Dietary carotenoids, connexins and cancer: what is the connection?

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Abstract
Carotenoids and retinoids are chemically related; indeed a major source of vitamin A in humans occurs through enzymic cleavage of \(\beta\)-carotene. However, most dietary carotenoids cannot be converted into retinoids. Retinoids have demonstrated cancer-preventive activities in humans and experimental models; however, their toxicity has precluded wide-scale clinical use. In contrast, carotenoids are essentially non-toxic and their cancer-preventive activities, although strongly supported by epidemiological studies, have only been satisfactorily demonstrated in experimental systems. We have shown that in an experimental cell culture system consisting of carcinogen-treated 10T1/2 cells, both retinoids and all dietary carotenoids examined can reversibly inhibit neoplastic transformation in the post-initiation phase of carcinogenesis. This activity strongly correlates with their ability to increase gap junctional intercellular communication by up-regulating the expression of the gene CX43 (connexin43). Connexins comprise the structural unit of gap junctions, organelles which allow direct transfer of signals, nutrients and waste products between contacting cells. CX43 is the most widely expressed member of the gap junction family of genes, and we have demonstrated that its expression is strongly down-regulated in human cancers and in several premalignant conditions. When several human tumour cell lines were genetically engineered to conditionally express CX43 under the influence of a tetracycline promoter, their neoplastic phenotype was strongly attenuated. Specifically, induced cells were inhibited from growing in an anchorage-independent manner and, additionally, growth as xenografts in immunocompromised animals was also strongly attenuated. Growth inhibition in suspension was associated both with increased G1 cell-cycle arrest and with increased apoptosis. We propose a model whereby junctional communication allows the transfer of growth inhibitory signals from normal to neoplastic cells and that retinoids and carotenoids, by increasing signal transfer, act to prevent cancer.

Diet and cancer
There is abundant epidemiological evidence that a diet rich in green leaves and yellow and red vegetables is associated with a reduced incidence of cancer [1]. Undoubtedly, some of this protection is due to substitution of high-fat animal products with low-fat, high-fibre vegetable products, but that is not the whole story. When the constituents of such a healthy diet were analysed individually, it was found that the carotenoid component of this diet was most strongly associated with decreased risk. At the time that these initial studies were published, there was a fairly detailed knowledge about levels of \(\beta\)-carotene to be found in many foods because of the obvious importance of this carotenoid as a precursor to vitamin A, the active molecule retinoic acid being vital for normal growth and immune competence. Moreover, because previous research had demonstrated that vitamin A derivatives, now called retinoids, were cancer-preventive agents, one of the first hypotheses was that the \(\beta\)-carotene content of these healthy foods was responsible for the observed protection. However, many more carotenoids exist in nature and the human diet is now known to contain up to 100 individual carotenoids of which approximately 20 can be detected in human serum (reviewed in [1]). Unfortunately, the presence of these carotenoids in frequently consumed foods had not been determined quantitatively prior to the diligent work of Dr Khachik at the USDA (US Department of Agriculture) [1a]. With this new information, it became obvious that not just \(\beta\)-carotene was associated with protection; of particular recent interest has been the association of frequent consumption of lycopene-containing foods, a carotenoid primarily obtained from tomatoes, with decreased incidence of prostate cancer [2]. Of significance here is that lycopene is a straight-chain polyunsaturated hydrocarbon without the necessary \(\beta\)-ionone ring required for conversion into retinoids (Figure 1).

Diverse carotenoids inhibit neoplastic transformation
To determine if the association between decreased risk and carotenoids could be confirmed experimentally, and if so which dietary carotenoids possessed this activity, we began studies in transformable C3H/10T1/2 cells (10T1/2). We had previously shown these cells to respond to chemical and physical carcinogens by the quantitative formation of

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Abbreviations used: CX43, connexin43; GJC, gap junctional communication; THF, tetrahydrofuran.
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neoplastically transformed foci [3], and had shown that cancer-preventive retinoids could inhibit neoplastic transformation in these cells [4]. Carotenoids are extremely lipophilic molecules and are, thus, difficult to deliver to cells in culture; even when consumed, their bioavailability is extremely low unless dissolved in fat. Because of these limitations, the first carotenoids to be tested were those available in a commercial ‘beadlet’ formulation; however, this limited our studies to β-carotene and canthaxanthin, the latter a non-provitamin A carotenoid. These studies successfully demonstrated that both molecules could inhibit neoplastic transformation at concentrations that were physiological [5].

To extend studies to a more diverse series of dietary carotenoids, we developed THF (tetrahydrofuran) as a delivery solvent. Use of THF results in the formation of a pseudo-solution of carotenoids in cell culture medium, a form which is highly bio-available. When carotenoids were added 1 week after removal of the chemical carcinogen, all carotenoids, regardless of their provitamin A activity, were capable of inhibiting the development of neoplastic transformation. Moreover, just as in our earlier studies with retinoids, removal of the carotenoid led to emergence of neoplastic-transformed foci some 3–4 weeks later [6]. This indicated that we were not dealing with selective cytotoxicity, but with a reversible inhibition of the process of neoplastic transformation. Because of the highly unsaturated nature of the carotenoid molecule, most of them contain at least 11 conjugated double bonds, and all carotenoids are potent antioxidants. There is much evidence that reactive oxygen species are highly toxic to cells inducing DNA damage and subsequent carcinogenic mutations [7]. Thus one immediate explanation for the ability of diverse carotenoids to protect against carcinogenesis was this shared antioxidant property. However, when we compared the ability of carotenoids to protect against oxidative damage of cell membranes, by measuring the formation of oxidation products, we found that, as expected, all were active but that activity did not correlate with their cancer-preventive properties. Moreover, α-tocopherol, vitamin E, was found to be more potent than the tested carotenoids, yet did not inhibit transformation even at 10-fold higher concentrations than that used for the carotenoids [8].

These studies demonstrated that, at least in the model cell-culture system employed, dietary carotenoids had the following properties: (1) they inhibited neoplastic transformation in the post-initiation phase, (2) their action was reversible and thus not a consequence of selective cytotoxicity, (3) their action was not the result of selective growth inhibition of transformed cells, (4) their action was independent of conversion into chemopreventive retinoids, since activity was observed even with straight-chain hydrocarbons such as lycopene, carotenoids did not increase expression of a known retinoid-responsive gene, RAR-β [8] and (5) activity did not correlate with their antioxidant properties.

Thus while the action of carotenoids was similar in many respects to the more potent retinoids, they appeared to have an independent mechanism of action. Further research however revealed that these independent mechanisms of retinoids and carotenoids converged on the ability of both compounds to up-regulate expression of CX43 (connexin43), a structural unit of gap junctions.

**GJC (gap junctional communication) and carcinogenesis**

A consistent finding in studies of human or animal tumour cell lines and in studies of neoplastic transformation *in vitro*, is that tumour cells communicate poorly, if at all, with their normal counterparts [9]. These results led to the original hypothesis of growth control through junctional communication, eloquently proposed by Loewenstein [10]. Gap junctions are water-filled pores called connexons that connect adjacent cells in most organs of the body. These pores allow direct cytoplasmic to cytoplasmic communication of water-soluble molecules and ions. Because of the limiting size of the pore, only molecules < approx. 1000 Da can pass, excluding molecules such as mRNA and protein and, thus, maintaining genetic identity of the cells. The existence of this network of communication creates a syncytium through which cells can exchange nutrients, waste products and signalling molecules such as cAMP, Ca²⁺ etc. [11]. There is evidence that gap junctions also serve to transmit growth-inhibitory signals that can inhibit the aberrant proliferation of carcinogen-initiated and fully transformed cells [12].

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Figure 2 | Topographical organization of connexins into connexons, the functional unit of gap junctions
Each communicating cell contributes six connexin proteins which are arranged in the plasma membrane to surround a water-filled pore. The pore allows passive diffusion of water-soluble small molecules and ions up to approx. 1000 Da. Tight binding in the extracellular domain, essential to maintain integrity of the plasma membrane, is achieved by three thiol linkages between each connexin molecule, a total of 18 per connexon. Modified from Bertram, J.S. (2000) Cellular communication via gap junctions. Sci. Med. (Philadelphia) 8, 18–27, with permission. © 2000 Science and Medicine Inc.

was derived in part from studies in the 10T1/2 cell line and was extended by the observation that the inhibitory action of retinoids on neoplastic transformation in 10T1/2 cells was closely linked to their ability to increase GJC through increased expression of CX43 at the mRNA and protein levels [13]. It is of interest that many classes of tumour promoters, agents that accelerate the process of carcinogenesis but are not themselves carcinogenic, inhibit communication through gap junctions [14].

Gap junctions are formed by the assembly of transmembrane proteins called connexins; six of these connexin molecules assemble radially in the plasma membrane to enclose a central pore which then docks with a similar structure on a contacting adjacent cell to form the complete connexon. Each connexon is thus composed of 12 connexin molecules, contributed equally by each of the communicating partners [15]. This arrangement is shown diagrammatically in Figure 2. Passage of molecules or ions through the central pore appears to be by passive diffusion along concentration gradients. At present, over 20 connexin family members have been recognized that are differentially expressed according to cell type and at different periods of development [16]. CX43 is the most widely expressed connexin and is the family member induced by retinoids, and as we later discovered, carotenoids.

**Carotenoids induce CX43 by transcriptional activation**

We have demonstrated that carotenoids will increase CX43 expression at the message and protein levels in human and mouse fibroblasts and in supra-basal layers of human keratinocytes grown in organotypic culture [17]. Most mechanistic studies have been conducted in 10T1/2 cells. Here we have shown that increases in CX43 mRNA levels, which are not inhibited by cycloheximide, indicate direct action, and that the half-life of this message does not change after treatment with retinoid or carotenoid. Moreover, utilizing a luciferase reporter coupled with the CX43 promoter, we were able to localize the responsive region to an area between −158 and +209 bp of the transcriptional start site. This region does not contain a retinoid-responsive element but does contain an Sp-1 site which, when mutated, abolished retinoid but not carotenoid-responsiveness (A.L. Vine, Y.M. Yee and J.S. Bertram, unpublished work). These results again emphasize the similar but separate actions of retinoids and carotenoids in up-regulating the expression of this gene.

**Forced expression of CX43 in human carcinoma cells reduces markers of malignancy**

The studies discussed above, relating connexin-mediated functional GJC with enhanced growth control and inhibition of neoplastic transformation, relied on correlations to prove the association. However, these correlations do not prove a cause and effect relationship. For example, the actions of carotenoids on CX43 gene expression may go hand-in-hand with actions of carotenoids on growth control but be functionally unrelated to these actions. To establish more firmly the role of up-regulated CX43 expression and enhanced junctional communication as central to the role of retinoids and carotenoids as anti-proliferative and cancer-preventive agents, we embarked on the development of cells in which CX43 was inducible, not by carotenoids or retinoids, but by using a bacterial-promoter system. In this construct, activity of the artificially introduced gene is controllable by picogram amounts of doxycycline, which at these concentrations is not known to produce other effects in mammalian cells. The major advantage of this approach is that cells in the non-induced situation can serve as their own controls; thus the effects of CX43 expression can be determined without interpretation problems due to clonal differences between transfected cells [18] and in the absence of concomitant exposure to carotenoids. Unfortunately, because of technical difficulties, these studies have been limited to the genetic engineering of established human tumour cell lines. Three such lines have now been created, one from a cervical carcinoma [19], one from a fibrosarcoma [20] and one from a breast adenocarcinoma (X.-L. Chen and J.S. Bertram, unpublished work). In all cell lines, CX43 has been shown to be rapidly inducible from the bacterial promoter, to be integrated into the plasma membrane and to form functional gap junctions with adjacent induced tumour cells. The consequences of this induction have also been consistent: in all three engineered cell lines, anchorage-independent growth, that is growth as spheroids suspended in a semi-solid medium, is significantly decreased in CX43-induced...
Expression of CX43 reduces the growth rate of human cervical carcinoma cells in the ‘nude’ mouse

HeLa cells were engineered to express CX43 under the influence of a bacterial promoter driven by doxycycline. Immunocompromised nude mice were injected subcutaneously with HeLa cells, then randomized to receive doxycycline (0.2 mg/ml in 5% sucrose) in drinking water, or sucrose alone as controls. Tumour volumes were measured by callipers at the indicated times. (O-O) Doxycycline treated, (●-●) sucrose controls. Numbers on each data point represent total number of tumours/number of tumour injections. Reproduced from King et al. [19] with permission from Oxford University Press.

CX43. Under these conditions, cancer cell proliferation was inhibited. The implication of these studies is that the breast carcinoma cells can no longer generate growth inhibitory signals but can still respond to signals supplied by the growth-inhibited rat cells (X.-L. Chen and J.S. Bertram, unpublished work).

The clinical significance of carotenoid-induced expression of CX43 is at present uncertain; however, we have shown that down-regulated expression of CX43 in both the human cervix and oral epithelium is an early event, being observed even in dysplasia, a pathology known to predispose to malignancy but is not yet malignant [19]. Our data demonstrating that up-regulated expression of CX43 achieved by pharmacological or molecular means results in decreased proliferation of normal and malignant cells, suggests that if the observed down-regulated expression of CX43 in dysplasia can be corrected, progression to malignancy may be delayed. Indeed, in clinical intervention studies, retinoids, for cervical dysplasia [24] and oral leukoplakia [25], and carotenoids, for oral leukoplakia [26], have been shown to retard significantly carcinogenic progression. The role of JGC in these responses has not been thoroughly evaluated but the results are strongly suggestive that agents capable of normalizing junctional communication would have cancer-preventive properties.

References


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