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Down-regulation of telomerase activity in DLD-1 human colorectal adenocarcinoma cells by tocotrienol

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Abstract

As high telomerase activity is detected in most cancer cells, inhibition of telomerase by drug or dietary food components is a new strategy for cancer prevention. Here, we investigated the inhibitory effect of vitamin E, with particular emphasis on tocotrienol (unsaturated vitamin E), on human telomerase in cell-culture study. As results, tocotrienol inhibited telomerase activity of DLD-1 human colorectal adenocarcinoma cells in time- and dose-dependent manner, interestingly, with δ-tocotrienol exhibiting the highest inhibitory activity. Tocotrienol inhibited protein kinase C activity, resulting in down-regulation of c-myc and human telomerase reverse transcriptase (hTERT) expression, thereby reducing telomerase activity. In contrast to tocotrienol, tocopherol showed very weak telomerase inhibition. These results provide novel evidence for the first time indicating that tocotrienol acts as a potent candidate regulator of telomerase and supporting the anti-proliferative function of tocotrienol.

Keywords: Telomerase inhibition; Tocotrienol; hTERT; c-myc; Protein kinase C

Human telomeres comprise long tandem repeats of hexanucleotide TTAGGG. Since conventional DNA polymerases cannot replicate the 5′-end of linear DNA, telomeres shorten with each cell division in normal human somatic cells [1]. When telomeres reach a critical length, cell senescence occurs.

Human telomerase is a ribonucleoprotein that adds TTAGGG repeats to telomere ends [2]. The enzyme comprises three main components, including human telomerase RNA component (hTR) [3], telomerase-associated protein 1 (TP1) [4], and human telomerase reverse transcriptase (hTERT) [5,6], the last of which plays a key role in catalytic activity. Telomerase is not detectable in most somatic tissues, whereas it is activated in the majority of cancer cells and malignant tumors [7], implying that telomerase is essential for cell immortalization and tumorigenesis. Therefore, telomerase is a novel and potentially highly selective target for cancer therapy. In such a context, there is considerable work being undertaken to screen potential telomerase inhibitors. The dietary constituents (i.e., flavonoids [8], ceramide [9], and curcumin [10]) have been shown to inhibit telomerase activity in vitro. In our recent studies, some lipids (i.e., polyunsaturated fatty acids [11,12] and sulfoquinvosyldiacylglycerol [13]) appeared to act as powerful telomerase inhibitors. Hence, these dietary compounds have potential use as therapeutic supplements for telomerase inhibition, which may contribute to cancer prevention.

Vitamin E occurs in nature as at least eight different isoforms that include α-, β-, γ-, and δ-isomers of both tocopherol and tocotrienol (Fig. 1). Structurally, these compounds are similar, except that tocotrienols have an unsaturated side chain with three double bonds, whereas tocopherols have a fully saturated side chain. Humans and animals are unable to synthesize vitamin E and therefore must obtain the compound from plant sources. Tocopherol is abundantly present in staple foods such as nuts and com-

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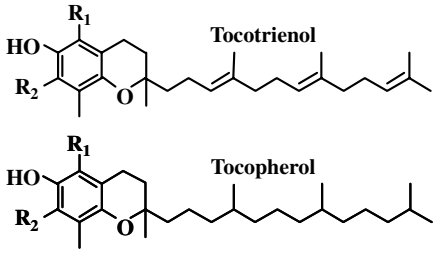

	R_1	R_2
α-	CH_3	CH_3
β-	CH_3	H
γ-	H	CH_3
δ-	H	H

Fig. 1. Chemical structures of vitamin E.

mon vegetable oils, whereas tocotrienol is only a minor constituent of plants with high levels occurring in palm oil, cereal grains, and rice bran.

A major physiological activity of vitamin E is its well-defined antioxidative action [14], with α-tocopherol having the most powerful antioxidant activity among vitamin E group. However, recent study suggested that tocotrienol might be a better bioactive compound than tocopherol [15], as it was found to inhibit 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase activity and thereby reduce cholesterol synthesis [16]. In addition, most interestingly, we and other researchers reported that tocotrienol has potential anti-cancer properties [17,18] including anti-angiogenesis [19–23]. However, the exact mechanism of anti-carcinogenic action by tocotrienol has remained poorly understood.

Tocotrienol has been reported to regulate cellular protein expression, such as Akt, nuclear factor κB, and protein kinase C (PKC) [24,25]. It has been reported that PKC is involved with the control of telomerase activity [26,27]. Hence, in this study, we hypothesized that tocotrienol may act as a candidate regulator of telomerase activity via PKC inhibition. In the present study, this hypothesis was investigated *in vitro* using the DLD-1 human colorectal adenocarcinoma cells.

Materials and methods

Materials. Tocotrienol and tocopherol were obtained from Wako (Osaka, Japan). RPMI 1640 medium (containing 0.3 mg/ml L-glutamine and 2.0 mg/ml sodium bicarbonate) was purchased from Sigma (St. Louis, MO). Fetal bovine serum (FBS) was from Dainippon Pharmaceutical (Osaka, Japan). Penicillin and streptomycin were products of Gibco BRL (Rockville, MD). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was produced by Dotite (Kumamoto, Japan).

Cell culture. DLD-1 (human colorectal adenocarcinoma) cells were obtained from the Cell Resource Center for Biomedical Research at Tohoku University School of Medicine (Sendai, Japan). Cells were precultured in growth medium (RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

MTT dye reduction assay. Test medium was prepared from growth medium supplemented with vitamin E (dissolved in ethanol at various concentrations). The final concentration of ethanol in test medium was 0.1% (v/v). DLD-1 cells were seeded onto a 96-well plate at a density of 1.0×10^4 cells/well in 100 μl of growth medium. After incubation for 24 h, the medium was removed, and the cells were cultured in 100 μl of test medium. Then, DLD-1 proliferation was assessed by the MTT method [28].

Lactate dehydrogenase (LDH) measurement. After cultivation of DLD-1 cells in test medium (5 ml/6-cm dish), a portion of the culture supernatant was harvested for cytotoxicity analysis by LDH detection kit (Takara, Ohtsu, Japan) under the conditions recommended by the manufacturer.

Detection of telomerase activity. Telomerase activity was determined by the stretch PCR assay [29] using a TeloChaser Kit (Toyobo, Osaka, Japan) which includes a 65-base pair internal control to allow quantification of activity [11]. Briefly, vitamin E-treated and non-treated cells, grown in 6-cm dishes, were rinsed twice with phosphate-buffered saline and suspended in lysis buffer (Toyobo). Then, the cell lysate (20 μl; equivalent to 1.5×10^4 cells) was subjected to stretch PCR assay. The PCR product was electrophoresed on a 10% non-denaturing polyacrylamide gel and visualized with SYBR Green I (Cambrex Bio Science, Walkerville, MD). Relative telomerase activity was determined by measuring the band intensities of all six-base ladders, and comparing them with those of internal standard. Band intensity was measured using Scion Image Beta 4.02 picture analyzing software for Windows (Scion Corporation, Frederick, MD), which is based on the NIH Image software.

Isolation of total RNA and analysis of mRNA expression. For real-time quantitative reverse transcriptase-PCR (RT-PCR), total RNA was isolated from the cells with RNeasy total RNA extraction kit (Qiagen, Hilden, Germany), and cDNA was synthesized from 1 μg of RNA using M-MLV reverse transcriptase (Takara) and oligo(dT) primer (Takara). The cDNA was subjected to PCR amplification using DyNAmo SYBR Green qPCR kit (Finnzymes, Espoo, Finland) and gene-specific primers for hTERT [30], c-myc [31], and β-actin [32]. Quantitative PCR was performed with the DNA engine opticon 2 system (MJ Research, Waltham, MA), which allows real-time quantitative detection of the PCR product by measuring the increase in fluorescence caused by the binding of SYBR Green to double-strand DNA. The thermal cycling conditions comprised an initial denaturation step at 95 °C for 5 min and 33 cycles of 95 °C for 10 s, 63 °C for 20 s, and 72 °C for 20 s.

PKC activity. Cellular PKC activity was determined using the PepTag non-radioactive PKC assay kit (Promega, Madison, WI) [11].

Statistical analysis. Data represent means ± SD of at least three independent experiments. Statistical differences between control and the corresponding vitamin E treatments were determined by one-way analysis of variance (ANOVA) followed by Dunnett's test. P-values < 0.05 were considered significant.

Results

Before the evaluation of telomerase activity, the growth inhibition of DLD-1 cells by tocotrienol was investigated. As shown in Fig. 2, tocotrienol dose-dependently inhibited DLD-1 proliferation. The inhibitory potency of each tocotrienol isomer varied as the following order: δ- > β- > γ- > α-tocotrienol. The ranked order is consistent with our previous report [19]. On the other hand, tocotrienol showed no cytotoxicity, except for 30–50 μM δ-tocotrienol when it was cultured with DLD-1 cells for 24 h (data not shown). This indicated that tocotrienol up to 20 μM does not induce cytotoxicity. From these results, tocotrienol at the concentrations of 20 μM or less was chosen for the telomerase experiments.

Telomerase activity was examined using a semi-quantitative telomerase assay (stretch PCR) [29]. In this assay, DNA amplified from telomerase products by the stretch PCR was separated by electrophoresis and detected on the gel as a ladder consisting of bands spaced six-base pairs apart (refer to the ladder presented for control in Fig. 3A). After culturing DLD-1 cells with β- and δ-tocotrienols for

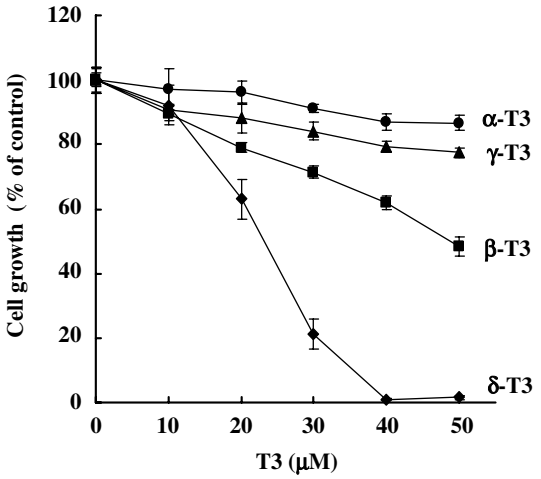

Fig. 2. Effect of tocotrienol on the DLD-1 proliferation. DLD-1 cells grown in 96-well plates were treated with 10–50 μM tocotrienol or without sample (control) for 48 h. The viable cells were then evaluated by MTT assay and are expressed as a percentage of the control. Values are means ± SD from five independent experiments. T3, tocotrienol.

24 h, a decrease in intensity of the six-base ladders was documented (Fig. 3A), indicating that these tocotrienols can modulate telomerase activity. Fig. 3B shows that treatment of DLD-1 cells with 5–20 μM β- and δ-tocotrienols for 24 h resulted in a dose-dependent decrease in telomerase activity. To examine the time-dependent telomerase inhibition, DLD-1 cells were cultured with β- and δ-tocotrienols (each 20 μM) for 6–72 h (Fig. 3C). Telomerase activity was time-dependently decreased, and the inhibitory potency of δ-tocotrienol was stronger than that of β-isomer. Interestingly, in contrast to tocotrienols, tocopherols (α-, β-, γ-, and δ-isomers) exhibited very weak telomerase inhibition (data not shown). It is therefore likely that the telomerase-inhibitory potency of tocotrienol is much higher than that of tocopherol.

hTERT is the catalytic subunit of telomerase, and it plays an important role in telomerase activation [33]. Therefore, to evaluate the mechanism for telomerase inhibition by tocotrienol, we investigated the effect of tocotrienol on hTERT expression in DLD-1 cells using a real-time RT-PCR. As shown in Fig. 4A, both β- and δ-tocotrienols effectively inhibited the expression of hTERT mRNA in a dose-dependent manner, indicating that telomerase activity is controlled at the transcriptional level. Since oncogene c-myc is a well-known regulator of hTERT expression [34], we then examined and found that β- and δ-tocotrienols caused a significant decrease in c-myc mRNA expression (Fig. 4B). At last, because PKC participates in the up-regulation of c-myc mRNA expression [35], we investigated and confirmed that β- and δ-tocotrienols inhibited cellular PKC activity (Fig. 4C). These observations suggested that β- and δ-tocotrienols reduce telomerase activity by down-regulating hTERT and c-myc expression through inhibition of PKC activity.

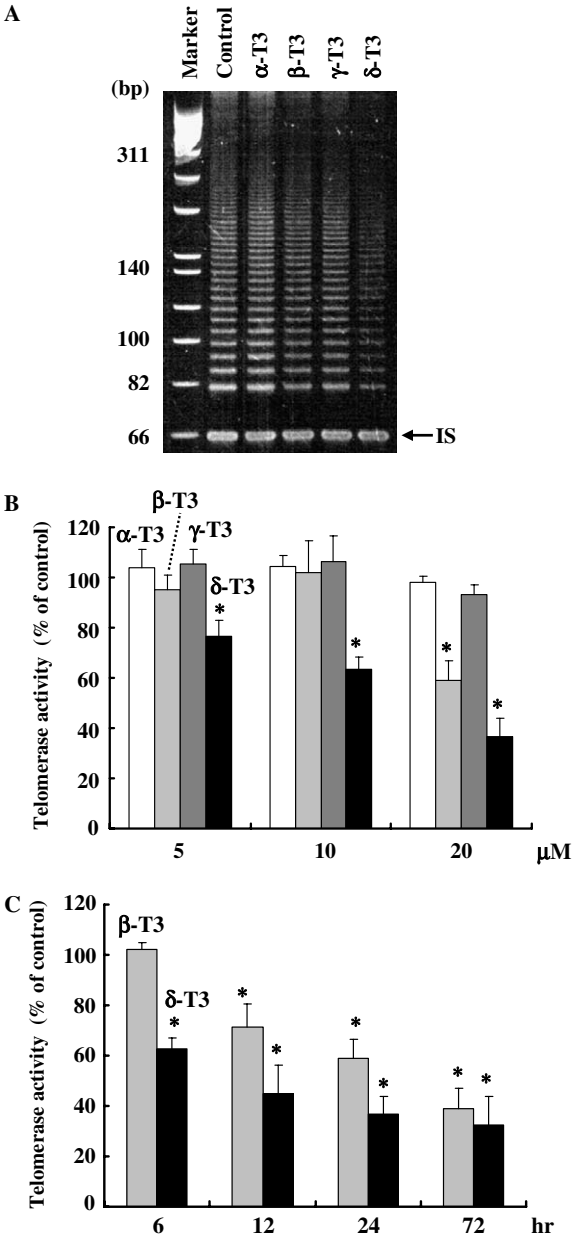

Fig. 3. Cellular telomerase activity is decreased by treatment with tocotrienol. (A) DLD-1 cells were cultured with or without 20 μM tocotrienol for 24 h. Subsequently, telomerase activity in the cell extracts was measured by the stretch PCR assay. The PCR product was separated by 10% non-denaturing polyacrylamide gel electrophoresis and visualized with SYBR Green I. IS, internal standard. (B) The dose-dependent inhibition of telomerase activity was evaluated by treating DLD-1 cells with 5–20 μM tocotrienol or without sample (control) for 24 h. (C) The time-dependent inhibition of telomerase activity was investigated by treating DLD-1 cells in the presence or absence of 20 μM tocotrienol for 6–72 h. Telomerase activity is expressed as a percentage of the control. Values are means ± SD from three independent experiments; $^{*}P < 0.05$ compared with control. T3, tocotrienol.

Discussion

To date, the notion of tocotrienol being a potent chemopreventive agent has been enhanced due to its anti-tumor property. A number of studies have shown the

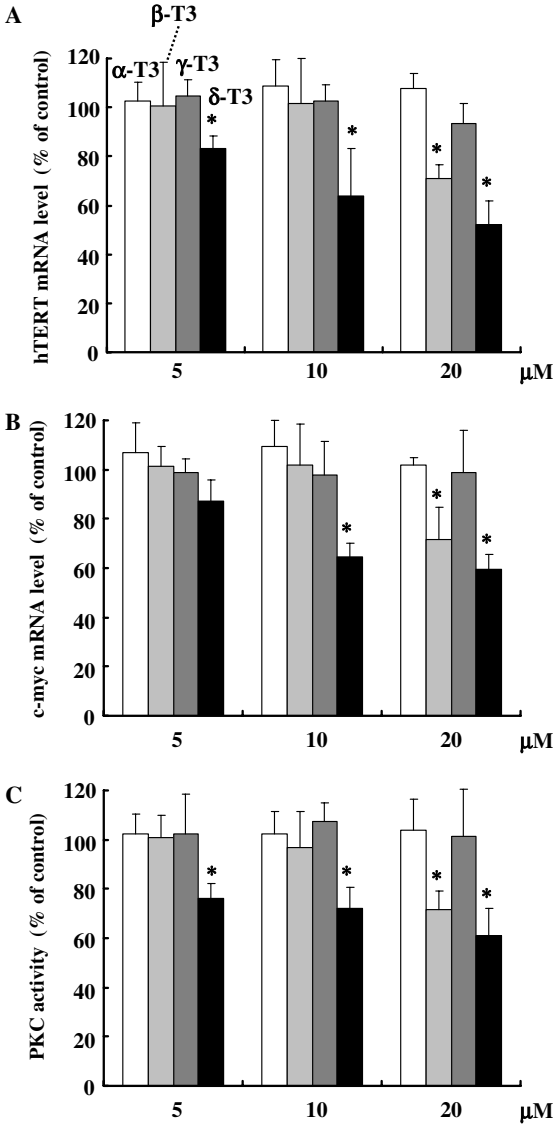

Fig. 4. Inhibitory effect of tocotrienol on mRNA expression of hTERT and c-myc, and on cellular PKC activity. DLD-1 cells were cultured with 5–20 μM tocotrienol or without sample (control) for 24 h. The mRNA expression levels of hTERT (A) and c-myc (B) were then measured by real-time RT-PCR and are expressed as percentages of the control. The mRNA level of β-actin was used as an internal control. (C) PKC activity in DLD-1 cells cultured in the presence or absence of 5–20 μM tocotrienol for 24 h was examined and is expressed as a percentage of the control. Values are means ± SD from three independent experiments; $^{*}P < 0.05$ compared with control. T3, tocotrienol.

growth inhibition of human, mouse, and rat tumor cells by tocotrienol [17]. Particularly, tocotrienol, especially δ-isomers, inhibited the proliferation of human breast cancer cells, irrespective of estrogen receptor status [18]. The inhibitory effect of tocotrienol on proliferation of breast cancer cells represented a potentially physiological role in the prevention of cancer [17]. In this study, we demonstrated for the first time that β- and δ-tocotrienols significantly inhibited the proliferation of DLD-1 cells as well as cellular telomerase activity. The inhibitory potency of tocotrienol on cell growth [18] was comparable with that on telomerase activity. Therefore, it is likely that the anti-cancer effect of tocotrienol may be attributable in part to the inhibition of telomerase activity.

Interestingly, we found that tocotrienol has much higher inhibitory action on telomerase activity than tocopherol (Figs. 2 and 3). Structurally, tocopherol and tocotrienol are distinguished by their side chains. It has been demonstrated that the unsaturated side chain of tocotrienol allows it to pass through cell membranes more efficiently and at a faster rate than the saturated side chain of tocopherol [14]. Therefore, it is possible that the greater telomerase-inhibitory effect of tocotrienol would be due, in part, to their effective incorporation into DLD-1 cells. In addition, among tocotrienols, β- and δ-isomers were shown to possess significant anti-telomerase activity. Both β- and δ-tocotrienols are structurally different from other isomers in lacking the 7-methyl substituent. Thus, tocotrienol without the 7-methyl group would play a key role in exhibiting a potent telomerase-inhibitory effect.

PKC is known to participate in both the post-transcriptional control of telomerase activity [26] and the modulation of hTERT expression [27]. As shown in Figs. 3 and 4, there were proportional correlations among telomerase activity, hTERT expression, and PKC activity. Therefore, telomerase inhibition via suppression of PKC activity by tocotrienol could be assigned to the transcriptional inhibition of hTERT expression. This result is in an agreement with recent studies showing that ceramide and curcumin, as inhibitors of PKC, can repress telomerase activity through down-regulation of hTERT [9,10]. Moreover, both direct activators of PKC and hormones which induce PKC via receptor-mediated phosphoinositide turnover elicit a rapid increase in c-myc mRNA expression, suggesting that c-myc expression is controlled through a PKC signaling pathway [35]. Thus, we deduced that down-regulation of c-myc (Fig. 4B) would be due to the inhibition of PKC activity by tocotrienol (Fig. 4C). Considering these findings (Figs. 3 and 4), the following mechanism is conceivable for telomerase inhibition by tocotrienol: tocotrienol inhibits PKC activity, leading to down-regulation of c-myc mRNA, resulting in repression of hTERT expression, thereby reducing telomerase activity.

It is reported that PKCα and ζ modulate telomerase activity in breast and nasopharyngeal cancer cells, respectively [26,36]. The findings indicate that different PKC isoforms are involved in the regulation of telomerase activity in different cancer cell types. In colorectal cancer cells (like DLD-1), it is not clear which PKC subtypes participate in the control of telomerase activity. However, Murray et al. [37] demonstrated that elevated expression of PKCβII is implicated in colon carcinogenesis. It is therefore plausible that PKCβII might regulate telomerase in DLD-1 cells and that PKCβII inhibition with tocotrienol may cause reduction of telomerase activity. Further studies are needed to clarify the inhibitory mechanism for the PKC isoform by tocotrienol.

Tocotrienol has been shown to post-transcriptionally attenuate HMG-CoA reductase activity [16]. Recent studies have revealed that the HMG-CoA reductase inhibitors such as lovastatin can induce cell cycle arrest and therefore have potential as novel chemotherapeutic agents [38]. Thus, it is likely that the anti-proliferative property of tocotrienol may partly participate in the inhibition of HMG-CoA reductase. Interestingly, lovastatin represses the E2F-1-regulated expression of several cell cycle genes including c-myc [39]. These findings raise the possibility that tocotrienol may inhibit telomerase through not only the reduction of PKC activity but also the suppression of HMG-CoA reductase. Further investigation of telomerase inhibition by tocotrienol will help to develop mechanism-based strategies for cancer prevention.

In conclusion, we demonstrated for the first time that tocotrienol exhibited the inhibitory effect on cellular telomerase activity and the inhibitory potency of tocotrienol is much superior to that of tocopherol. Tocotrienol modulated telomerase by repressing hTERT and c-myc expression via inhibition of PKC activity. These results support the anti-proliferative function of tocotrienol and indicate that tocotrienol is a potent candidate regulator of telomerase.

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