

The chemoprevention of cancer by dietary carotenoids: studies in mouse and human cells

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ABSTRACT. Dietary carotenoids, particularly beta-carotene, have in many epidemiologic studies been associated with a decreased risk of cancer. Experimental studies have been inhibited by difficulties in delivering these molecules to target cells. A novel delivery system has been developed in the mouse 10T1/2 cell line. With these cells several carotenoids of dietary and commercial interest have been shown capable of inhibiting carcinogen-induced neoplastic transformation. Their action appears qualitatively similar to the previously documented action of retinoids (vitamin A derivatives) in this cell system, in that inhibition occurs in the post-initiation phase of carcinogenesis, but higher concentrations (10-1000 fold) are required. Both types of compound were found to strongly up-regulate gap junctional intercellular communication (GJC) and these activities were statistically correlated. Up-regulation of gap junctional intercellular communication was caused by the increased expression of connexin 43 (Cx43) at the message and protein level. Cx43 is one member of a family of gap junctional structural proteins. While protection from carcinogen-induced neoplastic transformation cannot be directly studied in human cells, we have shown that Cx43 expression is also up-regulated in human fibroblasts, suggesting that carotenoids have chemopreventive action in humans. We have proposed that increased junctional communication is mechanistically linked to inhibition of transformation in 10T1/2 cells. In this model, the gap junction serves as a conduit for growth regulatory signals from normal to carcinogen-initiated cells, thereby suppressing their transformation.

INTRODUCTION

Carotenoids as cancer preventive agents ; Studies in Humans

The concept that carotenoids play a preventive role in human cancer has undergone much revision in recent years. The results to be presented here indicate that further revision may be necessary. For many years the only known function of carotenoids in human nutrition was the role of beta-carotene, and a few of the other carotenoids found in the human diet, as a source of vitamin A. Vitamin A, and more specifically retinoic acid, was known to be a potent regulatory factor of epithelial cell differentiation (ref. 1), and, in many studies in experimental animals and in cell cultures, had been shown to act as a potent cancer chemopreventive agent (refs. 2,3). Thus, it was not unexpected when several epidemiologic studies demonstrated an inverse correlation between consumption of foods rich in vitamin A and pro-vitamin A carotenoids and future incidence of cancer (reviewed in ref. 4). These findings appeared to confirm other studies which had revealed a similar inverse correlation between serum retinol levels and cancer risk (refs. 4,5).

Re-assessment of these conclusions occurred as a result of two events: first a re-evaluation of the blood retinol data which revealed that low retinol values occurred only in subjects whose blood was obtained within about 2 years of the diagnosis of cancer (ref. 6) (i.e. lowered serum retinol was most likely a consequence of pre-clinical disease); second, a seminal paper by Sporn and Peto (ref. 7) in which it was argued that it was the beta-carotene component of the diet that was responsible for the observed epidemiologic findings. Many subsequent epidemiologic studies have statistically separated the carotenoid component of the diet from the pre-formed retinoid component, and have repeatedly demonstrated, particularly in the case of lung cancer, that populations who consume diets rich in carotenoid-containing foods have a lower risk of cancer than those who do not (ref. 8). However, because such diets contain many other potentially protective substances, the cause-and-effect relationship remains to be proven. To test this hypothesis, multiple cancer chemoprevention intervention trials using beta-carotene are currently on-going. The largest involves 20,000 physicians in the U.S.A. who have been taking 25mg beta-carotene every other day for 10 years.

Experimental studies in animals and cell cultures

In experimental animals, certain carotenoids such as β -carotene and canthaxanthin have shown activity against carcinogenesis induced by chemicals or UV-light (ref. 2). However, most experimental animals, including the rat and mouse, differ dramatically from humans in their limited ability to absorb these compounds as intact molecules, and by the rapid metabolism of any absorbed carotenoid. Several alternative animal models have been suggested, but none has been evaluated for carcinogenesis studies (ref. 9). Definitive experiments have thus been severely hindered. Studies in cell culture have until recently been hindered also by difficulties in supplying the highly lipophilic carotenoids in a bioavailable form. We have overcome this problem by the use of tetrahydrofuran (THF) as solvent, allowing the delivery of diverse carotenoids to cultured cells at high concentration in a bioavailable, micelle-like, form. Using this delivery system, and the 10T1/2 line of transformable mouse fibroblasts, we have demonstrated that many dietary carotenoids can inhibit neoplastic transformation in the post-initiation phase of carcinogenesis (ref. 10). The 10T1/2 cell culture system has been widely employed in studies of chemical and physical carcinogenesis, and mirrors whole animal studies in many important respects (ref. 11). The cell culture studies presented here have allowed examination of the structure/activity relationships of diverse dietary carotenoids, and have suggested a novel mechanism of action of carotenoids as chemopreventive agents, i.e. the induction of gap junctional communication

CELL CULTURE PROCEDURES

Chemicals.

Tetrahydrofuran (THF) was analytical grade obtained from Fischer (Fair Lane, NJ). Butylated hydroxytoluene (BHT), 3-methylcholanthrene (MCA), Lucifer Yellow CH and other chemicals were obtained from Sigma Chemical Co (St. Louis, MO). Canthaxanthin was a gift from Hoffmann-La Roche, Basel.

Cells and cell culture.

C3H 10T1/2 cells were cultured as previously described (ref. 10) in basal Eagle's medium (GIBCO) with calf serum and 25 μ g/ml gentamycin. They were exposed to 3 μ g/ml methylcholanthrene (MCA) in acetone for 24 hr, one day after seeding then, 7 days after removal of the carcinogen, were given the stated concentration of carotenoid dissolved in tetrahydrofuran (THF). Cultures were re-fed and retreated with carotenoid every 7 days. Control cultures received acetone plus MCA or acetone plus THF. Numbers of morphologically transformed foci were evaluated 42 days after seeding the cultures as described (ref. 12).

Communication assays.

Cell cultures were seeded as above and treated with carotenoids as in the transformation experiments. After 14, 24 and 34 days of treatment, intercellular communication was assessed by microinjection of the junctionally permeable fluorescent dye Lucifer Yellow into approximately 20 randomly chosen cells. Communication, measured as the number of fluorescent cells surrounding each injected donor cell, was assessed 10 minutes after injection as described (ref. 13).

Molecular studies.

Western blotting and immunofluorescence studies on intact cells were performed with a rabbit polyclonal antibody raised against a synthetic peptide corresponding to the C-terminal 15 residues of the predicted sequence of connexin 43 (ref. 14). Northern blotting of total cellular RNA was performed at high stringency with 32 P-labelled full-length cDNA to connexin 43, a gift of E. Beyer (ref. 14).

CAROTENOIDS INHIBIT CHEMICALLY- AND PHYSICALLY-INDUCED NEOPLASTICALLY TRANSFORMED FOCI IN C3H10T1/2 CELLS.

In order to examine the chemopreventive action of the carotenoids, 10T1/2 cells were induced to undergo neoplastic transformation by exposure to either a chemical carcinogen, 3-methylcholanthrene, or to 600 rad X-irradiation. These cultures respond to carcinogenic insult by the production of foci of carcinogen-induced neoplastic transformed cells 4-5 weeks after exposure. Non-toxic concentrations of carotenoids, dissolved in THF, were added to 10T1/2 cells 7 days after removal of 3-methylcholanthrene or 8 days after X-irradiation, and were maintained in the cultures for the remaining 4-week duration of the experiment. Treatment resulted in a dose-dependent reduction in the formation of carcinogen-induced neoplastically transformed foci. As seen in Figs. 1 and 2, a

concentration of 10^{-5} M beta-carotene or canthaxanthin virtually completely eliminated transformed foci, but continuous treatment was required. If carotenoids were removed after four weeks treatment, foci appeared in carcinogen-treated cultures after a 3-5 week latent period. Addition of carotenoids prior to and during carcinogen exposure, or after neoplastic transformation had occurred, had little influence on the formation of carcinogen-induced transformed foci. It may therefore be concluded that carotenoids act in the post-initiation phase of carcinogenesis by reversibly suppressing the ability of carcinogen-initiated cells to undergo neoplastic transformation.

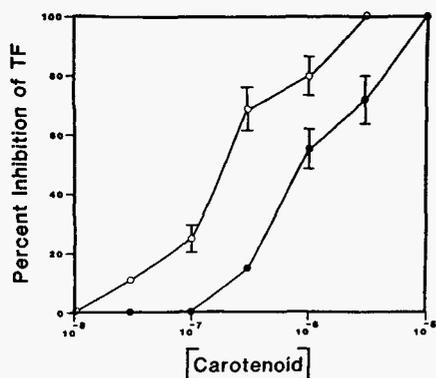


Figure 1. Inhibition of MCA-induced neoplastic transformation in 10T1/2 cells by carotenoids. Beta-carotene (●) or canthaxanthin (○) were added to cultures treated 7 days previously with MCA ($1\mu\text{g/ml}$). Control cultures received MCA then vehicle. Data are expressed as transformation frequency (TF) as a % of these controls. From ref. 36.

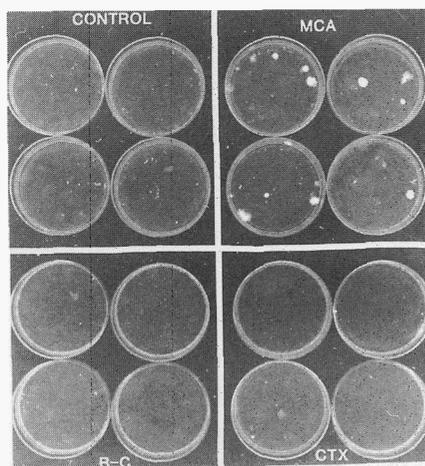


Figure 2. Appearance of culture dishes after 5 weeks incubation. Beta-carotene or canthaxanthin 10^{-5} M were added to MCA-treated cultures as in Fig. 1 for 4 weeks. Solvent control, top left; MCA top right; MCA followed by β -carotene, lower left; MCA followed by canthaxanthin, lower right.

The activity of carotenoids in this respect mirrors the actions of retinoids in C3H10T1/2 cells. Here too, transformation is reversibly inhibited when retinoids are added after exposure to carcinogen, yet transformation can be again expressed upon drug withdrawal, after a 4-5 week latent period (ref. 3). Might this similarity of biological action imply similar molecular action? Unfortunately the mode of action of carotenoids as cancer chemopreventives is poorly understood at the molecular level. Previously, carotenoids had been considered to have two possible functions in mammals: to be metabolized to retinoids, for that limited group of compounds capable of such conversion (the provitamin A carotenoids), and to act as lipid-phase antioxidants. This latter activity is shared by all carotenoids but to differing degrees. Our results presented above suggested that the action of carotenoids may be due to their conversion into retinoids in cell cultures. However, on a chemical basis, this explanation appears improbable; canthaxanthin, which is marginally more active than beta-carotene in 10T1/2 cells, is not a pro-vitamin source in mammals. We have furthermore demonstrated that lycopene, an acyclic carotenoid, (ref. 10), and a synthetic C-22 carotenoid analogue (8,13-dimethyl-2,2,19,19-tetramethoxy-eicosa-4,6,8,10,12,14,16-heptaene-3,18-dione (ref. 15), are active in 10T1/2 cells as inhibitors of transformation. Conversion of these compounds to retinoids seems most unlikely. Even for beta-carotene, the carotenoid with the highest pro-vitamin A activity in mammals, we were not able to detect the expected products of its conversion into retinoids after exposure of

10T1/2 cells to C-14 labelled material (ref. 16).

Since the pro-vitamin A properties of the tested carotenoids did not correlate with their activities as inhibitors of neoplastic transformation, we next examined their potential to act as lipid-phase antioxidants.

Carotenoid inhibition of oxidative damage.

To determine if the antioxidant properties of carotenoids correlated with their abilities to inhibit carcinogen-induced neoplastic transformation, we measured thiobarbituric acid-reactive material (TBA) in carotenoid-treated 10T1/2 cells. This assay, though non-specific for the type of oxidative damage, has been widely used to measure lipid peroxidation in biological samples (ref. 17). As shown in Figure 3, while all carotenoids tested inhibited the formation of TBA-reactive material in 10T1/2 cells, there was no correlation between the potency of individual carotenoids in this assay and their potencies as inhibitors of carcinogen-induced neoplastic transformation. For example, Me-bixin was among the most potent of the carotenoids tested in the TBA assay, yet was inactive in the transformation assay (ref. 13). Similar conclusions resulted from studies of the relative potencies of synthetic carotenoids to quench singlet oxygen in a chemical system (ref. 18) and to protect against carcinogen-induced neoplastic transformation (ref. 15). In addition, the potent lipid-phase antioxidant alpha-tocopherol demonstrated greater potency in the TBA assay than any of the carotenoids, yet was much less active than many of the carotenoids in the transformation assay (ref. 13). Thus, while prevention of oxidative damage by carotenoids and other antioxidants may play a role in protection from neoplastic transformation, we concluded that other factors must predominate.

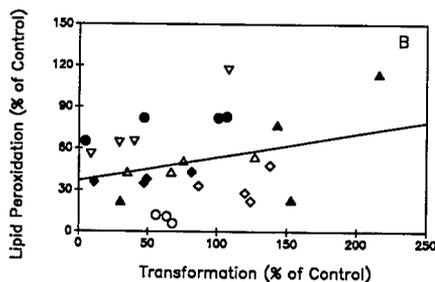


Figure 3. Lack of correlation between inhibition of transformation and of protection from lipid oxidation by diverse carotenoids. Beta-carotene, ∇; alpha-carotene, ●; alpha-tocopherol, ○; canthaxanthin, ◆; lutein, □; lycopene, Δ. Data from ref. 13

Do Carotenoids up-regulate gap junctional intercellular communication?

As discussed above, carotenoids inhibit carcinogen-induced neoplastic transformation in a manner qualitatively similar to the retinoids. For the retinoids, we have previously demonstrated that induction of gap junctional communication (GJC) is highly correlated with their chemopreventive activity (ref. 19). Furthermore there is evidence from our research, and that of others, that GJC is involved in growth control and is therefore of great interest to the study of carcinogenesis (ref. 20). Several lines of evidence lend support to this statement: For example: 1) when fully transformed 10T1/2 cells are forced into gap junctional communication with non-transformed cells the transformed cell becomes growth-arrested and the transformed phenotype latent (ref. 21); 2) GJC is inhibited by tumor promoters, which have biological effects opposite to the retinoids on carcinogenesis (ref. 22). 3) Transfection of genes coding for functional gap junctions into neoplastic cells partially restores the normal phenotype (refs. 23-25). 4) The chemopreventive retinoids (ref. 19), and as shown below, the carotenoids, up-regulate GJC.

To determine if carotenoids share with retinoids this ability to up-regulate junctional communication, 10T1/2 cells were exposed to carotenoids under the conditions of the transformation assay, and at various periods thereafter junctional communication was measured by dye-injection. As shown in Figure 4, carotenoid treatment caused a progressive increase in junctional communication after a delay of about 4 days. This increase was maintained over the 4 week experimental period; the same treatment period used in the transformation assays. Dose-response studies demonstrated that a good correlation existed between the ability to inhibit transformation and to induce gap junctional communication. Thus beta-carotene and canthaxanthin were approximately equipotent in both assays, while Me-bixin for example, was without activity in either of the assays (Figure 5). Although

the carotenoids produced a similar magnitude of induced GJC as did the retinoids, this activity required higher concentrations (up to 1000-fold, depending on the compound) and longer treatment times (3-4 days vs. 1 day for retinoids).

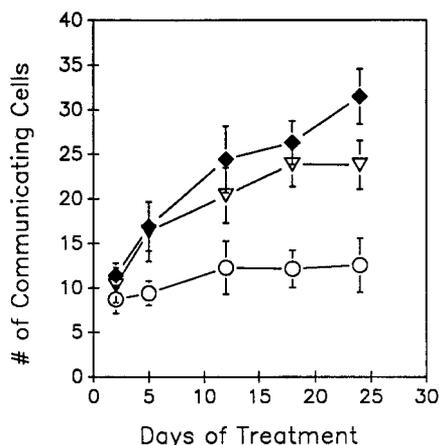


Figure 4. Time course for induction of gap junctional intercellular communication in 10T1/2 cells by carotenoids. Beta-carotene, ▽; canthaxanthin, ◆; THF solvent control, ○. All compounds were tested at 10^{-5} M. From ref. 13 with permission.

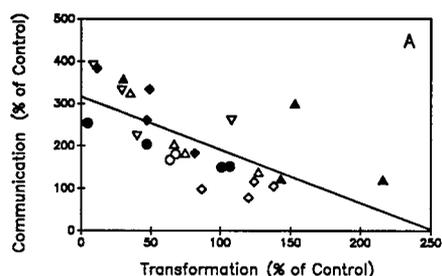


Figure 5. Correlation between induction of gap junctional communication as measured by dye-transfer and suppression of transformation. Symbols as in Fig. 3.

These data demonstrating the role of intercellular junctional signalling in growth control suggest that the action of retinoids, and perhaps the carotenoids, may be to maintain carcinogen-initiated cells in junctional communication with surrounding non-transformed cells thereby preventing their transformation. Before describing in more detail the molecular aspects of carotenoid action on gap junctional communication a brief discussion of gap junctions themselves seems in order.

Function and structure of gap junctions: Gap junctions have been shown to link virtually all cells within an organ to form a communicating syncytium. Genes coding for gap junctions have been highly conserved in evolution and are found in organisms as simple as hydra (ref. 26). They are known to: i) transmit the ionic signals for contraction in heart and myometrium, ii) act as substitutes for chemical synapses in neurons when speed is essential. Furthermore there is growing evidence that gap junctions have a role in regulating: iii) morphogenesis, iv) differentiation, v) growth control, vi) secretion of hormones, especially in the pancreatic islets, vii) transfer of nutrients and waste products in the avascular cornea and lens. The full spectrum of their functions is not yet fully understood (for reviews see refs. 27,28).

A family of closely related genes, with organ and developmental specific expression have been described (ref. 26). Connexin 43, first cloned from rat heart cDNA (ref. 14), and now known to be expressed in many tissues including 10T1/2 cells (ref. 29), codes for a transmembrane protein, six copies of which form a hexameric array surrounding a central water-filled pore. Two such arrays in adjacent cells create a gap junction capable of transferring molecules of up to about 1,000 daltons between communicating cells (ref. 27). The chemical nature of the signals and their physiological functions are beginning to be explored. It is known that intracellular messengers such as Ca^{++} , cAMP and inositol phosphates can travel through the junction (refs. 30,31). Perhaps one of these messages is responsible for the proposed transfer of growth regulatory signals from non-transformed cells to adjacent carcinogen-initiated cells thereby preventing their transformation

Mechanism of carotenoid-enhanced gap junctional communication

Measurements of connexin 43 gene expression. Previous studies had shown that the increased

gap junctional communication observed after retinoid treatment was a consequence of the up-regulated expression of connexin 43 at the protein and message level. This is the only connexin known to be expressed in 10T1/2 cells (ref. 29). When Northern and Western blots were performed on total mRNA and protein respectively, isolated from beta-carotene or canthaxanthin treated 10T1/2 cells, a major increase in connexin 43 gene products was observed in response to both carotenoids tested. On Western blots, an increased immunolabelling of two bands in the 43-45kD region by the anti-connexin 43 antibody was seen (Fig. 6). The higher Mr band, also seen after retinoid treatment of 10T1/2 cells, was shown to represent a phosphorylated form of connexin 43 (ref. 29). Northern blotting of total RNA extracted from 10T1/2 cells, probed with full length connexin 43 cDNA demonstrated a major increase in hybridization to a 3.1kb band in carotenoid treated cells. This corresponds to the reported transcript size of connexin 43 (ref. 14). Control cultures treated with THF as solvent expressed only low amounts of connexin 43 mRNA or protein. This is in accord with their low level of communication seen in the dye transfer experiments (ref. 13).

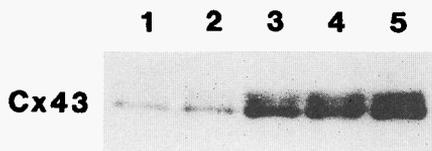


Figure 6. Canthaxanthin increases expression of connexin 43. 10T1/2 cells were treated with canthaxanthin or THF as solvent control as in Figure 4. After 4 days cultures were harvested and total cell protein prepared for Western blotting as described in ref. 33. Lanes: 1, control; 2, 0.3 μ M; 3, 1.0 μ M; 4, 3.0 μ M; 5, 10 μ M.

Immunofluorescence studies. When carotenoid treated cells were processed for indirect immunofluorescence using the same connexin 43 antibody as used in the Western blots, a major increase in immunofluorescent plaques was observed in regions of cell-cell contact (Figs. 7C,D). These plaques are the presumed sites of aggregation of individual connexons to form the junctional complexes which have been visualized by electron microscopy of other junctionally communicating cells. Thus the increased amounts of connexin 43 detected by Western blotting became localized in regions of the cell where they could contribute to the carotenoid-enhanced junctional communication detected by the dye transfer experiments.

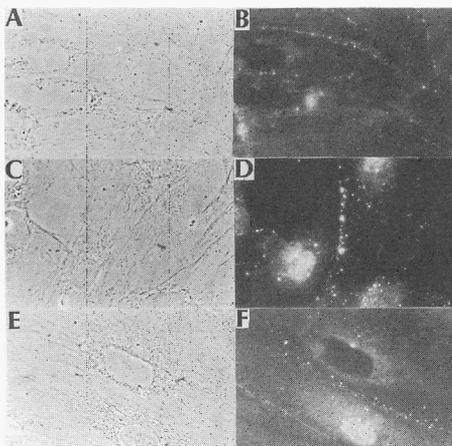


Figure 7. Carotenoids increase the number of junctional plaques recognized by anti-connexin 43 antibody 10T1/2 cells. A, B THF control; C, F after treatment for 4 days with canthaxanthin. A, C, E, phase contrast; B, D, F, immunofluorescence. A,B, THF control; C,D, CTX 10^{-5} M; E,F, CTX 10^{-6} M. From ref. 33, with permission.

Effects in human cells. Addition of beta-carotene, canthaxanthin or lycopene to early passage human dermal fibroblast cells has recently been shown to up-regulate gap junctional communication and the expression of Cx43. These effects were produced at concentrations between 10^{-6} and 10^{-5} M, as in 10T1/2 cells (data not shown). Effects on carcinogenesis could not be determined because human cells have so far resisted attempts to transform them with carcinogens

Do carotenoids up-regulate other retinoid-responsive genes? The conclusion that carotenoids do not act through conversion to retinoids was primarily based on chemical evidence: the absence of C-14 labeled retinoids after incubation with C-14 beta-carotene, and the activity of non pro-vitamin A carotenoids in our assay system. However the possibility exists that at high concentration, carotenoids may possess weak activity as retinoid agonists. To test this we investigated the potential for carotenoids to activate a known retinoid-responsive gene. We chose to examine RAR-beta. This

nuclear retinoid receptor(RAR), is retinoid-inducible in a wide variety of cell types and has been shown to possess a retinoic acid responsive element (RARE) in its promoter region (ref. 32). In both T1/2 and F9 teratocarcinoma cells retinoids up-regulated expression of both connexin 43 and RAR-beta as expected. However, when we added canthaxanthin to these cultures, chosen because of its chemical purity and stability, only Cx43 was up-regulated at the message level (ref. 33). These results imply that carotenoids and retinoids function through separate but overlapping pathways. We are currently investigating whether retinoid and/or carotenoid responsive elements exist in the promoter region of the Cx43 gene.

Proposed significance of up-regulated gap junctional communication

The consistent association between enhanced gap junctional communication, suppression of neoplastic transformation and augmented growth control of both normal and neoplastic cells (refs. 19,21,34), strongly supports the argument for a functional role for junctionally transmitted signals in these events. In our proposed model of carotenoid and retinoid action, the increased gap junctional communication caused by these compounds places carcinogen-initiated cells within an expanded communicating network. This will usually be dominated by normal cells since, both *in vitro* and *in vivo*, initiation appears to be a rare event. This increased communication, which as discussed above is associated with enhanced growth control, acts to stabilize the initiated cell and prevent its neoplastic transformation.

However, once neoplastic transformation has occurred, retinoids (carotenoids have not yet been tested), have been shown unable to enhance the low level of heterologous communication that exists between normal and transformed 10T1/2 cells (ref. 19). Accordingly, in most circumstances, retinoids do not act as chemotherapeutic agents, but as chemopreventive agents against preneoplastic cells. These conclusions, derived from studies in cell cultures, have received support from a recent clinical trial of 13-*cis*-retinoic acid. Here the drug failed to influence the growth of existing head and neck tumors but strongly suppressed the development of new primary tumors (ref. 35).

A major question not yet addressed in our studies, is the chemical nature of the putative growth regulatory signal(s) that is transferred through gap junctions. Because of the constraints of the junctional pore, such signals must be below about 1000 daltons in size; because the pore is water-filled the signals should be water-soluble, and, to exclude passive transfer through adjacent plasma membranes, the signals should be electrically charged. Clearly, properties of physiologically active ions such as Ca⁺⁺, or second messengers such as cAMP, satisfy these criteria and both are known to traverse the junction (refs. 30,31). One of the next phases of research in this area will be to devise appropriate methods to test the effects of such ions and molecules on growth control and neoplastic transformation.

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