Chemoprevention by the Oxygenated Carotenoid β-Cryptoxanthin of N-Methylnitrosourea-induced Colon Carcinogenesis in F344 Rats

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β-Cryptoxanthin (βCx), one of 4 major carotenoids in the blood, was investigated for anticarcinogenic activity in F344 rats. Four groups of 25 rats each received an intrarectal dose of 2 mg of N-methylnitrosourea 3 times a week for 5 weeks, and were fed the diet supplemented with 0 ppm (control), 25 ppm, 5 ppm or 1 ppm βCx throughout the experiment. The colon cancer incidence at week 30 was significantly lower in the βCx (25 ppm) diet group, but not in the βCx (5 ppm) and βCx (1 ppm) diet groups, than in the control diet group: 68%, 84%, 80% vs. 96%. The results suggested that dietary βCx may affect colon carcinogenesis after accumulation in the colonic mucosa, perhaps due to absorption from the colon as well as the small intestine.

Key words: β-Cryptoxanthin — Carotenoid — Orange — Colon cancer — Chemoprevention

Epidemiological studies have suggested a protective role of vegetables and fruits against certain types of cancer, including colon cancer.1,2) There is increasing evidence from epidemiological1–7) and animal7–13) studies that a high consumption of foods containing carotenoids such as β-carotene and lycopene, which possess potent antioxidant properties and ant cancercinogenic activity, reduces the risk of colon cancer, although there were negative results in human intervention studies14–16) and animal studies.17,18) A possible mechanism for the anticarcinogenic potential of plant carotenoids is their antioxidant functions (scavenging free radicals and quenching singlet oxygen), which are associated with lowered DNA damage, diminished membrane lipid peroxidation and inhibition of malignant transformation in vitro.19) An oxygenated carotenoid, β-cryptoxanthin (hydroxy-β-carotene, βCx), is one of the major carotenoids in the blood,6,20–23) after β-carotene, lycopene and lutein, even though intake is far smaller (<200 µg/day) than that of the other carotenoids (>1,500 µg/day for each of β-carotene, lycopene and lutein).5,24) βCx is enzymatically converted to retinol, which is involved in cell differentiation, in the intestine and the liver, like β- and α-carotenes. However, this carotenoid has received less attention as a possible protector against carcinogenesis. Recent epidemiological studies show a weak inverse association between βCx in the blood and in the diet, and the risk of cancer in various organs including the colon.5,6,21–24) βCx content is particularly high in orange juice (1 mg/100 ml). The present study was carried out to investigate whether or not βCx supplemented in the diet has a chemopreventive activity against chemically induced colon carcinogenesis in rats. βCx used was extracted from Citrus unshiu oranges by one of the authors. The distribution of βCx in the body and in the feces was also analyzed.

Female F344 rats (Shizuoka Laboratory Animal Center, Hamamatsu), 7 weeks of age at the start of the experiment, were used. The rats were housed in plastic cages with sterilized wood-chip bedding in a specific-pathogen-free animal room under constant environmental conditions with a 12 h light and dark cycle, a temperature of 22°C and a relative humidity of 50%. They had free access to drinking water and to a standard laboratory pelleted diet CE-2 supplemented with βCx (purity 95.0%) at a concentration of 0 ppm (control basal diet), 25 ppm, 5 ppm or 1 ppm, which was prepared at CLEA Co., Tokyo. It was confirmed by HPLC that the basal CE-2 diet contained 0.4 ppm βCx. The body weight and food intake were measured once a week. The rats were maintained according to the standards set forth in the Guidelines for the Care and Use of Laboratory Animals of the Experimental Animal Facility, Akita University School of Medicine.

Four groups of 25 rats each received an intrarectal dose of 0.5 ml of 0.4% aqueous solution of N-methylnitrosourea (MNU, Nacalai Tesque, Kyoto) 3 times a week during weeks 1 and 5 as described previously.25) Briefly, a metal feeding tube 8 cm long was inserted two-thirds of
the way into the colon lumen through the anal orifice, and the solution was injected. The solution filled the distal half of the colon, where tumors developed. The rats were given one of the 4 diets described above throughout the experiment. The feces were collected for 3 days in week 15. At week 30, all the rats were killed by exsanguination from the abdominal aorta after laparotomy under intraperitoneal pentobarbital anesthesia (40 mg/kg body weight). The blood was centrifuged, and the serum was collected. At autopsy, the colon was excised, cut open along its length, and inspected grossly. The mucosa of the colon without tumors was scraped with a blunt steel plate and collected after having been rinsed with saline solution to remove debris. The liver and the mucosa of the mid-jejunum were also collected. Then, the colon was fixed in 10% formalin solution. All tumors and grossly abnormal organs were examined histologically after standard processing, sectioning and staining with hematoxylin and eosin. The collected samples of the serum, liver, jejunum, colon and feces were stored at −80°C until βCx analysis.

The assay of βCx, β-carotene and retinol was performed by HPLC described previously.26) Briefly, the serum (0.2 ml) was mixed with ethanol (1.0 ml), then extracted twice with a solution of n-hexane and dichloromethane (4:1, v/v, 5.0 ml). The supernatant was evaporated to dryness under nitrogen gas, and the residue was reconstituted in a solvent mixture of methanol and acetonitrile (1:1, v/v) as a mobile phase for HPLC analysis. The liver, colonic mucosa, jejunal mucosa and feces were each homogenized and saponified by addition of 60% KOH and 3% butylated hydroxytoluene in ethanol, followed by heating at 50°C for 30 min, then extracted twice with n-hexane and dichloromethane (4:1, v/v). The supernatant was dried, and then reconstituted as described. The samples were analyzed by HPLC using a Shimadzu SPD-10AV spectrophotometric detector (Shimadzu, Kyoto) and a Lichrospher RP18-5 column (E. Merck, Darmstadt, Germany). The flow rate of the mobile phase was 2.0 ml/min.

The statistical significance of differences was tested by use of the χ² test and Student’s t test. The criterion of significance was taken as P<0.05.

Body weight gain (108–114 g after 108 g at the start of experiment) and food intake (9.6–9.8 g/day/rat) were similar in all the groups of rats. The mean amount of βCx intake from the diets throughout the 30-week experiment, and the data on colon tumor yield at week 30 are summarized in Table I. The tumor incidence in the βCx (25 ppm) group was significantly lower than that in the control group. Those in the βCx (5 ppm) and βCx (1 ppm) groups were lower, but not statistically significantly lower, than

Table I. Development of N-Methylnitrosourea-induced Colon Tumors in F344 Rats Treated with β-Cryptoxanthin

| Treatment groupsa | No. of rats examined | Amount of βCx intake (μg) | No. of rats with tumors (%) | No. of tumors per rat | No. of tumors per tumor-bearing rat |
|-------------------|----------------------|---------------------------|-----------------------------|----------------------|-----------------------------------|
| Control           | 25                   |                           |                             |                      |                                   |
| βCx (25 ppm)     | 25                   | 4b                        | 24 (96)                     | 1.7±0.9c             | 1.8±0.8e                          |
| βCx (5 ppm)      | 25                   | 247                       | 17 (68)                     | 1.4±1.2              | 2.1±0.8                           |
| βCx (1 ppm)      | 25                   | 14                        | 20 (80)                     | 1.7±1.3              | 2.1±1.1                           |

a) All rats received an intrarectal dose of 2 mg of N-Methylnitrosourea 3 times a week for weeks 1 to 5, and were fed a diet supplemented with β-cryptoxanthin (βCx) at the indicated concentration throughout the experiment. The experiment was terminated at week 30.
b) Mean amount/day/rat.
c) Mean±SD.
d) Significantly different from Control group: P<0.02.

Table II. Concentration of β-Cryptoxanthin in Serum, Tissues and Feces of N-Methylnitrosourea-treated and β-Cryptoxanthin-supplemented Diet-fed F344 Rats

| Treatment groupsa | Serum | Liver | Jejunum mucosa | Colon mucosa | Feces |
|-------------------|-------|-------|----------------|--------------|-------|
| Control           |       |       |                |              |       |
| βCx (25 ppm)     | 0.24±0.15c | 0.27±0.05 | 0.08±0.04 | 34.3±8.7 |
| βCx (5 ppm)      | 0.09±0.06 | 0.02±0.01 | 0.03±0.01 | 8.7±0.8  |
| βCx (1 ppm)      |       |       |                |              | 2.0±0.1 |

a) See Table I or text. Serum, liver, jejunal mucosa and colon mucosa were collected at week 30, and feces at week 15.
b) Not detected (<0.01 μg/ml or g).
c) Mean±SD, μg/g, of 8 samples.
the control. The tumor multiplicity was not significantly different among the groups. The tumors were located diffusely in the distal half of the colon, and were plaque-shaped or polyloid. Most of them were small, less than 10 mm in diameter. Histologically, all the tumors were well-differentiated adenocarcinomas with various degrees of invasive growth. No distinct differences among the groups were observed in the pathological findings of tumors. No metastases to the lymph nodes or other organs were found. There were no other noteworthy pathological findings, except for malignant thymomas in two rats of the control and βCx (1 ppm) groups, one rat of the βCx (5 ppm) group and three rats of the βCx (25 ppm) group, and mammary carcinomas in three rats of the βCx (5 ppm) group.

The results clearly demonstrated that βCx supplemented in the diet inhibited MNU-induced colon cancer development, as found in our preliminary study, in which a daily gavage of 1.2 mg or 0.24 mg of βCx per rat, but not 0.048 mg of βCx per rat, for 5 weeks suppressed the formation of MNU-induced rat colonic aberrant crypt foci (unreported data), putative preneoplastic lesions and an early intermediate biomarker of colon carcinogenesis. After this short-term assay for screening the potency and the effectiveness of βCx, the present long-term bioassay was designed. The doses of βCx taken in from the respective experimental diets were close to those of the short-term experiment.

The results of βCx analysis are shown in Table II. βCx was not detected in the serum of any rats, while it was detected at low levels in the liver, jejunum mucosa and colon mucosa, and at a high level in the feces, showing a close association with the dose of βCx in the diet. β-Carotene was detected in the feces, but not in other samples. The mean (SD) values/g dry feces for 8 samples each were 3.9 (0.3), 4.3 (0.4), 4.6 (0.2) and 4.3 (1.2) µg in the control, βCx (25 ppm), βCx (5 ppm) and βCx (1 ppm) groups, respectively. Retinol was assayed only in the serum. The mean (SD) values/ml for 8 samples each were 0.20 (0.03), 0.22 (0.06), 0.22 (0.04) and 0.22 (0.05) µg in the control, βCx (25 ppm), βCx (5 ppm) and βCx (1 ppm) groups, respectively. The levels of fecal β-carotene and serum retinol showed no great differences among the groups.

These results showed that a certain amount of βCx was absorbed and accumulated in the liver and colon in a dose-dependent fashion, and that a large amount was excreted in the feces. However, it was not detected in the serum, perhaps because of the species specificity of carotenoid metabolism. The serum concentration of carotenoids is low in rodents, compared to humans, but that of retinol is comparable in both species, while the tissue concentrations of carotenoids and retinol are similar in humans and rodents. Carotenoids may be absorbed from the colon as well as the small intestine via concentration-dependent passive transport and accumulate in a considerable amount in the colon and in a large amount in the liver. Carotenoids such as canthaxanthin and α- and β-carotenes suppressed the growth of human colon cancer cells in vitro, and the proliferation of the colon mucosa of high-risk subjects. Thus, it is probable that dietary βCx accumulated in the colon mucosa accounted for the protection against colon cancer development in the present study. However, the antioxidant potential of βCx is not strong in comparison with that of other carotenoids such as lycopene and β-carotene. Recent evidence suggests that other mechanisms such as modulation of intercellular communication via gap junctions, and alterations in intracellular signalling pathways and regulation of cell apoptosis could also contribute to their anticarcinogenic potential. Further studies are needed to clarify the mechanisms of the chemopreventive action of βCx demonstrated in the present study.

In conclusion, the current results suggest that dietary βCx may prevent colon carcinogenesis. To our knowledge, there are no previous reports on the anticarcinogenic effect of βCx in animal models.

The authors thank Mr. T. Toita for his excellent technical assistance, and Dr. John H. Weisburger, American Health Foundation, Valhalla, NY, USA for a critical reading of the manuscript and valuable comments. This work was supported by a Grant-in-Aid from BRAIN.

(Received June 28, 1999/Revised August 26, 1999/Accepted September 7, 1999)

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