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Inhibitory effects of different forms of tocopherols, tocopherol phosphates and tocopherol quinones on growth of colon cancer cells

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Abstract

Comparison of growth inhibitory activities of tocopherols and derivatives against colon cancer cells

Tocopherols are the major source of dietary vitamin E. In this study, the growth inhibitory effects of different forms of tocopherols, tocopheryl phosphates (TP) and tocopherol quinones (TQ) on human colon cancer HCT116 and HT29 cells were investigated. δ-T was more active than γ-T in inhibiting colon cancer cell growth, decreasing cancer cell colony formation and inducing apoptosis; however α-T was rather ineffective. Similarly, the rate of cellular uptake also followed the ranking order δ-T > γ-T >> α-T. TP and TQ generally had higher inhibitory activities than their parent compounds. Interestingly, the γ-forms of TP and TQ were more active than the δ-forms in inhibiting cancer cell growth; whereas the α-forms were the least effective. The potencies of γ-TQ and δ-TQ (showing IC₅₀ of ~0.8 and ~2 μM on HCT116 cells after a 72-h incubation, respectively) were >100 and >20 fold higher, respectively, than those of their parent tocopherols. Induction of cancer cell apoptosis by δ-T, γ-TP and γ-TQ was characterized by the cleavage of caspase 3 and PARP1 and DNA fragmentation. These studies demonstrated the higher growth inhibitory activity of δ-T than γ-T, the even higher activities of the γ-forms of TP and TQ, and the ineffectiveness of the α-forms of tocopherol and their metabolites against colon cancer cells.

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Keywords

tocopherol; tocopheryl phosphate; tocopherol quinone; uptake; apoptosis

Introduction

Tocopherols, existing as α -, γ -, δ -, and β -tocopherols (α -, γ -, δ -, and β -T), are the major forms of vitamin E. The structural difference between these forms lies in the methylation pattern on the chromanol ring, with α -T tri-methylated at the 5-, 7- and 8-positions, γ -T di-methylated at the 7- and 8-positions, and δ -T mono-methylated at the 8-position¹. Although these compounds differ by only one or two methyl groups, they have been shown to have distinct metabolic fates and activities¹. The major dietary sources of tocopherols are vegetable oils². Among tocopherols, γ -T is the most abundant tocopherol in human diet, but α -T is present at the highest levels in human blood and tissues¹. Among all the tocopherols, α -T has the highest activity in the classical “fertility restoration assay” and is considered to be the most active form of vitamin E in nutrition¹.

In the past few decades, tocopherols have received much attention for their role in the prevention of cancer. Nevertheless, most of these studies have used only α -T, and the results are inconsistent (reviewed by Ju *et al.*³). Many clinical trials have been conducted to determine the efficacy of α -T in the prevention of human cancer, but most recent results are disappointing. For example, in two recent large scale clinical trials on prostate cancer prevention, high dose of α -tocopheryl acetate did not reduce the risk for prostate or other cancers⁴⁻⁶. These disappointing results reflect our lack of understanding of the biological activities of different forms of tocopherols. There are different interpretations for these disappointing results. One interpretation is that γ -T is a cancer preventive agent, but α -T is not⁷⁻⁹. Our collaborative research group at Rutgers University has demonstrated that a γ -T-rich mixture of tocopherols (γ -TmT) inhibits carcinogenesis in animal models of colon, lung, breast, and prostate cancer¹⁰⁻¹⁶. With regard to the relative activity of different forms of tocopherols, two recently published studies from our group demonstrated that δ -T is more active than γ -T in inhibiting azoxmethane-induced colon carcinogenesis and lung xenograft tumor growth, whereas α -T is not effective^{7,8}.

In addition to the parent tocopherols, the metabolites of tocopherols may also contribute to the overall anti-cancer and anti-inflammatory activities of tocopherols. Several studies have reported that tocopherol metabolites from side-chain oxidation possess interesting anti-cancer activities^{17,18}. However, the activities of metabolites on the chromanol rings, including tocopheryl phosphates (TP) and tocopherol quinones (TQ), have not been systematically studied. α -TP has been reported to induce apoptosis, prevent inflammation and provide cardioprotection¹⁹⁻²¹. It has been suggested that α -TP exerts its effects by modulating the levels of AKT, VEGF, and the cell-membrane level of the scavenger receptor CD36^{20,22}. The mechanisms of the anti-cancer activity of TQ are not well understood. TQ were reported to affect mitochondria functions, form reactive oxygen species and release apoptotic signals²³. γ -TQ also induced apoptosis in cancer cells, possibly through caspase-9 activation and cytochrome c release^{24,25}. γ -TQ, but not α -TQ, induced adaptive response through upregulation of cellular glutathione and cysteine availability via activation of activating transcription factor 4²⁶. However, a systematic comparison of the anti-cancer activities of different forms of tocopherols, TP and TQ is still lacking. In the present study, we examined the growth-inhibitory activities of different forms of tocopherols and their phosphorylated and quinone derivatives in colon cancer and normal cell lines.

Material and Methods

Cell culture

HCT116 and HT29 human colorectal cancer cells, CRL-1831 normal human colon epithelial cells, and INT 407 human intestine epithelial cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). The cells were used at passage 10–30 for the experiments. HCT116 and HT29 cells were maintained in McCoy's 5A medium. INT 407 cells were maintained in DMEM medium. CRL1831 cells were maintained in DMEM/F12 medium containing extra 10 mM HEPES (for a final concentration of 25 mM), 10 ng/ml cholera toxin, 5 µg/ml insulin, 5 µg/ml transferrin, and 100 ng/ml hydrocortisone. The media were supplemented with 10% FBS and 1% of penicillin (10,000 I.U./ml)/streptomycin (10 mg/ml) solution. Media supplemented with 5% FBS were used for all treatments. All cells were maintained in a 37°C humidified incubator with 5% CO₂.

Purification of tocopherols

α-T, γ-T and δ-T were the naturally occurring RRR-forms (d-forms). Teledyne ISCO CombiFlash® Companion® XL Automated Flash Chromatographic System was used for the purification of individual tocopherols. To purify γ-T, 25 g of γ-tocopherol-rich mixture of tocopherols (γ-TmT, Cognis Inc; 1 gram γ-TmT contains 130 mg α-T, 15 mg β-T, 568 mg γ-T, and 243 mg δ-T) was applied to a 1500 g RediSep Rf Gold™ high performance flash silica gel column (20–40 µm in particle size). Different forms of tocopherols were separated by elution with a gradient of 0–5% ethyl acetate in hexane at a flow rate of 250 ml/min over a 150 min period of time. Thin layer chromatography was used to determine the purity of eluted fractions. The fractions containing only γ-T were pooled, and solvents were removed via rotary evaporation. With this procedure, the purity of γ-T was 96%, as determined by HPLC and spectrometry, and the yield was 70%. A similar procedure was used for the purification of δ-T and α-T from the crude material (Sigma, the purity is 90% and 69% for δ-T and α-T, respectively). The final purity of δ-T and α-T reached 99% and 98%, respectively.

Synthesis of TP and TQ

α-TQ and γ-TQ were synthesized by oxidation with FeCl₃ from parent tocopherols as described by Schudel *et al.*²⁷. A FeCl₃ solution (0.2 g in 2.5 ml methanol/water, 50/50, v/v) was added into a solution of tocopherols in diethyl ether (1 g in 10 ml). After 30 min of agitation, the aqueous phase was removed. The tocopherols in the ether phase were reacted with the FeCl₃ solution again four times and then the ether phase was extensively washed with water (10 times). The ether phase was dried and dissolved in hexane. δ-TQ was synthesized by a modification of the AgNO₃ oxidation procedure²⁸. δ-T (130 mg) and AgNO₃ (920 mg) were dissolved in 6 ml of ethanol/water (85:15, v/v), heated at 60–70°C for 30 min, and the products were then extracted with diethyl ether. The resulting δ-TQ was purified on a silica gel column using hexane/ethyl acetate as the elutant. The chromatography was performed with Teledyne ISCO CombiFlash® Companion® XL Automated Flash Chromatographic System and δ-TQ was detected at 260 nm and 292 nm. To synthesize TP, γ-, δ-, or α-T were phosphorylated with P₂O₅ at 90°C. After the reaction, NaOH was added to precipitate inorganic phosphate and to convert the phosphorylated tocopherols to disodium tocopheryl phosphates, which were then converted to tocopheryl phosphates by the addition of HCl²⁹. The resultant products were purified by flash chromatography to produce highly pure γ-, δ-, or α-TP. The purity of all the TP and TQ were examined by HPLC, and only one peak for each compound was observed. All tocopherols and their derivatives were stored at –80°C and dissolved in DMSO before use in experiments.

Cell proliferation assay

HCT116, INT407 and CRL-1831 cells were seeded in 96-well plates at a density of 2×10^3 cells/well in media supplemented with 10% FBS and 1% penicillin/streptomycin. After overnight incubation, cells were treated with tocopherols (5–100 μM), TP (5–100 μM) or TQ (0.5–5 μM) for 48 and 72 h. 0.2% DMSO was used as a negative control. Treatment was performed in six wells for each concentration tested. At the termination of treatment, culture media were removed and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Aldrich) was added to the cells at a concentration of 0.5 mg/ml. After 1 h incubation at 37°C, the MTT reagent was removed and DMSO was used to solubilize the formazan dye formed by viable cells. The absorbance of the dissolved dye was measured at 550 nm and the percentage of viable cells was compared with the non-treatment control.

Colony formation assay

HCT116 cells were seeded in 6-well plates at a density of 100 cells/well. After cells attached, they were treated in triplicate with tocopherols, TP or TQ for 10 days. Once colonies formed in the control wells, cells were stained with 1% crystal violet and colony numbers were counted.

Tocopherol levels in cells and media

HCT116 and HT-29 cells were seeded in 10 cm dishes at a density of 3×10^6 cells/dish and allowed to attach by overnight incubation. The cells were then treated with 100 μM α -T, γ -T, δ -T, and a combination of 100 μM α -T and 100 μM δ -T. After incubation for 3, 6, 12 and 24 h, cells were washed with PBS and trypsinized. The cells collected were analyzed for tocopherol and metabolite levels using HPLC according to a previous method ³⁰.

Flow cytometry

HCT116 cells were seeded in 6-well plates at a density of 8×10^4 cells/well. After overnight incubation, cells were treated with different concentrations of tocopherols, TP and TQ for 12, 24, or 48 h. 0.2% DMSO was used as a negative control, and 2 μM atorvastatin plus 10 μM γ -tocotrienol (γ -T3) was used as a positive control for apoptosis based on our previous report ³¹. The cells were trypsinized, washed, and then stained with FITC-annexin V and propidium iodide (PI) in the Alexa-fluor Dead Cell Apoptosis kit (Invitrogen, Carlsbad, CA). Stained cells were counted using the Coulter Cytomics FC500 Flow Cytometer.

DNA fragmentation

To test for fragmented DNA after tocopherol treatment, HCT116 cells were seeded at 1.5×10^6 cells in a 10 cm dish and then treated with corresponding tocopherols, TP and TQ for 12, 24 and 48 h. DNA was extracted from the cells as described previously ³¹, and resolved by agarose gel electrophoresis.

Western blots

HCT116 cells were treated as described for DNA fragmentation. The cells were collected, and protein was extracted using a total cell lysis buffer (Cell Signaling). Cell lysates were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was then blotted with primary antibodies followed by fluorescently labeled secondary antibodies. The labeled proteins were visualized using the Odyssey infrared imaging system (LI-COR, NE). The primary antibodies against cleaved caspase 3, cleaved PARP and cleaved caspase 9 (Cell Signaling) and β -actin (Sigma) were used with 1:500 and 1:5000 dilution, respectively.

Statistical Analysis

The comparison of means between the treatment groups and the control group was performed with one-way analysis of variance (ANOVA) followed by Dunnett's test. The significance level used for all the tests is 0.05.

Results

Effects of different forms of tocopherols on the growth of HCT116 and HT29 cells

To study the effect of tocopherols on HCT116 cell growth, cells were treated with increasing concentrations of pure tocopherols, γ -TmT, TP and TQ. δ -T significantly reduced the number of viable cells as compared to the control (IC_{50} of $\sim 45 \mu M$), as shown in the MTT assay after treatment for 72 h (Fig. 1A). However, α - and γ -T were not as effective ($IC_{50} > 100 \mu M$). The inhibitory activity of γ -TmT was between those of δ -T and γ -T (at $100 \mu M$). δ -T, the most effective form of tocopherol, was more effective in inhibiting cancer cells than in inhibiting normal colon (CRL-1831) and intestinal (INT 407) cells. The percentage of viable cells, when treated with $100 \mu M$ δ -T for 48 h, was 41% for HCT116 cells, as compared to 82% and 89% for INT407 and CRL-1831 cells, respectively (Fig. 1B).

When the effects of TP and TQ treatments in HCT116 cells were examined, the γ -forms of TP and TQ (IC_{50} of 30 and $0.8 \mu M$, respectively) were found to be more effective than the corresponding δ -forms (IC_{50} 55 and $2 \mu M$ for δ -TP and δ -TQ, respectively) (Fig. 1C and D). γ -TP were much more active than γ -T, while activity of δ -TP was comparable to that of δ -T. γ -TQ had the most potent inhibitory activity of all the compounds examined, and the activities of γ -TQ and δ -TQ were much higher than the corresponding TP and tocopherols. The α -forms of TP and TQ were the least inhibitory, but their activities were still higher than α -T. Similar results for tocopherol and TP treatments were observed with the HT29 cells, but they are not as susceptible to the inhibition as the HCT116 cells (data not shown). Therefore, subsequent experiments were performed mainly with HCT116 cells.

Effects of tocopherol treatment on the clonogenic potential of colon cancer cells

Colony formation assays were performed to determine the effect of tocopherols and their derivatives on the clonogenic potential of HCT116 cells. The result of a typical colony formation assay is shown in Fig. 2A. The colony size was decreased with increasing concentrations of the test compound, suggesting the inhibition of cell division (Fig. 2A). The ranking orders of the inhibitory potencies for tocopherols, TP and TQ were similar to those of MTT assays. Among the tocopherols, δ -T was most active, with an IC_{50} of $20 \mu M$, whereas the IC_{50} for γ -T was about $30 \mu M$ and the IC_{50} for α -T was above $100 \mu M$ (Fig. 2B). The activity of γ -TP (IC_{50} of $\sim 20 \mu M$) was more active than that of δ -TP (IC_{50} of $\sim 40 \mu M$), and α -TP was ineffective ($IC_{50} > 100 \mu M$) (Fig. 2C). TQ exhibited the most effective inhibition of colony formation among the compounds examined, with estimated IC_{50} values of $1 \mu M$, $2 \mu M$ and $8.5 \mu M$ for γ -TQ, δ -TQ and α -TQ, respectively (Fig. 2D).

Cellular uptake of different forms of tocopherol

α -T, γ -T or δ -T ($100 \mu M$) was added to HCT116 cells and the uptake of tocopherols was analyzed by HPLC (Fig. 3A). Tocopherol levels in the media did not change significantly during a 24-h incubation period (data not shown). The cellular uptake of tocopherols increased with time for all tocopherols (Fig. 3). The cellular level of δ -T after a 24-h treatment ($7.9 \text{ nmol/million cells}$) was 2-fold higher than γ -T, and more than 6-fold higher than α -T (Fig. 3A). A similar pattern in cellular tocopherol uptake was also observed in HT29 cells; after a 24-h incubation, the cellular level of δ -T was 1.6- or 6.7-fold higher than that of γ -T or α -T, respectively, while the absolute level in HT29 cells of each tocopherol

was lower than in HCT116 cells (Fig. 3B). After incubation with tocopherols, their metabolites such as phosphates and quinones, were not detected in the cells.

Induction of cell apoptosis by tocopherols, TP and TQ

The induction of apoptosis was examined using annexin V/PI staining followed by flow cytometry. Treatment with δ -T (50 and 100 μ M) for 48 h led to a dose-dependent increase in the percentages of cells in early and late apoptosis as demonstrated by annexin V positive and annexin V plus PI double positive cells, respectively (Fig. 4A). γ -T also induced apoptosis, but was less effective than δ -T. α -T, however, had no significant effect at 50 and 100 μ M (data not shown). Treatment of cells with γ -TP (50 and 100 μ M) for 24 h induced apoptosis, whereas the α - and δ -forms did not have significant effects at similar concentrations (Fig. 4B). γ - and δ -TQ were effective in inducing apoptosis at much lower concentrations (3 and 6 μ M) in a dose-dependent manner after a 24-h incubation, but α -TQ did not significantly induce apoptosis (Fig. 4C). The ranking orders of effectiveness for tocopherols, TP and TQ are the same as the results from the MTT and clonogenic assays (Fig. 1 and 2)

To further investigate the pro-apoptotic effects of tocopherols and their derivatives, signaling proteins known to be involved in apoptosis were analyzed by Western blotting. The cleavage of caspase 3, caspase 9 and poly(ADP-ribose) polymerase 1 (PARP1) was observed with δ -T treatment, peaking at 24 h (Fig. 5A). Similarly, γ -TP and γ -TQ induced cleavage of caspase 3 and PARP1 (Fig. 5A). DNA fragmentation in cells was also detected for δ -T, γ -TP and γ -TQ (Fig. 5B), supporting the occurrence of apoptosis. Taken together, the data suggest that δ -T, γ -TP and γ -TQ induced cell death through the induction of apoptosis.

Discussion

In this study, we demonstrated that δ -T has stronger activity than γ -T, and that α -T is rather ineffective, in the inhibition of colon cancer cell growth and colony formation. It is interesting that the levels of cellular uptake also follow the ranking order of δ -T > γ -T > α -T (Fig. 4). The molecular basis for this observation is unknown, but it may suggest that the difference in cellular uptake contributed to the relative potencies of tocopherols in inhibiting cell growth. Cancer cells (HCT116) were more susceptible than normal cells (human intestinal INT407 cells and human colon CRL-1831 cells) to the inhibition of δ -T (Fig. 1B) and perhaps of γ -T. These results agree with previous reports in that γ -T is more effective than α -T in inhibiting the growth of colon cancer cells³² and that δ -T is more effective in inhibiting the growth of neoplastic than preneoplastic (or normal) mouse mammary epithelial cells^{33,34}. Overall, this result agrees with our recent studies that demonstrated that δ -T is the most effective tocopherol in inhibiting the growth of H1299 lung cancer cell xenograft tumors⁸ and the formation of aberrant crypt foci (ACF) in an azoxymethane (AOM)-induced rat model⁷.

TP and TQ are more active than their parent tocopherols; γ -TQ is the most active compound studied in these experiments. Upon comparing the activities of TP and TQ, we found that the γ -forms of both TP and TQ were more active than their δ -form counterparts in inhibiting cell growth. Their relative activities were different from their parent tocopherols in that δ -T was more active than γ -T. For all tocopherols and derivatives tested, the α -forms had little to no activity.

α -T is phosphorylated by yet unidentified kinases to α -TP, which has been detected and extracted from animal and human tissues²⁹. α -TP is converted to α -T in cultured cells and in mice, although it is still not clear which phosphatase is responsible for this process³⁵.

Intact α -TP has been proposed to function as a signaling messenger; for example, it reduces the production of proinflammatory cytokines³⁶. The studies of other forms of TP are rather limited. Our results suggest that γ -TP is more potent than δ -TP and α -TP in inhibiting cancer cell growth.

The formation and distribution of TQ in animal and human tissues is not well-documented, but α -TQ has been detected in vegetables³⁷. The conversion of α -T to α -TQ has been observed during food storage and processing^{38,39}. American diets are usually more abundant in γ -T⁴⁰. It may be converted to γ -TQ in the human body, and this topic remains to be investigated. Our laboratory recently detected TQ in our experimental diet and animal tissues. For example, the AIN76A diet contains α -TQ at 0.185 nmol/g (0.1% of α -T) and no detectable amount of other TQ. Upon feeding this diet to mice for 10 days, the levels of α -TQ, γ -TQ, and δ -TQ in lung tissues were 0.435, 0.010, and 0.011 nmol/g, respectively. The level of 0.435 nmol/g was approximately 5% of α -T level in the same sample. Apparently, a small percentage of tocopherols are converted to TQs in animals.

The induction of apoptosis by δ -T, γ -TP and γ -TQ was demonstrated by flow cytometry, the cleavage of caspase 3 and PARP1, and DNA fragmentation. These changes are likely to be a consequence of the reduction of mitochondrial membrane potential and release of mitochondria cytochrome c, which subsequently activates caspase 9 and then caspase 3²⁴. However, the existence of other cell death mechanisms, such as necrosis, cannot be excluded.

The current study demonstrated the growth inhibitory activities of tocopherols against colon cancer cells, particularly the higher cellular uptake and more potent inhibitory effect of δ -T than other tocopherols. We also reported, for the first time, the activities of different forms of TP and TQ in inhibiting colon cancer cell growth, preventing colony formation and inducing apoptosis. Even though the effective inhibitory concentrations of these compounds are higher than those found *in vivo*, these findings contribute to the body of knowledge on the biological activities of tocopherols and their derivatives.

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Abbreviation

α-T, γ-T and δ-T	α -, γ -, and δ -tocopherol
γ-TmT	a γ -T-rich mixture of tocopherols
TP	tocopheryl phosphate
TQ	tocopherol quinone
MTT	3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
IC₅₀	inhibitory concentration that causes 50% inhibition
PARP1	poly[ADP-ribose]polymerase 1

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Fig. 1.

Effect of tocopherols, TP and TQ on the growth of colon cancer and intestinal cells. Cells were seeded in 96-well plates at a density of 2×10^3 cells per well in a growth medium. After overnight incubation, HCT116 cells were treated with different concentrations of α -T, γ -T, δ -T, and γ -TmT (A), TP (C), and TQ (D) for 72 h. Effects of δ -T on different types of cells were also analyzed (B). Viable cells were analyzed using the MTT assay by measuring the absorbance at 550 nm, and the results are shown as percent viable cells. The values are mean $\pm$ S.E. (n=6). ANOVA-Dunnnett's test was conducted, and significant differences among the treatment groups at a specific concentration are designated with different letters.

Fig. 2.

Effect of tocopherols, TP and TQ on clonogenic potential. HCT116 cells were seeded in a 6-well plate at a density of 100 cells per well. After cell attachment, the media were replaced with fresh media containing tocopherols (B), TP (C) or TQ (D). After incubation for 10 days, the cells were stained with 1% crystal violet and colony numbers were counted. Representative colony formation plates are also shown (A). The number of colonies was shown as a percentage of control wells. The data are presented as mean $\pm$ S.E. (n=3).

Fig. 3.

Cellular uptake of tocopherols in colon cancer cells. HCT116 and HT-29 cells were seeded in 10 cm dishes at a density of 3×10^6 cells/dish and allowed to attach overnight. The cells were then incubated with 100 μ M α -T, γ -T, or δ -T. At the time periods indicated, cells were washed and harvested by trypsinization. The levels of tocopherols in the cells were measured by HPLC. The tocopherol levels in HCT116 (A) and in HT29 (B) cells are shown as nmol/million cells. The values are means from duplicate analyses with error bar (A) or means $\pm$ S.E. ($n=3$) (B). ANOVA-Dunnnett's test was conducted, and significant differences among the different tocopherol groups are designated with different letters (B).

Fig. 4.

The induction of apoptosis by tocopherols, TP and TQ in HCT116 cells detected by annexin V and PI staining followed by flow cytometry. HCT116 cells were seeded in 6-well plates at a density of 8×10^4 cells/well. After overnight incubation, cells were treated with different concentrations of γ -T and δ -T (A), TP (B) and TQ (C). After incubation for the indicated time periods (A) or 24 h (B and C), the cells were trypsinized, washed, and then stained with FITC-annexin V and PI. Stained cells were then counted with the flow cytometer. The number of cells in each stage of apoptosis is shown as a percentage of total cells gated. The data are presented as mean $\pm$ S.E. (n=3). ANOVA-Dunnnett's test was conducted. A significant difference between the treatment group and the control group is designated with an asterisk.

Fig. 5. The induction of apoptosis by δ -tocopherol, γ -TP and γ -TQ as determined by the changes in caspase 3, PARP, and caspase 9 (A) and DNA fragmentation (B). HCT116 cells were seeded at 1.5×10^6 cells in a 10 cm dish and then treated with each compound for the indicated time periods. Western blot analysis was performed on cell lysates with antibodies against cleaved caspase 3, cleaved PARP, cleaved caspase 9 and β -actin. DNA was extracted from these cells and resolved by agarose gel electrophoresis. The results are from one experiment; similar results were obtained in another experiment.