Antitumor Activity of Astaxanthin and Its Mode of Action

Harumi Jyonouchi, Sining Sun, Koji Iijima, and Myron D. Gross

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 syngeneic 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 x 10^6 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

H. Jyonouchi, S. Sun, and K. Iijima are affiliated with the Department of Pediatrics, School of Medicine, and M. D. Gross with the Division of Epidemiology, School of Public Health, University of Minnesota, Minneapolis, MN 55455.
(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 syngeneic 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 syngeneic 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 CO₂ 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 × 10⁶ 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 attenuated growth of Meth-A tumor cells with doses of 3 × 10⁶ cells, but not with doses of 5–10 × 10⁶ Meth-A tumor cells. None of the mice rejected the tumor with a dose of 3 × 10⁶ cells. We thus chose to inoculate mice with 3 × 10⁶ 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 syngeneic 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 (100 U/I), streptomycin (100 mg/l), and L-glutamine (2 mmol/l). Cells were cultured overnight and inoculated subcutaneously into the right flank of syngeneic 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 (100 U/I), streptomycin (100 mg/l), L-glutamine (2 mmol/l), and 2-mercaptoethanol (10⁻⁴ mol/l). Then the cells were mixed with irradiated (3,000 R) Meth-A tumor cells (2 × 10⁶ 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 × 10^6 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 CO2 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-disopropylethylamine 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 ranked 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 at 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 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 × 10^6 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
IFN-\(\gamma\) production against Meth-A tumor cells (Figures 3 and 4). CTL activity and IFN-\(\gamma\) production were further augmented when astaxanthin supplementation was started at one and three weeks before tumor inoculation (Figures 3 and 4). Astaxanthin increased IFN-\(\gamma\) production by spleen cells but not by TDNL cells against Meth-A tumor cells when as-
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 as-
taxanthin 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 im-
une responses against Meth-A tumor cells.

The antitumor activity of astaxanthin is extremely un-
likely 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 con-
centrations 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 at-
tenuated 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 × 10^5 cells) were inoculated, such
astaxanthin-suppressive action was not observed (unpub-
lished 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 mito-
gen, and prolong the life span of mice fed a calorie-re-
stricted diet (27). It does not demonstrate antitumor activity
in the postinitiation or tumor progression phases. Ethoxy-
quin did not suppress proliferation of Meth-A tumor cells in
vitro at 10^{-2}–10^{-4} mol/l (unpublished observations); tissue
concentrations of ethoxyquin from beadlets are lower than
these levels. Ethoxyquin did not augment IFNy production
or CTL activity against Meth-A tumor cells in vitro (un-
published 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 con-
trol 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 medi-
ated immune responses, as shown in Results. Immune re-
ponses against tumor Ag may be induced when sufficient
quantity of tumor Ag enters the secondary lymphoid tissues
(TDLN) in a localized manner. In TDLN, tumor Ag is pre-
sented by Ag-presenting cells to T cells, and if appropriate
secondary signals are provided through costimulatory mole-
cules, 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 dom-
inate 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
phage cell appeared to be closely associated with aug-
mented IFN-γ production and CTL activity against tumor Ag
(17–21). However, tumor cells, including Meth-A tumor
phage, are capable of suppressing immune responses against
tumor Ag. Any agent that augments tumor immunity can po-
tentially suppress tumor development, especially in the early
tumor progression phase. This could well be a part of anti-
tumor 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 inoc-
ulation. 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 nec-
essary to determine the source and identity of each asta-
xanthin 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 asta-
xanthin de novo and must acquire it through diets. Asta-
xanthin is widely distributed in fish (e.g., salmon, trout, cavi-
 iar), 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 shrimp 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 carotenoid 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 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

This study was partly supported by grants funded through the Minnesota Medical Foundation (Minneapolis, MN) and the Tsumura Corp. (Tokyo, Japan). Address reprint requests to Harumi Jyonouchi, M.D., Dept. of Pediatrics, University of Minnesota, Box 610, UMC, 420 Delaware St. SE, Minneapolis, MN 55455. Phone: (612) 626-3412. FAX (612) 624-9188. E-mail: jyon001@umn.edu.

Submitted 14 January 1999; accepted in final form 20 September 1999.

References


