamous metaplasia as well as an increase in the level of a cell proliferation marker (proliferating-cell nuclear antigen, PCNA). In β -carotene-supplemented animals, this increase was enhanced further by tobacco smoke. Their report offers a possible explanation for the enhancing effect of β -carotene supplementation on lung carcinogenesis in smokers as has been reported in large intervention studies [27,28].

CONCLUDING REMARKS

As discussed in this review, the beneficial effect of a diet rich in fruits and vegetables appears to depend on the concerted action of multiple micronutrients, which act either additively or synergistically. We suggest that one of the mechanisms underlying such an action could be the activation of a network of transcription factors. In this model, carotenoids, their derivatives, and other phytonutrients may activate the same transcription factor, producing an additive effect. A synergistic effect can be achieved by the interaction with different transcription systems.

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