Changes in $\beta$-Carotene Levels by Long-Term Administration of Natural $\beta$-Carotene Derived from *Dunaliella bardawil* in Humans

Takao MORINOBU, Hiroshi TAMAI,* Takuji MURATA, Mitsuhiro MANAGO, Hiroyuki TAKENAKA, Katsuhiko HAYASHI, and Makoto MINO

Department of Pediatrics, Osaka Medical College, Takatsuki 569, Japan
1Nikken Sohonsha Corporation, Gifu 501-62, Japan

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Summary Long-term administration of a $\beta$-carotene preparation derived from *Dunaliella bardawil*, a $\beta$-carotene-rich algae, was studied in healthy young male volunteers. The daily administration of 60 mg of the $\beta$-carotene preparation (30 mg of all-trans $\beta$-carotene and 30 mg of 9-cis $\beta$-carotene) was performed and $\beta$-carotene concentrations were determined in the plasma, red blood cells (RBC), platelets (PLT), and mononuclear cells (MN). The all-trans $\beta$-carotene level increased four-, two-, and threefold the baseline in plasma, PLT, and MN, respectively. Basal levels of 9-cis $\beta$-carotene in plasma, PLT, and MN were low and found as one-tenth, one-fifth, and one-fifth of all-trans $\beta$-carotene, which increased three-, two-, and 1.5-fold the baseline, respectively. Plasma and RBC $\alpha$-tocopherol levels were not changed by the intake of $\beta$-carotene. No side effects or toxicities were documented in any of the subjects during the administration period. In conclusion, the bioavailability of $\beta$-carotene derived from *Dunaliella bardawil* was preferential for all-trans $\beta$-carotene, although a small amount of the 9-cis form was detected in the plasma and blood cells.

Key Words *Dunaliella Bardawil*, all-trans $\beta$-carotene, 9-cis $\beta$-carotene, platelets, mononuclear cells, $\alpha$-tocopherol, retinol

Megadose administration of $\beta$-carotene has been advocated to promote general well being and prevent the development of cancer because of a possible relationship between increased $\beta$-carotene intake and a low incidence of epithelial cancer in humans (1, 2). However, the precise mechanisms by which $\beta$-carotene and/or carotenoids act to prevent cancer are not known. The importance of $\beta$-carotene was first recognized as the ultimate source of vitamin A, which is also known to
have antitumor activity. However, many investigators have demonstrated the ability of β-carotene to protect against cancer in animals independent of its role in the formation of vitamin A, because carotenoids quench singlet oxygen and free radical formation (3, 4), which are clearly important in carcinogenesis. Accordingly, many individuals in developed countries prefer to take vitamin preparations, such as β-carotene, vitamin C, and vitamin E to maintain their health. The naturally occurring β-carotene derived from Dunaliella bardawil contains about 50% all-trans β-carotene with the rest being mostly 9-cis carotene and has attracted attention as a source of β-carotene. Previous investigations on the bioavailability of β-carotene relied on changes in plasma β-carotene levels (5–7), and there have been a few studies on cellular β-carotene levels which may actually be more important biologically (8, 9). Therefore, we determined the β-carotene levels in blood cells during the long-term administration of a natural β-carotene preparation in humans. Since β-carotene has been reported to interact with vitamin E in the rat liver (10, 11) and human plasma (12), the influence of β-carotene intake on the plasma α-tocopherol level was also investigated.

MATERIALS AND METHODS

1. Subjects. Twenty healthy male college student volunteers aged 20–25 years (median age: 22±1 years) were enrolled in this study after a health check, and were fully informed of the objectives and the procedures involved at the beginning of the trial. The hospital ethics committee also approved the study protocol. Ten of the subjects were randomly assigned to the β-carotene treatment group, while other ten served as the placebo group.

2. Administration. The components of the preparation of natural β-carotene used in this study are listed in Table 1. The β-carotene capsules contained 10 mg of the all-trans form (50%) and 10 mg of the 9-cis form (50%). Three

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<th>Table 1. Composition of a capsule preparation of natural β-carotene (Dunaliella bardawil).</th>
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<td>Lactate</td>
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Each volunteer took 3 capsules per day with breakfast. The placebo capsules contained 390 mg of lactate.
capsules (corresponding to 60 mg of β-carotene) were taken once a day immediately after breakfast. The control subjects received identical placebo capsules and the study was performed by the single-blind method. The subjects were not limited with respect to lifestyle and food intake during the experiment. No effort was made to control nutrient intake during the experiment, except for the avoidance of other vitamin supplements. The experiment took place over a period of 44 weeks from February to November 1992.

3. **Sample collection and preparation.** A fasting blood sample was obtained by venipuncture at 8:00 a.m. at the beginning of treatment (day 0) and once a week during the 1st month of administration. Thereafter, samples were obtained every 10 weeks during administration and at the end of the 44-week treatment periods. Blood samples were also taken at 2, 4, and 20 weeks after the cessation of administration. On each occasion, 10 ml of blood was collected in a tube containing EDTA-2Na.

4. **Health assessment.** Each subject was also asked to complete a simple questionnaire for the subjective evaluation of treatment and to obtain other information. The questionnaire included items regarding fatigue, muscle weakness, gastrointestinal symptoms (anorexia, nausea, diarrhea, and abdominal pain), headache, and abnormal vision.

5. **Preparation of blood samples.** The whole blood samples were centrifuged at 900 rpm for 10 min at room temperature and two layers were obtained: top, platelet-rich plasma (PRP), bottom, RBC containing white blood cells (WBC). PRP was centrifuged at 3,000 rpm for 10 min to obtain platelet pellet. This platelet pellet was suspended in a sterile plastic tube with 10 ml of normal saline and was washed three times with 10 ml of normal saline (2 × 5 min and 1 × 10 min). After the final washing, the pellet was suspended in 2.5 ml of normal saline and sonicated for 1 min at 20 kcyle with a Handy Sonic (Ultrasonic Disruptor UD-201, Tomy Co. Ltd., Tokyo). For the separation of the RBC and mononuclear (MN) cells in the bottom layer, “Lymphofrep” (NYCOMED PHARMA AS, Norway) was used according to the Ficoll-Hypaque methods. Onto a layer of 1.5 ml of the medium in a tube, 2–3 ml of the RBC-MN cell suspension was slowly placed with a syringe, and then centrifugation was performed at 10°C for 30 min at 1,200 rpm to yield to separate MN cell and RBC layers. Then the MN layer and bottom layer of RBC were used for the β-carotene assay. The RBC pellet was washed three times with 10 ml of normal saline, and after the final centrifugation for 10 min the cells were suspended in an equivalent volume of normal saline. Then the hematocrit of the suspension was measured. The MN cell-containing tubes were filled with 10 ml of normal saline and centrifuged at 10°C for 15 min at 1,000 rpm. To remove the inevitable contaminating RBC, the MN pellet was then washed for 40 s in 2 ml of hypotonic 0.2% NaCl solution, after which 2 ml of 1.6% NaCl was added. After refilling each tube with 10 ml of normal saline, centrifugation and decantation at 10°C for 15 min at 1,000 rpm was repeated twice more. After a final wash, the pellet was suspended in 2.5 ml of normal saline and the cells were counted with a

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Leukocyte Counter (Sysmex Platelet Counter, PL-110, Japan).

6. Assays. a) $\beta$-Carotene: The method used for the determination of $\beta$-carotene levels was described previously (13). One milliliter of ethanol containing 0.015% butylated hydroxytoluene was added to 0.2 ml of plasma or 0.4 ml of cell suspension followed by vigorous shaking under a stream of nitrogen gas. Then 5 ml of n-hexane was added to this mixture, which was then centrifuged at 3,000 rpm for 10 min, and 4 ml of the hexane layer was evaporated under nitrogen gas. The residue was dissolved in 100 $\mu$l of ethanol and a 20-$\mu$l aliquot was injected to HPLC for the assay of $\beta$-carotene, while another 20-$\mu$l aliquot was injected into another HPLC system for retinol (14). Assay of $\beta$-carotene was performed using an Irika $\Sigma$871 HPLC apparatus with a Vydac reverse phase C$_{18}$ column (Hesperia, CA), and detection was done with an Irika $\Sigma$875 amperometer. The column was eluted at a flow rate of 1 ml/min using methanol/acetonitrile (95/5, v/v) containing 50 mM NaClO$_4$. Authentic $\beta$-carotene was obtained from Nippon Roche K.K. (Tokyo, Japan) and was dissolved in ethanol to produce standard solutions. Then the concentrations of the standards were determined with a Hitachi U-2000 spectrophotometer (Hitachi Co. Ltd., Tokyo), using the absorption coefficient $E_{453nm}^{1\%\,cm}=2,620$ at 453 nm in ethanol.

b) Retinol: For the retinol assay, a Shimadzu LC-5A HPLC apparatus (Shimadzu Co. Ltd., Kyoto) with an Irika RP-18 (4×250 mm) column was used. The detector was a Shimadzu RF-550 fluorescence photometer, and detection was performed with excitation at 340 nm and emission at 460 nm. The column was eluted with ethanol/water (95/5, v/v) at a flow rate of 0.7 ml/min.

c) Tocopherols: Plasma and RBC $\alpha$-tocopherol levels were determined as described previously using HPLC with electrochemical detection (15). For HPLC, an Irika $\Sigma$871 apparatus equipped with an Irika RP-18 (4×250 mm) column (Irika Co. Ltd., Kyoto) was used. Electrochemical detection was performed with an Irika $\Sigma$875 amperometric detector. The column was eluted with methanol/water/NaClO$_4$ = 100/2/7 (v/v/w) at a flow rate of 1 ml/min.

d) Retinol-binding protein and prealbumin: Retinol-binding protein (RBP) and prealbumin levels were determined by a Behring nephelometer system (16), which method is closely correlated with the conventional immunodiffusion method (17).

Statistical analysis. All results are expressed as the M±SE. Statistical analysis included assessment of means and comparison of treatment results by repeated measures ANOVA and a post hoc test (Scheffe's F-test). Statistical significance was set at the $p < 0.05$ level.

RESULTS

1. Plasma all-trans $\beta$-carotene level

The plasma all-trans $\beta$-carotene level increased fourfold above the baseline after 2 weeks of administration and then formed a plateau (Fig. 1A). After the
cessation of administration, it took 8 weeks to return to the baseline. On the other hand, plasma 9-cis \( \beta \)-carotene only showed a small increase, although a natural \( \beta \)-carotene mixture contains equal amounts of 9-cis \( \beta \)-carotene and the all-trans form. This result suggests that 9-cis \( \beta \)-carotene was not absorbed or that it was rapidly consumed or immediately taken up by the tissues after being absorbed.

2. **MN cells, PLT, and RBC all-trans \( \beta \)-carotene levels**

The all-trans \( \beta \)-carotene levels in MN cells and PLT increased by twofold and threefold above the baseline after the fourth and 12 weeks of administration, respectively, and then reached a plateau (Fig. 1B and C). In contrast, no changes were observed in the RBC \( \beta \)-carotene level (Fig. 1D). The 9-cis form also increased in PLT and MN to a small extent, but not in RBC.

3. **Plasma retinol, retinol-binding protein, and prealbumin levels**

Plasma retinol levels (Fig. 2), as well as retinol-binding protein and prealbumin (data not shown), did not differ between the treated and placebo groups.
Fig. 2. Changes in the plasma retinol levels.

Fig. 3. Changes of α-tocopherol levels in plasma and red blood cells. A: plasma, B: RBC.
Thus, the retinol status was not affected by administration of the β-carotene preparation.

4. Plasma and RBC tocopherol levels

The α-tocopherol levels were also examined in plasma and RBC during the study (Fig. 3A, B). There were no significant changes in the α-tocopherol level during administration of the β-carotene preparation, and the placebo group also showed no significant changes.

5. Side effects and toxicity

There were no significant changes in the laboratory parameters assessed, including blood cell counts, and the serum GOT, GPT, γ-GTP, CPK, and BUN levels. The subjective questionnaire examined muscle weakness, work performance, gastrointestinal discomfort, headache, hypertension, visual changes, and general well-being. No changes in any of the items were documented.

DISCUSSION

Elevation of the all-trans form of β-carotene in plasma was observed during the first 2 weeks of administration of a natural β-carotene preparation which also contained 50% of the 9-cis form. After 2 weeks, no further increase in plasma levels was observed despite continuing administration. A similar change was also observed in the levels of PLT and MN cells, but the time required to reach a maximum level was 4–12 weeks, which may be because the transportation of β-carotene from plasma to the cells took this long. Costantino et al. (5) have reported a tenfold increase in plasma all-trans β-carotene levels after administration of a relatively low dose of all-trans β-carotene (15 mg), with the maximum level being seen after 4 months. These differences in the maximum level and time required to reach it might be due to racial differences in body stores of the vitamin.

Ingestion of a natural β-carotene mixture increased the all-trans β-carotene in MN cells and PLT as well as plasma. The decline of β-carotene levels after cessation of administration was slower when compared to that of vitamin E in a previous study (18), although the behavior of circulating vitamin E has been reported to be similar to that of β-carotene (19). This indicates that β-carotene is stored by the body cells for a long period. Recently, β-carotene has been suggested to improve the immune response of elderly individuals; Alexander (20) reported an increase of OKT4+ cell numbers, while van Poppel (21) reported an increase of PHA-induced lymphocyte proliferation. Thus, an elevated β-carotene level in MN cells might contribute to the improvement of immunity.

Body mass index, alcohol consumption, smoking habits, and plasma lipids can influence the plasma and cellular β-carotene levels. Our subjects did not have an abnormal body mass index and did not change their dietary habits during the course of the study. Thus, the elevation of the plasma β-carotene level that we detected...
was due to all-trans β-carotene administration of a natural β-carotene mixture. The lack of a marked increase in plasma 9-cis β-carotene levels suggests that it was not absorbed, was immediately consumed, or was taken up by various tissues. This finding is similar as showed by the other investigators. Mokady et al. (22) have reported on the accumulation of 9-cis β-carotene in chick liver after administration of β-carotene-rich *Dunaliella bardawil*. Stahl et al. have reported the predominant presence of all-trans β-carotene in human plasma, but relatively high concentrations of 9-cis β-carotene in liver, adrenal gland and testes (8). Furthermore, he reported the preferential increase of all-trans β-carotene in human serum after ingestion of a natural isomer mixture obtained from *Dunaliella salina* which contains equal amounts of all-trans and 9-cis form as well as *Dunaliella bardawil* (9). However, further studies are required to clarify the behavior of 9-cis β-carotene in humans.

There have been a few reports on the interaction of β-carotene with α-tocopherol. Blakely et al. (10, 11) reported that β-carotene decreased the α-tocopherol level in rat liver, while Xu et al. (12) noted a similar result in human plasma. An earlier study by Willett (23) also showed that excess vitamin E intake decreased the plasma carotene level in humans. However, we could not find any changes in the plasma α-tocopherol level, although a natural β-carotene mixture contains only a small amount of α-tocopherol.

In addition, although β-carotene is a provitamin A, the administration of large doses of β-carotene did not affect the vitamin A status defined by plasma retinol, RBP, and prealbumin levels. It is well known that the serum levels of β-carotene and retinol are regulated by two independent mechanisms. Although carotenoids are known to be rapidly converted to vitamin A in vitamin A-depleted fish, many investigations on the administration of β-carotene in human subjects have found no significant changes in the serum retinol level (24).

With respect to the side effects or toxicity of a natural β-carotene mixture, none of the volunteers showed yellowing of the skin. In addition, there were no abnormalities found by physical and laboratory examinations. Furthermore, β-carotene has been successfully used to treat patients with genetically inherited photosensitivities for more than 15 years, and the ingestion of large doses of pure β-carotene has not produced any side effects (25, 26).

In conclusion, the present study demonstrated that β-carotene derived from a natural β-carotene mixture could increase β-carotene levels in human plasma and blood cells. Recently, Coodley et al. has reported that a high-dose β-carotene (180 mg/day) supplement for 4 weeks increased total WBC count, % change in CD4 count, and % change in CD4/CD8 ratios compared to placebo, in human immunodeficiency virus (HIV)-infected patients (27). On the other hand, the intervention study in Finland showed unexpected results of long-term supplementation of α-tocopherol and β-carotene in smokers, which showed a slight increase of lung cancer incidence by ingestion of β-carotene (28). However, this study have raised many arguments about effects of β-carotene.

The precise mechanism of those functional effects of an elevated levels of β-carotene.

β-carotene as an antioxidant, anticancer, or immunomodulator need to be investigated in the future.

REFERENCES


