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γ-Tocotrienol inhibits cell viability through suppression of β-catenin/Tcf signaling in human colon carcinoma HT-29 cells☆

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Abstract

γ-Tocotrienol, a major component of the tocotrienol-rich fraction of palm oil, has been suggested to have antioxidant and anticancer activity as well as potent chemopreventive effects on tumor cells. In this study, the mechanisms underlying γ-tocotrienol-mediated growth inhibition of human carcinoma HT-29 cells were further investigated, especially in correlation with the involvement of β-catenin/T-cell factor (Tcf) signaling pathway. We found that γ-tocotrienol could strongly suppress the transcriptional activity of β-catenin/Tcf signaling pathway in HT-29 cells. γ-Tocotrienol inhibited the expression level of total β-catenin protein but did not significantly affect the phosphorylated β-catenin level. Meanwhile, γ-tocotrienol down-regulated the protein level of nuclear β-catenin and induced its redistribution to cell membrane. Furthermore, γ-tocotrienol suppressed the expression of downstream target genes such as c-*myc*, cyclin D1 and survivin. The results demonstrated that γ-tocotrienol-inhibited growth and -induced apoptosis in HT-29 cells were accompanied by significant inhibition of β-catenin/Tcf signaling. Blocking the expression of β-catenin with small interfering RNA significantly suppressed the ability of γ-tocotrienol to reduce viability and induce apoptosis in HT-29 cells. Thus, our data suggested that γ-tocotrienol exerts its anticancer activity through β-catenin/Tcf signaling, and β-catenin is a target for γ-tocotrienol in the Wnt/β-catenin signaling pathway.

Keywords: γ-Tocotrienol; Cancer chemoprevention; β-Catenin; Wnt signaling; HT-29 cells

1. Introduction

Cancer is a major health problem in both industrialized and developing nations [1], and its rates are expected to increase 50% by the year 2020 [2]. Colorectal cancer is the fourth most common cancer in men and the third in women worldwide [3]. For women, it is the third leading cause of cancer-related deaths behind lung and breast cancers. Women have the same risk as men, and the lifetime risk of the development of colorectal cancer is 6% [4].

Carcinogenesis for most cancers is a multifactorial and multistep process that involves various genetic alterations and several biological pathways. Numerous studies suggested that activation of the Wnt/β-catenin signaling pathway plays an important role in human tumorigenesis [5–7]. In the absence of a Wnt signal, β-catenin is mostly associated with the plasma membrane, where it is in conjunction with E-cadherin and α-catenin that promote cellular adhesion. Cytosolic β-catenin is normally bound to axin and the adenomatous polyposis coli (APC) protein, phosphorylated at the N-terminal Ser/Thr residues by casein kinase Iα and glycogen synthase kinase 3, and then degraded by the ubiquitination–proteasome system. In response to a Wnt signal, β-catenin accumulates in the cytoplasm and is translocated to the nucleus, where it is bound to proteins of the T-cell factor (TCF)/lymphoid enhancer factor family and regulates the expression of genes involved in proliferation, invasiveness and angiogenesis [8,9]. The Wnt signaling pathway is deregulated in over 90% of human colorectal cancers [10]. In the majority of colon cancers, mutation in either the APC or β-catenin gene leads to an increased expression level of β-catenin [5,6,8,11,12]. Therefore, the stabilization of β-catenin and its translocation into nucleus are the key steps in Wnt pathway activation, which may serve as potential target for colorectal cancer therapy.

Epidemiological studies indicate that the processes of carcinogenesis and tumorigenesis are mainly induced by environmental factors. About 75% of cancer-related deaths are due to diet and lifestyle [13]. Numerous studies have suggested that most colorectal cancers are neither purely genetic nor purely environmental. Environmental factors, both dietary and other environmental factors, appear to interact with genetic factors in the development of colorectal cancer [14–16]. Epidemiological studies have demonstrated that a reduced incidence of colorectal cancers has correlated with tocotrienol present in their diet [17–20]. Furthermore, accumulating evidence has

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indicated that tocotrienols can significantly reduce tumor growth *in vivo* and suppress cell viability and induce apoptosis of cancer cells *in vitro* [21–26]. Previous results from our laboratory have demonstrated that γ-tocotrienol inhibits cell proliferation and induces apoptosis in the human colon carcinoma HT-29 cells in a time- and dose-dependent manner [27] and confirmed that small interfering RNA (siRNA) targeted against β-catenin inhibited cell proliferation, and this process may be associated with switching-off cyclin D1 and c-*myc* expression by β-catenin siRNA in colon cancer HT-29 cells [28]. Nevertheless, the molecular mechanisms in γ-tocotrienol-mediated growth inhibition are still poorly elucidated.

In the present study, the colon cancer HT-29 cell line was used to investigate the effect of γ-tocotrienol on (1) phosphorylation of β-catenin and total β-catenin protein, (2) the subcellular localization of β-catenin and (3) protein expressions of c-*myc*, cyclinD1 and survivin. Furthermore, the role of β-catenin in γ-tocotrienol-mediated growth inhibition in HT-29 cells was studied using RNA interference to knockdown β-catenin.

2. Materials and methods

2.1. Materials

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 Lakes, NJ, USA). γ-Tocotrienol was from Davos (Biopolis Way, Singapore). 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide [MTT] and dimethyl sulfoxide were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). β-Catenin–siRNA (sc-29209), control siRNA (sc-37007), rabbit polyclonal antibody for β-catenin (sc-7199), mouse monoclonal antibody for c-*myc* (sc-7480), rabbit polyclonal antibody for survivin (sc-10811) and fluorescein isothiocyanate-conjugated fluorescent secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibody for phospho-β-catenin (9561) was bought from Cell Signaling Technology (Beverly, MA, USA). Rabbit polyclonal antibody for cyclin D1/Bcl-1(SP4) (RM-9104) was obtained from Neomarkers (Fremont, CA, USA). Goat antirabbit (w3960) and antimouse (w3950) secondary antibodies were purchased from Promega (Promega, Madison, WI, USA).

2.2. Cell culture

Human colon carcinoma HT-29 cells were maintained in RPMI 1640 (Gibco, Paisley, Scotland) in 75-cm^2 flasks at 37 °C in a 5% CO_2 atmosphere at constant humidity. The medium was supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (Gibco), 2 mmol/l L-glutamine (Gibco) and 1% antibiotic solution (Gibco) and was changed every other day. For subculturing, cells were rinsed once with phosphate-buffered saline (PBS) and incubated in 0.25% trypsin containing 0.02% EDTA (Gibco) in PBS for 3 min. For the γ-tocotrienol supplementation experiment, stock solutions of γ-tocotrienol were prepared in absolute ethanol and stored at −20°C. The ethanol vehicle was used in the control cell culture. The final ethanol concentration in all cultures was 0.15% [29].

2.3. Transfection and luciferase assay

Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Briefly, HT-29 cells were seeded into 6-well plates at 60% confluence and transfected with TOPflash or FOPflash reporter, and pRL-CMV *Renilla* control reporter (Promega) to control for transfection efficiency. After 4 h of posttransfection, cells were treated with various doses of γ-tocotrienol (0, 15, 30, 45 and 60 μmol/l) for 24 h. Luciferase assays were carried out using a dual Luciferase assay kit (Promega). Luciferase activity was normalized by *Renilla* luciferase activity.

2.4. Western blot

After treatment with γ-tocotrienol, HT-29 cells were detached in PBS containing 0.25% trypsin and 0.02% EDTA, collected and washed twice with PBS. The harvested cells were lysed in 20 mmol/l Tris-HCl buffer (pH 7.5) containing 2% sodium dodecyl sulfate (wt/vol), 2 mmol/l benzamidine and 0.2 mmol/l phenylmethanesulfonyl fluoride. To investigate the nuclear localization of β-catenin, both nuclear and cytoplasmic proteins from HT-29 cells before and after treatment with γ-tocotrienol were extracted by Nuclear-Cytosol Extraction Kit (Applygen Technologies Inc., Beijing, China), and their expression levels were investigate by Western blot method according to our previous study [27].

2.5. Immunofluorescent detection of β-catenin

HT-29 cells seeded onto glass coverslips placed in 6-well plates (Nunc, Wiesbaden, Germany) and incubated overnight were treated with γ-tocotrienol for desired time. The slips were washed with PBS and fixed in methanol for 4 min. The intracellular localization of β-catenin was determined as previously described [27].

2.6. MTT

The effect of γ-tocotrienol on cell viability was determined by MTT method as previously described with some modifications [30–33]. Briefly, cells were seeded in 96-well microtiter plates (Nunc) at 1.0×10^4 per well. After 24-h incubation, the medium was removed and the cells were treated with 200 μl of medium containing various concentrations (15, 30, 45 and 60 μmol/l) of γ-tocotrienol for the desired time. Control cells were supplemented with 0.15% ethanol vehicle. Each concentration of γ-tocotrienol was repeated in 5 wells. After incubation for 1, 2, 3, 4 and 5 days, 20 μl 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 μl of dimethyl sulfoxide 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., Winooski, VT, USA). 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.

2.7. Morphological observation of apoptosis

After treatment with γ-tocotrienol, morphological changes in HT-29 cells were assessed by inverted microscope. Changes in the nuclei were investigated by staining the cells with fluorescent DNA-binding dyes. Briefly, cells exposed to γ-tocotrienol for 48 h were harvested and washed with PBS, and 25 μl of cell suspension was mixed with 1 μl Hoechst 33258 (10 mg/ml). Nuclear morphology was assessed by fluorescence microscopy (Olympus IX70, Tokyo, Japan).

2.8. Flow cytometry analysis

HT-29 cells were harvested, washed three times with cool PBS, fixed with 70% cool 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, Franklin Lakes, NJ, USA). The proportions in G0/G1, S and G2/M phases were estimated using ModFit LT analysis software.

2.9. RNA Interference

HT-29 cells were cultured at a density of 60% confluence in the complete medium. Cell transfection with the siRNA at a final concentration of 100 nmol/l was performed using siRNA transfection reagent (Santa Cruz Biotechnology) according to the manufacturer's instructions. After 24-h incubation, the cells were treated with 60 μmol/l of γ-tocotrienol for 24 h, cell viability was determined by the MTT assay and nuclear morphology was assessed by fluorescence microscopy as described above.

2.10. Statistical analysis

Statistical analysis was performed using SPSS 13.0 software. The data were expressed as mean±S.D. Differences between the control and treated groups were evaluated by Student's *t* test, and *P* values less than .05 were considered statistically significant.

3. Results

3.1. γ-Tocotrienol inhibits β-catenin/Tcf signaling in HT-29 cells

To determine whether γ-tocotrienol modulates β-catenin/Tcf signaling, the effects of the various concentrations of γ-tocotrienol on this β-catenin-mediated transcriptional activity were analyzed. HT-29 cells were transiently transfected with TOPflash reporter encoding luciferase driven by a promoter containing either Tcf-binding sites or FOPflash reporter containing a mutated Tcf-binding sites, together with *Renilla* control reporter, and were treated with γ-tocotrienol at the indicated concentrations for 48 h. The results in Fig. 1 showed a dramatic decrease (60%–75%) in luciferase activity due to treatment of 45–60 μmol/l γ-tocotrienol. Meanwhile, FOPflash activity remained unchanged after γ-tocotrienol treatment, suggesting that the functional binding of β-catenin/Tcf might be important for the TOPflash. The results presented in Fig. 1

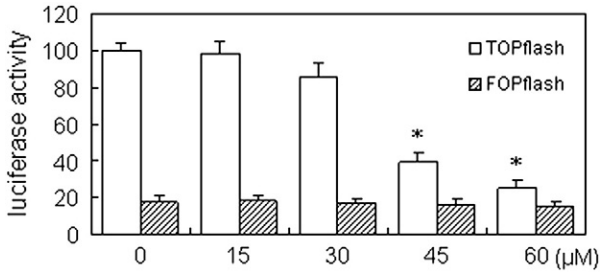

Fig. 1. γ-Tocotrienol inhibits the transcriptional activity of β-catenin/Tcf in HT-29 cells. HT-29 cells were transfected with either TOPflash reporter or FOPflash reporter, respectively. After 4 h of posttransfection, the cells were incubated with 0–60 μmol/l γ-tocotrienol for 24 h. Luciferase activity was determined and normalized to pRL-CMV *Renilla*. An equivalent volume of ethanol substituted for γ-tocotrienol was used as a vehicle control. Values represent the means±S.D. of five independent experiments. *P<.05, compared with the control group.

showed that γ-tocotrienol significantly suppresses the transcriptional activity of β-catenin/Tcf signaling.

3.2. γ-Tocotrienol down-regulates expression of total β-catenin and does not affect the level of phosphorylation of β-catenin

The effect of γ-tocotrienol on total β-catenin and phosphorylation of β-catenin protein expression was investigated by Western blot in HT-29 cells. Fig. 2A showed that 45- and 60-μmol/l γ-tocotrienol treatment significantly decreased the expressive level of whole β-catenin protein in HT-29 cells in a dose-dependent manner. However, no obvious changes were observed in the phosphorylation level of β-catenin protein in HT-29 cells treated with 15-60 μmol/l γ-tocotrienol for 48 h (Fig. 2B).

3.3. γ-Tocotrienol affects the intracellular localization of β-catenin

To determine whether this reduction of total β-catenin might also lead to reduction of β-catenin in the nucleus and cytosolic, the β-catenin distribution upon γ-tocotrienol treatment for 48 h was investigated by indirect immunofluorescence staining in HT-29 cells. Fig. 3A showed strong nuclear and relatively weaker cytoplasmic expression of β-catenin in control HT-29 cells. Fig. 3B and C showed that γ-tocotrienol at low concentrations (15 and 30 μmol/l) had no effect on the distribution of β-catenin staining, whereas Fig. 3D showed that treatment with 60 μmol/l γ-tocotrienol for 48 h markedly decreased nuclear β-catenin level and increased the level of membrane-associated β-catenin in HT-29 cells. Results of quantification of immunofluorescent signals indicated that about 8% of HT-29 cell nuclei were positive for β-catenin after supplementation of 60 μmol/l γ-tocotrienol (Table 1). In contrast, about 93% β-catenin-positive nuclei were observed in HT-29 cells.

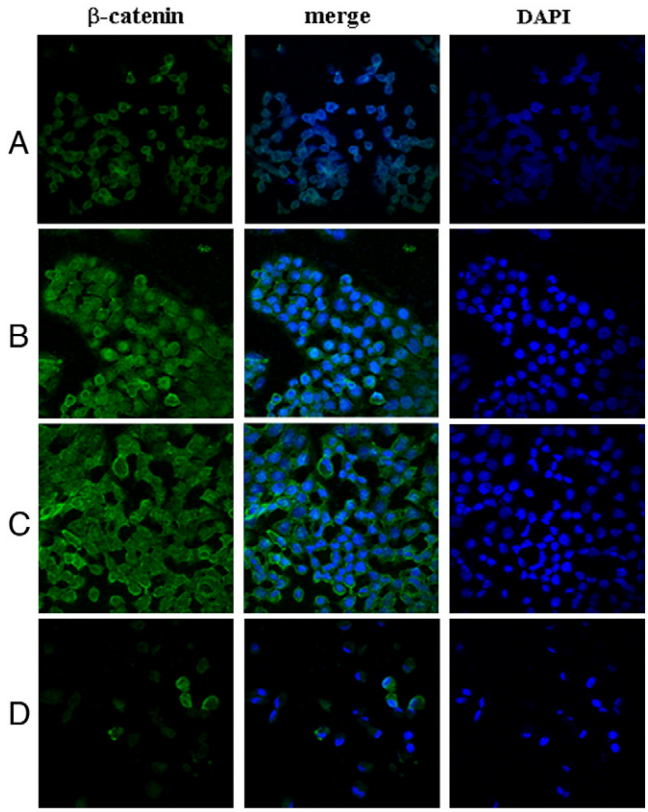

Fig. 3. Subcellular localization of β-catenin in HT-29 cells. Primary cultures were incubated for 24 h and stimulated with 15, 30 and 60 μmol/l γ-tocotrienol for 48 h. Detection of β-catenin signals was conducted as described in "Materials and methods." (A) Immunofluorescence of β-catenin (magnification ×200). Control (A), 15 μmol/l (B), 30 μmol/l (C) and 60 μmol/l (D). The experiments were repeated three times.

HT-29 cells treated with different concentrations of γ-tocotrienol were harvested, and then the nuclear and cytoplasmic extracts were prepared and analyzed using Western blot. The results showed that

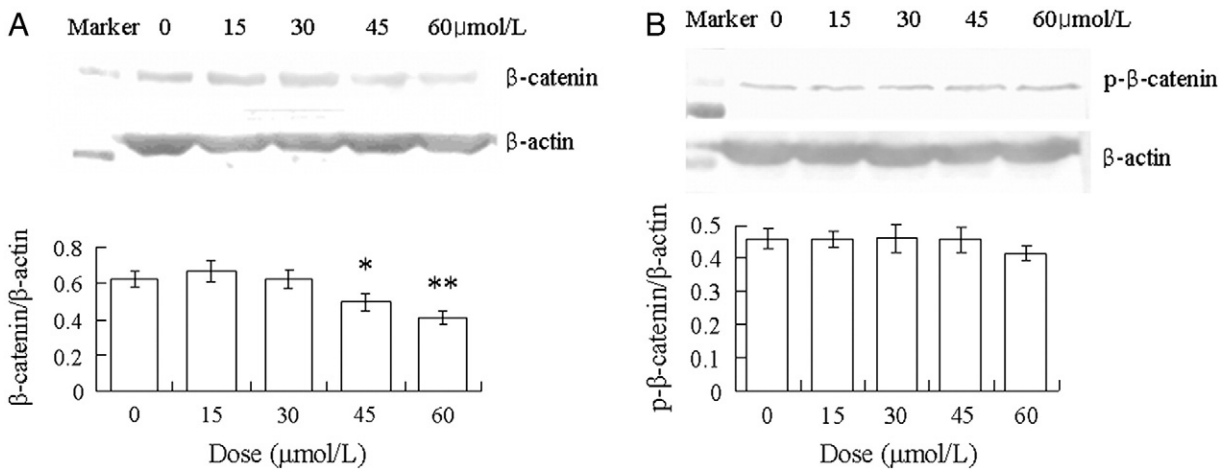

Fig. 2. The expression of total β-catenin (A), phosphorylation of β-catenin (B) and β-actin (A, B) in HT-29 cells treated with γ-tocotrienol for 48 h. Data are presented as mean±S.D. (n=3). *P<.05 and **P<.01, compared with the control group.

Table 1
Quantitative determination of β-catenin-positive nuclei in HT-29 cells treated with γ-tocotrienol

Concentration (μmol/l)	Total cells	Positive cells	Positive rate (%)
0	100	93.0±4.3	93.0
15	100	87.5±5.7	87.5
30	100	11.4±4.9**	11.4
60	100	8.1±2.3**	8.1

The data represent the means±S.D. (n=3).
** P<.01, compared with the control group.

γ-tocotrienol (45 or 60 μmol/l) significantly inhibited the nuclear β-catenin level in HT-29 cells in comparison with the control group (Fig. 4A; P<.05). Meanwhile, treatment with 15, 30, 45 and 60 μmol/l of γ-tocotrienol for 48 h did not significantly affect the expression level of cytosolic β-catenin in HT-29 cells (Fig. 4B). Our results suggested that the nuclear translocation of β-catenin was blocked by treatment with 60 μmol/l γ-tocotrienol; however, the expression of cytosolic β-catenin protein was not affected in HT-29 cells treated with 15-60 μmol/l γ-tocotrienol for 48 h.

3.4. γ-Tocotrienol treatment decreases Wnt/β-catenin target gene expression

CyclinD1, c-*myc* and survivin have been identified as a target of the β-catenin/Tcf signaling pathway [34]. We examined the expression of three cellular genes. As shown in Fig. 5, no significant changes were found in the expression levels of cyclinD1, c-*myc* and survivin in HT-29 cells treated with 15 μmol/l γ-tocotrienol for 48 h, while treatment with 45 and 60 μmol/l γ-tocotrienol for 48 h resulted in significantly lower endogenous expression of cyclinD1, c-*myc* and survivin than those of control group (P<.05).

3.5. γ-Tocotrienol inhibits HT-29 Cell viability

The effect of various doses of γ-tocotrienol on HT-29 cell viability was shown in Fig. 6. HT-29 cells grown in control media displayed a continuous increase in viable cell number over the 5-day culture period. There was no significant difference between solvent control cells and blank control cells. These results indicated that low concentration of ethanol (0.15 vol/vol) did not affect cell growth, whereas treatment with 15-60 μmol/l γ-tocotrienol decreased the HT-29 cell viability in a dose-dependent manner. After 2 days of culturing, 60 μmol/l γ-tocotrienol decreased viable HT-29 cell number by 90% as compared with those of untreated controls.

3.6. Morphological changes of HT-29 cells after exposure to γ-tocotrienol

Under the phase contrast microscope (Fig. 7A), untreated cells were cuboid and polygonal in normal shape. The considerable alteration in the morphology of HT-29 cells was observed after treatment with 60 μmol/l γ-tocotrienol for 24, 36 and 48 h, respectively. Compared with the untreated cells, the treated cells had more shrinking and floating cells, which lost their adhesive ability.

γ-Tocotrienol-treated cells and control cells were stained with 4,6-diamino-2-phenylindole (DAPI) fluorescence staining. As shown in Fig. 7B, the blue emission light in treated cells was much brighter than those of control cells. Cells treated with γ-tocotrienol showed typically apoptotic changes, such as chromatin condensation, deformation and nuclear fragmentation. The apoptotic rate of the cells was counted after treating with γ-tocotrienol (15, 30 and 60 μmol/l) for 48 h, and the results demonstrated that γ-tocotrienol treatment resulted in dose-dependent increase in apoptotic cells (Table 2).

3.7. Assessment of apoptosis using the flow cytometry

Flow cytometry analysis further confirmed that γ-tocotrienol induced apoptosis of HT-29 cells. It was found that the sub-G1 peak appeared before G1 phase, which represents apoptotic cell population. As shown in Fig. 7C, 60 μmol/l γ-tocotrienol supplements resulted in 1.62 %, 5.32%, 28.63% and 53.4% of apoptosis cells after 12, 24, 36 and 48 h, respectively. While induction of apoptosis was almost negligible (1.62% and 5.32% compared to 0.57% of control) at 12 and 24 h (data not shown), significant increase (P<.05) in apoptosis was observed after 36 and 48 h (28.63%, and 53.4%) with 60 μmol/l γ-tocotrienol treatment.

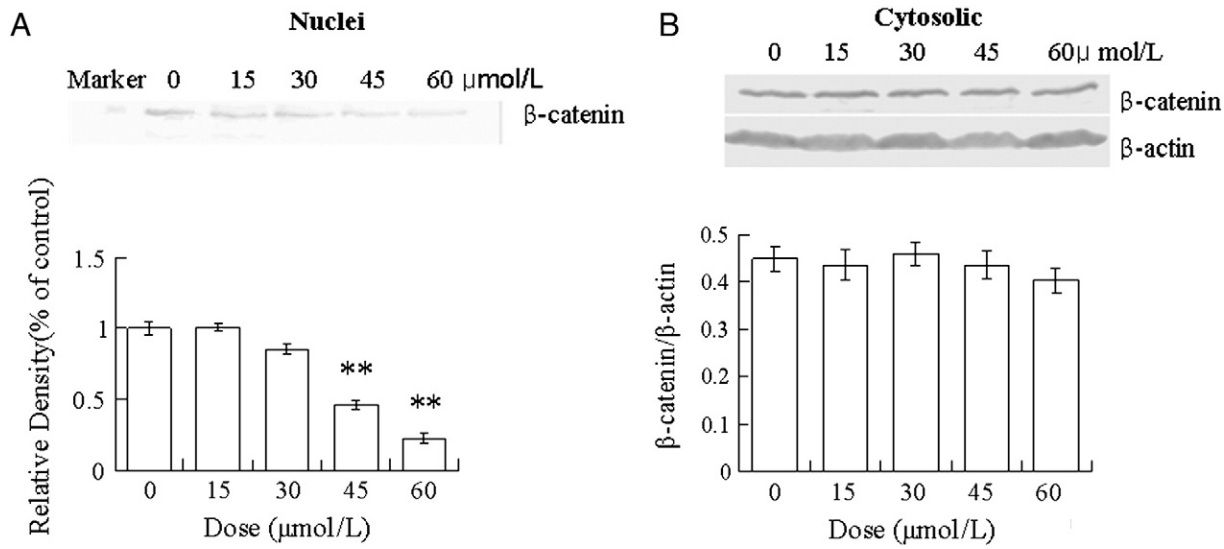

Fig. 4. The expression of nuclear β-catenin protein (A), cytosolic β-catenin (B) and β-actin (B) in HT-29 cells treated with 15, 30, 45, 60 μmol/l of γ-tocotrienol for 48 h. The cells were trypsinized and harvested. Their nuclear and cytosolic extracts were prepared for Western blot. Data are presented as mean±S.D. (n=3). *P<.05 and **P<.01, compared with the control group.

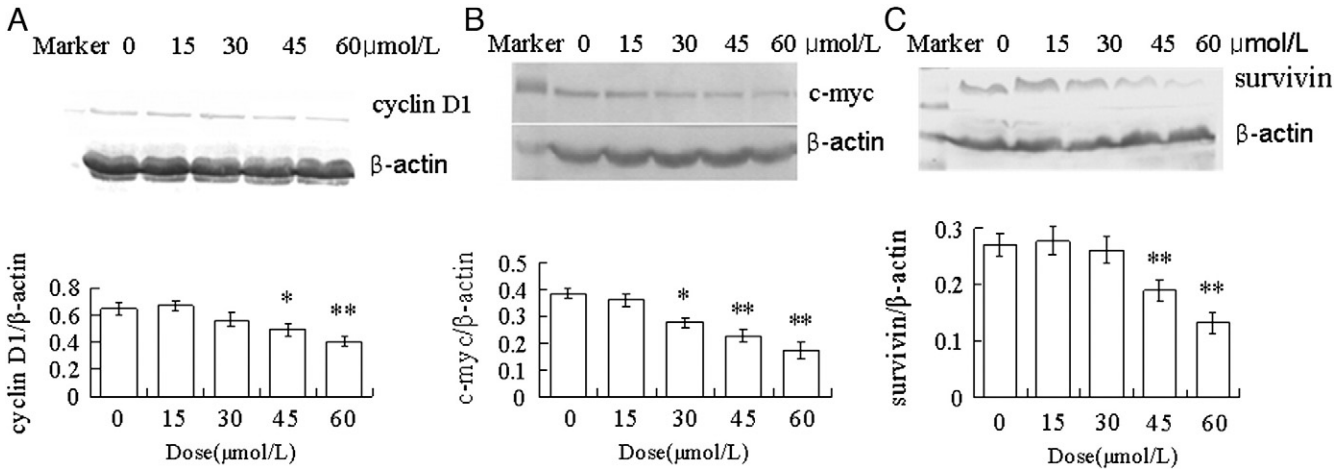

Fig. 5. Expression of cyclin D1 (A), c-myc (B), surviving (C) and β-actin (A–C) in HT-29 cells treated with γ-tocotrienol for 48 h. The lysates were separated on a 10% sodium dodecylsulfate polyacrylamide gel electrophoretic gel, transferred to a nitrocellulose membrane and probed with anti-β-actin and anti-cyclin D1 antibodies. Protein content was normalized by probing the same membrane with anti-β-actin. Data are presented as mean±S.D. (n=3). *P<.05 and **P<.01, compared with the control.

3.8. Role of β-catenin in γ-tocotrienol-inhibited cell growth in HT-29 cells

Because γ-tocotrienol treatment caused a significant decrease in β-catenin expression in HT-29 cells, the role of β-catenin in γ-tocotrienol-mediated decrease in colon cancer cell growth was determined. After 24 h of transfection with control siRNA or β-catenin–siRNA, HT-29 cells treated with γ-tocotrienol were analyzed by MTT method (Fig. 7D). After treatment with γ-tocotrienol for 24 h, the statistical analysis showed that cell viability did not differ between control group and control siRNA group, but the viability of HT-29 cells transfected with β-catenin–siRNA was significantly increased compared to the control group (P<.05). These results suggested that pretreatment of HT-29 cells with β-catenin–siRNA significantly suppressed the ability of γ-tocotrienol-decreased cell growth.

In addition, the Hoechst 33258 staining was used to investigate morphological changes of the cell nuclei, and the results are presented in Fig. 7E and Table 3. After treatment with γ-tocotrienol for 24 h, the cell apoptosis rate in the β-catenin–siRNA group, comparing with the control siRNA group and control group, was decreased by approximately 50%. The result indicated that blocking of β-catenin with RNAi technology significantly reduced γ-tocotrienol-induced apoptosis in HT-29 cells.

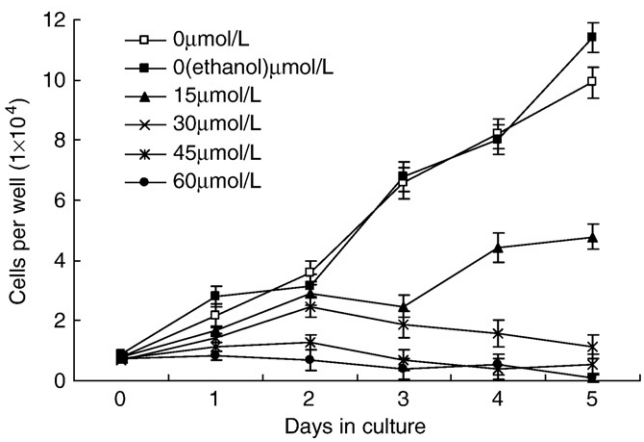

Fig. 6. Effects of various doses of γ-tocotrienol on HT-29 cell viability. Cells were exposed to different concentrations of γ-tocotrienol for 1, 2, 3, 4 and 5 days, and viability was determined by MTT assay. Data are presented as mean±S.D. (n=5). The results represent the mean of at least three independent experiments.

4. Discussion

Chemoprevention of natural compounds from plants or in diets appears to be an effective approach to prevent and treat carcinoma, and it is estimated that diets rich in phytochemicals can reduce the cancer risk by 20% [35]. γ-Tocotrienol is one of the most important components of vitamin E, which is enriched in high-lipid plants. γ-Tocotrienol has potent biological and pharmacologic activities. Previous studies have shown that γ-tocotrienol exerts antiproliferative activity on various types of cancer cells including prostate, breast, stomach, liver and colon cancers. Molecular mechanisms underlying antitumor activity of γ-tocotrienol were discussed, including mitochondria-dependent apoptosis pathway, interaction with proteins that control cell cycle progression, nuclear factor κB p65 protein and altered gene expression [27,36–39]. However, the molecular mechanism underlying anticancer activities of γ-tocotrienol in human colon cancer HT-29 cells has not been fully understood. The results of our previous studies demonstrated that γ-tocotrienol had an antitumor effect on colon cancer HT-29 cells through antiproliferative and apoptosis-inducing effects that related with rapid reduction in nuclear factor κB p65 [27].

Colorectal cancer is the second leading cause of cancer-related deaths in developed countries. Activation of the Wnt/β-catenin signaling pathway occurs in over 90% of human colorectal cancers, and thus, it is an attractive target for anticancer therapy and the chemoprevention of colon cancer. Since it is well known that Tcf signaling-activation results from an accumulation of nuclear β-catenin [40,41], down-regulation of β-catenin proteins may inactivate the transcriptional activity of β-catenin/Tcf. Many studies on the inhibitory agents against β-catenin/Tcf signaling in colon cancer cell lines have been reported. Hexachlorophene suppressed the transcriptional activity of β-catenin/Tcf signaling and decreased the level of β-catenin in HCT116, which express wild-type APC, but not in SW480, which express truncated APC. Similar effect of hexachlorophene was elucidated on LS174T and DLD-1, which contain mutant β-catenin and mutant APC, respectively [42]. In addition, murrayafoline A, a carbazole alkaloid, isolated from *Glycosmis stenocarpa*, was able to decrease intracellular β-catenin level in SW480 cells and HCT 116 cells

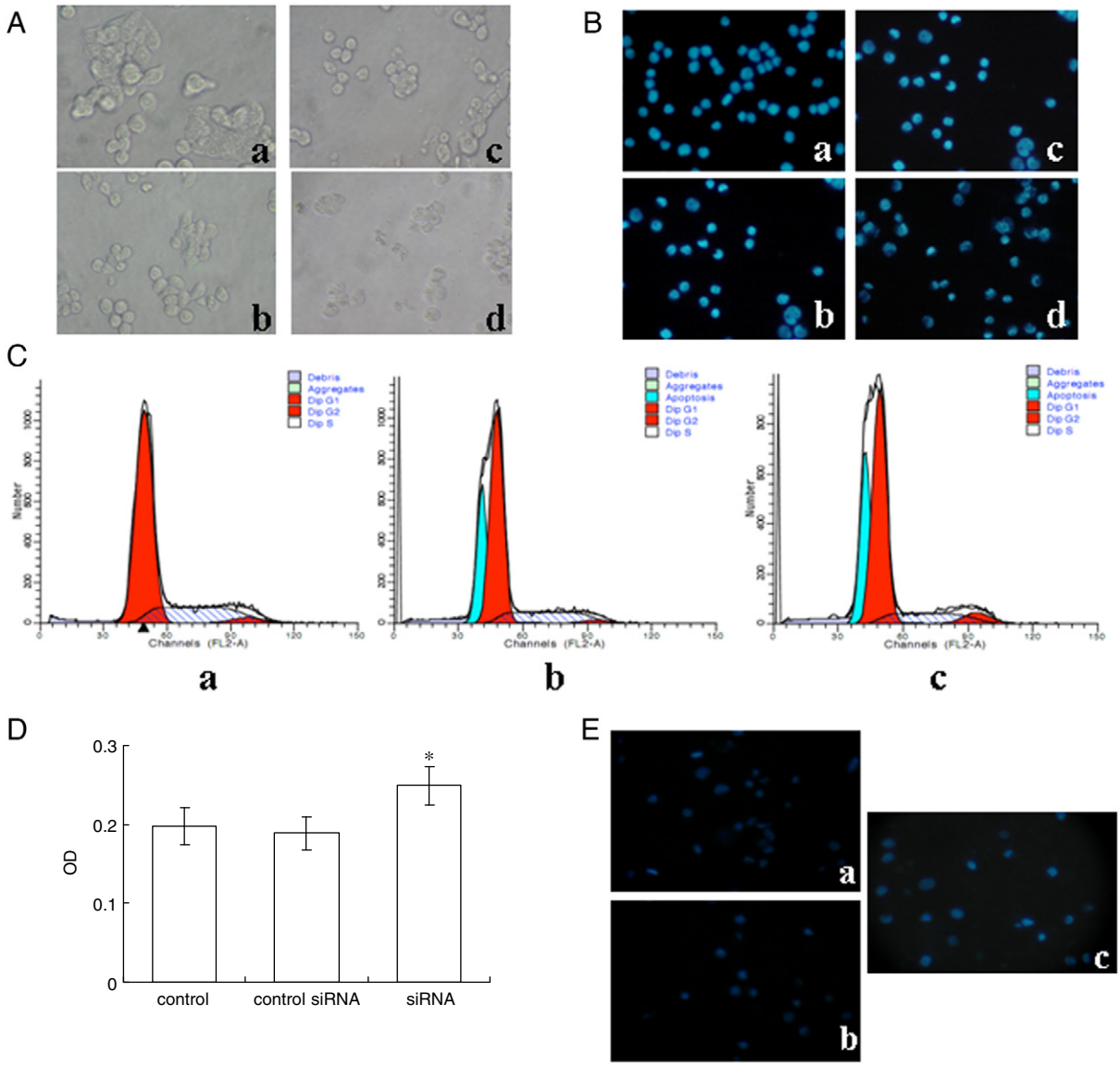

Fig. 7. γ-Tocotrienol inhibits cell growth and induces apoptosis in human colon carcinoma HT-29 cells. (A) The morphological changes of HT-29 cells treated by γ-tocotrienol (light microscope, ×200): control (a); 60 μmol/l, 24 h (b); 60 μmol/l, 36 h (c) and 60 μmol/l, 48 h (d). (B) Nuclear morphological changes of HT-29 cells treated with different doses of γ-tocotrienol for 48 h (stained by Hoechst 33258, ×200): control (a), 15 μmol/l (b), 30 μmol/l (c) and 60 μmol/l (d). (C) Flow cytometric analysis of apoptosis in HT-29 cells treated with 60 μmol/l γ-tocotrienol for 0, 36 and 48 h (n=3): control group (a), 36 h (b) and 48 h (c). (D) The growth of HT-29 cells treated with 60μmol/l γ-tocotrienol for 24 h by MTT assay. *P<.05, compared with the control group; the experiments were repeated three times. (E) The effect of Hoechst 33258 staining on HT-29 cells treated with 60μmol/l γ-tocotrienol for 24 h (200×): control group (a), control siRNA transfection (b) and β-catenin–siRNA transfection (c).

[43]. Curcumin could inhibit β-catenin associated with Tcf-4 in nuclei and decreased the transcriptional activity of β-catenin/Tcf-4 signaling pathway in HT-29 cells [44]. In the present study, we found that γ-tocotrienol could strongly suppress the transcriptional activity of β-catenin/Tcf signaling pathway in HT-29 cells with mutations in the APC gene, and the inhibitory mechanism of γ-tocotrienol was not related to the phosphorylation level of β-catenin. We further considered the possibility that the suppressed β-catenin/Tcf signaling might result from the reduction of nuclear β-catenin proteins due to γ-tocotrienol. In order to confirm this possibility, we determined the β-catenin distribution upon γ-tocotrienol treatment for 48 h by indirect immunofluorescence staining and Western blot with the nuclear and cytoplasmic

Table 2
The rate of apoptotic cells induced by γ-tocotrienol (%) for 48 h

γ-Tocotrienol (μmol/l)	Rate of apoptotic cells (%)
0	5.75±2.10
15	11.74±4.44
30	28.35±5.73 *,#
45	37.50±9.34 *,#
60	61.45±8.53 *,#

The data represent the means±S.D. (n=3).
* P<.05, compared with the control.
# P<.05, compared with 24 h.

Table 3
The apoptosis index of HT-29 cells of different treatment

Groups	Apoptotic index of HT-29 cells (%)
Control+60 μmol/l γ-tocotrienol	32.5±3.0
Control siRNA+60 μmol/l γ-tocotrienol	29.7±3.5
β-Catenin–siRNA+60 μmol/l γ-tocotrienol	17.7±2.9 **

The data represent the means±S.D. (n=3).
** P<.01, compared with the control+60 μmol/l γ-tocotrienol group.

extracts in HT-29 cells. Our results showed that middle–high doses of γ-tocotrienol significantly reduced β-catenin levels in nucleus but had no effects on the levels of β-catenin in cytoplasm. The above results suggested that the mechanism of reduced transcriptional activity of β-catenin/Tcf by γ-tocotrienol depended on the level of β-catenin protein in nuclear.

We also evaluated the effects of γ-tocotrienol on the expression of cyclin D1, c-*myc* and survivin. Our results indicated that γ-tocotrienol specifically decreased the expression of β-catenin-dependent genes. Similarly, hexachlorophene decreased the protein levels of cyclin D1 and c-*myc* and mRNA level of cyclin D1 in a concentration-dependent manner [42]. The protein expression levels of cyclin D1 and c-*myc* were significantly suppressed in response to murrayafoline A [43].

Several studies have reported that the disruption of β-catenin function specifically reduced the cell growth and induced apoptosis of human colon cancer cells [28,45–48]. Moreover, β-catenin/Tcf-dependent gene products are known to regulate cell proliferation and apoptosis [14,16]. For example, cyclin D1, which forms a complex with cyclin-dependent kinase 4/6, mediates growth factor-dependent G1 phase progression [49]. The proto-oncogene c-*myc* encodes a transcription factor, c-Myc, which is of great importance in controlling cell growth and vitality [50]. Survivin is a recently discovered protein belonging to the inhibitor of apoptosis gene family [51]. Given that γ-tocotrienol promotes β-catenin degradation, we examined whether γ-tocotrienol suppress the proliferation of colon cancer cells. The results of this study demonstrated that 45–60 μmol/l of γ-tocotrienol efficiently induced apoptosis and inhibited viability in HT-29 Cell, and these results are consistent with the previous reports [27]. Previous studies have shown that hexachlorophene efficiently inhibited the growth of HCT116 cells in a concentration-dependent manner. In contrast, the growth of WI38 cells, which are nontransformed cells, was less affected by hexachlorophene [42]. In addition, Murrayafoline A efficiently inhibits the growth of colon cancer cells (such as SW480, DLD-1, HCT-116 and LS174T) by promoting the degradation of intracellular β-catenin [43]. Various studies have demonstrated that the antiproliferative activities of the various curcuminoids on colon cancer cells correlate with their inhibitory effects on the β-catenin/Tcf pathway [52–54]. We further confirmed that γ-tocotrienol could not induce cell apoptosis and decrease the viability in HT-29 cells after blocking the expression of β-catenin. These results suggested that β-catenin was the main target point for regulation of the Wnt pathway by γ-tocotrienol, which inhibits proliferation and induces apoptosis of HT-29 cells.

In conclusion, our results indicated that the effect of inhibition on β-catenin/Tcf signaling by γ-tocotrienol was caused by reduction of nuclear β-catenin levels in HT-29 cells. Meanwhile, the antiproliferative effects of γ-tocotrienol were mediated by their ability to suppress the β-catenin/Tcf signaling, and these actions might contribute to the cancer chemoprevention efficacy of γ-tocotrienol.

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