Dietary Administration of the Proapoptotic Vitamin E Analogue α-Tocopheryloxyacetic Acid Inhibits Metastatic Murine Breast Cancer

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Abstract

The ability of the vitamin E (RRR-α-tocopherol) derivatives α-tocopheryl succinate (α-TOS) and α-tocopheryloxyacetic acid (α-TEA) to suppress tumor growth in preclinical animal models has recently led to increased interest in their potential use for treating human cancer. To make the use of these vitamin E analogues more clinically relevant, we compared the antitumor efficacy of orally and i.p. delivered forms of α-TEA and α-TOS against a murine mammary cancer (4T1) that bears resemblance to human breast cancer because of its poor immunogenicity and high metastatic potential. In cell culture studies, we showed that both compounds inhibited tumor colony formation and induced apoptotic death of tumor cells. To avoid solubility concerns associated with the hydrophobicity of α-TEA and α-TOS, we used the vesiculated forms of α-TEA (Vα-TEA) and α-TOS (Vα-TOS) for the *in vivo* tumor studies. Both compounds inhibited the growth of preestablished 4T1 tumors when given i.p. However, when given by oral gavage, only the esterase-resistant Vα-TEA was able to suppress primary tumor growth and reduce lung metastasis. To make this approach more translatable to the clinic, α-TEA was incorporated into the diet and fed to tumor-bearing mice. We report here for the first time that dietary α-TEA delivery significantly inhibited primary tumor growth and dramatically reduced spontaneous metastatic spread to the lung in prophylactic and therapeutic settings. This study suggests that dietary α-TEA could prove useful as a relatively easy and effective modality for treating metastatic breast cancer. (Cancer Res 2006; 66(19): 9374-8)

Introduction

Chemotherapy, which is a standard form of adjuvant cancer therapy, is plagued by undesirable adverse side effects, including toxicity to normal cells and suppression of the immune system. These drawbacks have fueled efforts to develop novel anticancer agents that are toxic to tumor cells while sparing normal host cells. In this respect, the antitumor properties of the vitamin E [α-tocopherol (α-TOH)] derivatives α-tocopheryl succinate (α-TOS) and α-tocopheryloxyacetic acid (α-TEA) have recently sparked interest (1) because they have been shown to exhibit selective toxicity toward tumor cells (1–5) and to suppress tumor growth in various rodent and human xenograft tumor models (2, 3, 5–9). α-TOS and α-TEA are semisynthetic derivatives of vitamin E that structurally share the phytyl tail and the chroman head with vitamin E (Fig. 1*A*). α-TOS differs from vitamin E in that the hydroxyl group at the number 6 carbon of the phenolic ring of the chroman head has been replaced by a succinic acid residue (Fig. 1*B*; ref. 4). Because this succinate group is linked to the chroman head by an ester bond, α-TOS is sensitive to hydrolytic cleavage by intestinal esterases (5). In contrast, α-TEA has an acetic acid moiety attached via a nonhydrolyzable ether bond to the chroman head at this position (Fig. 1*C*; ref. 3). However, translation of α-TOS and α-TEA chemotherapy to the clinic has been hampered by the hydrophobic nature of both compounds. To improve the bioavailability of α-TOS, Jizomoto et al. (10) developed a vesiculated formulation of α-TOS (Vα-TOS) that is formed by spontaneous self-assembly on addition of sodium hydroxide, rendering it more readily soluble in aqueous solvents. Vα-TOS generated by this method is effective at inhibiting tumor growth and prolonging survival of B16 melanoma and 4T1 mammary tumor-bearing mice (11, 12). The susceptibility of α-TOS to hydrolytic cleavage by intestinal esterases makes it unsuitable for oral delivery because only the intact molecule has antitumor activity (13). In contrast, α-TEA is resistant to esterase cleavage and has been shown to exhibit antitumor activity when given by oral gavage to tumor-bearing mice, whereas α-TOS was ineffective in this setting (3, 8). In this report, we evaluated the antitumor effect of Vα-TOS and the vesiculated form of α-TEA (Vα-TEA) delivered by i.p. injection or oral gavage against a highly metastatic murine breast cancer cell line (4T1). With an eye to moving α-TEA chemotherapy to the clinic, we incorporated α-TEA into mouse chow to prevent and treat spontaneous metastatic breast cancer. We report here for the first time that dietary α-TEA significantly inhibited primary tumor growth and dramatically reduced lung metastases before and after primary tumor establishment. This study shows that dietary administration of α-TEA is a relatively easy and effective treatment modality that could have translational potential for treatment of metastatic breast cancer.

Materials and Methods

Preparation of vitamin E analogues. α-TOS was purchased from Sigma (St. Louis, MO). α-TEA [(2,5,7,8-tetramethyl-(2R-(4R,8R,12-trimethyltridecyl) chroman-6-yloxy) acetic acid)] was synthesized using a combination of previously described methods (3, 14), and purity was confirmed by high-performance liquid chromatography and nuclear magnetic resonance analysis. Vα-TOS and Vα-TEA were generated as described previously (11, 12). Briefly, 40 mg of α-TOS or α-TEA were dissolved in methanol and a thin film was formed in a 50 mL round-bottomed flask by rotary evaporation under a nitrogen atmosphere. Approximately 1.9 mL of PBS

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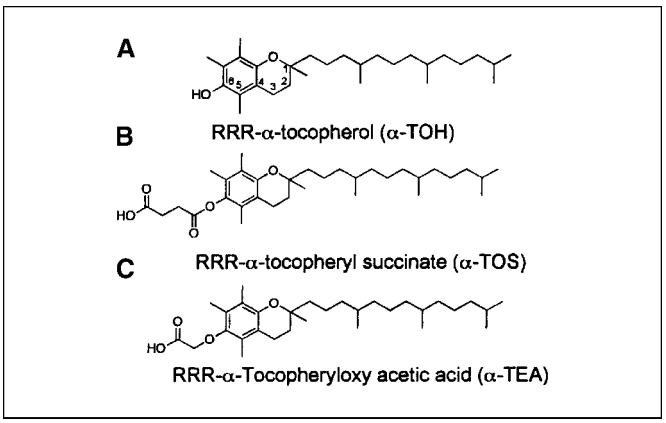

Figure 1. Structure of vitamin E (α-TOH; *A*) and the vitamin E derivatives α-TOS (*B*) and α-TEA (*C*). α-TOS possesses an ester-linked succinate moiety attached to carbon number 6 of the chroman head, making it susceptible to hydrolysis and cleavage by esterases. This is in contrast to α-TEA that possesses an ether-linked acetic acid moiety attached to the carbon number 6 of the chroman head.

(10 mmol/L; pH 8.0) were added and sonicated for 10 minutes. While sonicating (40 minutes), 10 μL of 1 N NaOH were added every 10 minutes (total, 40 μL). The pH was then adjusted to 7 using 1 mol/L HCl before sonication for 15 minutes.

Tumor cells and cell culture. The 4T1 cell tumor cell line is a variant of 410.4, a tumor subline that was isolated from a spontaneous mammary tumor in a BALB/cfC3H mouse. 4T1 is a poorly immunogenic and highly metastatic murine mammary tumor model that spontaneously metastasizes to the liver, lungs, bone marrow, and brain (15, 16), a characteristic that is shared with human breast cancers. The tumor cells were maintained in complete Iscove's modified Dulbecco's medium (IMDM; JRH Biosciences, Lenexa, KS) containing 100 units/mL penicillin, 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA), 0.75 μg/mL fungizone (Gemini Bio-Products, Woodland, CA), and 10% fetal bovine serum (Gemini Bio-Products).

Assessment of tumor cell viability, clonogenic potential, and apoptotic cell death. For the *in vitro* cell viability assays, 2×10^5 4T1 tumor cells per well were allowed to adhere in six-well tissue plates overnight in triplicate and then treated with Vα-TEA or Vα-TOS. After 24 hours, nonadherent and adherent cells were collected (200 × *g*, 5 minutes) and cell number and viability were determined by trypan blue dye exclusion (12). For the clonogenicity assay, 10^2, 10^3, 10^4, and 10^5 viable cells (trypan blue negative) from each treatment group were plated in triplicate in 100-mm dishes and incubated (7% CO_2, 37°C) for 10 days in complete IMDM. The resulting colonies were fixed with methanol, stained with Giemsa, and counted. The surviving cell fraction was then determined as described previously (8, 9) using the following formula: surviving fraction = [no. of colonies counted at a given concentration of Vα-TEA or Vα-TOS / no. of cells plated at that concentration] / [no. of control colonies counted (PBS) / no. of control cells plated (PBS)].

Determination of poly (ADP-ribose) polymerase cleavage. 4T1 tumor cells (2×10^6) were allowed to adhere overnight in 100-mm tissue culture plates (Sarstedt, Newton, NC) and then treated with Vα-TEA or Vα-TOS for 4, 8, 12, or 16 hours after which nonadherent and adherent cells were collected and lysed using Complete Lysis-M Buffer containing protease inhibitors (Roche, Indianapolis, IN) and homogenized by forcing through a 21-gauge needle and placing on ice for 10 minutes. Supernatant was recovered (14,000 × *g*, 4°C, 15 minutes), and 20 μg of protein per sample were resolved by 10% SDS-PAGE and analyzed using a poly (ADP-ribose) polymerase (PARP)-specific antibody (Cell Signaling Technology, Beverly, MA). To control for equal loading, the membranes were stripped using Restore Western Blot Stripping Buffer (Pierce, Rockford, IL) and reprobed using a glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific antibody (Chemicon, Temecula, CA).

Animal studies. Six- to eight-week-old female BALB/c mice (National Cancer Institute, Frederick Cancer Research Facility, Frederick, MD) were housed at the University of Arizona Animal Facilities (Tucson, AZ) in accordance with the Principles of Animal Care (NIH publication no. 85-23).

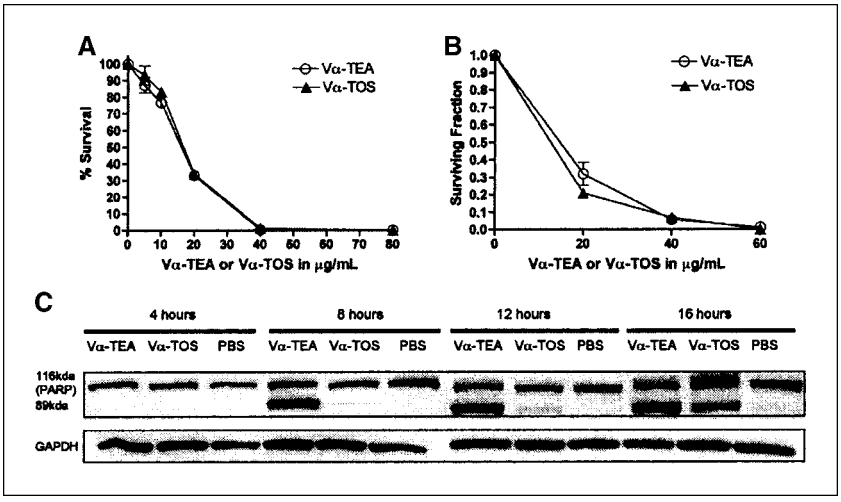

Figure 2. *A,* tumoricidal effect of Vα-TEA and Vα-TOS. 4T1 tumor cells were allowed to adhere overnight in six-well tissue culture plates in triplicate. The cells were then treated with none (PBS), 5, 10, 20, 40, or 80 μg/mL of Vα-TEA or Vα-TOS (in PBS). After a 24-hour exposure, nonadherent and adherent cells were collected and cell number and viability were determined by trypan blue dye exclusion. Data are representative of two independent experiments. *Points,* mean of triplicate samples; *bars,* SE. *B,* clonogenic potential of surviving cells following Vα-TEA or Vα-TOS treatment. 4T1 tumor cells were allowed to adhere overnight in six-well tissue culture plates. The cells were then treated with none (PBS), 20, 40, or 60 μg/mL of Vα-TEA or Vα-TOS. Nonadherent and adherent cells were collected and cell number and viability were determined by trypan blue dye exclusion 24 hours later. Subsequently, 10^2, 10^3, 10^4,and 10^5 viable cells from each treatment group were plated in triplicate in 100-mm tissue culture dishes and incubated (7% CO_2, 37°C) for 10 days in complete culture medium. The resulting colonies were fixed and Giemsa stained. Colonies were counted, and the surviving cell fraction was determined using the following formula: surviving fraction = [no. of colonies counted at a given concentration of Vα-TEA or Vα-TOS / no. of cells plated at that concentration] / [no. of control colonies counted (PBS) / no. of control cells plated (PBS)]. *Points,* mean of triplicate samples; *bars,* SE. *C,* induction of tumor cell apoptosis by Vα-TEA and Vα-TOS. 4T1 cells were allowed to adhere overnight in tissue culture plates. The cells were then treated with none (PBS) or 40 μg/mL of Vα-TEA or Vα-TOS for 4, 8, 12, or 16 hours after which nonadherent and adherent cells were collected and lysed using lysis buffer containing protease inhibitors. Proteins (20 μg/sample) were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked using TBS (Tris-buffered saline, 0.05% Tween 20, 5% nonfat dry milk) and stained with a PARP-specific antibody. Full-length PARP, 116 kDa; cleaved PARP, 89 kDa. To control for equal loading, the membranes were stripped and reprobed using a GAPDH-specific antibody.

α-TEA was incorporated into the AIN93G diet by Harlan Teklad (Madison, WI) at a concentration of 3.3 g α-TEA/kg. Tumor growth was monitored by measuring the tumor length and width with calipers and calculating the tumor volume [$V = (L \times W^2) / 2$]. Visible metastatic lung nodules were enumerated by staining with India ink and Fekete's solution (15).

Statistical analysis. Statistical significance of differences among data sets of treatment groups was assessed either by Student's t test, where applicable, or by one-way ANOVA, including Tukey-Kramer post-tests for multiple comparisons. To compare tumor growth rates, growth curves were transformed to linearity, and linear regression analysis was used to determine slopes that were then compared by Student's t test. All analyses were done using the Prism software (GraphPad, San Diego, CA). Ps ≤ 0.05 were considered indicative of significant differences between data sets.

Results and Discussion

Tumoricidal properties of Vα-TEA and Vα-TOS. We compared the growth-inhibitory and tumoricidal properties of Vα-TEA and Vα-TOS on the murine breast cancer cell line 4T1. Exposure to Vα-TEA and Vα-TOS for 24 hours killed 4T1 tumor cells in a dose-dependent manner with similar efficacy (Fig. 2*A*). Treatment of cells with 20 μg/mL of Vα-TEA or Vα-TOS caused 67% cell death, which increased to 99% and 100% when treated with 40 and 80 μg/mL of the drug, respectively. In addition, tumor cells that survived the Vα-TEA or Vα-TOS treatment were significantly impaired in their ability to proliferate and form colonies in a dose-dependent manner (Fig. 2*B*).

To determine the mechanism of Vα-TEA- and Vα-TOS-induced cell death, we evaluated tumor cells for signs of apoptosis. For this, we evaluated the cleavage of PARP, an event occurring late in the apoptotic program, by Western blot analysis (Fig. 2*C*). Whereas the p89 PARP cleavage product was strongly detected after 8 hours of Vα-TEA treatment, it was only weakly detectable at the 8- and 12-hour time points after Vα-TOS treatment. Furthermore, even 16 hours of Vα-TOS exposure resulted in less PARP cleavage than Vα-TEA treatment at shorter times. This finding shows that Vα-TEA induces apoptosis of 4T1 tumor cells more efficiently than Vα-TOS and suggests the potential superiority of Vα-TEA over Vα-TOS as an antitumor agent.

Oral administration of Vα-TEA inhibits tumor growth *in vivo*. Having shown that Vα-TEA and Vα-TOS kill tumor cells *in vitro*, we compared their efficacy as a single treatment modality when given by i.p. injection or oral gavage to control the growth of established 4T1 tumors. For this purpose 5×10^4 viable 4T1 tumor cells were injected orthotopically into the mammary fat pad of BALB/c mice. When tumors became palpable (~ 30 mm^3), mice received three i.p. injections of 4 mg of either Vα-TEA or Vα-TOS every 4 days as described previously (12). I.p. injection of both Vα-TEA and Vα-TOS significantly reduced the tumor growth rate ($P = 0.002$ and 0.005, respectively), resulting in over 2-fold lower average tumor volumes in the Vα-TEA-treated (400 ± 129 mm^3; $P < 0.001$) and Vα-TOS-treated (406 ± 109 mm^3; $P < 0.001$) groups compared with PBS-treated mice (987 ± 229 mm^3; Fig. 3*A*). When given by daily oral gavage, the esterase-resistant Vα-TEA significantly inhibited the tumor growth rate ($P = 0.017$) and reduced the average tumor volume 2-fold to 274 ± 53 mm^3 ($P < 0.001$) compared with PBS-treated mice (699 ± 66 mm^3). In contrast, Vα-TOS given by oral gavage had no effect on tumor progression as tumors grew to a final average tumor volume of 723 ± 67 mm^3 in

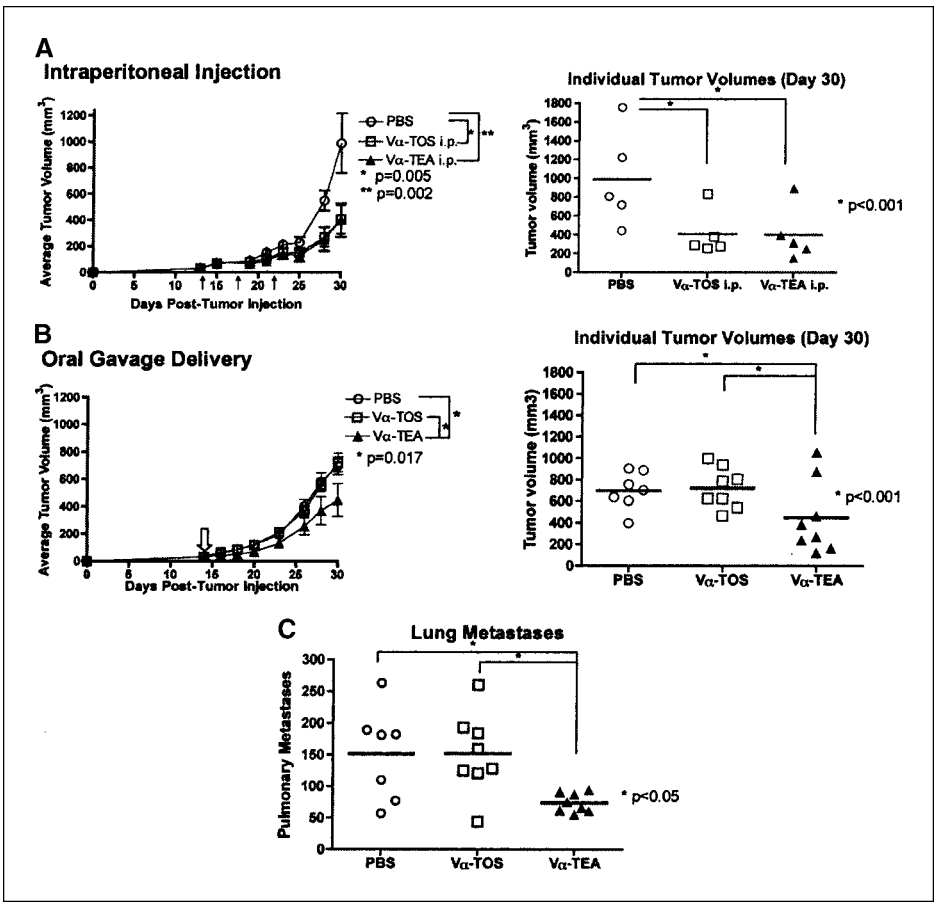

Figure 3. *A,* effect of i.p. injection of Vα-TEA and Vα-TOS on tumor growth. BALB/c mice were injected with 5×10^4 4T1 tumor cells into the right mammary fat pad. On tumor establishment (~ 30 mm^3, day 14 after tumor cell injection), mice received PBS (○) or 4 mg (in 200 μL) of Vα-TEA (▲) or Vα-TOS (□) by i.p. injections (↑) on days 14, 18, and 22. *Points,* mean tumor volumes or the tumor volumes of individual mice and average (—) on day 30 after tumor injection; *bars,* SE. *B,* effect of oral delivery of Vα-TEA and Vα-TOS by gavage on tumor growth and metastases formation. BALB/c mice were injected with 5×10^4 4T1 tumor cells into the right mammary fat pad. On tumor establishment (⇓, ~ 30 mm^3, day 14 after tumor cell injection), mice received PBS (○) or 4 mg (in 200 μL) of Vα-TEA (▲) or Vα-TOS (□) orally by daily gavage (days 14-30). *Points,* mean tumor volumes or the tumor volumes of individual mice and average (—) on day 30 after tumor injection; *bars,* SE. The values in (*C*) represent the number of lung metastases of individual mice and average (—) on day 30. *Asterisks,* significant differences between the groups.

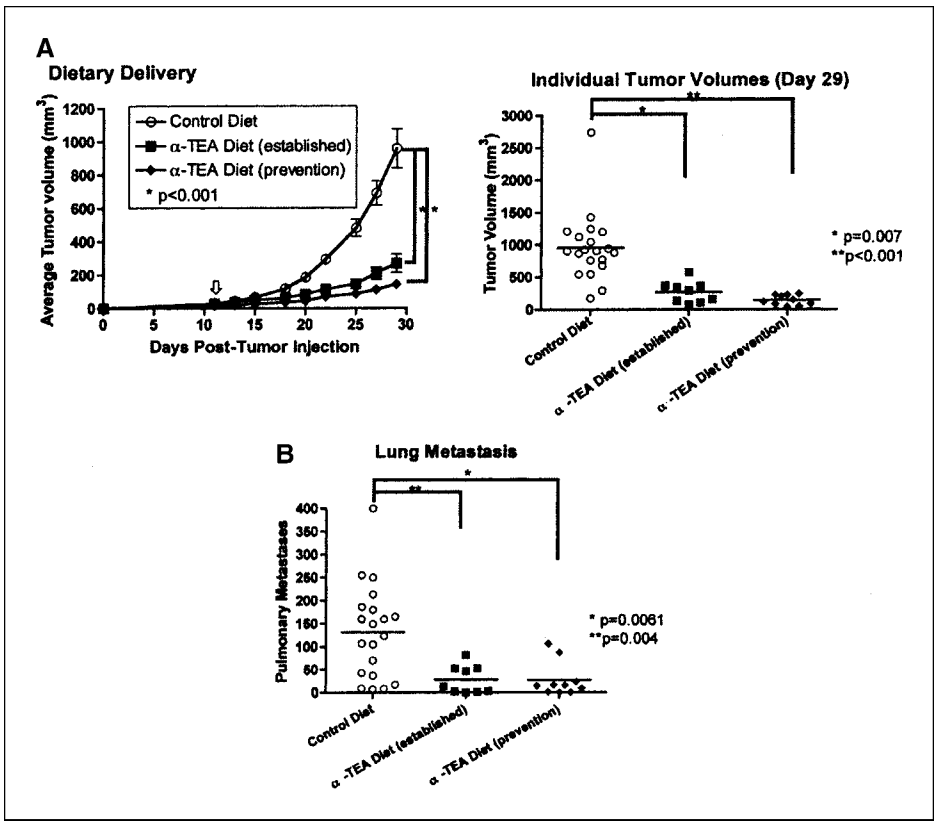

Figure 4. Effect of dietary delivery of α-TEA on primary tumor growth and metastases formation. BALB/c mice were injected with 5×10^4 4T1 tumor cells in the right mammary fat pad. *A,* mice received α-TEA diet (5.5 mg α-TEA per mouse daily) either from the day of tumor cell injection (♦, prevention) throughout the study or were switched to the α-TEA diet (■, established) after tumors became palpable (⇓, ~30 mm^3, day 11 after tumor cell injection). Untreated mice (○) received control diet throughout the study. *Points,* mean tumor volumes or the tumor volumes of individual mice and average (—) on day 29 after tumor injection; *bars,* SE. The values in (*B*) represent the number of lung metastases of individual mice and average (—) on day 29. *Asterisks,* significant differences between the groups.

this group (Fig. 3*B*). Increasing the oral gavage dose of Vα-TEA to 8 mg did not result in further tumor growth inhibition (data not shown). These findings corroborate studies by others in which liposomal α-TEA or α-TOS dissolved in peanut oil and ethanol given by oral gavage was shown to suppress lung metastasis of the 66cl murine breast cancer line (3, 8). In addition to its effect on established primary tumors, Vα-TEA given by oral gavage caused a 2-fold reduction in the average number of spontaneously arising, visible lung metastases (75 ± 5; $P < 0.001$) compared with Vα-TOS (152 ± 22) and PBS (151 ± 28; Fig. 3*C*).

Delivery of α-TEA in the diet significantly suppresses the growth of established 4T1 mammary tumors. Based on the thinking that translation of α-TEA chemotherapy to the clinic would be facilitated by an easier and more efficient mode of drug delivery, we evaluated the effectiveness of giving α-TEA in the diet for the prevention or treatment of established disease. For this purpose, α-TEA was composed in mouse chow that was fed to mice *ad libitum*. For the prevention study, BALB/c mice received α-TEA diet (3.3 g/kg food, ~5.5 mg α-TEA per mouse daily) starting on the day of tumor cell injection. To test α-TEA in the established disease setting, BALB/c mice received control diet until day 11 after tumor cell injection when mice with established tumors (~30 mm^3) were switched to the α-TEA diet. Dietary α-TEA therapy in the prophylactic (prevention) or therapeutic (established disease) settings resulted in a significant reduction of the tumor growth rate ($P < 0.0001$) compared with control mice. Compared with the final tumor volume of control diet-fed mice (959 ± 117 mm^3), the average final tumor volumes of mice that received the α-TEA diet in the prophylactic and therapeutic settings were reduced by 6.7-fold (144 ± 24 mm^3; $P < 0.001$) and 3.6-fold (270 ± 54 mm^3; $P = 0.0007$), respectively (Fig. 4*A*). The primary tumor size in the prevention study was 1.9-fold smaller than that in the therapeutic study, suggesting that the treatment was more effective before primary tumor establishment. Furthermore, dietary α-TEA therapy significantly reduced the average number of lung metastases >4.8-fold in both the prevention (27 ± 12; $P = 0.004$) and the established disease groups (28 ± 10; $P = 0.0061$) compared with the control group (132 ± 23; Fig. 4*B*). To assess the effect of α-TEA treatment on mice, close monitoring of the α-TEA diet-fed mice revealed no adverse effects, including weight loss. Whereas the average weight of mice before the diet switch was 18.7 ± 0.1 g, at the end of the study the average weight of mice in the prevention group (29 days of α-TEA diet) was 17.7 ± 0.4 g and that of mice in the established disease group was 17.4 ± 0.3 g (18 days of α-TEA diet). The goal of this study was to test the antitumor activity of dietary administration of the esterase-resistant vitamin E analogue α-TEA. Unlike oral gavage, dietary administration is easier to do and more readily translated to the clinic. These studies show for the first time the effectiveness of dietary α-TEA in controlling the growth and spread of a highly aggressive murine breast cancer. The combined characteristics of ease of delivery, relevance of route of delivery, and selectivity for killing tumor cells suggest that dietary α-TEA may be useful for treating metastatic breast cancer. Recognizing that the overall dose received per mouse in these studies [5.4 and 8.7 g/kg body weight (based on the average weight of 18.3 g per mouse) over 18 and 29 days, respectively] is relatively high, experiments to determine the effectiveness of lower doses of α-TEA alone or in combination with dendritic cell vaccines are warranted. In previous studies (9, 12),[4] we showed that, in addition

[4] Unpublished observation.

to their direct proapoptotic effect on tumor cells, α-TOS and α-TEA stimulate dendritic cell maturation and that the combination of Vα-TOS (4 mg given i.p. every other day for nine injections, equivalent to ~2 g/kg body weight) with dendritic cell vaccination displayed comparable antitumor efficacy as the dietary α-TEA monotherapy, suggesting that the dose of α-TEA may be significantly lowered if combined with dendritic cell immunotherapy. This dose of Vα-TOS (2 g/kg body weight) is lower than the dose of 2.9 and 4.1 g/kg α-TOS (based on the average human weight of 70 kg) that was administered to angina pectoris patients without apparent toxicity (17, 18). In ongoing studies, we are assessing the effectiveness of a chemoimmunotherapy regimen consisting of dietary α-TEA plus dendritic cell vaccination in treating metastatic breast cancer before and after primary tumor resection.

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