Circulating Endogenous Retinoic Acid Concentrations among Participants Enrolled in a Randomized Placebo-Controlled Clinical Trial of Retinyl Palmitate

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Abstract

Retinoids have been studied extensively for their chemopreventive properties. The biological activity of retinoids is acquired through their conversion to retinoic acid (RA). Characterization of endogenous circulating RA concentrations after supplementation with vitamin A over longer time periods has not been done previously. Our investigation was conducted to determine whether vitamin A (retinyl palmitate) supplementation significantly increases circulating RA concentrations of all-*trans*-, 9-*cis*-, and 13-*cis*-RA. Using plasma samples from 41 participants enrolled in a randomized clinical trial of placebo, 25,000, 50,000, or 75,000 IU supplemental retinyl palmitate daily, high-performance liquid chromatography analyses were conducted for concentrations of three RA isomers. Seven plasma samples were analyzed for each participant over a 16-month period. Based on an intention-to-treat analysis, results obtained using linear mixed models showed that supplementation with retinyl palmitate statistically significantly increased concentrations of all three RA isomers from baseline levels. This study suggests that supplementation with retinyl palmitate is an effective means to increase circulating all-*trans*, 9-*cis*-, and 13-*cis*-RA concentrations among humans. (Cancer Epidemiol Biomarkers Prev 2004; 13(11):1687–92)

Introduction

Over the last several decades, vitamin A has been used in the treatment of various skin disorders (1), although only relatively recently has the role of vitamin A in cancer prevention been addressed. During the past 20 years, retinoids including retinol, retinyl palmitate, all-*trans*, 13-*cis*-, and 9-*cis*-retinoic acid (RA) have been evaluated in several chemoprevention trials involving a wide variety of intraepithelial neoplasias (2-8). The retinoids have been evaluated as potential chemoprevention agents due to their role in cellular differentiation, an important factor for the prevention of malignancy. As reviewed previously (7-9), retinoids are chemopreventive in several cancer progression pathways, including promyelocytic leukemia, squamous cell cancers of the skin, and cancers of the head and neck, and may be involved in cancers of the breast, lung, and cervix.

Although numerous studies indicate that treatment with RA is effective, this chemoprevention strategy is severely limited for long-term use by reported adverse events and its teratogenic potential (4, 10-12). An alternative retinoid chemoprevention approach is to develop a method of increasing endogenous concentrations of RA, as the biological activity of retinoids is obtained through their conversion to RA (13). The low endogenous concentrations of RA isomers (14) have impeded measurement of circulating levels in population studies. Furthermore, there are only a few high-performance liquid chromatography (HPLC) detection methods that are able to analyze small quantities of either serum or plasma collected during population-based studies for the detection of endogenous concentrations of several isomers concurrently (15).

A previous study by Tang and Russell (16), conducted in seven participants, showed an increase in circulating concentrations of all-*trans*- and 13-*cis*-RA over a 24-hour period after a single p.o. administrated dose of all-*trans*-retinyl palmitate. However, characterization of endogenous circulating RA concentrations after supplementation with p.o. vitamin A over longer time periods has not been reported. Our investigation was conducted to determine whether vitamin A (retinyl palmitate) supplementation significantly increases circulating RA concentrations of all-*trans*-, 9-*cis*-, and 13-*cis*-RA.

Materials and Methods

Study Design. The primary study was a randomized placebo-controlled phase IIB clinical trial of retinyl

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palmitate as a chemopreventive agent in participants at high risk for nonmelanoma skin cancer (NMSC; ref. 17). Participants (n = 129) were randomized to one of four treatment groups: placebo, 25,000, 50,000, or 75,000 IU of daily retinyl palmitate for up to 2 years. Randomization of participants was stratified based on age (50-64 versus ≥65 years), prior number of resected NMSCs (0, 1, or 2), and specific type of any previous skin cancer (basal versus squamous cell carcinoma) and blocked on gender (because NMSC incidence is more prevalent among males). The study protocol was approved by the University of Arizona's Internal Review Board. Prior to study entry, all participants signed an informed consent form. Details of the clinical results are being published separately (18).

Participant Selection. Selection criteria were chosen to enroll a population at high risk for NMSC and who exhibited an absence of medical or immunologic factors that may elevate risk of adverse effects of long-term retinoid supplementation. Study eligibility included (*a*) ages ≥50 years and from Pima and adjoining southern Arizona counties; (*b*) high risk for NMSC, defined as moderately severe clinically diagnosed sun-damaged skin and/or actinic keratoses on each dorsal forearm and minimal NMSC history (no more than two resected basal cell or squamous cell carcinoma); (*c*) adequate renal function (as indicated by a normal nuclear liver scan and levels of serum creatinine, bilirubin, glutamic oxaloacetic transaminase (aspartate aminotransferase), glutamic pyruvic transaminase (alanine aminotransferase), triglycerides, WBC, and platelet counts within normal limits); (*d*) no acute or chronic debilitating or immunologic diseases; (*e*) no history of invasive cancer or melanoma; (*f*) not taking >5,000 IU of supplemental vitamin A daily; and (*g*) postmenopausal or have been sterilized surgically (women only). Additionally, potentially eligible participants agreed to discontinue all topical or systemic actinic keratoses therapy for at least 3 months prior to study initiation. Data were collected on participants for 1 or 2 years. After all of the randomized participants reached 1 year, the study was closed early because there was no evidence of significant toxicity at 12 months on-study treatment. Data from the 41 participants, recruited the earliest into the study and therefore who had completed year 2 end points, were used for the present analyses. RA analyses, however, were only conducted for time points up to 16 months (~1 year on-study treatment).

Study Protocol

Baseline. After completion of informed consent procedures and initial dermatologic and medical history assessments indicating potential eligibility, liver nuclear scans were done and fasting blood was drawn for liver function tests, lipid determinations, and circulating nutrients. Placebo supplements were distributed and compliance was assessed monthly during the 3-month run-in period. At the end of the placebo run-in period, the participant received a more extensive dermatologic examination including photographs of forearm sun damage, collection of skin biopsies, and collection of fasting blood.

Follow-up Clinical Visits. The 41 participants visited the clinic monthly for fasting blood draws, treatment compliance assessments, and study medication for the first 7 months after randomization. After the first 7 months, these assessments were completed every 3 months for the duration of the treatment period. Participants received monetary compensation to defray travel costs for their participation in this study ($200) at the end of each treatment year. Compensation was prorated for any participants who were followed for shorter time periods.

Treatment Intervention. Study pills were supplied by Knoll Pharmaceuticals (a subsidiary of BASF, Whippany, NJ) as gelatin capsules of 25,000 IU retinyl palmitate doses and matched placebo of vegetable (soybean) oil for p.o. administration. Study drug and placebo were packaged in blister packs each containing a 31-day supply. To meet the specific assigned dosage, four capsules were taken daily by participants. Blister packs consisted of four placebo capsules for the placebo treatment, one 25,000 IU retinyl palmitate and three placebo capsules for the 25,000 IU treatment, two 25,000 IU retinyl palmitate and two placebo capsules for the 50,000 IU treatment, and three 25,000 IU retinyl palmitate and one placebo capsule for the 75,000 IU treatment.

Plasma Sample Collection. Fasting blood samples for retinoid determinations were collected using a 10 mL green-top tube containing sodium heparin, wrapped with aluminum foil to limit the UV light exposure, and stored on ice for a maximum of 30 minutes. After centrifugation, aliquots of plasma (1 mL) were stored at −20°C for up to 1 month and then transferred to liquid nitrogen until analyses.

β-Carotene Analyses. Baseline plasma β-carotene levels were measured on samples collected at the end of the placebo run-in period. Analysis was conducted at the Arizona Cancer Center, University of Arizona (Tucson, AZ).

Analytic quantitation was done using the Standard Reference Material 968b (fat-soluble vitamins and cholesterol in human serum) supplied by the National Institute of Standards and Technology (Gaithersburg, MD). This standard was used for assigning values to in-house control materials. Extinction coefficients were used to spectrophotometrically validate the final solution concentrations. The β-carotene value (at 452 nm, in hexane) was 2,592 dL/g/cm. The following solvents were used in the HPLC analysis. Solvent A was acetonitrile-tetrahydrofuran (85:15, v/v) with 250 ppm butylated hydroxytoluene and 0.05% triethylamine, and solvent B was 50 mmol/L ammonium acetate in methanol with 0.05% triethylamine.

To precipitate proteins, ethanol (250 μL, containing 0.1% butylated hydroxytoluene antioxidant) was added to an aliquot of plasma (250 μL). After vortexing, analytes were extracted into hexane, evaporated under nitrogen, and redissolved in mobile phase (solvent A). Extractant (50 μL) was injected directly into the HPLC system. The separation was carried out with a 5 μm Ultrasphere ODS column (4.6 × 250 mm, Beckman Instruments, San Ramon, CA) and detected at the wavelengths of 300 and 452 nm by use of the method of Xu et al. (19). The solvent system was isocratic and consisted of 95% solvent A and 5% solvent B and delivered at a flow rate of 2.5 mL/min. The retention time for β-carotene was 10.13 minutes. The total run time for a single analysis of sample was 13 minutes.

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Table 1. Baseline characteristics by treatment group

	Dose level of vitamin A				P
	Placebo	25,000 IU	50,000 IU	75,000 IU	
n	9	10	13	9	
Demographics					
Gender, % male	55.6	60.0	69.2	66.7	0.918*
Age (y), mean (SD)†	64.0 (6.6)	63.6 (5.6)	63.0 (7.9)	66.2 (9.7)	0.967‡
Body mass index (kg/m^2), mean (SD)†	28.2 (5.6)	26.3 (3.9)	27.3 (4.0)	28.5 (4.4)	0.693‡
Smoking, % ever	33.3	30.0	69.2	55.6	0.209*
Circulating blood values†					
Cholesterol (mg/dL), mean (SD)	206.1 (45.9)	209.0 (59.4)	196.8 (23.8)	212.8 (52.8)	0.932‡
Triglycerides (mg/dL), mean (SD)	109.3 (71.0)	134.0 (81.6)	146.5 (51.6)	121.6 (35.9)	0.346‡
Retinol (μg/mL), mean (SD)	0.609 (0.168)	0.713 (0.139)	0.680 (0.101)	0.611 (0.124)	0.164‡
β-Carotene (μg/mL), mean (SD)	0.288 (0.285)	0.196 (0.091)	0.134 (0.063)	0.230 (0.190)	0.342‡
9-*Cis*-RA (ng/mL), mean (SD)	2.7 (0.6)	2.7 (0.7)	2.5 (0.6)	2.7 (0.6)	0.756‡
13-*Cis*-RA (ng/mL), mean (SD)	2.1 (0.4)	2.1 (0.6)	1.8 (0.5)	1.9 (0.4)	0.402‡
All-*trans*-RA (ng/mL), mean (SD)	1.3 (0.4)	1.3 (0.5)	1.1 (0.3)	1.2 (0.3)	0.575‡

*Fisher's exact text for categorical data.
†Means are presented as untransformed values, but *P*s were calculated from log-transformed values.
‡One-way ANOVA for continuous data.

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RA Analyses. RA analyses were conducted on one baseline sample and six serial samples collected at months 3 (randomization), 4, 5, 6, 10, 13, and 16. Plasma samples were batch analyzed so that all samples from the various time points of each individual were sequentially ordered and processed in the same batch.

The 9-*cis*- and all-*trans*-RA standards were purchased from Sigma Chemical Co. (St. Louis, MO) and 13-*cis*-RA was obtained from ICN Biomedicals (Aurora, OH). The following reagents were used for sample preparation and analysis: butylated hydroxytoluene, hexane, ethanol, methanol, acetonitrile, isopropyl alcohol, HCl, NaOH, and acetic acid. All solvents were HPLC grade or equivalent and were used without further treatment.

After thawing, aliquots of plasma (500 μL) were deproteinated with acetonitrile/methanol (9:1, 500 μL) and made alkaline with 2 N NaOH (100 μL). The samples were extracted by vortex mixing for 45 seconds with hexane (1.5 mL) containing 0.025% butylated hydroxytoluene as an antioxidant. The organic phase was discarded. Samples were acidified with 2 N HCl (200 μL) and extracted three times with hexane (1.5 mL) with butylated hydroxytoluene. The combined supernatant was evaporated under nitrogen. The residue was dissolved by vortex mixing with mobile phase (120 μL). The injection volume was 90 μL.

HPLC analysis was done using a ThermoSeparation Products liquid chromatograph with the following components: P4000 solvent delivery system, vacuum degasser, AS3000 autosampler, Spectra FOCUS scanning UV-visible detector, and PC1000 computer-controlled data system (Fremont, CA). Samples were refrigerated at 10°C and the column was maintained at 30°C. RA isomers were monitored at 350 nm. The analytic column was a Spherisorb ODS2 (3 μm, 4.0 × 250 mm) with Javelin guard column containing Keystone ODS2 (3 μm, Keystone Scientific, Inc., Bellefonte, PA). The mobile phase consisted of 75% acetonitrile, 5% methanol, and 20% of 1% acetic acid at a flow rate of 1 mL/min. The retention times for 13-*cis*-, 9-*cis*-, and all-*trans*-RA were 12.7, 16.4, and 19.5 minutes, respectively.

Linear calibration curves were prepared consisting of three concentrations of RA isomers that spanned the physiologic levels in serum. Quantification was done by external standard calibration using peak area ratios. In-house quality control samples were analyzed at the beginning and end of each sample queue. The relative SD of analytes in the quality control samples ranged from 10% to 15%. For each RA isomer, the detection limit of this assay is ~0.5 ng/mL.

Statistical Analysis. Analyses were done on plasma collected from 41 participants using seven time points for each participant over 16 months. Four plasma samples were unavailable, resulting in a total of 283 person-visits. Baseline demographic characteristics were compared by treatment group using Fisher's exact test or one-way ANOVA. These tests along with mean and SD were calculated using Stata version 7.0 (20).

Preliminary graphics suggested that concentrations of all three RAs increased with time as a function of the vitamin A dose. To assess the statistical analysis of the increases for each dose level relative to placebo, a mixed model approach was adopted to determine whether vitamin A (retinyl palmitate) supplementation significantly increased serum RA concentrations over time. This allowed the explicit modeling of within-subject correlations inherent in repeated measurements over time. Modeling was done using the PROC MIXED procedure in SAS version 8.1. (21).

Based on graphic analysis and the Akaike information criterion, a linear model with an unstructured correlation matrix was chosen to maximize model fit. The unstructured correlation matrix has been proven far superior to a spatial power function that was used in an attempt to model structured temporal autocorrelation. Treatment and time were modeled as fixed effects, whereas subject status was treated as a random effect. RA concentrations were log transformed due to right skewness of the original distributions. Furthermore, due to the skewness of the concentration distributions,

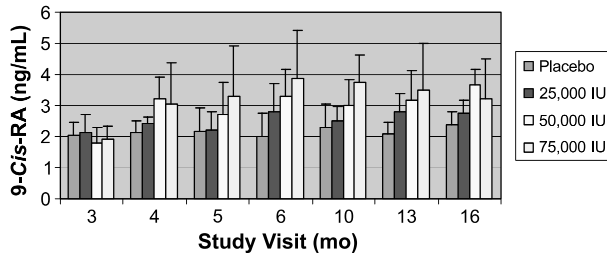

Figure 1. 9-*Cis*-RA concentrations by study visit and treatment group.

a nonparametric Wilcoxon sign rank test was used to determine if all-*trans*-RA was more responsive to supplementation than 9-*cis*-RA. All analyses used the intention-to-treat scheme.

Results

The distributions of baseline demographic characteristics, potential covariates, and circulating nutrient levels by treatment group are reported in Table 1. The demographic characteristics are consistent with a population at high risk for NMSC, an older adult group with slightly more men than women. No significant differences were observed for gender, age, body mass index, smoking status, or serum cholesterol, triglycerides, retinol, β-carotene, and RA levels at baseline among the four randomized treatment groups.

Figures 1, 2, and 3 graphically depict the mean (SD) plasma concentrations of each RA isomer by treatment group over each study visit. Overall, 9-*cis*-, 13-*cis*-, and all-*trans*-RA plasma concentration levels increased with increasing dosages of retinyl palmitate supplementation. Furthermore, levels of all three isomers of RA reached their highest concentration at 6 months of vitamin A dosing, after which little change was observed. At baseline, the plasma concentrations of RA were highest for 13-*cis* followed by 9-*cis* and lowest for all-*trans* isomers. Mean (SD) baseline endogenous concentrations among the 41 participants were 1.21 (0.39), 1.96 (0.48), and 2.66 (0.60) μg/mL for all-*trans*, 9-*cis*, and 13-*cis*-RA concentrations, respectively. After retinyl palmitate supplementation, the highest plasma concentrations were observed for the 13-*cis* isomer followed by all-*trans* and 9-*cis*-RA.

On average, concentrations of 9-*cis*-RA increased from baseline to end of study by 69% (median increase of 57%), whereas concentrations of all-*trans*-RA increased during the same time period by 186% (median increase of 114%). Because RA concentrations were skewed, the nonparametric Wilcoxon sign rank test was used to show the two proportional increases to be statistically significantly different from each other (P = 0.0001). The significance of this difference was confirmed using a paired t test to compare differences of the log-transformed concentrations (P < 0.0001). Additionally, 13-*cis*-RA increased from baseline to 16 months by an average of 140% (median increase of 89%) and was statistically different from all-*trans* (P = 0.0326) and 9-*cis* (P = 0.0001) using the Wilcoxon sign rank test.

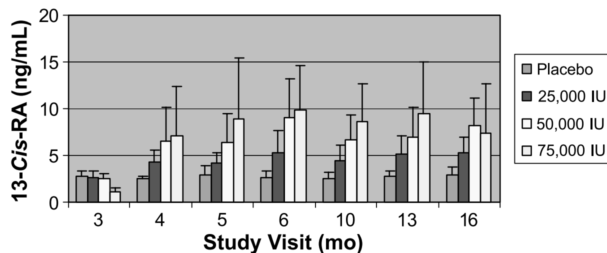

Figure 2. 13-*Cis*-RA concentrations by study visit and treatment group.

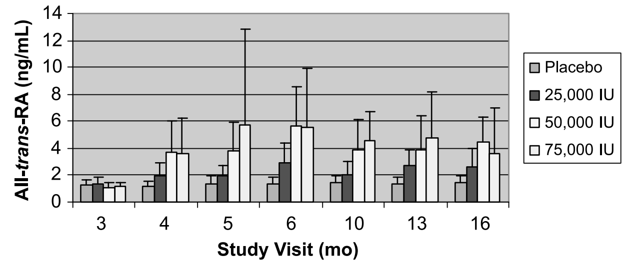

Figure 3. All-*trans*-RA concentrations by study visit and treatment group.

Results from the linear mixed models for all three RA isomers are presented in Table 2. For each dose level, the difference in least square mean (SE) for the log concentration of the RA between that dose level and the placebo group is given. For example, the 25,000 IU group had a least square mean log concentration that was 0.288 higher than was found in the placebo group. This difference was statistically significant at P < 0.01. Table 2 shows a dose response that increased monotonically for all three RAs. All increases in RA concentrations were statistically significant at P < 0.05 for the 25,000 IU group and at P < 0.001 for the 50,000 and 75,000 IU groups.

Discussion

In this cohort of participants at high risk for skin cancer defined as clinically assessed moderate to severe

Table 2. Difference in least square means (SE) of log-transformed concentrations of RA between treatment group and placebo adjusted for time and treatment × time interaction effects

RA	Dose level of vitamin A		
	25,000 IU	50,000 IU	75,000 IU
9-*Cis*-RA	0.288* (0.093)	0.436† (0.088)	0.464† (0.096)
13-*Cis*-RA	0.165‡ (0.088)	0.223† (0.062)	0.265† (0.068)
All-*trans*-RA	0.304‡ (0.141)	0.522† (0.133)	0.532† (0.145)

NOTE: P < 0.05, treatment × time interaction was statistically significant for 25,000 and 50,000 IU doses of 13-*cis*-RA only.
*P < 0.01.
†P < 0.001.
‡P < 0.05.

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sun-damaged skin and/or actinic keratoses, we showed a statistically significant dose-response increase for circulating all-*trans*, 9-*cis*-, and 13-*cis*-RA concentrations associated with retinyl palmitate supplementation.

These findings are consistent with a previous study conducted by Tang and Russell (16) that showed an increase in circulating concentrations of all-*trans*- and 13-*cis*-RA after a single p.o. administrated dose of all-*trans*-retinyl palmitate in humans. In the single p.o. dose study, a greater increase was associated with the pharmacologic dose (2,250 retinol equivalents/kg body weight) as compared with the physiologic dose (3,000 retinol equivalents/kg body weight). However, the study measured all-*trans*- and 13-*cis*-RA levels in only seven subjects over a 24-hour period and did not measure 9-*cis*-RA concentrations. Our study is the first, to our knowledge, to measure an increase in blood concentrations of RA over an extended period after supplementation with p.o. vitamin A administration. The data show that supplementation with all three doses of retinyl palmitate significantly increased plasma concentrations of all-*trans*, 13-*cis*, and 9-*cis*-RA levels from baseline levels. All three doses of vitamin A administered in this study are well above the current Recommended Dietary Allowance of 900 or 700 g retinol activity equivalent for men and women, respectively (22), and cannot be obtained through dietary intake alone.

Vitamin A supplementation has been reported to be safe at daily dosages of 25,000, 50,000, and 75,000 IU (18). For the 116 participants completing 1 full year of retinyl palmitate treatment, no significant differences in clinical or laboratory-related toxicities were reported across treatment groups (17, 18). Among the four treatment arms of the current analyses, no statistically significant differences were observed in triglyceride levels after ~1-year on-treatment (month 16; $P = 0.2697$). Whereas no reported toxicities were observed, caution should be taken when administering vitamins at pharmacologic doses (23).

Although these mean baseline plasma concentrations of all-*trans*, 9-*cis*-, and 13-*cis*-RA were higher than those values published previously (14), this is likely due to the difference between comparing plasma and serum values and/or improvements in the sensitivity of HPLC methods over the past 10 years. In our study, the baseline concentrations of the RA isomers were as follows: 13-*cis*-RA > 9-*cis*-RA > all-*trans*-RA; however, after supplementation with retinyl palmitate, the 13-*cis* isomer continued to have the highest circulating concentration with the all-*trans* isomer concentration found in higher concentrations than that of the 9-*cis* isomer. On further testing, it was determined that the all-*trans* isomer is more responsive to supplementation than the 9-*cis* isomer.

The chemoprevention action of RAs is generally thought to be obtained through their ability to alter gene transcription via the RA receptor (RAR) and retinoid X receptor (8, 24). As reviewed previously, each receptor includes three subtypes, and several isoforms are possible (8, 9, 24). Our data suggest that administration with vitamin A supplementation can increase circulating levels of RAs to concentration that will interact with their respective receptors. For example, the all-*trans*-RA concentrations observed after ~1-year on treatment with daily supplementation of 25,000, 50,000, or 75,000 IU of retinyl palmitate are sufficient to interact with the RARs (β and γ) based on evidence from *in vitro* studies (25, 26). Although the all-*trans*-RA concentrations reached the *in vitro* 50% effective concentrations for the RARs in some studies, care should be taken when comparing *in vitro* and *in vivo* concentrations, as the reported levels varied possibly due to the tissue and cell type specificity of the RAR subtypes.

Interestingly, not only did supplementation with retinyl palmitate increase plasma concentrations of all-*trans*, 9-*cis*, and 13-*cis* isomers, but supplementation also has been shown to significantly up-regulate expression of both RAR and retinoid X receptors in sun-damaged skin (17), suggesting that the metabolism of vitamin A can be altered. Previous studies have shown similar findings that RARβ expression can be up-regulated with 13-*cis*-RA treatment (27-30).

The strengths and limitations of this study should be noted when interpreting these data. This was a randomized clinical trial of three dose levels of retinyl palmitate supplementation and a placebo. This study collected and analyzed repeated plasma samples from 41 participants over a 1-year time period. Our findings of higher RA levels with increasing dosages of retinyl palmitate are consistent with what was expected based on the treatment group assignment, indicating that the participants were compliant with the study protocol. However, the participants in this trial were specifically selected based on factors associated with high risk of NMSC incidence and factors expected to minimize noncompliance. Individuals who participate in a randomized clinical trial may not be representative of the general population.

In conclusion, these data provide evidence that supplementation with retinyl palmitate is an effective means to increase plasma concentrations of all-*trans*-, 9-*cis*-, and 13-*cis*-RA concentrations among relatively normal, healthy humans. Further research is needed to establish the relationship between plasma concentrations of RA isomer concentrations and expression of the retinoic receptor type expression. Given that retinyl palmitate is safe and extremely well tolerated even at daily doses of 50,000 and 75,000 IU (17, 18), these data suggest that skin and other epithelial cancers can be treated successfully through increasing RA concentration by supplementation with retinyl palmitate.

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