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Effects of Topical and Oral Vitamin E on Pigmentation and Skin Cancer Induced by Ultraviolet Irradiation in Skh:2 Hairless Mice

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Abstract: *This study investigates whether supplementation with topical RRR-α-tocopherol (Eol), topical RRR-α-tocopheryl succinate, and oral RRR-α-tocopheryl acetate can reduce the incidence of acute and chronic damage to the skin (i.e., sunburn and pigmentation and skin cancer, respectively) induced by ultraviolet (UV) irradiation to mice. Groups of twenty Skh:2 female hairless pigmented mice were treated with 1) lotion vehicle, 2) 5% Eol lotion, 3) 5% topical RRR-α-tocopheryl succinate lotion, or 4) lotion vehicle and oral RRR-α-tocopheryl acetate. Within each group, 15 mice were exposed to 0.24 J/cm² of UV-B radiation three times per week. The animals' weights and food intakes were monitored, and the vitamin E concentrations of skin, liver, and adipose tissue were measured to determine whether the topical Eol resulted in significant tissue levels. Skin pigmentation was scored, and the total number of clinically detectable skin tumors per animal was counted weekly. Results showed that the skin concentrations of Eol, as well as levels in the adipose tissue, were increased after topical application. Mice treated with each form of vitamin E showed no signs of toxicity and had significantly less acute and chronic skin damage induced by UV irradiation, as indicated by reduced inflammation and pigmentation and by later onset and lesser incidence of skin cancer.*

Introduction

The skin is regularly exposed to ultraviolet (UV) radiation, which damages the skin acutely and chronically (1). Acute damage is characterized by inflammation, commonly known as "sunburn" when it is more extensive. Clinically, the acute reaction ranges in severity from a mild, asymptomatic erythema to a more intense erythema accompanied by tenderness, edema, and sometimes vesiculation or bulla formation. The acute reaction heals with tanning due to oxidation of the melanin already in the epidermal keratinocytes. With continued exposure, additional melanin is synthesized and transported to the keratinocytes.

Chronic exposure to UV radiation leads not only to acceleration of the skin's aging (2) but also, more seriously, to skin cancer (3). Skin cancer is the most prevalent type of human malignancy, with the incidence among Americans nearly equaling the annual incidence of all other malignancies combined. Especially with the rapid rise in occurrence of skin cancer (4), presumably due to increased recreational exposure to sunlight and to increasing solar UV-B radiation reaching the Earth's surface (5), it is important to investigate possible means to decrease photocarcinogenesis.

The purpose of this study was to determine whether topical RRR-α-tocopherol (Eol), topical RRR-α-tocopheryl succinate (Esuc), or oral RRR-α-tocopheryl acetate (Eac) can reduce acute and chronic UV radiation-induced damage to the skin. This is the first investigation using these natural isomers of vitamin E in topical application.

Natural vitamin E is the most important lipid-soluble, membrane-bound antioxidant in the body. Several forms of vitamin E exist in nature. The form that is found in mammalian tissues and has by far the greatest biological activity is pure, nonesterified Eol (6,7), which has three methyl groups on the 6-chromal ring. The other natural forms are β, γ, and δ, which contain only one or two methyl groups on the 6-chromal ring. Relative to the α-form, the β-, γ-, and δ-RRR-tocopherols have only 42%, 72%, and 40%, respectively, of the protection against post-UV edema (8). The synthetic form is "*dl*" or "all-*rac*," a mixture of eight stereoisomers. The synthetic isomers are esterified for use in commercial vitamins and some topical formulations because the esters are far more stable.

Most previous studies, especially those using topical vitamin E, have used synthetic ester forms, which have been shown to be absorbed by the skin. Quantitative penetration studies with ^{14}C-labeled all-*rac*-α-tocopheryl acetate on human scalps (9) demonstrated that this form of vitamin E is

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absorbed through the stratum corneum by two routes: 1) directly through the epidermis and 2) through the philosebaceous glands. However, in test volunteers, absorption of the Eac was lower than that of Eol (10,11). Also, the skin has only a limited capacity to cleave the acetate and succinate esters to the free tocopherol form (12,13), so that the antioxidant potential of the small amount absorbed is minimal. Furthermore, the all-*rac* form of vitamin E has been reported to cause allergic contact dermatitis (14) and erythema multiforme (15) when applied topically. No such adverse reactions have been reported with natural Eol.

Previous studies have demonstrated protection from acute UV-induced damage to the skin even by various forms of vitamin E that are less metabolically potent when applied topically than the nonesterified Eol used in the studies presented here (16–21). When all-*rac*-α-tocopheryl linoleate was applied before UV irradiation, electron microscopy showed it to help repair epidermal cell damage and to have anti-inflammatory effects (16). Application of various doses of topical all-*rac*-α-tocopheryl acetate to the backs of guinea pigs before UV-B irradiation reduced the acute formation of "sunburn cells" (17). When applied before UV irradiation, all-*rac*-α-tocopherol and two shortened side-chain analogs were found to protect from psoralin + UV-A erythema and impedance of human and rabbit skin, but these effects were not observed with treatment after UV exposure (18). An elegant study by Berton and co-workers (19) demonstrated that topical Eac decreased acute DNA photo damage when applied before or after UV irradiation and decreased p53 expression when applied after UV irradiation. Topical all-*rac*-α-tocopheryl acetate applied for three weeks before UV exposure protected the epidermis in hairless mice against the decrease in DNA thymidine incorporation and lipid peroxidation caused by UV irradiation; dietary all-*rac*-α-tocopheryl acetate protected only against UV-induced lipid peroxidation (20). These benefits were confirmed only when the all-*rac*-α-tocopheryl acetate was applied before UV exposure (21). Because tocopherol and tocopheryl acetate can partially block UV-B, it is possible that such protective effects might be due only to the sunscreen effect, rather than an antioxidant action. However, Trevithick and associates (22) found that topical application of Eac immediately after UV-B exposure significantly reduced the erythema, edema, and sensitivity to pressure, suggesting a true antioxidant effect. Other research has demonstrated anti-inflammatory activity of vitamin E that might decrease the degree of erythema and edema after UV exposure (23,24).

UV radiation also directly alters DNA and induces free radicals (25,26) and lipid peroxidation (27) in the epidermis, thus initiating and promoting skin cancer (28). Vitamin E may protect the skin from this chronic UV damage by several mechanisms: 1) quenching free radicals, by acting as an antioxidant to inhibit oxidation of unsaturated lipids [as confirmed by *in vitro* experiments which demonstrated that α-tocopheryl succinate protects against radiation-induced lipid peroxidation (29)], and 2) protecting specific membrane proteins that contain selenium or sulfur (30). Indeed, all-*rac*-α-tocopherol has been shown to prevent epidermal chemical carcinogenesis (31–33) as well as UV-induced photocarcinogenesis (34–37).

In a study of hairless mice exposed to various levels of UV radiation, oral treatment with a combined supplement containing all-*rac*-α-tocopherol, ascorbic acid, butylated hydroxytoluene, and reduced glutathione increased the latency period and decreased the number of tumors (34). In further research with hairless mice exposed chronically to suberythemal doses of UV radiation, 70% of the control animals had at least one visible skin tumor after 20 weeks, while only 10% of the mice treated topically with a combination of all-*rac*-α-tocopherol with vitamin C and an anti-inflammatory agent (hydrocortisone, naproxen, or ibuprofen) before exposure to UV-B irradiation had visible skin tumors (35).

Berton and others (19) demonstrated that topical Eac delayed UV-induced skin tumor development initially in Skh:1 mice, but in their protocol this protective effect was lost by 30 weeks. In contrast, Gensler and Magdaleno (36) showed that topical all-*rac*-α-tocopherol reduced UV-induced skin cancer incidence from 81% in controls to 42% in treated C3H/HeN mice receiving topical all-*rac*-α-tocopherol, even at the termination of the experiment at 34 weeks (36). Topical vitamin E treatment also prevented the development of UV radiation-induced immunosuppression (36).

These same researchers further demonstrated that UV-B-induced skin cancer was inhibited significantly in inbred C3H/HeN mice treated with dietary supplements containing Eac (37); in contrast, topical application of all-*rac*-α-tocopheryl succinate did not prevent UV-B-induced skin cancer in hairless mice and may indeed have enhanced the process (12). The esterified forms of vitamin E accumulated in the skin, but α-tocopherol levels remained low. Possibly the limited capacity of skin to cleave esterified forms of vitamin E to α-tocopherol explains the inability of vitamin E acetate or succinate to prevent UV-induced skin cancer in that protocol.

In the present study, natural nonesterified Eol or Esuc was applied topically or Eac was added to the diet. The effect on the acute UV-induced damage to the skin (erythema, blistering, and pigmentation) as well as on the UV-induced incidence of skin cancer was evaluated.

Materials and Methods

Animals and Treatments

Ninety-two female Skh:2 hairless pigmented dark-eyed mice (initial weight 20–25 g, 6–8 wk old) were purchased from the Skin and Cancer Hospital of Temple University Health Sciences Center (Philadelphia, PA). They were housed five or six mice per cage in a temperature- (24°C) and light-controlled (14 h/day) room. Their care was in accordance with the institutional guidelines of the Animal Care Committee of Cornell University. The mice were path-

Table 1. Treatment of Skh:2 Mice

RRR-α-Tocopherol Supplementation			UV Treatment, no. of mice	
Source	Route	Dose	+	–
Vehicle lotion (control)			15[a]	5
RRR-α-tocopherol	Topical	22.4[b]	15[a]	5
RRR-α-tocopheryl succinate	Topical	18.2[b]	15	5
RRR-α-tocopheryl acetate	Oral	1.76[c]	15	5

a: 6 additional mice were treated topically for 10 wk and then sacrificed for measurement of tissue levels of vitamin E.
b: Calculated topical in IU equivalents of RRR-α-tocopherol/wk/mouse (note that only ~10% is absorbed).
c: Calculated oral intake application in IU equivalents of RRR-α-tocopherol/wk/mouse.

ogen free, as confirmed by bacteriology and parasitology. All animals were fed a nutritionally adequate diet (Purina Rodent Laboratory Chow 5001, Ralston Purina, St. Louis, MO), a cereal-based chow that contained 32 IU of vitamin E as all-*rac*-α-tocopheryl acetate per kilogram and 0.23 ppm of selenium. Diets were stored refrigerated, and food was replenished daily. Diets were prepared by Dyets (Bethlehem, PA) approximately every three months. Throughout the studies, the weights of the mice and the feed and water intakes were monitored weekly. The mice were separated into treatment groups as indicated in Table 1.

Beginning two weeks before the UV exposure and during the full subsequent duration of the experiment (46 wk), aliquots (100 µl) of an oil-in-water emulsion skin lotion base were applied three times per week to the backs, heads, and ears of each mouse before the initiation of irradiation with UV light. Each application of skin lotion appeared to penetrate the skin within 10 minutes. During the period of irradiation, the lotion was applied ≥30 minutes before each UV exposure. Immediately after application and for at least two hours thereafter, the mice were placed in cages with dividers so that each was housed and later exposed to the UV radiation individually with no contact with other mice. Each mouse was wiped before being brought into the maintenance cages with multiple animals to prevent oral ingestion of topical lotions from other animals.

To check that the cutaneous supplementation gave increased tissue levels of vitamin E, after 10 weeks of topical application of Eol, six mice from the nonsupplemented and from the topical Eol group (exposed to UV-B) were killed by cervical dislocation. Vitamin E was measured in each of triplicate samples from dorsal skin, ventral skin, liver, and omental adipose tissue. The surviving mice were killed at 44 weeks because of the high mortality of the mice not supplemented with vitamin E. The level of vitamin E was determined in tumors excised from each of the mice in the nonsupplemented group and in the group treated with topical Eol at the termination of the experiment.

Lotion and Supplementation

The lotion was a standard cosmetic base, which contained C12-15 alkyl benzoate (FineTex, Spencer, NC), glyceryl stearate SE, stearic acid (Continental, Charlotte, NC), triethanolamine (Continental), trialkyl ammonium lauryl sulfate (SouthChem, Charlotte, NC), carbomer 934 (CA Specialties, Chester, SC), vegetable oil (WorthChem, Charlotte, NC), methylparaben (SouthChem), propylparaben (SouthChem), imidazodinyl urea (ISP/Sutton, Cranbury, NJ), and water. This lotion and the vitamin E-supplemented lotions were mixed by Harmony Laboratories (Landis, NC).

Eol and Esuc (Henkel, LaGrange, IL) were mixed into separate portions of lotion carrier at a concentration of 5%. For those mice supplemented with oral Eac, 62.5 IU of Eac (Covitol 625 CG, Henkel) were added per kilogram of chow by Dyets. The amount of vitamin E provided by topical application was calculated to be about equal to that provided by oral supplementation.

Analysis of Tissue Levels of Vitamin E

The tissue levels of vitamin E were measured in the mice ≥24 hours after the last application of lotion, after the skin was thoroughly washed.

Aliquots of liver were homogenized (5% wt/vol) with a Polytron homogenizer in 0.85% NaCl solution (wt/vol) containing 1% ascorbic acid (wt/vol). Aliquots of omental adipose tissue and skin tumors were homogenized (25% wt/vol) as described for liver. Aliquots of skin were ground in a mortar and pestle under liquid nitrogen and homogenized (25% wt/vol) as described for liver. The homogenates were flash frozen in liquid nitrogen and stored frozen at −80°C until analyzed for vitamin E according to the method of Driskel and associates (38).

UV Irradiation

The irradiation was conducted using four Westinghouse FS40 sunlamp bulbs. The spectral power distribution has maximal output of 310-nm wavelength (λ) with an output range of 265- to 440-nm λ, reported by the manufacturer to be comparable to sunlight in the Southern United States during summer. The output was monitored monthly with an L-1350 radiometer (International Light, Newbury, MA) and SED 240 detector (which measures λ = 280–320 nm). The mice were irradiated with the lights suspended 45 cm above

their backs in empty plastic cages with compartments separated by non-UV-absorbing Plexiglas dividers. The cages were rotated under lights so that each group of animals experienced all the different fields of the UV bulbs. The bulbs provided a homogeneous field of irradiation. The maintenance exposure time was 15 minutes per session, three times a week. The irradiation was initiated at 5 minutes per session (which was equal to ~75% of the average minimal erythema dose as measured on 5 mice) and increased by 2.5 minutes per session until the maintenance exposure time was reached. The maintenance dose was 0.24 J/cm^2/exposure. This irradiation was continued for 24 weeks to give a total exposure of ~15 J/cm^2. This dose of UV radiation has been shown to induce pigmentation and skin cancers in this mouse breed (39).

Evaluation of Skin Damage Induced by UV Irradiation

All animals were examined weekly to determine the degree of short-term sun damage (i.e., inflammation as indicated clinically by erythema, blistering, and pigmentation). Skin pigmentation was graded weekly until 20 weeks, when maximal pigmentation was observed. To assess the degree of skin pigmentation, a scoring system was constructed: 0 = no pigmentation and 4 = maximal darkening. The scoring was done by two independent observers, each of whom knew from previous experience the maximal pigmentation to be expected. Each observer scored each animal "blind" (i.e., without knowing the animal's supplementation group). Also, the numbers and sizes of tumors on each animal were noted weekly. Tumors ≥2 mm and ≤5 mm and those >5 mm were counted separately. Occasionally, small tumors enlarged to co-join, to appear as one large tumor, in those cases, the tumor count remained two. The diagnosis of tumor was confirmed by biopsy and histological examination of clinically representative tumors from each animal. One or more tumors from each tumor-bearing mouse was biopsied for histological examination.

Autopsy

Because previous experiments had demonstrated an increased incidence of leukemia and malignant lymphoma (~3.3%) in mice exposed to UV irradiation (40), as evidenced by enlarged mediastinal and peripheral lymph nodes and a varying degree of perivascular infiltrate of the liver and kidneys, complete autopsies were done on three mice from each UV-irradiated group after death or after they were killed.

Statistical Analysis

The mean number of tumors per animal was compared across treatment groups using a repeated-measures analysis of variance (41). The other main outcomes, time of onset of first tumor ≥2 mm and survival time, were compared across groups using Kaplan-Meier methods and the log-rank test (42). In addition, pairwise group comparisons were evaluated using the sign test to compare trend curves for outcome measures over time (43). All analyses were performed using SAS software (44,45).

Results

Mouse Growth Rate and Water and Food Intake

The body weight of the mice was not affected by exposure to UV radiation or by treatment with any of the forms of vitamin E. The average weight per mouse was 28.9 ± 2.0 g at Week 12, 31.8 ± 2.3 g at Week 24, and 33.5 ± 2.5 g at Week 34. Topical application of Eol or Esuc and oral intake of Eac, as well as the exposure to UV-B radiation, had no effect on the food or water intake (data not shown). In all cases, treated and untreated mice thrived; there was no clinical indication of any toxicity or adverse effects due to the topical or oral supplementation. All mice showed normal activity. No mice not exposed to UV radiation died.

Throughout the experiment, each mouse ate ~4.42 g of chow per day, giving an oral intake of 0.276 IU/day of Eac (0.251 IU equivalents of Eol/day). This is comparable to the supplementation recommended for humans equal to 400–800 IU/day. Also, the normal human diet of 2,000 kcal/day gives an intake of α-tocopherols of ~7 mg (46).

Each topical application of 0.1 ml of Eol (5%) or Esuc (5%) delivered 5 mg or 7.45 or 6.05 IU equivalents of Eol, respectively. This was distributed evenly over the backs, heads, and ears of each mouse. The lotion appeared to be absorbed within 10 minutes. The UV exposure was not begun until ≥30 minutes after the application.

Levels of Vitamin E

As shown in Table 2, topical application of Eol increased the concentration of Eol in the skin of the Skh:2 mice, with a preferential increase in the dorsal skin, the localized area actually treated. The liver showed a minimal increase in vita-

Table 2. Tissue Concentrations of RRR-α-Tocopherol in Skh:2 Mice After Application of Topical RRR-α-Tocopherol[a]

Treatment	Dorsal Skin	Ventral Skin	Liver	Adipose Tissue	Tumor
Vehicle, μg/g tissue	13.6 ± 0.8	17.5 ± 1.6	118.9 ± 15.1	66.8 ± 10.1	43.6 ± 23.5
RRR-α-tocopherol, μg/g tissue	144.6 ± 13.4	100.1 ± 11.0	158.1 ± 19.6	193.9 ± 47.0	444.0 ± 131.6
Magnitude increase	10.6	5.7	1.3	2.9	10.2

a: Values (means ± SE) were determined on 3 specimens from each of 6 Skh:2 mice. Measurements were done after topical application of vehicle or 5% RRR-α-tocopherol lotion and ultraviolet (UV)-B exposure for 10 wk. Animals had no topical application for ≥24 h before measurement; they were washed thoroughly before measurement to remove any excess vitamin E from the skin. Tumor levels were determined on 3 tumors excised from each animal when the mice were sacrificed after 44 wk.

Table 3. UV-Induced Blistering of Skh:2 Mice After Exposure to UV Irradiation

Treatment	Mice with Blistering, n[a]	
RRR-α-tocopherol lotion (5%)	4	(27)
RRR-α-tocopheryl succinate lotion (5%)	10	(67)
Oral RRR-α-tocopheryl acetate[b]	11	(73)
Vehicle	15	(100)

a: Number of mice in each treatment group was 15. Values in parentheses are percentages.
b: 0.251 IU equivalents of α-tocopherol/day/mouse.

min E after topical application, while omental adipose tissue demonstrated a substantial increase. Interestingly, the level of Eol was higher in the tumors than in any of the other tissues analyzed.

Skin Damage Induced by UV Irradiation

Despite the fact that the initial exposure to UV radiation was equal to only ~75% of the minimal erythema dose for these animals and the increase in UV exposure was gradual, all the mice not given vitamin E supplementation developed at least one blister within the second or third week of irradiation. As shown in Table 3, all forms of vitamin E were protective against acute blistering after UV exposure. Of the forms of vitamin E studied, Eol was the most protective: 73% of the mice had no blistering. Esuc was less protective, with only 33% of the mice totally protected from blistering. Oral Eac protected only 27% of the animals.

As shown in Figure 1, topical Eol and oral Eac as well as topical Esuc, protected against pigmentation induced by UV irradiation. Topical Eol and oral Eac were about equally protective; both of these forms were more protective than topical Esuc. After 20 weeks of UV irradiation, the animals did not become tanner.

Skin tumors were induced in the animals exposed to UV radiation; none of the control mice (not exposed to UV radiation) developed any tumors. The Skh:2 mice characteristically developed multiple tumors. In such tumors, many multinucleated, anaplastic, spindle-shaped cells were noted. Some animals were riddled with tumors, and others had only one or two large tumors. Most of the tumors were spindle-cell squamous cell carcinomas confirmed by histological sections. These were usually marked by invasion of the dermis. Some tumors were histologically similar to keratoacanthomas, with ulceration and marked hyperkeratosis and acanthosis of the epithelium and with invasive endophytic papillary projections. Whorls of cornified cells and atypical keratinocytes were noted with dense dermal inflammatory infiltrates. All tumors biopsied were squamous cell carcinomas varying from well differentiated to poorly differentiated. No benign papillomas were noted.

Figures 2 and 3 show that long after the exposure to UV radiation was terminated, the mice continued to develop tumors. By the termination of the experiment at 44 weeks, almost all mice had at least one tumor ≥2 mm. Figures 2 and 3 clearly show that oral and both forms of topical vitamin E protect against skin cancers. Repeated-measures analysis of variance (41) for the mean number of tumors ≥2 mm per animal indicated that the nonsupplemented group had significantly more tumors than any of the other three vitamin E-treated groups ($p = 0.0005$). Multiple comparison procedures failed to detect significant pairwise differences among the three supplemented groups.

Although the results of the repeated-measures analysis suggest that the three supplemented groups are equally protective, further analysis using the sign test (43) refined this conclusion. Pairwise group comparisons were made by ex-

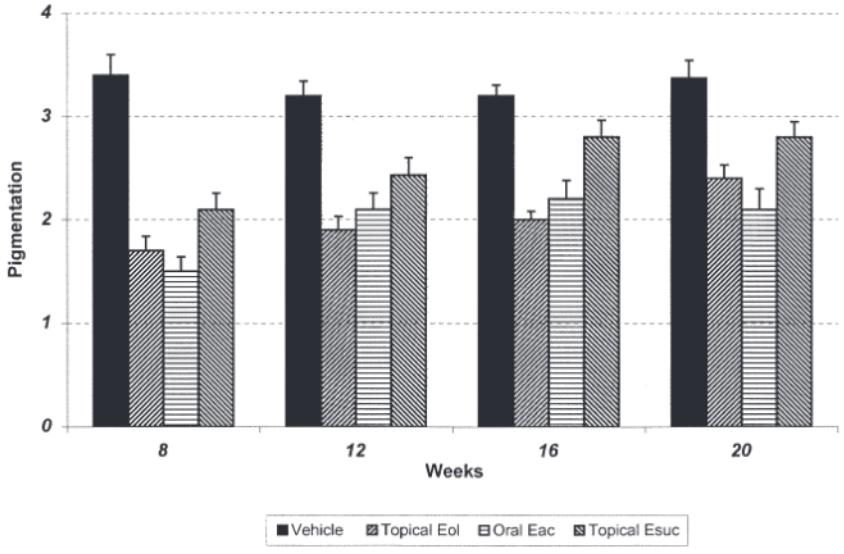

Figure 1. Ultraviolet (UV)-induced pigmentation of Skh:2 mice after exposure to UV irradiation. Values (means ± SE) are averages of all 15 mice in each treatment group. 0, No hyperpigmentation; 4, maximal hyperpigmentation. Eol, RRR-α-tocopherol; Esuc, RRR-α-tocopheryl succinate; Eac, RRR-α-tocopheryl acetate.

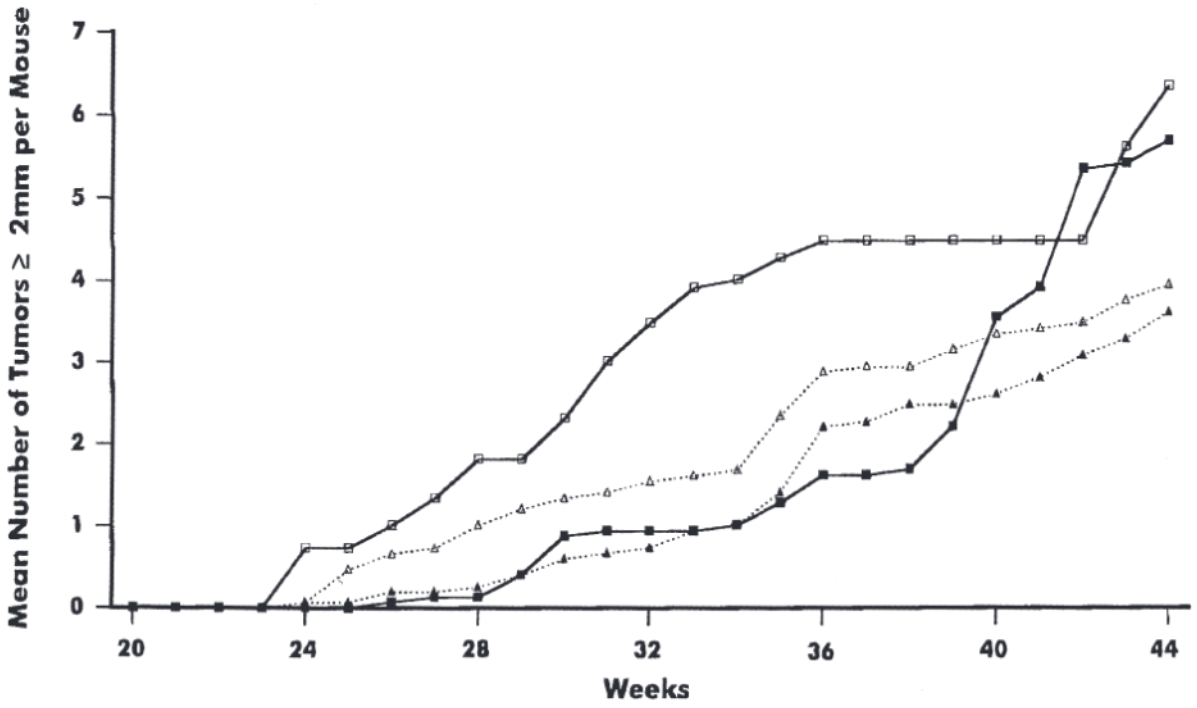

Figure 2. Mean number of tumors ≥2 mm in UV-irradiated Skh:2 mice. Beginning 2 wk before UV exposure, 15 mice in each treatment group were treated thrice weekly throughout duration of experiment with vehicle lotion (*open squares* and *filled squares*), topical Eol (*filled triangles*), or topical Esuc (*open triangles*) ≥30 min before UV exposure. In addition, 1 group (*filled squares*) was fed a diet supplemented with Eac. Animals were exposed to UV radiation thrice weekly for 24 wk, and topical and oral treatments were continued. Number of tumors ≥2 mm on each mouse was counted, and mean number of tumors per mouse was calculated based on total number of 15 mice in each treatment group.

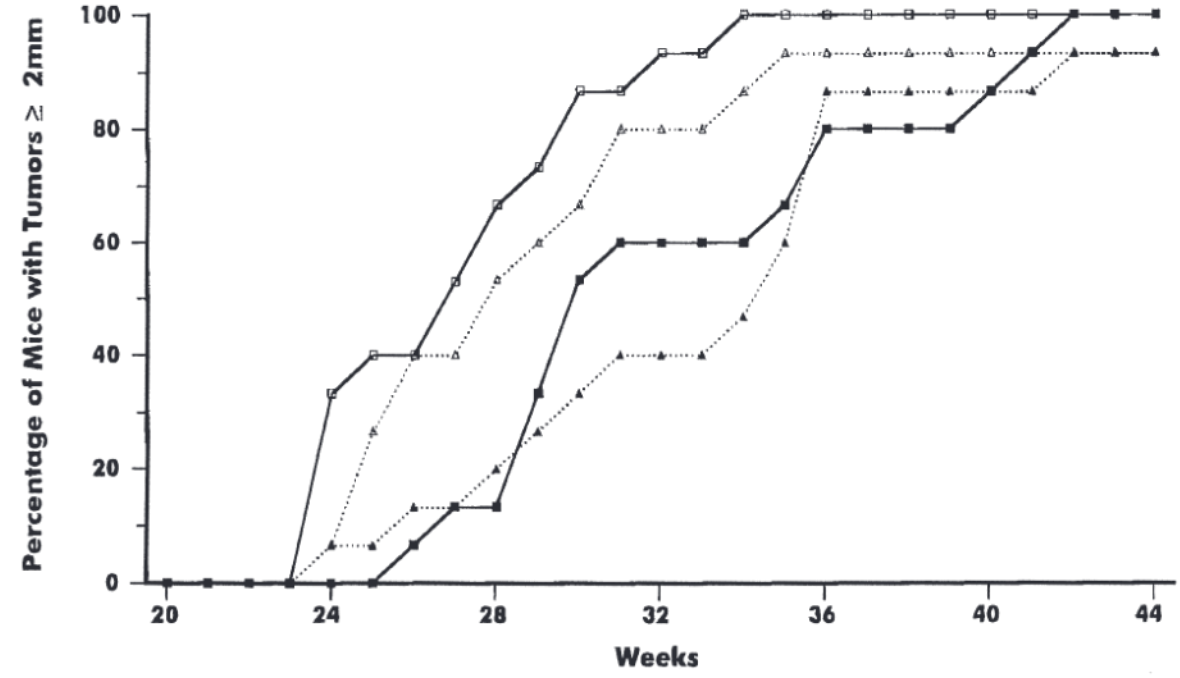

Figure 3. Percentage of total of 15 Skh:2 mice in each treatment group with a tumor ≥2 mm. Treatments were as summarized in Figure 2 legend.

amining differences in the mean number of tumors ≥2 mm per animal over four time periods: Weeks 24–44 inclusive; the earlier period alone, Weeks 24–35; and the later periods, Weeks 30–44 and Weeks 35–44. (These time periods were chosen because the first tumors were noted in Week 24, the last week of survival of all UV-exposed mice was Week 30, and the first major decrease in survival was noted in non-supplemented mice and Esuc-treated mice in Week 35.) Treatment with topical Esuc as well as with topical Eol and oral Eac resulted in lower mean number of tumors per animal than treatment with only vehicle lotion over all four time periods ($p < 0.001$). In addition, the topical Eol and oral Eac treatment groups had lower means than the topical Esuc treatment group in each of the four periods ($p < 0.001$). This protection by topical Eol and oral Eac was particularly significant in the earliest time period of Weeks 24–35 (Figure 2). There was no significant difference between the topical Eol and oral Eac groups in any of the four time periods ($p > 0.08$).

Evaluation of comparisons between pairs of groups (43) showed fewer mice with tumors ≥2 mm in all three vitamin E treatment groups than in the nonsupplemented group during each of the four time periods ($p < 0.001$; Figure 3). The number of animals with tumor(s) was significantly higher in the group treated with Esuc than in each of the topical Eol and oral Eac groups over Weeks 24–44, 24–35, and 30–44 ($p < 0.001$), but not over Weeks 35–44 ($p > 0.12$). This is probably because many of the topical Esuc-treated animals died during this last time period (Figure 4) so they could not develop more tumors, unlike those in the live vitamin E-supplemented mice. No differences were observed between the oral and topical vitamin E treatment groups for any of the time periods.

As seen in Figure 2, topical Eol and oral Eac significantly retarded the onset of skin cancers compared with nonsupplementation ($p = 0.0003$) or Esuc treatment ($p = 0.0016$). In analyzing the time of tumor onset, the vehicle group did not differ significantly from the topical Esuc group ($p = 0.1810$), and the topical and oral vitamin E groups did not differ significantly ($p = 0.5420$).

Figure 4 shows that UV irradiation resulted in decreased life span of Skh:2 mice. A comparison of the survival times of the four treatment groups was done using Kaplan-Meier survival curves and the log-rank test (42). There was no protection against the decreased survival time observed in non-supplemented mice exposed to UV radiation with topical Esuc ($p = 0.6796$). However, topical Eol and oral Eac protected against the increased UV-induced mortality ($p = 0.003$ and 0.0019, respectively, compared with nonsupplementation and $p = 0.0087$ and 0.0047, respectively, compared with topical Esuc treatment). This protection by topical Eol and oral Eac was confirmed in pairwise comparison with nonsupplemented and topical Esuc-treated animals for Weeks 30–45 and 35–45 ($p < 0.001$ in both cases). The topical Eol- and oral Eac-treated groups did not differ significantly ($p = 0.7191$) in survival time when compared using Kaplan-Meier survival curves, the log-rank test, and pairwise comparison for each of the four time periods.

The relative increase in the number of tumors after 36 weeks in the topical Eol- and oral Eac-supplemented animals in comparison with the nonsupplemented and the topical Esuc-treated animals is due to the fact that, in the latter two groups, many mice had died. No extrapolative or statistical analysis was done to take into account the effect of this decreased survival on the number of tumors. Since, clearly,

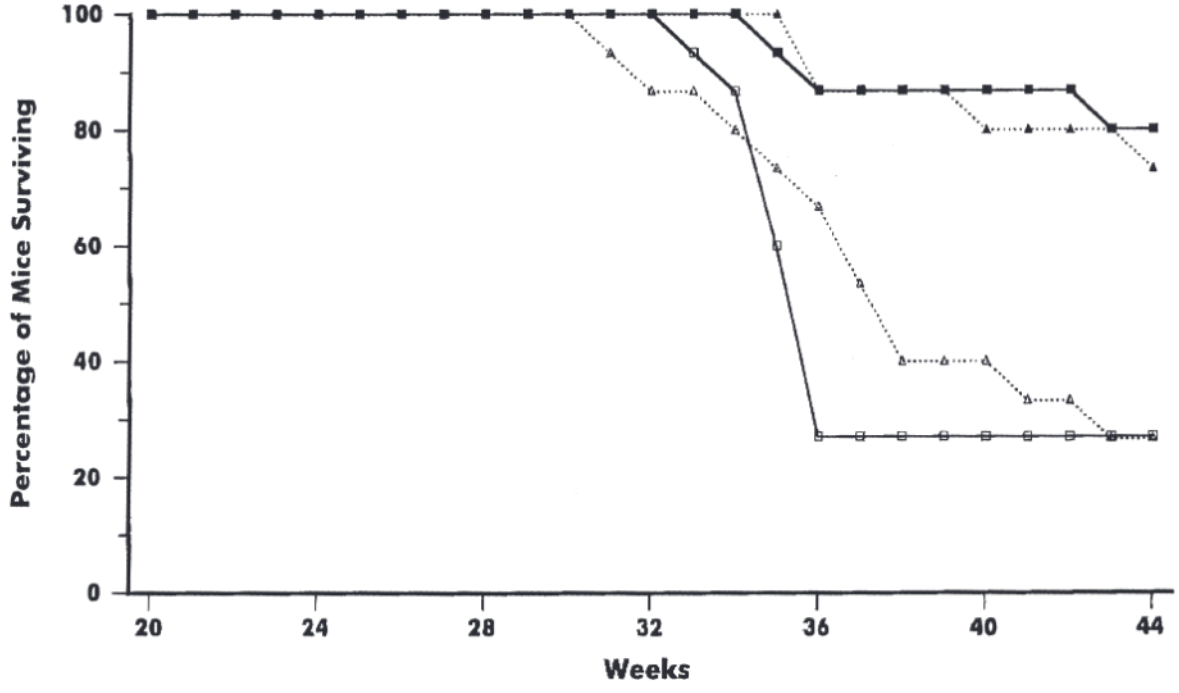

Figure 4. Percentage of total of 15 Skh:2 mice surviving in each treatment group. Treatments were as summarized in Figure 2 legend.

deceased animals do not acquire more tumors, the increase in the number of tumors per animal after 36–38 weeks is relatively less for the nonsupplemented and the topical Esuc-treated mice than for the oral Eol- and topical Eac-treated mice. The nonirradiated control group had no tumors and no deaths during the experimental period.

Autopsy

No case of leukemia or malignant lymphoma was detected in the limited number of cases autopsied.

Discussion

Mice have been used extensively in studies of photocarcinogenesis, because tumors can be so readily induced (47). Obviously, the hairless mouse has been used more, because shaving the hair is not required. The vulnerability to skin cancer may be a result of a limited capacity to repair UV-induced pyrimidine dimers (48,49) and the thin stratum corneum (50). Skh:2 hairless mice are ideal, inasmuch as they are not immunologically compromised and they pigment with UV exposure, allowing this parameter of acute UV damage to be evaluated. The authors have had prior experience with the induction of UV-induced skin cancer in this breed of mice (39). Thus this model is ideal for evaluating the efficacy of vitamin E in protecting against UV-induced damage to the skin.

These results show that each of the three forms of vitamin E was effective in reducing the acute damage induced by UV irradiation: inflammation (sunburn), blistering, and pigmentation (tanning). Although UV irradiation was begun at only 75% of the minimal erythema dose for these mice and incremented sequentially before the maintenance dose was reached, all the mice with no vitamin E supplementation developed at least one observable blister during the initial exposure. All forms of vitamin E studied protected against this initial blistering. The topical Eol was by far the most effective: Only 27% of the mice so treated developed at least one observable blister during the early weeks of UV irradiation. About two-thirds of the mice treated with topical Esuc and three-fourths of those supplemented orally with Eac developed at least one observable blister with the initial exposure to the UV irradiation. A direct anti-inflammatory effect of vitamin E has been demonstrated previously: 1) Topical application of all-*rac*-α-tocopheryl decreased the severity and duration of croton oil dermatitis in rabbits (23), and 2) intramuscular injection of α-tocopherol had a significant anti-inflammatory effect on dextran-mediated edema in rabbits (24). Since the UV-induced inflammatory reactions may be mediated *via* membrane damage, the decreased inflammation with vitamin E supplementation might also be due to vitamin E functioning as an antioxidant to prevent damage to skin cell membranes.

All forms of vitamin E studied here were effective in reducing the pigmentation of the UV-irradiated Skh:2 mice, especially at the onset of exposure. Topical Eol and oral Eac were equally protective; both of these forms were more protective than topical Esuc. This protection by vitamin E was expected, since it has been demonstrated that there is a direct relationship between free radical concentration and epidermal pigmentation (51). UV-induced tanning is caused by some combination of several mechanisms: division of melanocytes, activation of pigment formation in amelanogenic melanocytes, migration of dermal melanocytes into the epidermis, and increased transfer of melanosomes to keratinocytes (52). With prolonged UV exposure, all these actions occur. After 20 weeks, UV-irradiated mice did not tan further.

Clearly, these experiments demonstrate that, in Skh:2 mice, topical Eol and topical Esuc as well as oral Eac are effective in protecting against skin cancer induced by UV irradiation. Statistical analysis showed topical Eol and oral Eac to be about equally effective; these forms were somewhat more effective than topical Esuc not only in decreasing the incidence of skin tumors but also in prolonging the onset. This protection was statistically significant even at the termination of the experiment, in contrast to the results reported by Berton and others (19), who, in a similar experiment in which Skh:1 mice were irradiated with a 15% higher dose of UV light, observed no decrease in tumor multiplicity after 30 weeks (despite initial increased latency and decreased tumor incidence) after treatment with topical Eac. This difference is possibly because Eac is not metabolized to Eol as effectively when administered topically as when given orally, thus emphasizing the importance of the type and form of vitamin E in determining its effectiveness as a chemopreventive agent in UV-induced carcinogenesis (19).

The protection against UV-induced epidermal carcinogenesis by topical and oral vitamin E was expected, because vitamin E is known to act as a free radical quencher and an antioxidant that directly protects all membranes. Indeed, previous research has demonstrated that treatment with topical all-*rac*-α-tocopherol (35,36), as well as oral all-*rac*-α-tocopherol (34) and oral all-*rac*-α-tocopheryl acetate (37), does inhibit UV-induced skin cancer. However, the protection against UV-induced skin cancers seen in the experiments reported here by Esuc is in contrast to the observations of Gensler and associates (12), who found that topical all-*rac*-α-tocopheryl acetate and all-*rac*-α-tocopheryl succinate applied at various concentrations three times per week for three weeks before UV irradiation and throughout the 24-week experiment actually enhanced photocarcinogenesis. Skin cancer developed in 70% of the control BALB/cAnNTacfBR mice and in 90%, 73%, and 90% of mice treated with 12.5, 25, and 50 mg of all-*rac*-α-tocopheryl acetate in 2.0 ml of acetone, respectively; skin cancers developed in 59% of the control mice and in 82%, 100%, and 82% of mice receiving 2.5, 12.5, and 25 mg, respectively, of all-*rac*-α-tocopheryl succinate. These authors found that the esterified forms of vitamin E accumulated in the skin, but α-tocopherol levels remained low. This study suggests that the limited capacity of the skin to cleave esterified forms of vita-

min E to α-tocopherol may explain the inability of topical Esuc or Eac to prevent UV-induced skin cancer (12). Alternatively, this result might be a consequence of a metabolic peculiarity in this particular inbred strain of mice.

A difficulty in these experiments was to ensure that the mice supplemented topically with vitamin E did not ingest the vitamin from themselves or other animals. Although each mouse was isolated for several hours after application of the lotion and wiped before being placed into the maintenance cage, the possibility of oral intake from the topical supplementation could not be precluded. Topical application to the back did result in an increased concentration not only in dorsal skin, but also (to a lesser extent) in ventral skin, indicating that some topical vitamin E was likely transferred by rubbing. Topical supplementation did not increase the vitamin E concentration in the liver. However, the marked increased concentration in the adipose tissue demonstrated that the vitamin E was indeed absorbed topically. This increased concentration was to be expected for this fat-soluble vitamin. The measurement of a high concentration of vitamin E in the skin tumors was surprising. This interesting result is being pursued to ascertain whether indeed skin tumors sequester vitamin E and/or other antioxidants and whether there are differences in the effect on tumor growth with supplementation before vs. after tumor onset.

In the experiment reported here, supplementation topically with natural Eol or Esuc and orally with natural Eac showed no toxicity or adverse reactions. The food and water intake was not changed by application of the various forms of vitamin E or by exposure to UV radiation; the average weights of each mouse increased with aging but were comparable in all treatment groups at each time. All animals appeared healthy and were normally active (even when they acquired skin tumors). In fact, topical Eol and oral Eac (but not topical Esuc) actually protected against the increased mortality noted here and in previous experiments (39) in Skh:2 mice exposed to UV-B radiation.

With one exception (37), there have not been reports of adverse reactions or toxicity in previous animal studies using various forms of vitamin E with UV exposure (34–37). However, Gerrish and Gensler (37) noted that supplementation with Eac at 100 or 200 IU/kg diet, although reducing the incidence of UV-induced skin cancer from 68% in nonsupplemented C3H/HeN mice to 46% and 19%, respectively, resulted in premature death of 25% and 40%, respectively, of UV-B-irradiated mice. There was no premature death of nonirradiated, supplemented mice. Also, the relative spleen weight was decreased in the UV-irradiated mice fed this high-vitamin E-supplemented diet (37).

As discussed above, there are several possible mechanisms by which vitamin E may protect the skin from photocarcinogenesis. As a free radical quencher and an antioxidant, vitamin E may prevent lipid peroxidation and subsequent DNA damage (25–29). At a molecular level, Perchellet and co-workers (53) demonstrated that topical all-*rac*-α-tocopherol can inhibit ornithine decarboxylase induction by 12-*O*-tetradecanoylphorbol-13-acetate, suggesting that vitamin E may also prevent cutaneous ornithine decarboxylase induction by UV irradiation (54). Also, local administration of vitamin E to hairless mice before and after exposure to UV radiation resulted in a 60% reduction in the skin's production of malonyl dialdehyde (55) (the end product of peroxidation of unsaturated fatty acids by oxygenated free radicals). Vitamin E may also decrease photocarcinogenesis by preventing the development of UV-induced immunosuppression. Gensler and Magdaleno (36) found that the splenocytes from UV-irradiated mice treated with topical all-*rac*-α-tocopherol allowed recipient naïve mice to reject antigenic UV-induced tumors, whereas splenocytes from UV-irradiated mice without vitamin E treatments did not allow recipients to reject similar tumor challenges.

In the dermatological literature, extensive quantitative data indicate that UV-induced skin cancer formation is a cumulative process that begins with initial exposure. In fact, it has been estimated that one blistering sunburn in a child doubles the potential to develop basal cell or squamous cell skin cancers as an adult (56). If mouse tumorigenesis, with its short latent period, can be inhibited effectively with topical or oral vitamin E or topical vitamin E succinate, as the experiments presented here demonstrate, a similar effect might be expected for humans with their long latent period. Indeed, clinical experience suggests that regular use of a sunscreen with a sun protection factor of 15 during the first 18 years of life may reduce the lifetime incidence of nonmelanoma skin cancer by 78% (57). Therefore, the protection that might be provided by topical or oral vitamin E is a compelling reason for everyone to consider such supplementation.

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