Antiproliferative and Apoptotic Effects of Tocopherols and Tocotrienols on Preneoplastic and Neoplastic Mouse Mammary Epithelial Cells (44544)

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Abstract. **Studies were conducted to determine the comparative effects of tocopherols and tocotrienols on preneoplastic (CL-S1), neoplastic (-SA), and highly malignant (+SA) mouse mammary epithelial cell growth and viability *in vitro*. Over a 5-day culture period, treatment with 0–120 μ*M* α- and γ-tocopherol had no effect on cell proliferation, whereas growth was inhibited 50% (IC_{50}) as compared with controls by treatment with the following: 13, 7, and 6 μ*M* tocotrienol-rich-fraction of palm oil (TRF); 55, 47, and 23 μ*M* δ-tocopherol; 12, 7, and 5 μ*M* α-tocotrienol; 8, 5, and 4 μ*M* γ-tocotrienol; or 7, 4, and 3 μ*M* δ-tocotrienol in CL-S1, -SA and +SA cells, respectively. Acute 24-hr exposure to 0–250 μ*M* α- or γ-tocopherol (CL-S1, -SA, and +SA) or 0–250 μ*M* δ-tocopherol (CL-S1) had no effect on cell viability, whereas cell viability was reduced 50% (LD_{50}) as compared with controls by treatment with 166 or 125 μ*M* δ-tocopherol in -SA and +SA cells, respectively. Additional LD_{50} doses were determined as the following: 50, 43, and 38 μ*M* TRF; 27, 28, and 23 μ*M* α-tocotrienol; 19, 17, and 14 μ*M* γ-tocotrienol; or 16, 15, or 12 μ*M* δ-tocotrienol in CL-S1, -SA, and +SA cells, respectively. Treatment-induced cell death resulted from activation of apoptosis, as indicated by DNA fragmentation. Results also showed that CL-S1, -SA, and +SA cells preferentially accumulate tocotrienols as compared with tocopherols, and this may partially explain why tocotrienols display greater biopotency than tocopherols. These data also showed that highly malignant +SA cells were the most sensitive, whereas the preneoplastic CL-S1 cells were the least sensitive to the antiproliferative and apoptotic effects of tocotrienols, and suggest that tocotrienols may have potential health benefits in preventing and/or reducing the risk of breast cancer in women.**

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Vitamin E is a generic term representing two groups of chemically related, lipid-soluble compounds, the tocopherols and tocotrienols (1, 2). Tocopherols are commonly present in a variety of foods, whereas tocotrienols are relatively rare and found in appreciable levels only in a few specific vegetable fats, such as palm oil (3, 4). Earlier studies showed that high dietary intake of crude palm oil, in contrast to other high-fat diets, suppressed carcinogen-induced mammary tumorigenesis in experimental animals (4–7). Although palm oil also contains modest amounts of α-tocopherol, it is unlikely that α-tocopherol is responsible for mediating the antitumor effects of dietary palm oil because other dietary fats containing higher levels of α-tocopherol than palm oil stimulate mammary tumorigenesis (4, 5). Furthermore, high-palm oil diets stripped of tocotrienols were found to stimulate, whereas dietary supplementation with the tocotrienol-rich fraction (TRF) of palm oil significantly inhibited mammary tumor development and growth (7). Although tocopherols and tocotrienols are potent antioxidants, the antitumor activity of these compounds is not dependent on their antioxidant activity (1, 2). Available evidence suggests that these compounds inhibit

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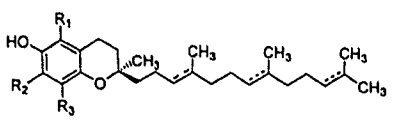
tumor development and growth by modulating multiple intracellular signaling pathways involved in mitogenesis (8–11) and apoptosis (12–15). Nevertheless, the majority of studies have shown that tocotrienols display greater antitumor activity than tocopherols (16–22).

The exact reason why tocotrienols are more potent antitumor agents than tocopherols is presently unknown. Although tocopherols and tocotrienols have the same basic chemical structure characterized by a long phytyl chain attached at the 1-position of a chromane ring, the major difference between these vitamin E subgroups is that tocopherols have a saturated, while tocotrienols have an unsaturated, phytyl chain (Fig. 1). In addition, specific tocopherol and tocotrienol isoforms differ from each other based on the number of methyl groups bound to their chromane ring (Fig. 1). It is possible that the level of phytyl chain saturation and/or chromane ring methylation may be critical in determining the antiproliferative and apoptotic activity of individual tocopherol and tocotrienol isoforms.

In the present study, experiments were conducted using preneoplastic and neoplastic mouse mammary epithelial cell lines that were derived from the hyperplastic D1 cell line that spontaneously arose in BALB/c mice (23). The CL-S1 preneoplastic cell line is immortal in culture, but does not grow in soft agarose or form solid tumors upon transplantation back into the mammary gland (23). The -SA and +SA cell lines were derived from adenocarcinomas that developed spontaneously from the original D1 cell line (24, 25). The major difference between these neoplastic cell lines is that -SA cells do not grow in soft agarose, whereas +SA cells display anchorage-independent growth when cultured in soft agarose gels (24, 25). When injected back into the mammary gland fat pad of syngeneic female mice, -SA cells grow to form well-differentiated tumors, whereas +SA cells form anaplastic adenocarcinomas (24, 25). In addition, both -SA and +SA cells metastasize to the lung, but +SA tumors are much more aggressive in their growth and metastatic characteristics than -SA tumors (25).

Taken together, these cell lines representing a gradient of transformed states provide an ideal experimental model for study of mammary neoplasia and tumor progression. The following experiments were conducted to characterize the differential antiproliferative and apoptotic effects of specific tocopherol and tocotrienol isoforms on these preneoplastic and neoplastic mammary epithelial cell lines grown in culture and maintained on serum-free media. Additional studies were conducted to determine the relationship between biopotency and the magnitude of cellular accumulation of individual tocopherol and tocotrienol isoforms in each cell line.



Compound	R_1	R_2	R_3	Phytyl Chain
α-tocopherol	CH_3	CH_3	CH_3	Saturated
γ-tocopherol	H	CH_3	CH_3	Saturated
δ-tocopherol	H	H	CH_3	Saturated
α-tocotrienol	CH_3	CH_3	CH_3	Unsaturated
γ-tocotrienol	H	CH_3	CH_3	Unsaturated
δ-tocotrienol	H	H	CH_3	Unsaturated

Figure 1. Generalized structure of vitamin E compounds.

Materials and Methods

Cell Culture. All materials were purchased from Sigma Chemical Company (St. Louis, MO) unless otherwise stated. Preneoplastic CL-S1 and neoplastic -SA and +SA mammary epithelial cell lines were serially passaged at subconfluent cell density. All cell lines were maintained in serum-free control medium consisting of DMEM/F12 containing 5 mg/ml bovine serum albumin (BSA), 10 μg/ml transferrin, 100 U/ml soybean trypsin inhibitor, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 10 ng/ml EGF, and 10 μg/ml insulin. For subculturing, cells were rinsed twice with sterile Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS), and then incubated in 0.05% trypsin containing 0.025% EDTA in PBS for 5 min at 37°. The released cells were then diluted in DMEM/F12 medium, pelleted by centrifugation, and cell pellets were then resuspended in serum-free medium, and counted by hemocytometer. CL-S1 cells were plated at a density of 1×10^5 cell/well in 24-well culture plates for growth and viability studies, and at a density of 1×10^6 cells/100 mm culture plates for DNA fragmentation studies. Because -SA and +SA cells have a more rapid doubling time, these cell lines were plated at a density of 5×10^4 cells/well in 24-well culture plates (growth and viability studies) and 5×10^5 cells/100 mm culture plates (DNA fragmentation studies). For tocopherol and tocotrienol uptake studies, CL-S1 cells were plated at a density of 3×10^5 cells/well, whereas -SA and +SA cells were plated at a density of 1.5×10^4 cells/well in 6-well culture plates. Cells were divided into different treatment groups and fed serum-free control or treatment medium every other day and maintained in a humidified incubator at 37°C in an environment of 95% air and 5% CO_2.

Medium Vitamin E Supplementation and Experimental Treatments. To dissolve the highly lipophilic vitamin E compounds in aqueous culture medium, these compounds were conjugated to bovine serum albumin (BSA) as previously described (26). Briefly, an appropriate amount of α-, γ-, δ-tocopherol, α-, γ-, δ-tocotrienol, or tocotrienol-rich fraction of palm oil (TRF) was placed into a 1.5-ml screw-top glass vial and dissolved in 100 μl of 100% ethanol. Once dissolved, this ethanol/vitamin E solution was added to a small volume of sterile 10% BSA in water and incubated overnight at 37°C. This solution of vitamin E conjugated to BSA was used to prepare various concentrations (0–250 μ*M*) of tocopherol-, tocotrienol-, or TRF-supplemented treatment media such that all control and treatment media had a final concentration of 5 mg/ml BSA. Ethanol was added to all treatment media such that the final ethanol concentration was the same in all treatment

groups within a given experiment and was always less than 0.1%.

Measurement of Viable Cell Number. Preneoplastic and neoplastic mammary epithelial cell number was determined in 24-well culture plates (6 wells/group) by the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay as described previously (26, 27). On the day of assay, treatment medium was replaced with fresh growth medium containing 0.83 mg/ml MTT, and the cells were returned to the incubator for 4 hr. Afterward, the medium was removed, and the MTT crystals were dissolved in 0.5 ml of dimethyl sulfoxide. The optical density of each sample was read at 570 nm on a microplate reader (model 7520 Cambridge Technology, Inc., Watertown, MA), against a blank prepared from cell-free cultures. The number of cells/well was calculated against a standard curve prepared by plating various concentrations of cells, as determined by hemocytometer, at the start of each experiment (26, 27). In separate control studies, various doses (0–250 μM) of TRF or specific tocopherol and tocotrienol isoforms were not found to affect the specific activity of the MTT colorimetric assay.

Determination of Treatment-Induced DNA Fragmentation. Cells in each treatment group were grown in 100-mm plates (2–3 plates/group) and treated with various doses of specific tocopherols, tocotrienols, or TRF for 0–48 hr. Fragmentation of chromatin into units of single or multiple nucleosomes that form the nucleosomal DNA ladder in agarose gels is an established hallmark of programmed cell death or apoptosis (28). To determine treatment-induced programmed cell death, as indicated by DNA fragmentation, cells were isolated from culture with trypsin, rinsed three times, pooled, and DNA was then isolated from cells in each treatment group by phenol/chloroform extraction (28). Isolated DNA was then fractionated on a 1.2% Tris/acetic acid/EDTA (TAE) agarose gel, and visualized with an ultraviolet transluminator.

Cellular Accumulation of Tocopherols and Tocotrienols. Preneoplastic and neoplastic mammary epithelial cell lines were cultured in serum-free control media for 5 days, then treated with various doses of specific tocopherol or tocotrienol isoforms for 0, 6 hr, 12 hr, or 24 hr. In each treatment group, adherent cells were isolated from culture plates by trypsin digestion, and then combined with cells floating in the culture media. Cells were then pelleted, twice washed, and resuspended in PBS; an aliquot was removed for protein determination using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA), according to the manufacturer's directions. Cells were then pelleted and extracted for assay of tocopherol and tocotrienol content by reverse phase HPLC fluorometric detection, by a modification of methods previously described (29, 30). Briefly, an internal standard (1.6 nmol of α-tocopherol for determination of δ-tocopherol levels or 1.6 nmol of δ-tocopherol for quantitation of all other tocopherol and tocotrienol isoforms) was added to the appropriate treatment group of isolated mammary epithelial cells. The same amount of the corresponding internal standard (1.6 nmol) was also added to the appropriate tocopherol and tocotrienol isoform standards. Cells in each treatment group were then resuspended by sonication in 0.3 ml of 1% ascorbate in 0.1 M SDS and 0.45 ml 100% ethanol. Hexane (0.8 ml) was then added to each sample, followed by vortexing for 30 sec, and the resulting hexane extracts were dried under nitrogen. The dried extracts were then resuspended in 1 ml methanol containing 2.5% ascorbate. Extracted and nonextracted standards of each tocopherol and tocotrienol isoform (0.05–5 nmol/sample) were run with each assay, and expressed as nmol/mg. Samples were injected on a Hewlett-Packard 1050 HPLC equipped with an autosampler, Chem Station software, McPherson 749 fluorescence detector, and Spherisorb ODS II column (250 × 4.6 mm I.D., 5 μm; Alltech, Avondale, PA). The mobile phase was 96% methanol, which was run isocratically at a flow rate of 1.8 ml/min. Excitation and emission wavelengths of 210 nm and 300 nm, respectively, were used for all tocopherol and tocotrienol isoform determinations. Samples and standards were assayed by HPLC on the same day of extraction. Cellular concentrations were expressed as the average of four replicates in each treatment group. TRF was assayed by HPLC prior to use in experimentation, and determined to have a composition of 20.2% α-tocopherol, 16.8% α-tocotrienol, 44.9% γ-tocotrienol, 14.8% δ-tocotrienol, and 3.2% of a nonvitamin E lipid-soluble contaminant. Treatment doses of TRF were then calculated on the basis of percentage composition and molecular weights of individual vitamin E isoforms within TRF.

Statistical Analysis. Differences among the various treatment groups were determined by analysis of variance, followed by Duncan's multiple-range test. A difference of $P < 0.05$ was considered to be significant, as compared with controls or as defined in the figure legends. Linear regression analysis of treatment effects on viable cell number in growth and cytotoxicity studies was used to determine the 50% growth inhibition concentration (IC_{50}) and 50% lethal dose (LD_{50}) for individual treatments.

Results

The effects of various doses of TRF on preneoplastic CL-S1 and neoplastic -SA and +SA mammary epithelial cell proliferation are shown in Figure 2. CL-S1, -SA and +SA cells grown in serum-free control media displayed a continuous increase in viable cell number over the 5-day culture period (Fig. 2). Supplementation of culture medium with 10–20 μM (CL-S1) or 2–8 μM (-SA and +SA) TRF significantly inhibited EGF-induced cell proliferation in a dose-responsive manner, as compared with controls (Fig. 2).

Since TRF contains a number of vitamin E isoforms, it was not possible to determine if one or all of these isoforms was responsible for mediating inhibitory effects of TRF on CL-S1, -SA and +SA cell growth described in Figure 2.

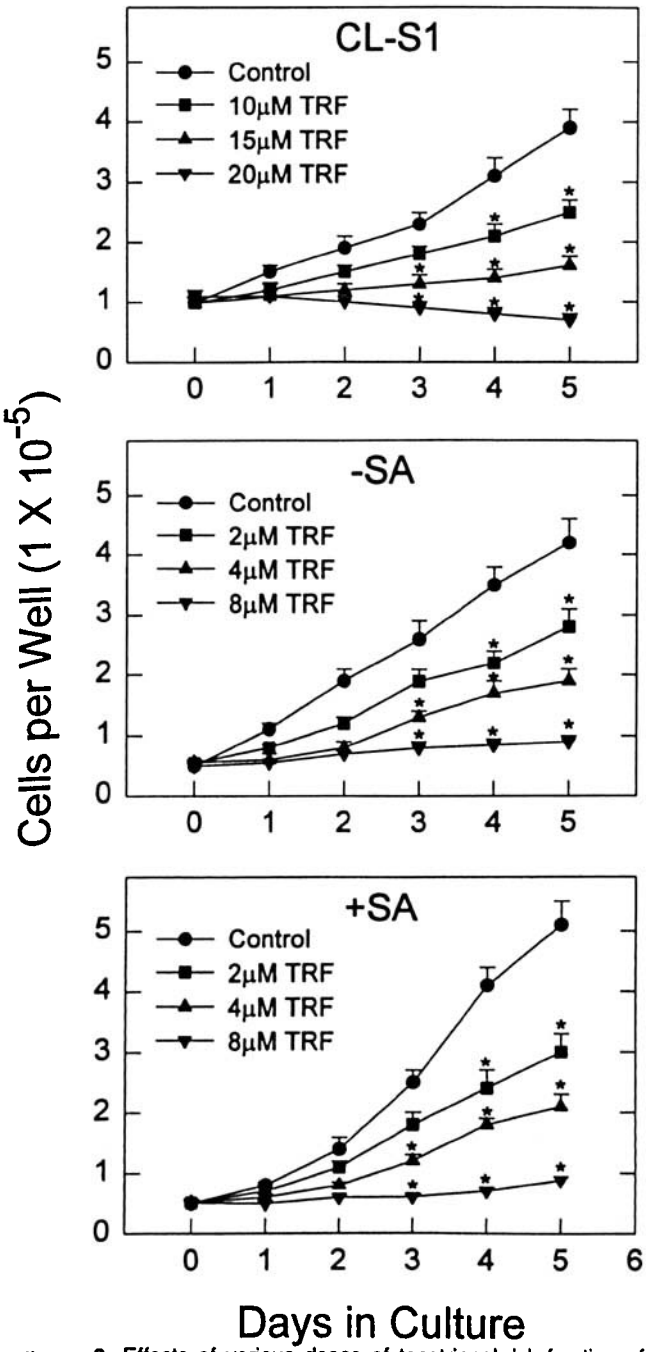


Figure 2. Effects of various doses of tocotrienol-rich-fraction of palm oil (TRF) on preneoplastic (CL-S1), neoplastic (-SA), and highly malignant (+SA) mammary epithelial cell proliferation in culture. Data points indicate the mean cell count/well ± SEM for six replicates in each treatment group after 5 days in culture. *$P < 0.05$, as compared with controls.

Therefore, additional studies were conducted to determine the antiproliferative effects of specific tocopherol and tocotrienol isoforms on these preneoplastic and neoplastic mammary epithelial cell lines. After 5 days of culture, treatment with 0–120 μ*M* α- or γ-tocopherol had no effect, whereas treatment with 40–120 μ*M* (CL-S1) or 30–120 μ*M* (-SA and +SA) δ-tocopherol significantly inhibited cell growth, as compared with the respective controls (Fig. 3). Treatment with 8–40 μ*M* α-tocotrienol, or 5–20 μ*M* γ- or δ-tocotrienol significantly inhibited CL-S1 cell growth, as compared with controls (Fig. 3). Treatment with 5–40 μ*M* α-tocotrienol, or 4–10 μ*M* γ- or δ-tocotrienol significantly inhibited -SA cell growth, whereas treatment with 4–30 μ*M* α-tocotrienol, or 3–10 μ*M* γ- or δ-tocotrienol significantly inhibited +SA cell growth, compared with the controls (Fig. 3).

The effects of acute 24-hr exposure to various concentrations of tocopherols, tocotrienols, or TRF on viable CL-S1, -SA, and +SA cell number are shown in Figure 4. Acute treatment with 6–250 μ*M* α-, γ-, or δ-tocopherol had no affect on CL-S1 preneoplastic mammary epithelial cell viability (Fig. 4). Similar treatment with 30–80 μ*M* TRF, 20–60 μ*M* α-tocotrienol, 15–60 μ*M* γ-tocotrienol, or 10–60 μ*M* δ-tocotrienol significantly decreased CL-S1 viable cell

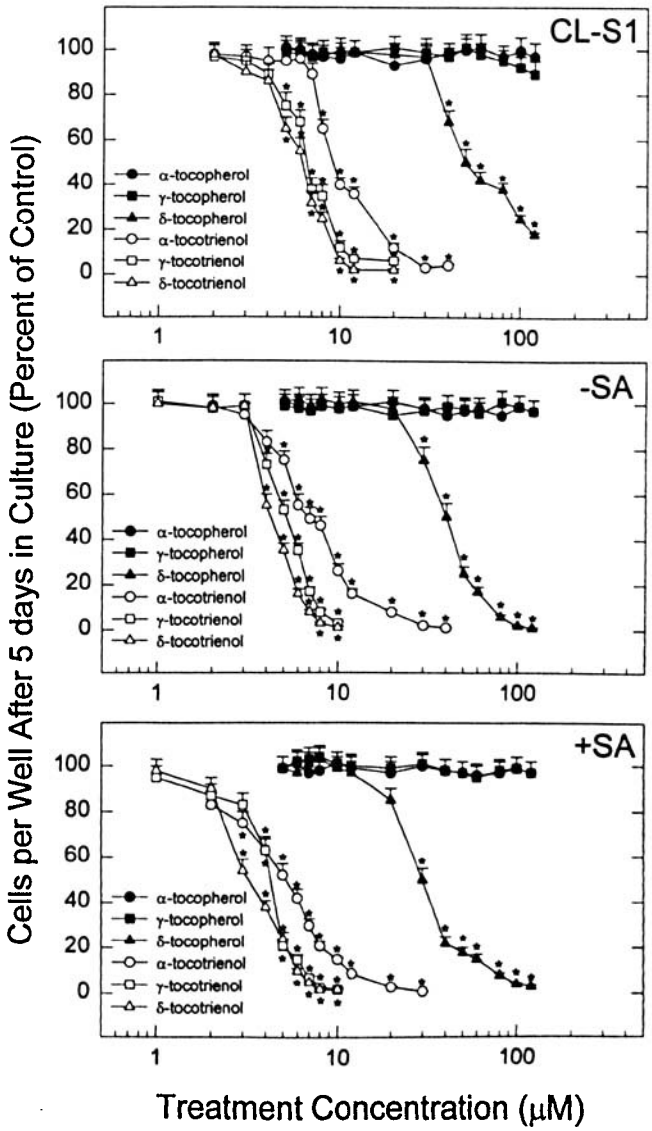


Figure 3. Effects of various doses of individual tocopherol and tocotrienol isoforms on preneoplastic (CL-S1), neoplastic (-SA), and highly malignant (+SA) mammary epithelial cell proliferation in culture. Data points indicate the percentage of viable cells/well ± SEM for six replicates in each treatment group, as compared with controls. *$P < 0.05$, as compared with controls.

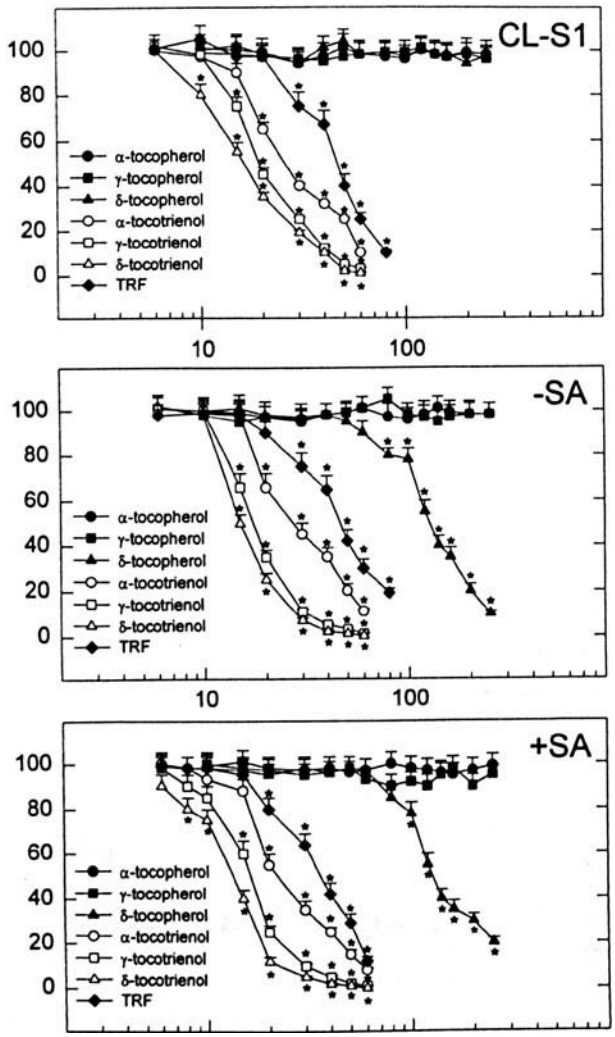


Figure 4. Preneoplastic (CL-S1), neoplastic (-SA), and highly malignant (+SA) mammary epithelial cell viability after a 24-hr exposure period to various doses of tocotrienol-rich-fraction of palm oil (TRF), or individual tocopherol and tocotrienol isoforms. Cells in each treatment group were grown in culture and maintained on control medium for 5 days prior to exposure to their respective treatments. Data points indicate the percentage of viable cells/well ± SEM for six replicates in each treatment group, as compared with controls. $^*P < 0.05$, as compared with controls.

number in a dose-responsive manner (Fig. 4). Acute treatment with 6–250 μ*M* α- or γ-tocopherol had no effect, whereas treatment with 70–250 μ*M* δ-tocopherol, 30–80 μ*M* TRF, 20–60 μ*M* α-tocotrienol, or 15–60 μ*M* γ- or δ-tocotrienol significantly decreased -SA cell viability in a dose-responsive manner (Fig. 4). Treatment for 24 hr with 6–120 μ*M* α- or γ-tocopherol had no affect, whereas treatment with 100–250 μ*M* δ-tocopherol, 20–60 μ*M* TRF, 20–60 μ*M* α-tocotrienol, 15–60 μ*M* γ-tocotrienol, or 8–60 μ*M* δ-tocotrienol significantly decreased +SA viable cell number in a dose-responsive manner, as compared with controls (Fig. 4).

Table I summarizes the relative antiproliferative and

Table I. Effects of Various Vitamin E Compounds on Preneoplastic (CL-S1), Neoplastic (-SA), and Malignant (+SA) Mammary Epithelial Cell Growth (IC_{50}) and Viability (LD_{50})

Vitamin E		CL-S1	-SA	+SA
TRF	IC_{50}	13 μ*M*	7 μ*M*	6 μ*M*
	LD_{50}	50 μ*M*	43 μ*M*	38 μ*M*
α-Tocopherol	IC_{50}	>120 μ*M*	>120 μ*M*	>120 μ*M*
	LD_{50}	>250 μ*M*	>250 μ*M*	>250 μ*M*
γ-Tocopherol	IC_{50}	>120 μ*M*	>120 μ*M*	>120 μ*M*
	LD_{50}	>250 μ*M*	>250 μ*M*	>250 μ*M*
δ-Tocopherol	IC_{50}	55 μ*M*	47 μ*M*	23 μ*M*
	LD_{50}	>250 μ*M*	166 μ*M*	125 μ*M*
α-Tocotrienol	IC_{50}	12 μ*M*	7 μ*M*	5 μ*M*
	LD_{50}	27 μ*M*	28 μ*M*	23 μ*M*
γ-Tocotrienol	IC_{50}	8 μ*M*	5 μ*M*	4 μ*M*
	LD_{50}	19 μ*M*	17 μ*M*	14 μ*M*
δ-Tocotrienol	IC_{50}	7 μ*M*	4 μ*M*	3 μ*M*
	LD_{50}	16 μ*M*	15 μ*M*	12 μ*M*

Note. IC_{50} indicates treatment dose that induced a 50% growth inhibition as compared with controls over the 5-day culture period. LD_{50} indicates treatment dose that induced a 50% reduction in viable cell number as compared with controls after a 24-hr exposure period. Cells in LD_{50} studies were grown in culture and maintained on control media for 5 days prior to being exposed to their respective treatments. Each treatment group contained six replicates initially plated in 24-well plates at a density of 1×10^5 (CL-S1) or 5×10^4 (-SA or +SA) cells/well.

cytotoxic potencies of TRF, and individual tocopherol and tocotrienol isoforms on preneoplastic Cl-S1, neoplastic -SA, and highly malignant +SA mammary epithelial cells. Treatment doses of TRF that inhibited CL-S1, -SA and +SA cell growth by 50% (IC_{50}) were 13, 7, and 6 μ*M*, respectively. Acute exposure to treatment doses of TRF that reduced CL-S1, -SA and +SA viable cell number by 50% (LD_{50}) were 50, 43, and 38 μ*M*, respectively (Table I). Over the 0–250 μ*M* dose-range tested, treatment with α- and γ-tocopherol had no effect on CL-S1, -SA or +SA cell growth or viability. Therefore, the IC_{50} and LD_{50} doses were not determined for these compounds. Although treatment with δ-tocopherol inhibited CL-S1, -SA and +SA cell proliferation, acute treatment with δ-tocopherol reduced only -SA and +SA viable cell number (Table I). All tocotrienol isoforms tested were found to decrease CL-S1, -SA and +SA cell proliferation and viability. However, the highly malignant + SA cells were found to be the most sensitive, and the preneoplastic CL-S1 cells were found to be the least sensitive to these treatment effects (Table I). In addition, individual tocopherol and tocotrienol isoforms displayed differential antiproliferative and cytotoxic potencies in the preneoplastic and neoplastic cell lines that were characterized as δ-tocotrienol ≥ γ-tocotrienol > α-tocotrienol > δ-tocopherol > α- and γ-tocopherol (Table I).

DNA isolated from untreated CL-S1, -SA, and +SA mammary epithelial cells maintained in control media for 5 days did not exhibit appreciable levels of fragmentation (Fig. 5). Following exposure to 50 μ*M* TRF, preneoplastic and neoplastic mammary epithelial cell lines displayed de-

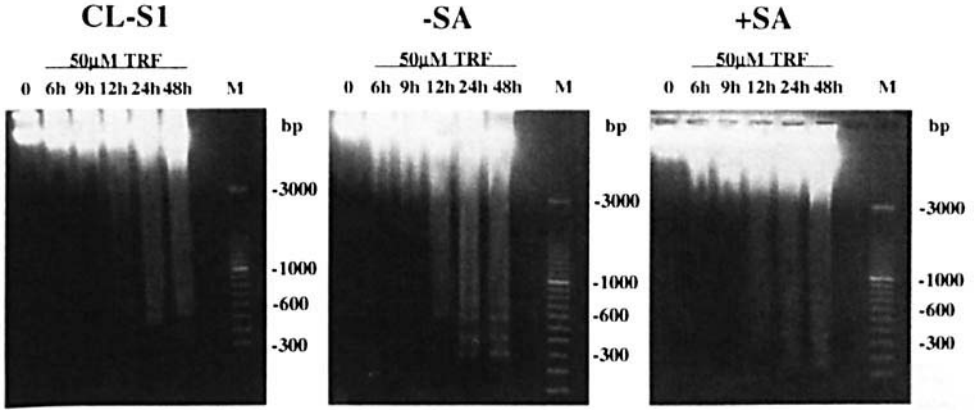


Figure 5. Preneoplastic (CL-S1), neoplastic (-SA), and highly malignant (+SA) mammary epithelial cell internucleosomal DNA fragmentation during a 48-hr period following exposure to 50 μ*M* tocotrienol-rich-fraction of palm oil (TRF). Cells were cultured in control medium for 5 days and then exposed to their respective treatments. Lane M contains DNA base pair (bp) laddering markers.

tectable levels of DNA fragmentation between 12 and 48 hr after initiation of treatment (Fig. 5).

The effects of 24-hr exposure to IC_{50} or maximum doses previously tested for individual tocopherols and tocotrienols on DNA fragmentation in CL-S1, -SA, and +SA mammary epithelial cells are shown in Figure 6. Cells were maintained on control medium for 5 days prior to treatment exposure. DNA isolated from untreated controls showed little fragmentation or laddering in CL-S1, -SA or +SA cells (Fig. 6, Lane C). Treatment for 24 hr with 120 μ*M* α- or γ-tocopherol in CL-S1, -SA or +SA cells had no effect on DNA fragmentation (Fig. 6, Lanes α-T and γ-T, respectively). However, 24 hr treatment with IC_{50} doses of δ-tocopherol or α-, γ-, and δ-tocotrienol induced intense DNA fragmentation in preneoplastic (CL-S1) and neoplastic (-SA and +SA) mammary epithelial cell lines (Fig. 6, Lanes δ-T, α-T^3, γ-T^3, and δ-T^3, respectively).

Figure 7 shows cellular accumulation of specific tocopherol and tocotrienol isoforms in CL-S1, -SA, and +SA mammary epithelial cells treated for 24 hr with 120 μ*M* α-, γ-, or δ-tocopherol, or 5 μ*M* α-, γ-, or δ-tocotrienol. CL-S1, -SA and +SA cellular levels of α-, γ-, and δ-isoforms of tocopherol and tocotrienol were undetectable prior to treatment exposure (Fig. 7). However, a dose- and time-responsive increase in cellular levels of these compounds was observed over the 24-hr treatment period (Fig. 7). Similar treatment with 5 μ*M* α-, γ-, or δ-tocopherol did not produce detectable levels of these compounds in any of the cell lines (data not shown). However, similar cellular levels of individual tocopherol and tocotrienol isoforms could be produced in the different cell lines when they were treated with 120 μ*M* α-, γ-, or δ-tocopherol versus 5 μ*M* α-, γ-, and δ-tocotrienol (Fig. 7). Results also showed that the relative cellular accumulation of individual tocopherol and tocotrienol isoforms was not equal and characterized as δ > γ > α for both tocopherol and tocotrienol isoforms in each cell line (Fig. 7).

Discussion

Results from these studies demonstrate that preneoplastic (CL-S1), neoplastic (-SA), and highly malignant (+SA) mammary epithelial cells display differential sensitivities to antiproliferative and apoptotic effects of tocopherols and tocotrienols. In general, +SA cells, which exhibit the greatest degree of tumor progression or malignancy, were found to be the most sensitive, whereas nonmalignant CL-S1 cells were shown to be least sensitive to the growth inhibitory and apoptotic effects of these compounds. Direct comparisons between the two vitamin E subclasses showed that tocotrienols were significantly more potent in suppressing growth and inducing cell death than tocopherols, and that the relative biopotency of specific isoforms displayed a consistent relationship corresponding to δ-tocotrienol ≥ γ-tocotrienol > α-tocotrienol > δ-tocopherol > γ- and α-tocopherol. Although preneoplastic and neoplastic mammary epithelial cells were found to accumulate tocotrienols with greater ease or preference than tocopherols, those findings do not fully explain the greater biopotency of tocotrienols versus tocopherols. Treatments that induced similar cellular levels of tocopherols and tocotrienols did not produce similar antiproliferative and cytotoxic effects in the preneoplastic and neoplastic cell lines. The mechanism(s) mediating the growth inhibitory effects of δ-tocopherol and α-, γ-, and δ-tocotrienol in preneoplastic and neoplastic mammary epithelial cells is presently unknown. However, tocotrienol-induced cell death results from the initiation of apoptosis, as indicated by DNA fragmentation.

The use of CL-S1, -SA, and +SA mammary epithelial cell lines in the present study provides a distinct gradient of transformed states that is ideal for evaluating the relative antitumor effects of specific tocopherol and tocotrienol isoforms. Previous studies have shown differential effects of these compounds in various cell lines (16–22). However, direct comparisons of the effects of tocopherols and tocotrienols on syngeneic cells characterized by varying degrees of tumor progression have never before been investigated. Although α- or γ-tocopherol treatments did not significantly affect cell proliferation or viability in any cell line, over the dose-range tested, it should not be concluded that these compounds lack bioactivity. Treatment with m*M* doses of α-tocopherol has been reported to exert antiproliferative and cytotoxic effects on other cell types (15, 31). However, the

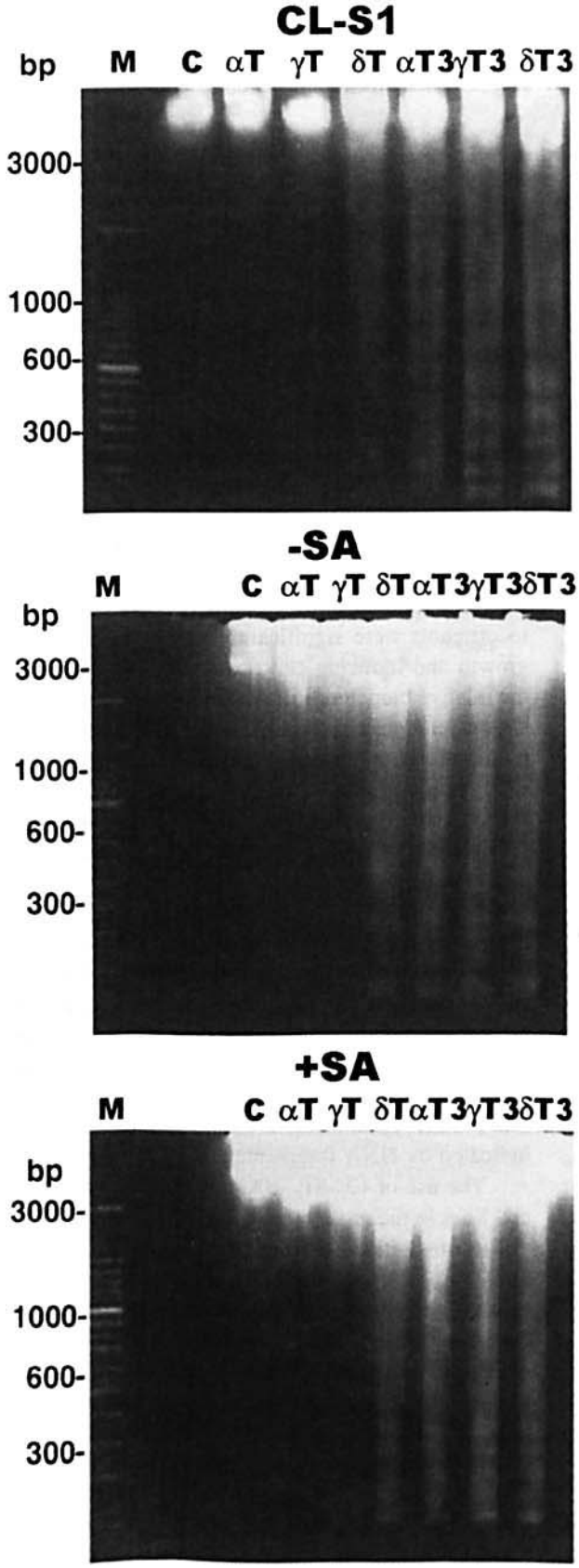


Figure 6. Preneoplastic (CL-S1), neoplastic (-SA), and highly malignant (+SA) mammary epithelial cell internucleosomal DNA fragmentation following a 24-hr exposure period to the following: Control medium (Lane C); 120 μ*M* α-tocopherol (Lane αT); 120 μ*M* γ-tocopherol (Lane γT); 55, 47, and 23 μ*M* δ-tocopherol (Lane δT, CL-S1, -SA and +SA, respectively); 12, 7, and 5 μ*M* α-tocotrienol (Lane αT^3, CL-S1, -SA, and +SA, respectively); 8, 5, and 4 μ*M* γ-tocotrienol (Lane γT^3, CL-S1, -SA and +SA, respectively); or 7, 4, and 3 μ*M* δ-tocotrienol (Lane δT^3, CL-S1, -SA and +SA, respectively). Lane M contains DNA base pair (bp) laddering markers.

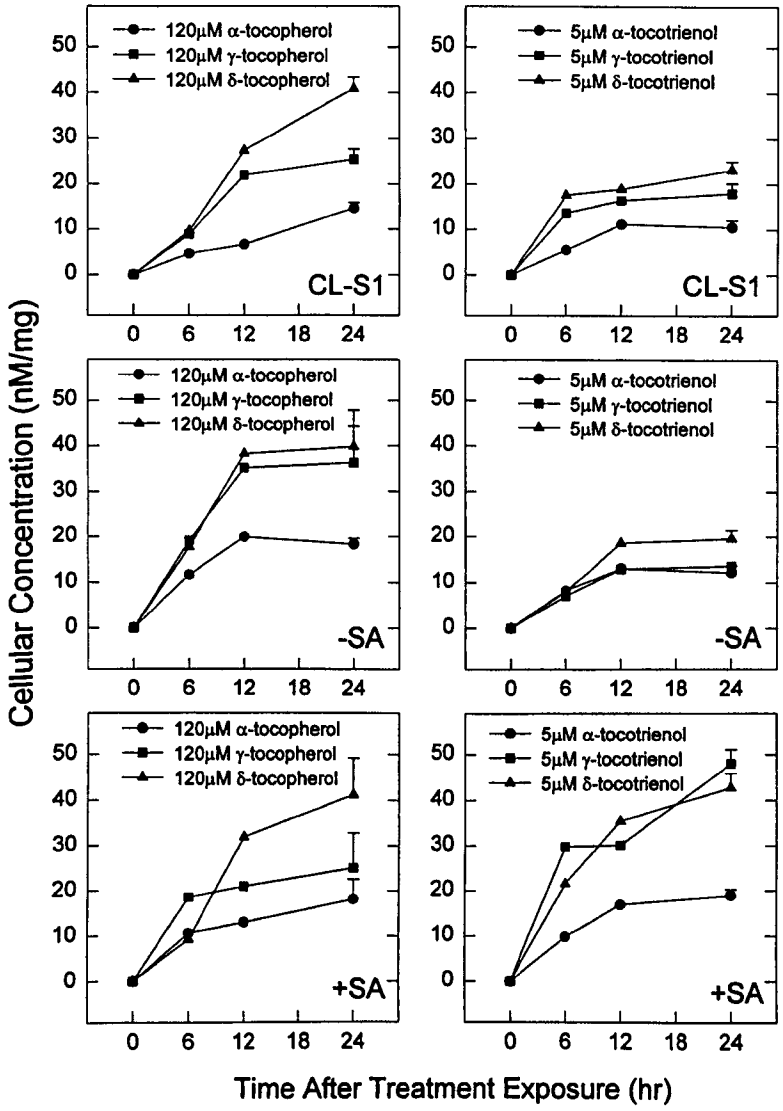


Figure 7. Cellular accumulation of individual tocopherol and tocotrienol isoforms in preneoplastic (CL-S1), neoplastic (-SA), and highly malignant (+SA) mammary epithelial cells during a 24-hr exposure to 120 μ*M* α-, γ-, or δ-tocopherol, or 5 μ*M* α-, γ-, or δ-tocotrienol. Data points indicate mean cellular concentration ± SEM for four replicates in each treatment group containing 1×10^7 cells per each time point.

physiological significance of these findings is unclear. The present findings clearly demonstrate and contrast the very high relative biopotency of tocotrienols versus tocopherols in reducing mammary tumor cell growth and viability.

Tocopherols and tocotrienols have been shown to inhibit several mitogenic signaling pathways, including protein kinase C, adenylate cyclase, and cyclic AMP-dependent protein activation in other cell types, and it is possible that one or more of these effects may be responsible for mediating the inhibitory effects of these compounds on CL-S1, -SA, and +SA mammary epithelial cell proliferation (11, 32–36). Similarly, multiple signaling pathways have been implicated in mediating tocopherol- and tocotrienol-induced apoptosis (13–15). However, the present results do not provide evidence that the antiproliferative and apoptotic effects of these compounds occur through independent intracellular mechanisms. Studies showed that IC_{50} doses of δ-tocopherol, and α-, γ-, and δ-tocotrienol induced substantially large amounts of DNA fragmentation within 24 hr after exposure. Therefore, it is possible that the growth-inhibitory effects of these compounds reflect an increase in the number of cells undergoing programmed cell death, and do not reflect the inhibition of EGF-dependent mitogenesis. Additional studies are needed to determine whether the antiproliferative and apoptotic effects of δ-tocopherol and α-, γ-, and δ-tocotrienols are mediated by similar or different mechanisms.

One possible explanation for the greater biopotency of tocotrienols versus tocopherols is suggested by the finding that tocotrienols are more easily or preferentially taken up by preneoplastic and neoplastic mammary epithelial cells. Prior to treatment, tocopherol and tocotrienol isoform levels were undetectable in each cell line, reflecting the absence of vitamin E in the culture medium. However, treatment with 120 μ*M* α-, γ-, or δ-tocopherol was required to obtain cellular concentrations similar to those obtained with 5 μ*M* α-, γ-, and δ-tocotrienol in the preneoplastic and neoplastic mammary epithelial cell lines. Since tocotrienols differ from tocopherols in that they contain an unsaturated phytyl chain, the presence of these three double bonds might result

in a less planar molecular conformation that facilitates less restricted transmembrane passage of tocotrienols into the cell, as compared with tocopherols. Since cellular accumulation of tocotrienols was greater than that of tocopherols in each cell line, higher concentrations of tocotrienols would occur at intracellular sites of action, thereby inducing a biological response of greater magnitude. Nevertheless, observations that comparable intracellular levels of α-, γ-, or δ-tocopherol and α-, γ-, or δ-tocotrienol did not elicit similar antiproliferative and cytotoxic effects suggest that specific tocotrienol isoforms are inherently more potent than their corresponding tocopherol isoforms in reducing mitogenic responsiveness and/or inducing apoptosis in these cell lines.

A direct correlation was also observed between the relative biopotency and cellular accumulation of individual tocotrienol isoforms in all three mammary epithelial cell lines, characterized as α > γ > δ. Various tocotrienol isoforms differ according to level of chromane ring methylation; α-tocotrienol is more highly methylated, and has a higher partition coefficient than the γ- and δ-isoforms. Therefore, it is possible that reductions in tocotrienol lipophilicity enhance cellular uptake and biopotency. This suggestion is further supported by observations that less lipophilic derivatives of α-tocopherol, such as α-tocopheryl succinate or hemisuccinate, display significantly greater cellular accumulation and bioactivity than α-tocopherol (30, 37). It has not yet been determined if succinate or other less lipophilic derivatives of tocotrienols also display significantly greater cellular accumulation and biopotency than corresponding naturally occurring α-, γ-, and δ-isoforms.

Although tocotrienols display greater biopotency than tocopherols *in vitro*, absorption and transport of individual tocopherol and tocotrienol isoforms *in vivo* are influenced by selectivity and saturability of specific transfer proteins and transport mechanisms that exhibit significant preference for α-tocopherol (38). Additional studies are required to determine if the potent antiproliferative and apoptotic activities displayed by individual tocotrienol isoforms in culture can be observed in the intact animal. Further studies characterizing intracellular mechanisms responsible for mediating the antiproliferative and apoptotic effects of tocotrienols could also provide essential information necessary for understanding the potential health benefits of these compounds in preventing and/or reducing the risk of breast cancer in women.

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