α-Tocopherol Dietary Supplement Decreases Titers of Antibody against 5-Hydroxymethyl-2′-Deoxyuridine (HMdU)[1]

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Abstract

This study evaluated the effects of vitamin E (α-tocopherol) on oxidative DNA damage in a randomized double-blind Phase II chemoprevention trial. Oxidative DNA damage was measured by the level of auto-antibody (Ab) against 5-hydroxymethyl-2′-deoxyuridine (HMdU) in plasma. After the baseline screening, eligible subjects ($n = 31$; plasma samples from 28 subjects were available for this study) were randomized to receive 15, 60, or 200 mg of α-tocopherol per day for 28 days. Biomarkers were measured twice at baseline—on day 1 (visit 1) and day 3 (visit 2)—and twice after intervention—on day 17 (visit 3) and day 31 (visit 4). At baseline, there was a highly significant inverse correlation between anti-HMdU Ab titer and plasma vitamin E level ($r = -0.53$; $P = 0.004$; $n = 28$). Smoking did not affect baseline anti-HMdU Ab titer; however, anti-HMdU Ab titer levels at baseline were significantly lower in subjects with above-median (0.75 ounce/day) alcohol consumption ($P = 0.008$). No significant change in anti-HMdU Ab level occurred at either visit 3 or visit 4 for subjects on the lowest dose, 15 mg α-tocopherol per day. Subjects receiving 60 mg of α-tocopherol per day had a significant decrease in anti-HMdU Ab level at visits 3 and 4 compared with baseline ($P = 0.049$ and $P = 0.02$, respectively). However, subjects receiving the highest dose, 200 mg/day, had less consistent results: a significant decrease in anti-HMdU Ab level was seen at visit 4 ($P = 0.04$) but not at visit 3. Our results demonstrate an inverse relationship between α-tocopherol and anti-HMdU Abs in plasma; oxidative DNA damage can be modulated by short-term dietary supplementation of α-tocopherol in some subjects.

Introduction

ROS[3] are formed as part of the normal metabolic pathways by ionizing and UV radiations, activation of chemical carcinogens, and heavy metal carcinogens (1–4). An imbalance between oxidants and antioxidants may result in a chronic state of oxidative stress that could contribute to various human diseases including autoimmune (lupus), cardiovascular disease, and cancer (1–13). Elevated DNA damage and defective DNA repair have been associated with the development of human cancers (14–16). Although genetic factors contribute to genomic instability and defective DNA repair, environmental exposures and lifestyle/dietary factors may also play a role in regulating DNA damage and repair, in part, by modulating the oxidant/antioxidant balance (17). Antioxidants protect cells from DNA damage by directly removing reactive free radicals, thus reducing the amount of DNA damage that leads to tumorigenesis.

Tumor promoters and inflammation increase the formation of oxidized bases, such as 8-hydroxyl-2′-deoxyguanosine (8-OHdG) and HMdU (11, 18). Increased oxidant production causes oxidation of DNA bases, which provides antigenic determinants that elicit antioxidized DNA Abs (19). An ELISA was developed to measure anti-HMdU Abs in human sera, the titer of which may reflect levels of oxidative DNA damage (19). The titers of anti-HMdU Abs are significantly higher in sera from patients with systemic lupus erythematosus and other inflammatory autoimmune diseases and in subjects with occupational exposure to cadmium, nickel, and chromium as well as in subjects with increased risk for breast, colon, or rectal cancer (20–22). Elevated anti-HMdU Ab titers may be an early signal of cancer risk because they were significantly increased in (*a*) in otherwise healthy women who had a family history of breast cancer; (*b*) in those who had benign breast disease or benign gastrointestinal tract diseases; and (*c*) most importantly, in those who at 0.5–6 years after the initial blood donation developed breast or colorectal cancer (22). Anti-HMdU Ab titers showed a remarkable stability over a period of 6 years, with a low (14%) intraindividual variance (22). In addition, in a trial of chromium (III) picolinate, results showed that the HMdU antibody titers did not change at 4 or 8 weeks. The titers were very stable within individuals, and those of one individual rarely crossed over values of other subjects, which was reflected in an intraclass correlation coefficient of 0.99 (95% confidence interval, 0.96–1.00; Ref. 23). Data suggest that anti-HMdU Ab titers may be a useful cancer risk biomarker.

Under normal circumstances, ROS are controlled by the

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[3] The abbreviations used are: ROS, reactive oxygen species; Ab, antibody; HMdU, 5′-hydroxymethyl-2′-deoxyuridine; GSH, glutathione; LIPP, lipid protection ratio; GEE, generalized estimating equation.

antioxidant/antioxidant enzyme defense system in the body. Data from animal and epidemiological studies suggest that dietary antioxidants may protect against cancer (24–31). Vitamin E (α-tocopherol), a well-studied lipid-soluble antioxidant, has been associated with a reduced risk of cancer in two epidemiological studies but not in the other two studies (24–27). The use of α-tocopherol to prevent human cancer has been evaluated for lung cancer, oral leukoplakia, colorectal polyps, and prostate cancer (28–31). The antimutagenic and antiproliferative effects of α-tocopherol may contribute to its chemopreventive properties (32). Intracellularly, α-tocopherol is localized in the lipid-rich membrane and may serve to protect the membrane from lipid peroxidation (LPO) by reacting with lipid peroxyl and alkoxyl radicals (33). α-Tocopherol is a major peroxyl-radical scavenger in biological lipid phases, such as membranes or low-density lipoproteins. Dietary supplements of α-tocopherol prevent exercise-induced DNA damage (34). Furthermore, vitamin C, vitamin E, and β-carotene supplementation has resulted in a highly significant decrease in endogenous oxidative DNA damage in lymphocytes and an increased resistance to oxidative damage induced by H_2O_2 (35).

This study aimed to evaluate the effects of short-term supplementation of α-tocopherol on anti-HMdU Ab titers in a pilot chemoprevention trial. Frozen plasma samples were derived from a randomized double-blind chemoprevention trial of α-tocopherol. Detailed elements of the study's design have been described previously (36).

Materials and Methods

Materials. Dulbecco's PBS (PBS) was obtained from Life Technologies, Inc. (Gaithersburg, MD). All of the other general laboratory chemicals were obtained from Sigma Chemical Company (St. Louis, MO). The d-l-α-tocopherol powder was provided by Hoffman LaRoche (Nutly, NJ), and capsules with three-dose levels were prepared by Pasteur Pharmacy (New York, NY).

Vitamin E Trial. Study samples were derived from a randomized trial ($n = 31$) designed to test the effects of vitamin E supplementation on DNA repair and plasma antioxidant levels (36). Frozen plasma samples were available from 28 subjects. During their first visit, all of the subjects signed informed consent form as approved by the Institutional Review Board of the Cancer Prevention Research Institute (closed in 1994). Inclusion criteria were: (*a*) willing to participate and able to give informed consent; (*b*) willing and able to return for a second visit on day 3; and (*c*) over 18 years old. Exclusion criteria were: (*a*) history of, or active, cancer; (*b*) history of abnormal white cell count (less than 3.0); (*c*) treatment with chemotherapy, radiation, or hormones within the past month; (*d*) life-threatening condition of any type; and (*e*) use of α-tocopherol or other micronutrient supplements. Information collected from the baseline questionnaire included diet, alcohol consumption, smoking, medications, and vitamin use. Subjects ranged from 24 to 78 years of age and consisted of 22 females and 6 males.

During the first visit, subjects completed a baseline questionnaire that included demographic information, medical history, family history of cancer, and (for women) reproductive history. Dietary intake was assessed by the National Cancer Institute Food Frequency Questionnaire (37). All of the biochemical measurements were performed twice at baseline (days 1 and 3) and twice after intervention (days 17 and 31). After blood was drawn on day 3 (visit 2), the subjects were randomized to receive 15, 60, or 200 mg of α-tocopherol per day. Neither the study participants nor the laboratory technicians had knowledge of the dose level assigned. Compliance was measured at visits 3 and 4 by calendar and pill count. Heparinized whole blood samples were collected in a nonfasting state between 9:00 and 11:00 a.m., stored in a dark container, transported to the laboratory within 2 h of phlebotomy, and immediately processed for plasma storage. Plasma levels of LIPP, GSH, and vitamin E were described in the original study (36). Plasma samples were stored in liquid nitrogen until assayed for anti-HMdU Abs.

ELISA. Antigen was prepared by periodate coupling of 5-hydroxymethyl uracil (HMUra) riboside to BSA followed by reduction with borohydride, according to the method of Erlanger and Beiser (38). This procedure led to the formation of a conjugate of HMUra 2′-deoxyribonucleoside (HMdU) with BSA. ELISA was carried out according to the standard procedures, as described previously (19, 39). To determine specific and nonspecific binding, an oxidized thymidine (HMdU) coupled to BSA as well as mock-coupled BSA (M-BSA) were used as antigens for coating the wells of microtiter plates. Antigen-coated 96-well plates were incubated at 37°C for 2 h with plasma diluted 10,250-fold and washed three times with PBS containing 0.05% Tween 20. Then the wells were incubated for 1 h with a goat antihuman IgM (1:1,000 dilution; nonaffinity purified; Sigma Chemical Company), labeled with horseradish peroxidase, and washed three times again. *o*-Phenylenediamine was used as a substrate for H_2O_2 oxidation mediated by horseradish peroxidase bound to the wells through the antihuman Ab during incubation at 37°C for 0.5 h. Development of color, measured at 492 nm (at acid pH) in ELISA microplate reader (Anthos Labtec Instruments, Model 2001), was proportional to the amount of human Abs bound to the coated plates. In this study, each plate contained wells incubated only with buffer instead of the subjects' plasma (negative control) or with an aliquot of plasma with a known high titer of anti-HMdU Ab (positive control). This allowed elimination of batch-to-batch variability of antigens and secondary antihuman Abs. Each plasma specimen was analyzed 4–6 times. The results are presented as mean absorbance (*A*) at 492 nm/μl of undiluted plasma ± SD.

Statistical Analysis. The characteristics of study subjects at baseline were compared using Fisher's exact test for dichotomous characteristics and the Kruskal-Wallis test for quantitative characteristics. The association between plasma anti-HMdU Ab level and other plasma antioxidant levels at baseline were estimated using Pearson's correlation coefficient (*r*). Changes in plasma anti-HMdU Ab level from baseline was tested separately at each subsequent patient visit (visits 3 and 4) and at each dose level of α-tocopherol, using the Wilcoxon signed-rank test. An overall test of a dose effect over time was performed by fitting a GEE. The GEE approach was preferred to repeated measures ANOVA because GEE did not require the data to be normally distributed (40). In the GEE regression model, each dose group was allowed its own intercept, and the results were adjusted for patient characteristics by including age, sex, and baseline GSH, LIPP, and vitamin E levels as covariates. In the GEE model, the within-patient correlation in anti-HMdU Ab level was assumed to follow an autoregressive process. In addition to this GEE model, we fit an addition GEE model adjusting for both smoking and alcohol use. We also examined dose by time by alcohol and dose by time by smoking interactions to determine whether there were differential effects of dose depending on baseline alcohol or smoking levels. Re-

Table 1 Baseline characteristics and measurements by supplementation group

Characteristics	A (15 mg)	B (60 mg)	C (200 mg)	*P*
Total	10	10[a]	8[a]	
Age[b]	41.0 ± 15.1	35.2 ± 8.5	34.4 ± 10.7	0.53[c]
Sex				
Female	8	9	5	
Male	2	1	3	0.44[d]
Smoking				
No	6	6	5	
Yes	4	4	3	0.99[d]
Alcohol				
<0.75 oz/day	6	8	2	
≥0.75 oz/day	4	2	6	0.06[d]
Plasma α-tocopherol (μM)[b]	26.5 ± 7.9	28.5 ± 13.7	26.3 ± 4.6	0.68[c]
Plasma GSH (μM)[b]	31.7 ± 13.4	33.9 ± 7.4	34.7 ± 10.5	0.80[c]
LIPP[b]	6.2 ± 0.9	6.4 ± 1.6	6.3 ± 0.9	0.91[c]
Anti-HMdU Ab (A_{492}/μl plasma)[b]	20.3 ± 11.3	29.4 ± 20.6	20.4 ± 19.3	0.54[c]

[a] In the original study, there were 11 and 10 participants in groups B and C, respectively. However, plasma samples were only available from 10 and 8 subjects in groups B and C, respectively.
[b] Data are presented as mean ± SD.
[c] *P* from Kruskal-Wallis test.
[d] *P* from Fisher's exact test.

sults were considered significant if the 2-sided *P* was less than 0.05.

Results and Discussion

Data were available on a total of 28 (22 females and 6 males) healthy volunteers. As shown in Table 1, there were no significant differences among three dose groups in age, sex, race, baseline plasma α-tocopherol levels, baseline plasma GSH, baseline LIPP, and baseline anti-HMdU Abs. At baseline, the mean anti-HMdU Ab level was higher in the 60 mg/day group than in the other dose groups; however, this difference was not significant ($P = 0.54$; Table 1). The distribution of study subjects in three groups was not different on gender, smoking status, or plasma antioxidant levels. However, the percentage of subjects with above-median alcohol consumption significantly differed among the three study groups.

To test the hypothesis that oxidative DNA damage is related to antioxidant level, we evaluated the association between plasma antioxidant level and anti-HMdU Ab level at baseline. At shown in Fig. 1, there was a highly significant inverse correlation between α-tocopherol level and anti-HMdU Ab level ($r = -0.53$; $P = 0.004$). However, there was no evidence of a correlation between anti-HMdU Ab level and either LIPP ($r = -0.20$; $P = 0.31$) or GSH ($r = -0.03$; $P = 0.89$).

Study subjects receiving 60 mg of α-tocopherol per day exhibited a significant decrease in anti-HMdU Ab level on visits 3 and 4 compared with baseline ($P = 0.049$ and 0.02, respectively; Table 2). However, subjects receiving the highest dose of α-tocopherol, 200 mg/day, had less consistent results: a significant decrease in anti-HMdU Ab level was seen on visit 4 ($P = 0.04$) but not on visit 3. No significant change in anti-HMdU Ab level occurred on either visit 3 or 4 for patients on the lowest dose of α-tocopherol. The overall test of a dose effect over time was not significant ($P = 0.49$; Table 2).

Table 3 shows the within-subject changes in anti-HMdU Ab levels by dose group and demonstrates the variations in each individual's response to α-tocopherol. Data suggests that drinking (≥0.75 ounce/day) is significantly associated with lower anti-HMdU Ab levels at baseline (30.78 and 13.94 in nondrinker and drinker, respectively; $P = 0.008$) but smoking is not (anti-HMdU Ab levels at baseline: 24.78 and 21.69 in nonsmoker and smoker, respectively; $P = 0.65$). To evaluate whether alcohol or smoking may affect an individual's response to α-tocopherol, we also examined dose by time by alcohol and dose by time by smoking interactions. Although there appeared to be large differences in anti-HMdU Ab levels based on alcohol consumption within different supplementation levels, these differences did not reach statistical significance because of the small sample sizes. However, these effects suggest that further research needs to be performed to assess the impact of alcohol on anti-HMdU Ab levels. This is the first report to suggest that alcohol consumption may influence anti-HMdU Ab levels. It is not clear whether alcohol affects the extent of oxidative DNA damage or the production of Abs by affecting immune response as suggested in other report (41).

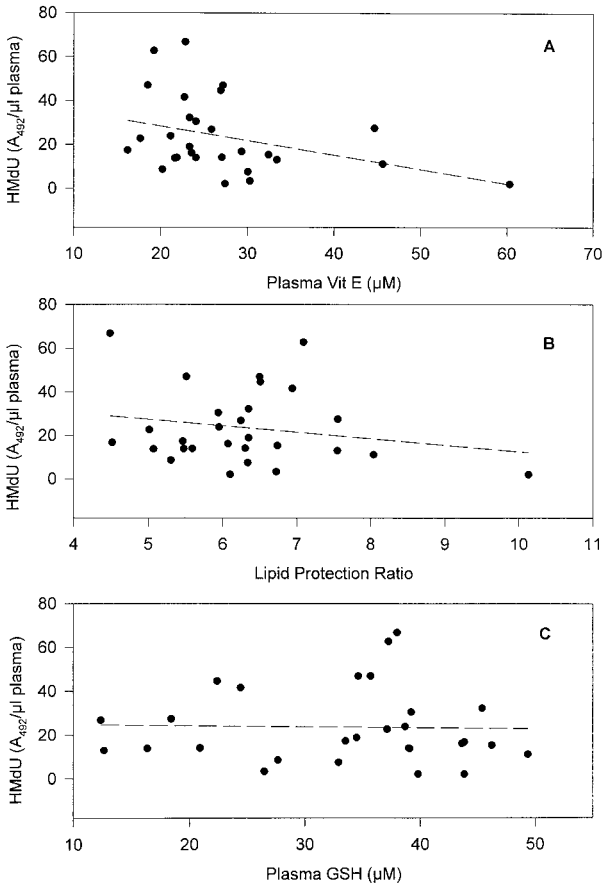

Fig. 1. Correlation between plasma anti-HMdU Ab level (A_{492}/μl plasma) and antioxidants at the baseline. *A*, plasma vitamin E level (μM); *B*, LIPP (*Lipid Protection Ratio*); *C*, plasma GSH level (μM).

ROS are constantly generated in cells. They can damage DNA, proteins, carbohydrates, and lipids; α-tocopherol can eliminate prooxidants, decrease oxidative DNA damage, and function as a part of an antioxidant defense system. Several studies have suggested that the chemopreventive properties of α-tocopherol include reducing micronuclei frequencies (29) and preventing peroxidative damage to DNA by dietary polyunsaturated fat (38). In the original study, we demonstrated a

Table 2 The effects of α-tocopherol supplements on plasma anti-HMdU Ab levels[a]

α-Tocopherol (mg/day)	*n*	Baseline ($A_{492}/\mu l$)	Visit 3 ($A_{492}/\mu l$)	% of patients with decrease	Visit 4 ($A_{492}/\mu l$)	% of patients with decrease
15	10	20.3 ± 11.3[b] (14.7)	17.8 ± 12.0 (14.8)	80[c]	18.1 ± 13.0 (13.8)	60
60	10	29.4 ± 20.6 (25.6)	26.9 ± 19.3[d] (23.8)	80	28.0 ± 20.5[e] (23.2)	90
200	8	20.4 ± 19.3 (16.4)	20.7 ± 18.5[f] (17.4)	29[f]	19.5 ± 19.9[g] (15.6)	88

[a] $P = 0.49$ for overall test of a dose effect over time.
[b] Data are presented as mean ± SD (median).
[c] % of patients with a decrease in HMdU Ab level compared to baseline.
[d] $P = 0.049$, visit 3 compared with baseline.
[e] $P = 0.02$, visit 4 compared with baseline.
[f] $n = 7$ at visit 3.
[g] $P = 0.04$, visit 4 compared with baseline.

Table 3 Plasma anti-HMdU Ab levels by alcohol consumption

Dose	Alcohol	Subject no.	Sex	Age	Baseline	Visit 3	Visit 4
15	<0.75	5[a]	M	45	8.55	8.05	8.57
		19	F	31	26.77	14.87	11.78
		20	F	43	44.58	48.87	51.92
		34[a]	F	78	11.09	7.40	8.17
		40	F	33	32.14	23.78	26.42
		44	F	40	22.62	21.00	19.98
					24.29 ± 13.45[b]	20.66 ± 15.32	21.14 ± 16.68
	≥0.75	9	F	50	13.88	14.67	14.13
		17	M	28	14.03	13.08	14.55
		36[a]	F	36	15.28	15.03	13.42
		37[a]	F	26	13.94	11.28	12.25
					12.22 ± 3.00	10.44 ± 3.50	10.60 ± 2.63
60	<0.75	6[a]	F	50	27.43	24.68	27.25
		24[a]	F	46	41.49	31.20	37.83
		26	F	25	17.28	14.95	16.87
		30	F	27	46.90	49.17	44.77
		31	F	25	7.51	5.92	5.72
		38[a]	F	37	46.86	46.22	47.55
		41[a]	F	33	13.75	11.33	13.48
		45	F	38	66.77	59.18	65.97
					33.50 ± 20.33	30.33 ± 19.51	32.43 ± 20.27
	≥0.75	32	F	32	23.80	22.83	19.08
		35	M	39	1.97	3.08	1.87
					12.89 ± 15.44	12.96 ± 13.97	10.48 ± 12.17
200	<0.75	27	F	36	62.73	55.85	63.58
		42	F	28	16.08	17.40	15.68
					39.41 ± 32.99	36.63 ± 27.19	39.63 ± 33.87
	≥0.75	22[a]	F	28	13.00	16.30	11.68
		25	M	51	3.32	2.55	1.68
		28[a]	F	50	16.78	ND	15.43
		29[a]	F	24	30.40	31.00	29.07
		33	M	33	2.05	2.15	1.90
		39	M	25	18.88	19.53	16.80
					14.07 ± 10.57	14.31 ± 12.21	12.76 ± 10.3

[a] Current smoker.
[b] Data are presented as mean ± SD.

dose-dependent increase in plasma α-tocopherol and GSH after short-term supplementation of α-tocopherol. Our current findings provide additional support for the hypothesis that supplementation with α-tocopherol protects cells from oxidative DNA damage. However, several factors may affect our study results. Obviously, a sample of less than 10 in each dose group with a trial period of 4 weeks allows only for preliminary conclusions and must be considered as a pilot project for a larger confirmatory study in the future. Furthermore, under our experimental conditions, the assay for anti-HMdU Abs may be influenced not only by the initial DNA damage levels but also by cellular immune responses, which could have contributed to the nonsignificant results when testing the overall effect of α-tocopherol dosage over time.

Three factors may influence our study results: (*a*) supplementation with α-tocopherol at high dosage (*i.e.*, 200 mg/day) may also stimulate cellular immune responses that increase Ab production, which may counteract the decreasing effects of α-tocopherol on anti-HMdU Ab levels; (*b*) smoking may influence an individual's response to α-tocopherol (a larger sample size is needed to test this possibility further); and (c) if there is a delayed response to α-tocopherol, a longer trial period may be required to obtain a more consistent effect on anti-HMdU Ab levels. Handelman *et al.* (42) raised important issues concerning the steady-state after a change of α-tocopherol intake. It was estimated that more than 2 years are required for the α:γ-tocopherol ratio to reach a new steady state after a change in α-tocopherol intake. Furthermore, the adipose α:γ-tocopherol ratio may be a valuable tool to estimate an individual's long-term α-tocopherol intake (42).

The normal intake of vitamin E in diets in the United States ranges between 4 and 22 IU/d in adults who are not taking vitamin E supplements (average values are between 11 and 13 IU/d). This intake maintains an average blood level of approximately 23 μM, which is somewhat lower than the mean baseline plasma α-tocopherol level of 27.9 μM among our subjects. In healthy individuals, a daily intake of about 15–30 mg of α-tocopherol is recommended to obtain "optimal plasma α-tocopherol concentrations" (30 μM or greater; Ref. 43). In general, the cause of vitamin E deficiency is usually malabsorption, which is a result of fat malabsorption or genetic abnormalities in lipoprotein metabolism. When selecting an appropriate cohort for dietary chemoprevention studies, it is important to keep in mind that α-tocopherol supplementation in subjects on a nutritionally adequate diet does not always provide additional benefit or protection (43). However, micronutrient supplementation in subjects with even slight vitamin E deficiency may provide maximum benefit and protection from cancer. In summary, our results demonstrate that short-term supplementation of α-tocopherol may protect cells against oxidative DNA damage and suggest that the underlying mechanism of this effect may involve the up-regulation of a network of an antioxidant-defense system (36). On the basis of the results from this pilot study, future confirmatory studies should address issues related to the selection of the study cohort, the optimal dose of α-tocopherol, the duration of the trial, and the specificity of oxidative DNA damage assays.

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