Antitumor Activity of Astaxanthin and Its Mode of Action

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Abstract: Astaxanthin, a carotenoid without vitamin A activity, may exert antitumor activity through the enhancement of immune responses. Here, we determined the effects of dietary astaxanthin on tumor growth and tumor immunity against transplantable methylcholanthrene-induced fibrosarcoma (Meth-A tumor) cells. These tumor cells express a tumor antigen that induces T cell-mediated immune responses in syngenic mice. BALB/c mice were fed astaxanthin (0.02%, 40 μg/kg body wt/day in a beadlet form) mixed in a chemically defined diet starting zero, one, and three weeks before subcutaneous inoculation with tumor cells (3 × 10⁵ cells, 2 times the minimal tumorigenic dose). Three weeks after inoculation, tumor size and weight were determined. We also determined cytotoxic T lymphocyte (CTL) activity and interferon-γ (IFN-γ) production by tumor-draining lymph node (TDLN) and spleen cells by restimulating cells with Meth-A tumor cells in culture. The astaxanthin-fed mice had significantly lower tumor size and weight than controls when supplementation was started one and three weeks before tumor inoculation. This antitumor activity was paralleled with higher CTL activity and IFN-γ production by TDLN and spleen cells in the astaxanthin-fed mice. CTL activity by TDLN cells was highest in mice fed astaxanthin for three weeks before inoculation. When the astaxanthin-supplemented diet was started at the same time as tumor inoculation, none of these parameters were altered by dietary astaxanthin, except IFN-γ production by spleen cells. Total serum astaxanthin concentrations were approximately 1.2 μmol/l when mice were fed astaxanthin (0.02%) for four weeks and appeared to increase in correlation with the length of astaxanthin supplementation. Our results indicate that dietary astaxanthin suppressed Meth-A tumor cell growth and stimulated immunity against Meth-A tumor antigen.

Introduction

Growing evidence indicates that pharmacological or naturally occurring agents inhibit the development of invasive cancer by preventing initiation of carcinogenesis or by arresting or reversing processes of tumor progress. Antitumor activity of such agents can be exerted through augmentation of tumor immunity against cancerous cells. Many nutrients appear to possess antitumor activity. Although any individual nutrient is unlikely to be "a magic bullet," studies of individual compounds are important for identification of the most potent agents and their mode of action. Our present study focuses on the potent antitumor activity of astaxanthin. This ketocarotenoid demonstrates antitumor and immunomodulating activities that are distinctly different and more potent than those of β-carotene (1–8).

Potent antitumor activity was reported for astaxanthin in rodent models. Astaxanthin attenuated development of murine urinary bladder tumors when given subcutaneously (6). Dietary astaxanthin (30 μg/g body wt/day) also exerted antitumor activity in the postinitiation phase of carcinogen-induced colon and oral cancer models (7,8). Others also reported the suppressive effects of dietary astaxanthin on transplantable tumor cells: 1) methylcholanthrene-induced fibrosarcoma (Meth-A tumor) cells with dietary astaxanthin at 80 μg/g body wt/day (10) and 2) murine mammary tumor cells with dietary astaxanthin at 50–200 μg/g body wt/day (9). The latter study found serum astaxanthin levels at 13.5–18 μmol/l.

Antitumor activity of astaxanthin may be exerted through several different mechanisms. These include the prevention of oxygen-mediated cytotoxicity and genotoxicity and induction of xenotoxic-metabolizing enzymes in the liver (11–15). Another possible mode of action is the modulation of tumor immunity (8,10). Our previous studies found potent effects of astaxanthin on T cell-mediated immune responses, which were distinctly different from those of β-carotene (1–5,16). Meth-A tumor cells express a tumor antigen (Ag) associated with major histocompatibility (MHC) class I molecules and induce significant T cell-mediated immune responses in tumor-draining lymph node (TDLN) cells. Such responses include interferon-γ (IFN-γ) production and cytotoxic T cell (CTL) activity against Meth-A tumor cells (17–21). We thus hypothesized that dietary astaxanthin exerts antitumor activity against inoculated Meth-A tumor cells by augmenting tumor immunity. In the preliminary study we found that dietary astaxanthin

(0.02–0.04% of the diet in a beadlet form, approx 40–80 μg/g body wt/day) suppressed the growth of Meth-A tumor cells inoculated in syngenic BALB/c mice (unpublished observation).

This study evaluated the effects of dietary astaxanthin on the growth of inoculated Meth-A tumor cells and tumor immunity in syngenic BALB/c mice. The results revealed that dietary astaxanthin suppressed Meth-A tumor cell growth when mice were fed astaxanthin (40 μg/kg body wt/day) starting at least one week before tumor inoculation. The antitumor activity of astaxanthin appeared to be associated with augmented CTL activity and IFN-γ production against Meth-A tumor cells.

Materials and Methods

Mice

B6 female mice (5–6 wk old) were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained in the animal facility at the University of Minnesota (Minneapolis, MN). Our facility includes areas for housing, breeding, necropsy, and sterile tissue collection. Animal care was provided by full-time staff supervised by veterinarians. The mice were housed in groups of four to five per cage. Mice were killed in a CO_2 chamber, as approved by the Institutional Animal Care and Use Committee, University of Minnesota.

Reagents

Astaxanthin (beadlet form, 8 g astaxanthin/100 g beadlet) was kindly provided by Hoffmann-La Roche (Basel, Switzerland). The beadlet is composed of sucrose, starch, and gelatin, with 1% ethoxyquin as an antioxidant. Astaxanthin was mixed with a powder form of a casein-based synthetic diet (Research Diets, New Brunswick, NJ) consisting of (in g/kg) 200 sodium caseinate, 3 DL-methionine, 150 corn starch, 500 sucrose, 50 cellulose, 50 corn oil, 35 salt mix (AIN 76A), 10 vitamin mix (AIN 76A), 2 choline bitartrate, and 4.74 nucleotides (2.2 CMP, 0.9 UMP, 0.9 GMP, and 0.7 AMP), with gross energy of 15.89 kJ/g at the recommended amount (Recommended Dietary Allowance) of vitamins A, C, and E for mice.

Experimental Design

In vivo antitumor activity of astaxanthin: In preliminary experiments we determined a minimum subcutaneous tumorigenic dose of Meth-A tumor cells for young BALB/c mice: around 1.5×10^5 cells. When mice were injected with this tumor dose, <50% of BALB/c mice rejected tumor cells. In two preliminary experiments (4 mice in each diet group in each experiment), mice were fed the astaxanthin-supplemented diet starting one week before inoculation attenu-

ated growth of Meth-A tumor cells with doses of 3×10^5 cells, but not with doses of $5–10 \times 10^5$ Meth-A tumor cells. None of the mice rejected the tumor with a dose of 3×10^5 cells. We thus chose to inoculate mice with 3×10^5 tumor cells.

Young BALB/c mice were fed the astaxanthin-supplemented diet starting zero, one, and three weeks before tumor inoculation in the first, second, and third experiments, respectively. In each experiment, control mice were fed the same diet without astaxanthin. Each diet group consisted of 10 mice in the astaxanthin-fed group and 6–7 mice in the control group of each experiment. The amount of astaxanthin supplementation (0.02%) was selected on the basis of our finding that this amount of astaxanthin yields a 1 μmol/l serum concentration after four weeks of dietary supplementation, a concentration that is similar to the serum levels of dietary carotenoids observed in humans (22).

Three weeks after tumor inoculation, we determined tumor weight, tumor size, and serum astaxanthin concentrations in addition to immune responses against Meth-A tumor cells. Markers of tumor immunity included CTL activity and IFN-γ production by TDLN and spleen cells. Meth-A tumor cells formed a single tumor mass without macroscopic dissemination of tumors.

Analytic Methods

Preparation of lymph node, spleen, and Meth-A tumor cells: Single-cell suspensions were prepared by gentle squeezing of lymph nodes or spleen with a rubber scraper and suspending cells in RPMI 1640 with heat-inactivated fetal calf serum (FCS, 50 ml/l). Debris was removed by passing cell suspensions through coarse filters. The cell suspensions were then used for IFN-γ production and CTL assays. Fibrosarcoma cells induced by methylcholanthrene in the BALB/c strain (Meth-A tumor cells, catalog no. WEHI 164, American Type Culture Collection, Rockville, MD) were maintained by a passage through syngenic BALB/c mice with inoculation of cells intraperitoneally. Meth-A tumor cells were harvested from peritoneal fluid by centrifugation, washed once, and resuspended in RPMI 1640 with FCS, penicillin (10^5 U/l), streptomycin (100 mg/l), and L-glutamine (2 mmol/l). Cells were cultured overnight and inoculated subcutaneously into the right flank of syngenic BALB/c mice.

IFN-γ production assay: Harvested TDLN and spleen cells were resuspended in RPMI 1640 supplemented with serum-replacement agent (2%, TM235, Hopkins) N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (20 mmol/l), sodium pyruvate (1 mmol/l), penicillin G (10^5 U/l), streptomycin (100 mg/l), L-glutamine (2 mmol/l), and 2-mercaptoethanol (10^{-6} mol/l). Then the cells were mixed with irradiated (3,000 R) Meth-A tumor cells (2×10^4 cells/well) in a 96-well microtiter plate (Costar, Cambridge, MA; 1:1–64:1). The plate was incubated for four days, and IFN-γ levels in the culture supernatant were measured by enzyme-

linked immunosorbent assay (Endogen, Cambridge, MA), as reported previously (16).

CTL assay: Harvested cells were diluted with RPMI 1640 with 1% bovine serum albumin (BSA; Sigma Chemical, St. Louis, MO) to 1×10^5 cells/ml and washed once. Freshly harvested Meth-A tumor cells were then mixed with TDLN or spleen cells at 1:1–1:64 in a 96-well microtiter plate (Costar) and incubated in a CO_2 incubator at 37°C overnight. The plate was centrifuged for 10 minutes, and culture supernatant (100 μl) was transferred to a new plate. A reaction mixture (100 μl/well; cytotoxicity detection kit, lactate dehydrogenase, Boehringer-Mannheim) was added and developed in the dark for 15–30 minutes at room temperature. Lactate dehydrogenase activity was measured as optical density at 492 nm with an enzyme-linked immunosorbent assay reader. Cytotoxicity was calculated per instructions from the company and expressed as a lytic unit.

Measurement of astaxanthin concentration: The measurement of astaxanthin was a modification of the method of Bieri and co-workers (23). Carotenoids were extracted with hexane, dried under nitrogen, resolubilized in high-performance liquid chromatography solvent (acetonitrile, methanol, and methylene chloride), and applied to a C_{18} reverse-phase column. Carotenoids were separated with an isocratic solvent system. The assay was standardized by the use of crystalline carotenoid standards. Since the original publication of the method, we have standardized the method for the measurement of astaxanthin and retinol and included the use of N,N-diisopropylethylamine in the high-performance liquid chromatography solvent (0.01%). These analyses can be quantitated by absorption at 325 and 450 nm and have retention times of 1.7 min (astaxanthin) and 2.25 min (retinol). The recovery of added carotenoids from these samples was >90%.

Statistics: Equality of two means was evaluated by Student's t-test or by Mann-Whitney test, depending on a distribution pattern of samples (normal vs. skewed) (24). Comparison of multiple values was done by Kruskal-Wallis test or Wilcoxon weighed ranks test (24). Correlation of two parameters was assessed by Kendall's τ-b test (24). Differences with $p < 0.05$ were considered significant.

Results

Serum Astaxanthin Concentrations

An analysis of the serum from mice fed astaxanthin revealed two analytes that were not present in control sera. The analytes had retention times and spectra similar, but not identical, to those of astaxanthin. Distinguishing characteristics were that each analyte had one additional absorption maximum 323 nm for Peak 1 and 366 nm for Peak 2, as well as a blue shift of a few nanometers (3 for Peak 1 and 8 for
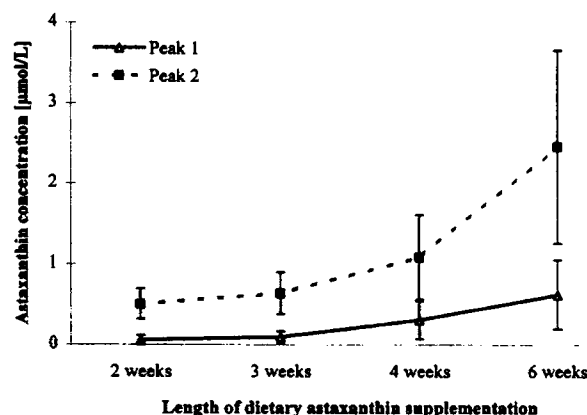

Figure 1. *cis*-Astaxanthin serum concentrations (Peaks 1 and 2) by high-performance liquid chromatography in BALB/c mice 2, 3, 4, and 6 wk after dietary astaxanthin supplementation (0.02%, 40 μg/kg body wt/day). Astaxanthin was undetectable in serum samples from control mice.

Peak 2), compared with a single absorption maximum at 473 nm, which is characteristic of astaxanthin. The additional absorption bands are characteristic of *cis*-forms of carotenoids and suggested the presence of *cis*-astaxanthin in the beadlet preparation or the conversion of *trans*-astaxanthin to *cis*-astaxanthin during absorption and metabolism. In subsequent analysis of astaxanthin beadlets, a single *cis*-astaxanthin compound and *trans*-astaxanthin were found. The single *cis*-astaxanthin had a spectrum that was identical to Peak 2 (absorption maxima at 366 and 465 nm). An analogous analyte was not found for Peak 1. The analyte identified as Peak 1 appears to be a metabolic product of *trans*-astaxanthin or the *cis*-astaxanthin found in beadlets. Peaks 1 and 2 serum concentrations increased in parallel to the length of astaxanthin supplementation (Figure 1).

Effects of Dietary Astaxanthin on Tumor Growth in BALB/c Mice Inoculated With Meth-A Tumor Cells

When the astaxanthin-supplemented diet was started on the day of Meth-A tumor cell inoculation (3×10^5 Meth-A tumor cells), there were no significant differences in tumor weight and size between controls and the astaxanthin-fed mice (Figure 2). However, tumor size and weight were significantly lower in the astaxanthin-fed mice when the astaxanthin diet was started one and three weeks before tumor inoculation. Tumor size and weight were not significantly different between the groups that started the astaxanthin diet one or three weeks before tumor inoculation (Figure 2).

Effects of Dietary Astaxanthin on Tumor Immunity Against Meth-A Tumor Cells

TDLN and spleen cells from unchallenged BALB/c mice had undetectable levels of CTL activity (<0.1 lytic unit) and IFN-γ production (<0.1 μg/l) after incubation with Meth-A tumor cells. In contrast, TDLN and spleen cells from tumor-inoculated mice revealed significant CTL activity and
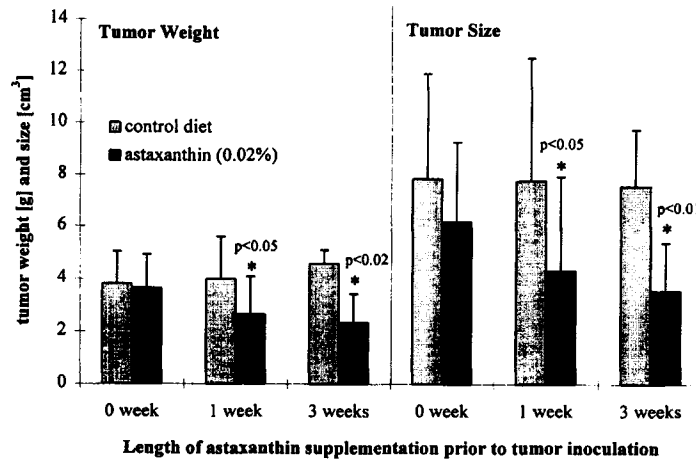
Figure 2. Tumor weight and size 3 wk after inoculation of methylcholanthrene-induced fibrosarcoma (Meth-A tumor) cells (3×10^5 cells/dose) in syngenic BALB/c mice. In each experiment, mice were fed a control diet or an astaxanthin-supplemented diet starting at 0, 1, and 3 wk before tumor inoculation. Values are means ± SD; $n = 10$ for astaxanthin-fed groups and 7 for controls. *, Significantly lower than controls (Mann-Whitney test).

IFN-γ production against Meth-A tumor cells (Figures 3 and 4). CTL activity and IFN-γ production were further augmented when astaxanthin supplementation was started at one and three weeks before tumor inoculation (Figures 3 and 4). Astaxanthin increased IFN-γ production by spleen cells but not by TDLN cells against Meth-A tumor cells when as-
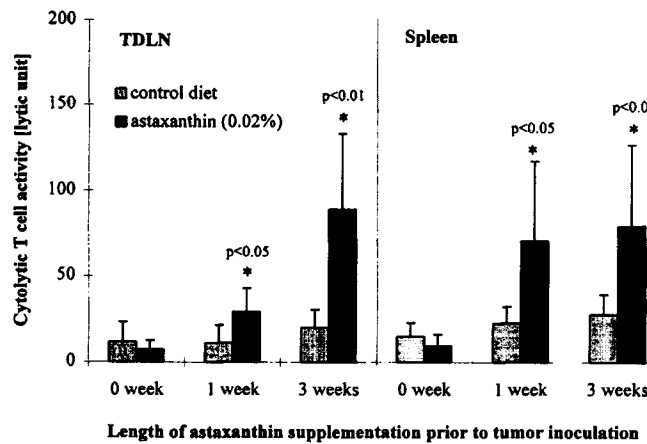

Figure 3. Cytotoxic T lymphocyte (CTL) activity of tumor-draining lymph node (TDLN) and spleen cells against Meth-A tumor cells determined 3 wk after inoculation of Meth-A tumor cells (3×10^5 cells/dose). CTL activity was measured using freshly isolated TDLN and spleen cells in same sets of mice shown in Figure 2. Values are means ± SD; $n = 10$ for astaxanthin-fed groups and 7 for controls. *, Significantly higher than controls (Mann-Whitney test).

Figure 4. Interferon-γ (IFN-γ) production by TDLN and spleen cells against Meth-A tumor cells 3 wk after inoculation of Meth-A tumor cells (3×10^5 cells/dose). IFN-γ production was measured by incubating TDLN and spleen cells for 4 days with irradiated Meth-A tumor cells in same sets of mice shown in Figure 2. Values are means ± SD; $n = 10$ for astaxanthin-fed groups and 7 for controls. *, Significantly higher than controls (Mann-Whitney test).
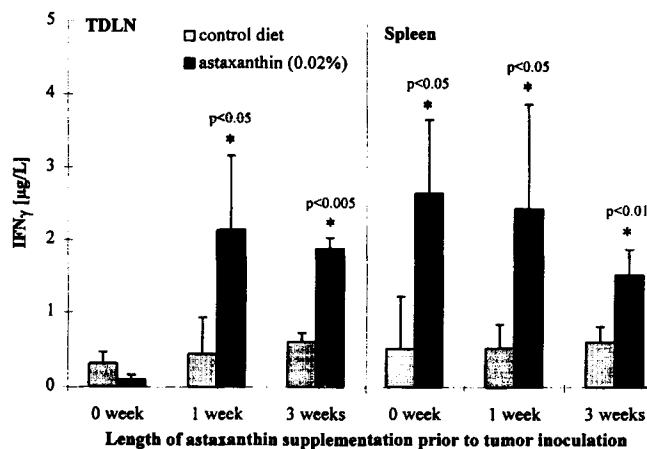
taxanthin supplementation was started on the same day as tumor inoculation. Astaxanthin augmented CTL activity of TDLN cells more significantly when the astaxanthin diet was started three weeks before inoculation than when it was started one week before inoculation (Figures 3 and 4).

Discussion

This study evaluated the direct antitumor activity of astaxanthin on transplantable Meth-A tumor cells in parallel with its augmenting action on tumor immunity. Our results indicate that dietary astaxanthin can exert antitumor activity at physiologically achievable serum concentrations and that astaxanthin may exert antitumor activity by modulating immune responses against Meth-A tumor cells.

The antitumor activity of astaxanthin is extremely unlikely to be the result of toxicity. No carotenoid has been shown to have a significant toxicity in humans or animals. Astaxanthin and several other carotenoids at dietary concentrations as high as 2% did not induce symptoms of toxicity in rats, mice, or ferrets (25). Thus the maximum tolerated dose is unknown at this time but, undoubtedly, is very high and above the amount (40 µg/kg body wt/day) used in this study.

When a relatively small dose of Meth-A tumor cells (3×10^5 cells, approximately twice the minimal tumorigenic dose) was inoculated into syngenic mice, our results revealed that dietary astaxanthin (40 µg/kg body wt/day) significantly attenuated tumor growth, as evidenced in decreased tumor size and weight in the astaxanthin-fed mice compared with controls. This astaxanthin action was observed when the astaxanthin-supplemented diet was started at one and three weeks, but not at zero week, before tumor inoculation. When larger tumor doses ($5-10 \times 10^5$ cells) were inoculated, such astaxanthin-suppressive action was not observed (unpublished observation). These results indicate that astaxanthin may prevent tumor development in the early stages of tumor development; also astaxanthin may not effectively suppress tumor growth in the late progression or metastasis phases.

Astaxanthin beadlets contain ethoxyquin, an antioxidant. Ethoxyquin, at concentrations ≥100 times higher than those used in this study, can exert antitumor activity by inducing phase I and II detoxifying enzymes against carcinogens (26), augment lymphocyte-proliferative responses against mitogens, and prolong the life span of mice fed a calorie-restricted diet (27). It does not demonstrate antitumor activity in the postinitiation or tumor progression phases. Ethoxyquin did not suppress proliferation of Meth-A tumor cells *in vitro* at $10^{-7}-10^{-8}$ mol/l (unpublished observations); tissue concentrations of ethoxyquin from beadlets are lower than these levels. Ethoxyquin did not augment IFN-γ production or CTL activity against Meth-A tumor cells *in vitro* (unpublished observations). Thus it is unlikely that ethoxyquin intake through the beadlets contributed to the findings of this study, although the limitation of this study is a lack of control mice fed a beadlet without astaxanthin.

Meth-A tumor cells express a tumor Ag associated with MHC class I molecules and induce significant T cell-mediated immune responses, as shown in **Results**. Immune responses against tumor Ag may be induced when a sufficient quantity of tumor Ag enters the secondary lymphoid tissues (TDLN) in a localized manner. In TDLN, tumor Ag is presented by Ag-presenting cells to T cells, and if appropriate secondary signals are provided through costimulatory molecules, tumor-Ag-specific T cells will be activated (28,29). Activated T helper (Th) and cytotoxic (Tc) cells demonstrate polarized cytokine production patterns [types 1 and 2 (Tc1 and Tc2)] (30,31). Environmental and genetic factors dictate type 1 and type 2 responses. Tc1 responses are likely to dominate tumor immunity, exhibiting strong CTL activity and IFN-γ production, but Tc2 responses may also be associated with antitumor activity in certain cancer models (31–33). Others reported that immune responses against Meth-A tumor cells appeared to be closely associated with augmented IFN-γ production and CTL activity against tumor Ag (17–21). However, tumor cells, including Meth-A tumor cells, are capable of suppressing immune responses against tumor Ag. Any agent that augments tumor immunity can potentially suppress tumor development, especially in the early tumor progression phase. This could well be a part of antitumor activity exerted by astaxanthin.

The results of the present study demonstrated for the first time that dietary astaxanthin augments CTL activity and IFN-γ production against Meth-A tumor cells in parallel to suppression of tumor growth when astaxanthin was started one and three weeks before tumor inoculation. Augmenting action of astaxanthin on these immune parameters is more evident when dietary astaxanthin supplementation was started at three weeks than at one week before tumor inoculation. However, astaxanthin-suppressive action on tumor growth did not differ between the mice fed astaxanthin for one week and those fed astaxanthin for three weeks before tumor inoculation. This may be partly due to the fact that tumor cells can counterregulate immune responses (34).

Most of the astaxanthin in the beadlets was in a *cis*-form, and all the serum astaxanthin was present in *cis*-forms. The formation of *cis*-carotenoids occurs readily with heat (35) and is known to occur in the metabolism of carotenoids. Our observation thus suggests that *cis*-astaxanthin was formed during preparation of beadlet astaxanthin and also was formed as a metabolic product of *trans*-astaxanthin or the *cis*-astaxanthin found in the beadlet. Further studies are necessary to determine the source and identity of each astaxanthin species. Also, we do not know which species are biologically active in the prevention of tumor formation.

Astaxanthin (3,3'-dihydroxy-4,4,'diketo-β-carotene) is synthesized by a number of marine bacteria, microalgae, and certain yeasts from β-carotene by the addition of two keto groups to carbons C-4 and C-4' and two hydroxyl groups to C-3 and C-3' (36–38). Mammals cannot synthesize astaxanthin *de novo* and must acquire it through diets. Astaxanthin is widely distributed in fish (e.g., salmon, trout, caviar), shellfish (e.g., lobster, crab, shrimp), and certain algae

commonly consumed in a diet rich in seafood, such as a traditional Oriental diet (39). Astaxanthin is responsible for inducing a pink color of the flesh of fish and shellfish and the feathers of some birds (40). Another source of astaxanthin is foods that are supplemented with astaxanthin as a food-coloring agent (40). Astaxanthin is very stable in processed or raw food (39) and does not have vitamin A activity in mammals, including humans.

In several human populations, including the native Japanese, Taiwanese, and certain US populations living in coastal areas, exposure to high amounts of astaxanthin is routine. Astaxanthin is found in numerous edible sea animals, including ocean fish and crustaceans. It is a major component of these animals and is found at high concentrations (5–10 mg/100 g of their total mass). Importantly, a large amount of the astaxanthin is found in the edible portion of crayfish, salmon, prawns, and lobsters. A typical edible serving of a commercially available prawn muscle (100 g) contains approximately 0.5 mg of astaxanthin (41). Crayfish and other crustaceans contain similar amounts (42). Because astaxanthin is used as a colorant for salmon, this food can contain even higher concentrations than prawns. These concentrations are comparable to the carotene concentrations in most vegetables. Consequently, populations with routine seafood intake (3–5 servings/wk) are exposed to large amounts of astaxanthin (1.5–2.5 mg). This amount of intake is similar to that estimated for α-carotene in the US population (22). Given these data and results of our our present study, astaxanthin can be an important chemopreventive agent. Others have also shown that other antioxidants demonstrate various immunomodulating actions in other experimental systems (43–45). Combination treatment of astaxanthin and other immunomodulating antioxidants may further help prevent tumorigenesis.

In summary, this study revealed a potent antitumor activity of dietary astaxanthin on transplantable Meth-A tumor cells at physiologically achievable levels, and this astaxanthin action may be partly associated with its augmenting action on tumor immunity against Meth-A tumor Ag.

Acknowledgments and Notes

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