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Journal of Nutritional Biochemistry 23 (2012) 800–807

Journal of Nutritional Biochemistry

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

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Received 26 October 2010; received in revised form 25 March 2011; accepted 6 April 2011

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

☆ This project was supported by the National Natural Science Foundation of China (No. 30471444).
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0955-2863/$ - see front matter.
doi:10.1016/j.jnutbio.2011.04.003

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