Basic nutritional investigation

Inhibition of proliferation and induction of apoptosis by γ-tocotrienol in human colon carcinoma HT-29 cells

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Objective: γ-Tocotrienol is a major component of the tocotrienol-rich fraction of palm oil, but there is limited evidence that it has antitumor activity. In particular, the effects of γ-tocotrienol on human colon carcinoma cells have not been reported. To investigate the chemopreventive effects of γ-tocotrienol on colon cancer, we examined its capacity to inhibit proliferation and induce apoptosis in HT-29 cells and explored the mechanism underlying these effects.

Methods: We cultured HT-29 cells in the presence of γ-tocotrienol. The effect of γ-tocotrienol on cell proliferation was investigated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, mitotic index, and colony formation. The cell-cycle distribution was investigated by flow cytometry. We measured apoptosis by nuclear staining, transmission electron microscopy, and DNA fragmentation. Apoptosis-related proteins and the nuclear factor-κB p65 protein were determined by western blotting and immunofluorescence.

Results: γ-Tocotrienol inhibited cell growth and arrested HT-29 cells in G0/G1 phase. The 50% inhibitory concentration was 31.7 μmol/L (48 h). γ-Tocotrienol–induced apoptosis in HT-29 cells was accompanied by downregulation of Bcl-2, upregulation of Bax, and activation of caspase-3. Furthermore, we found that γ-tocotrienol reduced the expression level of total nuclear factor-κB p65 protein and inhibited its nuclear translocation.

Conclusion: The results indicated that γ-tocotrienol inhibits cell proliferation and induces apoptosis in HT-29 cells in a time- and dose-dependent manner, and that this process is accompanied by cell-cycle arrest at G0/G1, an increased Bax/Bcl-2 ratio, and activation of caspase-3. Our data also indicated that nuclear factor-κB p65 protein may be involved in these effects.

Keywords: γ-Tocotrienol; Cell proliferation; Cell cycle; Apoptosis; Colon cancer cells
exist in four different forms or isomers, i.e., \( \alpha \)-, \( \beta \)-, \( \gamma \)-, and \( \delta \)-tocotrienol, which contain different numbers of methyl groups on the chromanol ring. Although all the isomers are effective antioxidants because a hydrogen atom from the hydroxyl group on the chromanol ring can readily be donated to reduce free radicals, each has its own biological activity. In particular, \( \gamma \)-tocotrienol is one of the most abundant forms of tocotrienol in foods [7]. Furthermore, various studies have indicated that \( \gamma \)-tocotrienol has significant anticancer activity [14–17]. In vivo, dietary \( \gamma \)-tocotrienol suppressed murine melanoma growth and increased host survival time [7]. In vitro, \( \gamma \)-tocotrienol proved cytotoxic to various human tumor cell lines [18–20] but had no toxic effect on the proliferation of normal cells [21,22]. For instance, it inhibits proliferation and induces apoptosis in MDA-MB-231 [17] and Hep3B [19] cells. Recent results from our laboratory have demonstrated that \( \gamma \)-tocotrienol induces apoptosis and metastasis in the human gastric adenocarcinoma cell line SGC-7901 by downregulation of the extracellular signal-regulated kinase signaling pathway [14,20]. However, details of the mechanism by which \( \gamma \)-tocotrienol inhibits proliferation and induces apoptosis in tumor cell lines remain unclear.

Colon carcinoma is a serious health problem and one of the leading causes of cancer mortality worldwide, especially in developed countries [23]. Chemoprevention is a major strategy in cancer prevention, because therapies have not proved effective to date in controlling the high incidence or the low survival rate of human colon carcinoma [24]. Dietary factors that inhibit cell proliferation are an exciting prospect for cancer prevention and treatment. A 4-y study provided convincing data that a high vitamin E intake was particularly for women younger than 65 y [25]. It has been reported recently that the TRF has antiproliferative effects and induces apoptosis in human colon carcinoma RKO cells [8]. However, the effects of individual TRF components on human colon carcinoma cell proliferation and the possible mechanisms involved remain unclear. \( \gamma \)-Tocotrienol has potent biological and pharmacologic activities. The objectives of our present study were to evaluate the effects of \( \gamma \)-tocotrienol on proliferation and apoptosis in HT-29 cells and to investigate the underlying molecular mechanism. Our results suggest that a possible molecular mechanism involves the suppression of nuclear factor-\( \kappa \)B (NF-\( \kappa \)B) p65 protein expression and its nuclear translocation, resulting in a direct effect on cell-cycle progression and activation of the proapoptotic pathway.

### Materials and methods

#### Materials

The human colon carcinoma HT-29 cell line was obtained from the Cancer Institute of the Chinese Academy of Medical Science. The Cycle Test PLUS DNA reagent kit was bought from Becton-Dickinson (Franklin Lanes, NJ, USA). \( \gamma \)-Tocotrienol was from Davos (Singapore). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethylsulfoxide were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Giemsa stain was purchased from Amresco (USA). Rabbit polyclonal antibodies for \( \beta \)-actin (sc-1616-R), Bel-2 (sc-492), caspase-3 (sc-7148), and NF-\( \kappa \)B p65 (sc-372) were bought from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal antibody for Bax (sc-7480) was obtained from Santa Cruz Biotechnology. Goat anti-rabbit (w3960) and anti-mouse (w3950) secondary antibodies were purchased from Promega (Madison, WI, USA). Fluorescein isothiocyanate–conjugated fluorescent secondary antibody was purchased from Santa Cruz Biotechnology.

#### Cell culture

Human colon carcinoma HT-29 cells were maintained in RPMI-1640 medium (Gibco, Paisley, Scotland) in 75-cm² flasks at 37°C in a 5% CO₂ atmosphere at constant humidity. The medium was supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco), 2 mmol/L of L-glutamine (Gibco), and 1% antibiotic solution (Gibco) and was changed every other day. The same dose of ethanol vehicle was used in the control cell culture. For subculturing, cells were rinsed once with phosphate buffered saline (PBS) and incubated in 0.25% trypsin containing 0.02% ethylene-diaminetetra-acetic acid (EDTA; Gibco) in PBS for 3 min. For the \( \gamma \)-tocotrienol supplementation experiment, stock solutions of \( \gamma \)-tocotrienol were prepared in absolute ethanol and stored at −20°C. The final ethanol concentration in all cultures was 0.15% [15].

#### Cell viability

The effect of \( \gamma \)-tocotrienol on cell viability was determined by an MTT assay. Briefly, cells were seeded in 96-well microtiter plates (Nunc, Wiesbaden, Germany) at 1.0 × 10⁴ per well. After 24 h of incubation, the medium was removed and the cells were treated with 200 \( \mu \)L of medium containing various concentrations (15, 30, 45, and 60 \( \mu \)mol/L) of \( \gamma \)-tocotrienol for the desired time. Control cells were supplemented with 0.15% ethanol vehicle. Each concentration of \( \gamma \)-tocotrienol was repeated in five wells. Twenty microliters of MTT (5 mg/mL in PBS) was added to each well and incubated at 37°C for 4 h. The medium was carefully removed and 150 \( \mu \)L of dimethylsulfoxide was added to each well. The plates were shaken for 10 min and the absorbance at 490 nm was measured in an ELx800 Universal microplate reader (Bio-Tek Instruments, Inc., USA). Growth inhibition by \( \gamma \)-tocotrienol was calculated as percentage of cell viability, taking the viability of the vehicle-treated cells as 100%.
Mitotic index

The HT-29 cells were seeded in 24-well plates at 1.0 × 10^5 cells/well and incubated for 24 h to allow the cells to attach the bottom of plate. The cells were treated with 15, 30, 45, and 60 μmol/L of γ-tocotrienol for 24 or 48 h. After incubation, the cells were fixed with methanol and stained with 1% Giemsa. The number of cells in mitosis was counted among 2000 cells under an inverted microscope (Olympus CK40, Japan). The mitotic index was calculated using the equation:

mitotic index = number of mitotic cells/total number of cells × 100%

Colony formation

The assay of colony formation was conducted as previously described with some modifications [26]. Briefly, exponentially growing cells were seeded at 400 cells/well in six-well plates and allowed to attach for 36 h. The medium was removed and the cells were treated with 15, 30, 45 and 60 μmol/L of γ-tocotrienol. After 24 or 48 h, the media containing γ-tocotrienol were discarded, the cells were washed three times with PBS, and fresh medium was added to each well. The plates were incubated for 12 d under the same conditions used for culturing, and then the cells were fixed with methanol and stained with Giemsa. Colonies, containing more than 50 cells originating from single cells, were counted under the inverted microscope (Olympus CK40, Japan).

Cell-cycle analysis

The HT-29 cells were harvested, washed three times with cool PBS, fixed with 70% cold ethanol for 2 h, and stained with propidium iodide (Cycle Test Plus DNA Reagent Kit). For each concentration, at least 2.5 × 10^4 cells were analyzed by FAC Sort flow cytometry (BD Biosciences, USA). The proportions in G0/G1, S, and G2/M phases were estimated using ModFit LT analysis software.

Detection of DNA fragmentation

DNA fragmentation was determined by agarose gel electrophoresis as described previously, with some modifications [27,28]. Briefly, cells were treated with various concentrations of γ-tocotrienol (15, 30, 45, or 60 μmol/L) for the desired time and then washed twice with PBS. Total DNA was isolated using a Wizard Genomic DNA Purification Kit (Promega). DNA agarose electrophoresis was executed at 50 V on a 1.2% agarose gel in 1× TAE buffer (40 mmol/L of Tris, 2 mmol/L of EDTA, 20 mmol/L of acetic acid). DNA ladder markers (100 bp; Promega) were added to each gel as a reference for the analysis of internucleosomal DNA fragmentation. The gel was stained with ethidium bromide (20 μg/mL) and photographed under ultraviolet illumination.

Western blotting

The HT-29 cells were treated with various concentrations of γ-tocotrienol for 48 h and then collected, washed twice with PBS, and detached in PBS containing 0.02% EDTA. Whole cells from the different treatment groups were lysed in 20 mmol/L of Tris-HCl, pH 7.5, 2% sodium dodecylsulfate (w/v), 2 mmol/L of benzamidine, and 0.2 mmol/L of phenyl-methane-sulfonyl fluoride. The protein concentrations in each sample were determined using a nucleic acid and protein analyzer (DUw 640; Beckman Coulter, Inc., Fullerton, CA, USA) according to the manufacturer’s directions. For western blotting, 50–80 mg of protein was resolved on 12% polyacrylamide gels and transferred to a nitrocellulose membrane. After blocking with 1% bovine serum albumin, 0.1% Tween 20 in 20 mmol/L of Tris-buffered saline (TBST), pH 7.6, for 1 h at 37°C in a hybridization oven (Amersham Life Science, Little Chalfont, Bucks, United Kingdom), the membrane was incubated with appropriate monoclonal or polyclonal primary antibodies in blocking buffer for 2 h at 37°C or overnight at 4°C. The membrane was washed 3 × 5 min with TBST followed by incubation with anti-mouse or anti-rabbit secondary antibody at 37°C for 1 h. The membrane was washed 2 × 5 min with TBST and once with TBS and then incubated with alkaline phosphatase until an appropriate signal level was obtained. Protein bands were detected by FluorChem Imaging Systems (Alpha Innotech, Corp., San Leandro, CA, USA).
Immunofluorescent detection of NF-κB p65

The HT-29 cells were seeded onto glass coverslips placed in six-well plates (Nunc) and incubated overnight. Then the media were removed, and fresh medium containing 30 or 60 μmol/L of γ-tocotrienol or ethanol as vehicle control was added to each well. After 48 h, the cells were washed with PBS and fixed in methanol for 4 min. After repeated washings with PBS, the coverslips were incubated for 30 min at room temperature with rabbit anti–NF-κB p65 antibody and then incubated with a fluorescein isothiocyanate–conjugated secondary antibody. Nuclei were counterstained with 4',6-diamidine-2'-phenylindole dihydrochloride (Roche). Slides were analyzed by confocal microscopy (TE2000U, Nikon, Japan) and digitally photographed. Each group of four different fields of 150–300 cells was counted, and the ratio of NF-κB p65–positive nuclei to 4',6-diamidine-2'-phenylindole dihydrochloride–stained nuclei was evaluated.

Statistical analysis

Statistical analysis was performed using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). The data were expressed as mean ± SD. Differences between the control and treated groups were evaluated by Student’s t test and P < 0.05 was considered statistically significant.

Results

Effect of γ-tocotrienol on growth of HT-29 cells

The effects of γ-tocotrienol on the viability of HT-29 cells are shown in Figure 1. After treatment with 15, 30, 45, and 60 μmol/L of γ-tocotrienol for 48 h, cell viability decreased by 26.8%, 52.3%, 64.2%, and 68.4% (Fig. 1A), respectively. The viability of cells cultured in 30 μmol/L of γ-tocotrienol for 24, 48, 72, and 96 h decreased with time (Fig. 1B) compared with the control group. These results showed that γ-tocotrienol inhibits the growth of HT-29 cells in a dose- and time-dependent manner. The 50% inhibitory concentration, the dose of γ-tocotrienol required to inhibit or kill 50% of the cells tested, was 31.7 ± 1.6 μmol/L at 48 h.

Effect of γ-tocotrienol on mitotic index of HT-29 cells

The effect of γ-tocotrienol treatment on the mitotic index of HT-29 cells is presented in Table 1. The mitotic index was decreased in comparison with the control group as the time and the γ-tocotrienol concentration increased. The lowest mitotic index was observed in HT-29 cells supplemented with 60 μmol/L of γ-tocotrienol (Table 1). The inhibitions (percentages) of mitosis were 3.8–26.3% at 24 h and 17.1–68.4% at 48 h.

Effect of γ-tocotrienol on colony formation in HT-29 cells

γ-Tocotrienol decreased colony formation by HT-29 cells compared with controls supplemented with ethanol (Fig. 2). Inhibition ranged from 22.8% to 85.3% at 24 h and from 38.9% to 93.2% at 48 h with different concentrations

Table 1

<table>
<thead>
<tr>
<th>Groups (μM)</th>
<th>No. of cancer cells in mitosis (mean ± SD)</th>
<th>Mitotic index (%)</th>
<th>Inhibition (%)</th>
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<tr>
<td></td>
<td>24 h</td>
<td>48 h</td>
<td>24 h</td>
</tr>
<tr>
<td>0</td>
<td>426 ± 2.1</td>
<td>620 ± 4.1</td>
<td>21.3</td>
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<tr>
<td>15</td>
<td>410 ± 3.4</td>
<td>514 ± 3.2</td>
<td>20.5</td>
</tr>
<tr>
<td>30</td>
<td>392 ± 2.5</td>
<td>388 ± 2.4*</td>
<td>19.6</td>
</tr>
<tr>
<td>45</td>
<td>350 ± 3.7*</td>
<td>248 ± 2.2*</td>
<td>17.5</td>
</tr>
<tr>
<td>60</td>
<td>314 ± 4.7*</td>
<td>222 ± 3.1*</td>
<td>1.0</td>
</tr>
</tbody>
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* P < 0.05 versus control group.
of γ-tocotrienol. These results demonstrated that γ-tocotrienol significantly inhibited colony formation in HT-29 cells in a dose-dependent manner (P < 0.05).

g-γ-Tocotrienol induces cell-cycle arrest in HT-29 cells

HT-29 cells supplemented with 60 μmol/L of γ-tocotrienol showed strong G1 arrest at 36 and 48 h, although no changes were observed after 12 or 24 h. The proportion in G1 phase changed from 60.1% and 59.2% in untreated cells to 68.1% and 74.7% in treated cells at 36 and 48 h, respectively. γ-Tocotrienol arrested the cell cycle of HT-29 cells in a time-dependent manner (P < 0.05); the percentage of cells in the S phase decreased (Fig. 3A–C). As shown in Figure 3D, HT-29 cells treated with 15, 30, and 60 μmol/L of γ-tocotrienol for 48 h resulted in a significant increase of the proportion in G1/G0 phase and a decrease of the proportion S phase in a dose dependent manner (P < 0.05).

Effect of γ-tocotrienol on HT-29 cell morphology

After treatment with 60 μmol/L of γ-tocotrienol for 48 h, HT-29 cells began to show shrinkage, rounding, and fragmentation when compared with the control cells (Fig. 4A). Morphologic changes in the nuclei were observed by Hoechst 33258 (Fig. 4B) and acridine orange/ethidium bromide (Fig. 4C) staining. Typical morphologic changes of apoptosis including chromatin condensation, nuclear fragmentation, and apoptotic body formation appeared in HT-29 cells treated with 60 μmol/L of γ-tocotrienol for 48 h. These ultrastructural changes are characteristic of apoptosis and further details are shown in Figure 5. Chromatin condensation, irregular nuclear envelopes, and vacuoles in the cytoplasm were observed by transmission electron microscopy (Fig. 5B–D). Similarly, the characteristic morphology of apoptosis was also observed in those cells treated with 30 μmol/L of γ-tocotrienol. Cells supplemented with ethanol vehicle showed normal morphology with randomly distributed organelles and nuclei with finely granular and uniformly dispersed chromatin and a single large electron-dense nucleolus (Fig. 5A).

DNA fragmentation

The effect of γ-tocotrienol on apoptosis in HT-29 cells was further assessed by a DNA fragmentation assay. As shown in Figure 6, clear ladders appeared after treatment with 45 and 60 μmol/L of γ-tocotrienol for 48 h and after treatment with 60 μmol/L for 48 h and 72 h, whereas no DNA ladders appeared in the control group or in cells supplemented with 15 and 30 μmol/L of γ-tocotrienol for 48 h or with 60 μmol/L of γ-tocotrienol for 24 h. Consistent with our previous observations of cellular morphology, the DNA fragmentation assays demonstrated that γ-tocotrienol induces apoptosis in HT-29 cells.

Western blot analysis of Bax, Bcl-2, and caspase-3 expression

Bcl-2 family proteins are required for their functions as inhibitors (e.g., Bcl-2, Bcl-XL, and MCL-1) or promoting factors (e.g., Bax, Bcl-XS, Bad, and Bak) of cell death. Bcl-2 proteins can interact with each other and subsequently modulate apoptosis. The proapoptotic protein Bax can heterodimerize with the antiapoptotic protein Bcl-2 and homodimerize with itself. The balance between the respective dimers (Bcl-2/Bcl-2, Bcl-2/Bax, and Bax/Bax) determines if apoptosis is suppressed or induced. Overexpressed Bax protein can bind competitively with Bcl-2 protein to form Bcl-2/Bax heterodimers, and the amount of Bcl-2/Bcl-2 homodimers reduced, at the same time, the amount of Bax/Bax homodimers increased [29,30]. We examined the effect of γ-tocotrienol on expression levels of the apoptosis-regulating proteins Bcl-2 and Bax. As shown in Figure 7, the expression levels of Bax and Bcl-2 of HT-29 cells treated with 15 μmol/L of γ-tocotrienol for 48 h did not change in comparison with the negative control. There were significant differences in the expressions of Bax and Bcl-2 between HT-29 cells treated with 45 and 60 μmol/L of γ-tocotrienol when compared with the control group (P < 0.05). Thus, the results indicated that γ-tocotrienol–mediated apoptosis in HT-29 cells may depend on the overexpression of Bax and suppression of Bcl-2.

In response to apoptotic stimuli, procaspase-3 becomes cleaved into a 20-kDa fragment, and the subsequent autocatalytic reaction leads to the formation of the active 17-kDa fragment [31,32]. To obtain direct evidence for the relation between caspase activation and
apoptosis, procaspase-3 cleavage was examined in HT-29 cells after γ-tocotrienol treatment. As shown in Figure 8, 45 or 60 μmol/L of γ-tocotrienol induced the cleavage of 32-kDa procaspase-3 to its active form. These results suggested that Bcl-2 family proteins participate in the induction of apoptosis by γ-tocotrienol in HT-29 cells.

**Effect of γ-tocotrienol on NF-κB p65 in HT-29 cells**

Rel/NF-κB is constitutively activated in colorectal cancer, where it contributes to the transcriptional activation of a variety of genes involved in proliferation, survival, and chemoresistance of the tumor cells [33]. Therefore, we also tested the effect of γ-tocotrienol on NF-κB p65 expression in HT-29 cells, which express constitutively active NF-κB [34]. HT-29 cells were treated with different concentrations of γ-tocotrienol and then analyzed for NF-κB p65. There was no change in the expression of total NF-κB protein after treatment with 15 μmol/L of γ-tocotrienol in HT-29 cells for 48 h. γ-Tocotrienol (45 or 60 μmol/L) significantly inhibited the constitutive expression of NF-κB in HT-29 cells in comparison with the control group (Fig. 9; P < 0.05).

To determine whether this reduction in total NF-κB p65 might also lead to reduced NF-κB p65 in the nucleus and cytosol, the experimental models (treatment of HT-29 cells with γ-tocotrienol) were examined using an anti–NF-κB p65 antibody. There was a strong nuclear staining of NF-κB p65 in HT-29 cells (Fig. 10A-1). However, no obvious changes were observed in nuclear NF-κB p65 after 48 h of incubation with 15 μmol/L of γ-tocotrienol (data not shown), but there was a significant reduction in nuclear NF-κB p65 appearing after 48 h of incubation with 60 μmol/L of γ-tocotrienol (Fig. 10A-2). Quantification of immunofluorescent signals indicated that 12.3% of HT-29 cell nuclei were positive for NF-κB after supplementation of γ-tocotrienol (Fig. 10B). In contrast, 93.7% p65-positive nuclei were observed in HT-29 cells. The results suggested that the nuclear translocation of NF-κB p65 was blocked by treatment with 60 μmol/L of γ-tocotrienol. Meanwhile, treatment with γ-tocotrienol for 48 h in 15, 30 (data not shown), and 60 μmol/L, respectively, did not significantly affect the cytosolic expression level of NF-κB p65 in HT-29 cells.
Discussion

Chemoprevention by natural compounds from plant or in diets appears to be a practical approach to preventing and treating carcinoma, and it is estimated that diets rich in phytochemicals can reduce the cancer risk by 20% [35,36]. γ-Tocotrienol is one of the most important components of vitamin E, which is enriched in high-lipid plants. Previous studies have shown that γ-tocotrienol in very low doses exerts antiproliferative and apoptotic activities on various types of cancer cells including prostate, breast, stomach, and liver cancers (4–20 μmol/L) [11,14,20,37,38]. Such cytostatic effects could be attained by the ingestion of tocotrienol in the diets of healthy individuals who are at increased risk of developing cancer [9].

In the present study, the results showed that treatments with 30–60 μmol/L of γ-tocotrienol significantly inhibited HT-29 cell proliferation in a dose- and time-dependent manner (P < 0.05). The 50% inhibitory concentration of γ-tocotrienol was estimated to be 31.7 μmol/L (2 d). These results partly support previous observations regarding inhibition of cell proliferation [20,37,38], but the 50% inhibitory concentrations for γ-tocotrienol in our study are higher than those for other cell lines, such as 4–5 μmol/L (5 d) in mammary malignant epithelial cells [11], 20 μmol/L (1 d) in murine melanoma cells [7], and 7.4 μmol/L (3 d) in human hepatocellular carcinoma cells [18]. This disparity may be due to the time of treatment, cell numbers seeded, or varieties of cell line. In the present study, we demonstrated that the mitotic index was decreased compared with the control group as the incubation time and concentration of γ-tocotrienol increased. Moreover, γ-tocotrienol significantly inhibited colony formation in HT-29 cells in a dose-
dependent manner. Accordingly, we found that γ-tocotrienol inhibits proliferation of HT-29 cells.

To test the influence of γ-tocotrienol on HT-29 cell proliferation further, we conducted cell-cycle distribution experiments. Our data showed that γ-tocotrienol affected the cell-cycle distribution in HT-29 cells, resulting in appreciable arrest in the G0/G1 phase and decreased numbers in S phase at 48 h compared with the control group. It has been reported that treatment with 20 μmol/L of γ-tocotrienol for 3 h induces G1-phase arrest in murine melanoma cells [39]. Cell-cycle analysis showed that the TRF (10–40 μg/mL) resulted in dose-dependent G0/G1 phase arrest and sub-G1 accumulation in all three human prostate cancer cell lines but not in normal human prostate epithelial cells [9]. In

Fig. 5. Transmission electron microscopic images. (A) Untreated HT-29 cells (original magnification 4000×). (B) HT-29 cells treated with 30 μmol/L of γ-tocotrienol for 24 h. The treated cells became small and round. The nuclear volume decreased (original magnification 5000×). (C) HT-29 cells were treated with 60 μmol/L of γ-tocotrienol for 24 h. The chromatin became condensed and diffused in the nuclei (original magnification 5000×). (D) HT-29 cells were treated with 60 μmol/L of γ-tocotrienol for 48 h (original magnification 5000×). The chromatin concentrated into masses. Cytoplasmic vacuoles were observed in some cells. The cells showed morphologic changes and pseudopods.

Fig. 6. γ-Tocotrienol–induced DNA fragmentation in HT-29 cells. The control group was treated with 0.15% ethanol. (A) Cells were treated with various concentrations of γ-tocotrienol for 48 h. (B) Cells were treated with 60 μmol/L of γ-tocotrienol for 24, 48, and 72 h. The cells were harvested and lysed. DNA was isolated and subjected to 1.2% agarose gel electrophoresis.
contrast, the TRF had no effect on the cell cycle in human RKO colon carcinoma cells [8]. These observations suggest that \( \gamma \)-tocotrienol–induced G1 arrest might also be involved in the inhibition of HT-29 cell proliferation and show that cell lines may differ in \( \gamma \)-tocotrienol–induced cell-cycle arrest.

Many chemotherapeutic agents, radiation therapy, and therapeutic cytokines are known to induce apoptosis [40–42]. Thus, induction of apoptosis has been recognized as an approach to cancer therapy. In our present studies, morphology and DNA fragmentation demonstrated that 30–60 \( \mu \)mol/L of \( \gamma \)-tocotrienol induced apoptosis in HT-29 cells and these effects were dose and time dependent.

Apoptosis is a complex process regulated by a variety of factors [43,44]. Two important groups of proteins involved in apoptotic cell death are members of the Bcl-2 family [45] and a class of cysteine proteases known as caspases [46]. Activation of caspase-3 is regarded as a primary mechanism of apoptosis [46,47]. Caspase-3 can be activated through cytosolic release of cytochrome c by Bax protein [48]. The results of this study demonstrated that \( \gamma \)-tocotrienol upregulated the expression of Bax and downregulated the expression of Bcl-2 in HT-29 cells. Similar results have been obtained in Hep3B [19] and SGC-7901 [20] cells. In addition, alteration of the Bax:Bcl-2 ratio in favor of apoptosis has been reported in human colon carcinoma RKO cells treated with TRF [8]. However, expression of Bax and Bcl-2 (mRNA and protein) did not change significantly in MDA-MB-231 cells [17] treated with \( \gamma \)-tocotrienol. A subsequent event might be caspase-3 activation. In the present study, we found that \( \gamma \)-tocotrienol at 45 and 60 \( \mu \)mol/L
activated caspase-3 to produce cleaved caspase-3 (p17 and p20) fragments in HT-29 cells at 48 h. These results indicate that \( \gamma \)-tocotrienol increases caspase-3 activity in cancer cells and caspase-3 is involved in the apoptotic process. Our previous studies showed that \( \gamma \)-tocotrienol increased caspase-3 activity in SGC-7901 cells [9]. A similar increase has been reported in Hep3B [19], RLh-84 [49], and highly malignant + SA mouse mammary epithelial cells [37] in the presence of \( \gamma \)-tocotrienol. In addition, it has been reported that the TRF of palm oil enhances caspase-3 activity in human colon carcinoma RKO cells [8]. However, caspases are not involved in the \( \gamma \)-tocotrienol–induced apoptosis in MDA-MB-231 cells [17]. This suggests that the participation of caspase-3 activation in apoptosis is cell line dependent.

Nuclear factor-\( \kappa \)B consists of homodimers and heterodimers formed by several subunits: NF-\( \kappa \)B1 (p50/p105),

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**Fig. 10.** Subcellular localization of NF-\( \kappa \)B p65 in HT-29 cells. Primary cultures were incubated for 24 h and stimulated with 15, 30, and 60 \( \mu \)mol/L of \( \gamma \)-tocotrienol for 48 h. Detection of p65 signals and quantitative analyses of the percentage of p65-positive nuclei were done as described in MATERIALS AND METHODS. (A) Immunofluorescence of NF-\( \kappa \)B p65 (magnification 200×). (1a–c) Control, green channel, blue channel, and green/blue channel; (2a–c) \( \gamma \)-tocotrienol 60 \( \mu \)mol/L, green channel, blue channel, and green/blue channel. The experiments were repeated three times. (B) Quantification of p65-positive nuclei. In each chamber the percentage of p65-positive nuclei was determined by counting four representative areas under the fluorescence microscope. Data are presented as mean ± SD (n = 3). *P < 0.05 versus the control group. NF-\( \kappa \)B, nuclear factor-\( \kappa \)B.
NF-κB2 (p52/100), Rel A (p65), Rel B, and c-Rel [50]. The inactive form of NF-κB is restricted to the cytoplasm and consists of three subunits: DNA-binding p50 and p65 subunits and an inhibitory subunit, IκB, which is bound to p65. IκB masks the nuclear location sequence and its release initiates activation of NF-κB and its subsequent translocation to the nucleus, where it binds to target sites on the DNA [51]. Activation of NF-κB results in the induction of a large number of genes that affect cell proliferation and survival [52]. NF-κB is an important regulator of cell proliferation through its direct role in cell-cycle progression [53]. It may inhibit apoptosis by enhancing the expression of the apoptosis genes Bcl-2 and Bcl-xL [54]. The NF-κB p65 subunit is specifically associated with the regulation of apoptosis [55] and increased expression of this subunit is important in the pathogenesis of colorectal carcinoma [54]. Studies using various experimental systems have demonstrated that p65-containing NF-κB complexes have an antiapoptotic effect after treatment with diverse stimuli including inflammatory cytokines, ionizing radiation, and chemotherapeutic drugs [56]. Previous studies have shown that the antiproliferative effects of γ-tocotrienol are correlated with a reduction in NF-κB activity [22,38]. In this study, the cellular levels of NF-κB p65 protein was significantly decreased in HT-29 cells treated with 45–60 μmol/L of γ-tocotrienol for 48 h (P < 0.05). Furthermore, γ-tocotrienol inhibited the NF-κB p65 nuclear translocation in a dose-dependent manner, but did not significantly affect the NF-κB p65 cytosolic expression in HT-29 cells. These findings indicated that γ-tocotrienol is an inhibitor of NF-κB p65 nuclear translocation in HT-29 cells. Thus, the data in this study showed a correlation between the antiproliferative and apoptosis-inducing effects and rapid reduction in NF-κB p65 expression of γ-tocotrienol in tumor cells.

Conclusion

Our data suggest that γ-tocotrienol may potently inhibit cell growth and induce apoptosis of HT-29 cells by up- or downregulation of the expression of Bcl-2 family proteins. Furthermore, these results indicate that NF-κB p65 protein may participate in the regulation of cell survival and induction of apoptosis by γ-tocotrienol.

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