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# Differential Dose-dependent Effects of α-, β-Carotenes and Lycopene on Gap-junctional Intercellular Communication in Rat Liver *in vivo*

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**In order to examine the relevance of alteration of gap-junctional intercellular communication (GJIC) to chemopreventive activity against carcinogenesis, the effects of α- and β-carotene as well as lycopene, typical chemopreventive carotenoids, on cell coupling *via* gap junctions in rat liver *in vivo* were studied using a direct functional dye-transfer technique. We found that all three test compounds given at a dose of 50 mg/kg-body weight (b.w.) daily, 5 times by gavage, inhibited GJIC, while similar treatment with 5 mg/kg b.w. caused enhancement, especially in the β-carotene- and lycopene-treated groups. At the dose level of 0.5 mg/kg b.w., the three compounds had no effect. The findings show that all three agents differentially modulate GJIC depending on the dose, with beneficial effects on cell communication only detected at the one dose. The result suggests that determination of the dose of chemicals to be used is crucial for human intervention studies.**

Key words: Carotenoid — Gap junction — Chemoprevention

Cancer chemoprevention is a rapidly developing field of cancer research and many compounds have been found to possess inhibitory potential. However, rather little is known concerning the mechanisms responsible for chemical inhibition of tumor development. Basically, however, cancer preventive action is based upon modulation of cell proliferation or apoptosis. Although many processes are known to be involved in the regulation of cell growth and therefore could play a role in chemoprevention, only a few are ubiquitous. A promising example is gap-junctional intercellular communication (GJIC).[1–4)]

An etiologic role for GJIC impairment in carcinogenesis has been established experimentally.[5–10)] In particular, a long-lasting decrease in cell communication appears to be a strong tumor-promoting factor with a great variety of known promoters specifically inhibiting GJIC both in cultured cells[11)] and *in vivo*.[12)] The reversibility of induced GJIC disorders,[13, 14)] however, provides a possible approach to chemoprevention. In fact, it has already been shown that certain chemopreventive chemicals, such as carotenes and retinoic acid, specifically increase GJIC *in vitro*.[15–18)] However, in order to validate up-regulation of GJIC as a tumor chemopreventive factor, corroborative *in vivo* evidence is needed.

The aim of this study was therefore to analyze the ability of well-known tumor chemopreventive agents, α- and β-carotenes and lycopene to modulate GJIC *in vivo*. To address this issue experimentally, we employed a direct functional dye-transfer technique, which has been successfully used to demonstrate inhibitory effects of different hepatopromoters on GJIC in rat liver.[12)]

Groups of twenty-four 5-week-old F344 male rats were treated for 5 days, daily by gavage, with α- and β-carotenes and lycopene at doses of 50, 5, 0.5 and 0 mg/kg body weight in corn oil and 24 hours after the last treatment GJIC was evaluated in the liver by the direct dye transfer technique.

GJIC was assayed by fluorescent dye coupling essentially as described previously.[19)] Briefly, 1 mm-thick slices from freshly removed rat livers were injected in multiple sites with Lucifer Yellow CH fluorescent dye (5% in 0.1 *M* LiCl) through glass capillary micropipettes. Five minutes later, after brief rinsing in phosphate-buffered saline (PBS), the slices were embedded in 7% gelatin solution in PBS and frozen in liquid nitrogen. The spread of dye from the injected sites into the surrounding tissue was evaluated microscopically using 5 μm cryosections. At least 20 injections per liver were evaluated in terms of mean areas of dye spots. The assays were performed under the same conditions (one glass capillary micropipette, constant dye pressure) for each treatment dose, including the control group, because these conditions affect the control value significantly. The values were statisti-

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Table I. Differential Dose-dependent Effects of α-, β-Carotenes and Lycopene on Gap-junctional Intercellular Communication in Rat Liver *in vivo*

| | Doses | | | | | |
|---|---|---|---|---|---|---|
| | 50 mg/kg | | 5 mg/kg | | 0.5 mg/kg | |
| | No. of spots | Area ($mm^2$) | No. of spots | Area ($mm^2$) | No. of spots | Area ($mm^2$) |
| α-Carotene | 55 | 0.125±0.076[a] | 43 | 0.474±0.118[b] | 40 | 0.158±0.060 |
| β-Carotene | 25 | 0.114±0.073[a] | 40 | 0.664±0.137[b] | 33 | 0.197±0.118 |
| Lycopene | 34 | 0.075±0.040[a] | 43 | 0.635±0.116[b] | 49 | 0.200±0.095 |
| Control | 39 | 0.372±0.269 | 43 | 0.391±0.116 | 50 | 0.169±0.095 |

*a*) The area of dye spread through gap junctions of the treated livers was significantly smaller than that of the control.
*b*) The area of dye spread through gap junctions of the treated livers was significantly larger than that of the control.

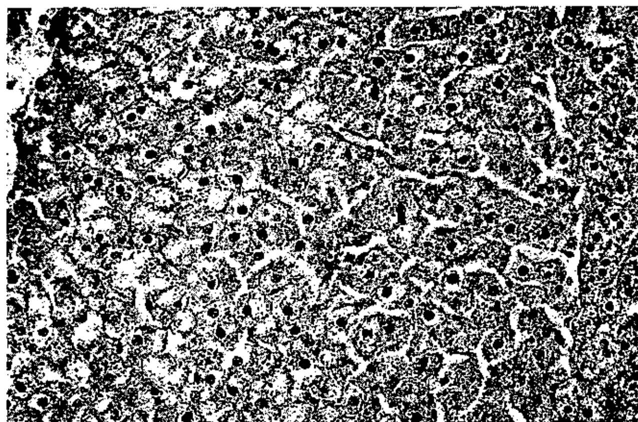

Fig. 1. Immunostaining of cx32 in β-carotene-treated liver. There is little change in the number of cx32-positive spots per hepatocyte compared to the control liver.

cally analyzed by ANOVA analysis followed by post-hoc tests (StatView 4.0, Abacus Concepts Inc., Berkeley, CA).

The major liver-specific gap-junctional protein connexin 32 (cx32) was also detected by indirect immunostaining on cryosections as described previously.[20] The primary antiserum was obtained by immunization of rabbits with a synthetic peptide corresponding to residues 98–124, located in the cytoplasmic loop of native cx32.[21] The secondary antibody was anti-rabbit peroxidase-conjugated mouse monoclonal IgG (Sigma Immunochemicals, St. Louis, MO). DAB-$NiCl_2$ coloring subsequently enriched by silver development[22] was used to reveal a positive reaction. Hematoxylin-eosin counterstaining was applied to facilitate orientation.

Treatment of rats with three different carotenoids at three different dose levels within a range of two orders of magnitude caused different effects on GJIC in the liver. Thus, administration of all three tested compounds at a dose of 50 mg/kg for 5 days resulted in inhibition of cell coupling (Table I). On the other hand, treatment with the 5 mg/kg dose clearly enhanced GJIC, particularly in β-carotene- and lycopene-treated rats (Table I), while the 0.5 mg/kg dose level failed to modulate GJIC in rat liver (Table I). Immunostaining of the livers of the treated animals revealed little change in the number of cx32-positive spots per hepatocyte (Fig. 1), and there were no obvious morphological signs of toxicity or aberrant localization of cx32 protein inside the cytoplasm of hepatocytes. Abnormalities of GJIC may be caused by several mechanisms. A missense mutation of the *cx32* gene in a rat hepatocarcinoma has been reported.[23] More common mechanisms for decreased GJIC are connexin mRNA instability[24, 25] and abnormal localization of connexin proteins due to post translational modification of the protein.[26] In all of the above cases, immunohistochemical techniques can reveal abnormalities. Our results in this paper are in contrast to our previous observation of decreased cell coupling in rat liver after treatment with different hepatospecific tumor promoters, mostly due to translocation of cx32 from the plasma membrane into the cytoplasm of hepatocytes, and the associated prominent hepatotoxicity,[12] and suggest that carotenoids change the permeability of gap junctions without affecting the protein localization.

Retinoids have been well studied for chemopreventive activities, and are known to have differential effects on gap-junctional capacity[27, 28] like the carotenoids examined in this study. The available data indicate that beneficial effects on GJIC can be achieved if an appropriate dose is employed. The finding that high doses of carotenoids could be rather harmful in terms of GJIC, on the other hand, is very much in line with the recent results of a human intervention study; after four years of supplementation of high-risk individuals with high doses of β-carotene in combination with vitamin A, adverse effects on the incidence of lung cancer and associated

mortalities became evident,[29] which caused the investigators to abort the trial.

Clearly, comparison of experimental data obtained in the rat liver with human observations must take into consideration the tissue specificity of effects of carotenes. Unfortunately, so far no adequate experimental approach to evaluate the function of GJIC directly in tissues other than liver has been developed. However, it has been shown experimentally that the rat liver is also a responsive tissue to carotenes in terms of modulation of experimental carcinogenesis. Thus, $\beta$-carotene, given to rats, delayed the formation of putative preneoplastic enzyme-altered lesions as compared to the control.[30, 31] In contrast, long-term $\beta$-carotene treatment (0.4 mg three times, weekly) in mouse hepatocarcinogenesis enhanced tumor formation (our unpublished data). Therefore, we believe that our present finding that different doses of carotenes have differential effects on GJIC rat liver is mechanistically relevant to carcinogenesis and should be taken into consideration in future long-term chemopreventive experiments with carotenoids and human applications. We are now investigating the dose dependence of carotenoid effects on rat hepatocarcinogenesis.

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## REFERENCES

1) Kumar, N. M. and Gilula, N. B. The gap junction communication channel. *Cell,* **84,** 381–388 (1996).
2) Trosko, J. E. and Goodman, J. I. Intercellular communication may facilitate apoptosis: implications for tumor promotion. *Mol. Carcinog.,* **11,** 8–12 (1994).
3) Mesnil, M. and Yamasaki, H. Cell-cell communication and growth control of normal and cancer cells: evidence and hypothesis. *Mol. Carcinog.,* **7,** 14–17 (1993).
4) Yamasaki, H. and Naus, C. C. G. Role of connexin genes in growth control. *Carcinogenesis,* **17,** 1199–1213 (1996).
5) Yotti, L. P., Chang, C. C. and Trosko, J. E. Elimination of metabolic cooperation in Chinese hamster cells by a tumor promoter. *Science,* **206,** 1089–1091 (1979).
6) Yamasaki, H. Gap junctional intercellular communication and carcinogenesis. *Carcinogenesis,* **11,** 1051–1058 (1990).
7) Trosko, J. E., Madhukar, B. V. and Chang, C. C. Endogenous and exogenous modulation of gap junctional intercellular communication: toxicological and pharmacological implications. *Life Sci.,* **53,** 1–19 (1993).
8) Klaunig, J. E. Biology of disease. Role of inhibition of intercellular communication in carcinogenesis. *Lab. Invest.,* **62,** 135–146 (1990).
9) Holder, J. W., Elmore, E. and Barrett, J. C. Gap junction function and cancer. *Cancer Res.,* **53,** 3475–3485 (1993).
10) Yamasaki, H., Mesnil, M., Omori, Y., Mironov, N. and Krutovskikh, V. Intercellular communication and carcinogenesis. *Mutat. Res.,* **333,** 181–188 (1995).
11) Budunova, I. V. and Williams, G. M. Cell culture assays for chemicals with tumor-promoting or tumor-inhibiting activity based on the modulation of intercellular communication. *Cell Biol. Toxicol.,* **10,** 71–116 (1994).
12) Krutovskikh, V. A., Mesnil, M., Mazzoleni, G. and Yamasaki, H. Inhibition of rat liver gap junction intercellular communication by tumor-promoting agents *in vivo.* Association with aberrant localization of connexin proteins. *Lab. Invest.,* **72,** 571–577 (1995).
13) Mehta, P. P., Bertram, J. S. and Loewenstein, W. R. Growth inhibition of transformed cells correlates with their junctional communication with normal cells. *Cell,* **44,** 187–196 (1986).
14) Yamasaki, H. and Katoh, F. Further evidence for the involvement of gap-junctional intercellular communication in induction and maintenance of transformed foci in BALB/c 3T3 cells. *Cancer Res.,* **48,** 3490–3495 (1988).
15) Zhang, L. X., Acevedo, P., Guo, H. and Bertram, J. S. Upregulation of gap junctional communication and connexin43 gene expression by carotenoids in human dermal fibroblasts but not in human keratinocytes. *Mol. Carcinog.,* **12,** 50–58 (1995).
16) Acevedo, P. and Bertram, J. S. Liarozole potentiates the cancer chemopreventive activity of and the up-regulation of gap junctional communication and connexin43 expression by retinoic acid and beta-carotene in 10T1/2 cells. *Carcinogenesis,* **16,** 2215–2222 (1995).
17) Bex, V., Mercier, T., Chaumontet, C., Gaillard-Sanchez, I., Flechon, B., Mazet, F., Traub, O. and Martel, P. Retinoic acid enhances connexin43 expression at the post-transcriptional level in rat liver epithelial cells. *Cell Biochem. Funct.,* **13,** 69–77 (1995).
18) Brummer, F., Zempel, G., Buhle, P., Stein, J. C. and Hulser, D. F. Retinoic acid modulates gap junctional permeability: a comparative study of dye spreading and ionic coupling in cultured cells. *Exp. Cell Res.,* **196,** 158–163 (1991).
19) Krutovskikh, V. A., Oyamada, M. and Yamasaki, H. Sequential changes of gap-junctional intercellular communications during multistage rat liver carcinogenesis: direct measurement of communication *in vivo. Carcinogenesis,*

12, 1701–1706 (1991).

20) Krutovskikh, V., Mazzoleni, G., Mironov, N., Omori, Y., Aguelon, A. M., Mesnil, M., Berger, F., Partensky, C. and Yamasaki, H. Altered homologous and heterologous gap-junctional intercellular communication in primary human liver tumors associated with aberrant protein localization but not gene mutation of connexin 32. *Int. J. Cancer,* **56,** 87–94 (1994).
21) Milks, L. C., Kumar, N. M., Houghten, R., Unwin, N. and Gilula, N. B. Topology of the 32-kd liver gap junction protein determined by site-directed antibody localizations. *EMBO J.,* **7,** 2967–2975 (1988).
22) Merchenthaler, I., Stankovics, J. and Gallyas, F. A highly sensitive one-step method for silver intensification of the nickel-diaminobenzidine endproduct of peroxidase reaction. *J. Histochem. Cytochem.,* **37,** 1563–1565 (1989).
23) Omori, Y., Krutovskikh, V., Mironov, N., Tsuda, H. and Yamasaki, H. *Cx32* gene mutation in a chemically induced rat liver tumour. *Carcinogenesis,* **17,** 2077–2080 (1996).
24) Neveu, M. J., Hully, J. R., Babcock, K. L., Hertzberg, E. L., Nicholson, B. J., Paul, D. L. and Pitot, H. C. Multiple mechanisms are responsible for altered expression of gap junction genes during oncogenesis in rat liver. *J. Cell Sci.,* **107,** 83–95 (1994).
25) Budunova, I. V., Carbajal, S. and Slaga, T. J. The expression of gap junctional proteins during different stages of mouse skin carcinogenesis. *Carcinogenesis,* **16,** 2717–2724 (1995).
26) Musil, L. S. and Goodenough, D. A. Biochemical analysis of connexin43 intracellular transport, phosphorylation, and assembly into gap junctional plaques. *J. Cell Biol.,* **115,** 1357–1374 (1991).
27) Shuin, T., Nishimura, R., Noda, K., Umeda, M. and Ono, T. Concentration-dependent differential effect of retinoic acid on intercellular metabolic cooperation. *Gann,* **74,** 100–105 (1983).
28) Guo, H., Acevedo, P., Parsa, F. D. and Bertram, J. S. Gap-junctional protein connexin 43 is expressed in dermis and epidermis of human skin: differential modulation by retinoids. *J. Invest. Dermatol.,* **99,** 460–467 (1992).
29) Omenn, G. S., Goodman, G. E., Thornquist, M. D., Balmes, J., Cullen, M. R., Glass, A., Keogh, J. P., Meyskens, F. L., Valanis, B., Williams, J. H., Barnhart, S. and Hammar, S. Effects of a combination of beta carotene and vitamin A on lung cancer and cardiovascular disease. *N. Engl. J. Med.,* **334,** 1150–1155 (1996).
30) Moreno, F. S., Rizzi, M. B., Dagli, M. L. and Penteado, M. V. Inhibitory effects of beta-carotene on preneoplastic lesions induced in Wistar rats by the resistant hepatocyte model. *Carcinogenesis,* **12,** 1817–1822 (1991).
31) Tsuda, H., Uehara, N., Iwahori, Y., Asamoto, M., Iigo, M., Nagao, M., Matsumoto, K., Ito, M. and Hirono, I. Chemopreventive effects of $\beta$-carotene, $\alpha$-tocopherol and five naturally occurring antioxidants on initiation of hepatocarcinogenesis by 2-amino-3-methylimidazo[4,5-*f*]quinoline in the rat. *Jpn. J. Cancer Res.,* **85,** 1214–1219 (1994).