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δ-Tocotrienol Suppresses VEGF Induced Angiogenesis whereas α-Tocopherol Does Not

Akira Shibata,[†] Kiyotaka Nakagawa,*[,†] Phumon Sookwong,[†] Tsuyoshi Tsuduki,[‡] Shinichi Oikawa,[§] and Teruo Miyazawa[†]

[†]Food & Biodynamic Chemistry Laboratory and [‡]Laboratory of Food and Biomolecular Science, Graduate School of Agricultural Science, Tohoku University, Sendai 981-8555, Japan, and [§]Department of Medicine, Nippon Medical School, Tokyo 113-8603, Japan

Recently, tocotrienol (T3), a less well-known form of vitamin E, has gained considerable attention as a potent antiangiogenic agent. However, the majority of vitamin E research has focused on tocopherol (Toc), with some studies indicating α-Toc may prevent tumor angiogenesis. In this study, we aimed to clarify the differences in antiangiogenic potential between δ-T3 and α-Toc. We showed δ-T3 (2.5−5 μM) completely abolished proliferation, migration and tube formation of human umbilical vein endothelial cells (HUVECs), whereas a similar dose of α-Toc had no such effects. δ-T3 suppressed VEGF receptor 2 (VEGFR-2) signaling, and activated caspases in HUVECs. In addition, via an in vivo mouse Matrigel plug angiogenesis assay, we found that δ-T3 (30 μg), but not α-Toc, inhibited tumor cell-induced vessel formation. In summary, our results demonstrate δ-T3 has superior antiangiogenic activities to α-Toc, and provide insights into the different mechanisms responsible for this effect of T3 and Toc.

KEYWORDS: Antiangiogenesis; tocotrienol; vitamin E; endothelial cell; VEGF

INTRODUCTION

Angiogenesis, the formation of new blood vessels, plays an important role in many physiological and pathological processes, especially cancers (*1*). The new-formed blood vessels can promote cancer growth by supplying nutrients and oxygen and removing waste products (*2*). As a consequence, well-vascularized tumors grow rapidly and have greater tendency to metastasize to other organs (*3*).

It is well-known that various growth factors have a major role in neovascularization, and one of the most important factors is vascular endothelial growth factor (VEGF) (*4*). The majority of tumors express and secrete high levels of VEGF, which is enhanced by hypoxia and/or genetic changes in cancer cells (*5*). The secreted VEGF binds to specific trans-membrane receptors such as VEGF receptor 1 (VEGFR-1) and VEGF receptor 2 (VEGFR-2) located on endothelial cells. This binding consequently activates endothelial mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K)/phosphoinositide-dependent protein kinase (PDK)/Akt pathways, which in turn initiate mitogenic and survival signaling. VEGF may therefore initiate a number of key endothelial angiogenic responses, such as proliferation, migration, and differentiation, as well as protection from apoptosis, via activating PI3K/PDK/Akt pathway (*6*). In this aspect, there is evidence that PI3K/PDK/Akt signaling is elevated in tumors in breast cancer patients, with levels correlating with tumor progression (*7*).

*Corresponding author. Tel: +81-22-717-8906. Fax +81-22-717-8905. E-mail nkgw@biochem.tohoku.ac.jp.

Understanding of the fundamental mechanisms of angiogenesis has allowed the discovery of inhibitors targeting VEGF for use in cancer therapy and in prevention of angiogenic disorders, such as diabetic retinopathy (*8*). Several antiangiogenic drugs (e.g., ZD6474 and Vatalanib/PTK787) are currently being studied in clinical trials involving patients with a wide variety of cancers. Some of these agents have considerable promise as future treatments (*9*, *10*). Recently, we demonstrated tocotrienol (T3, unsaturated vitamin E) as a potent natural antiangiogenic compound, since it suppresses angiogenesis by inhibiting proliferation, migration and tube formation of endothelial cells (*11*). The inhibitory effect was speculated to the regulation of growth factor-dependent PI3K/PDK/Akt signaling (*12*).

Vitamin E is a generic name for two subgroups of eight different fat-soluble compounds, α-, β-, γ-, δ-tocopherols (Toc) and α-, β-, γ-, δ-T3. The structural difference between Toc and T3 is that Toc has a saturated phytyl side chain attached to its chroman ring, whereas T3 possesses an unsaturated isoprenoid side chain. A major physiological activity of vitamin E is its well-defined antioxidative action and protective effect against lipid peroxidation in biological membranes (*13*), with α-Toc having the most activity of all the vitamin E isomers. However, T3 has recently gained increasing scientific interest due to its eminent antioxidative (*14*), anti-hypercholesterolemic (*15*), and neuroprotective (*16*) activities that differ somewhat from those of Toc. Further, the potent abilities of T3 to induce cell cycle arrest (*17*), to regulate HMG-CoA reductase (*18*), to activate p53 and caspase-8 (*19*, *20*), to suppress adhesion molecules (*21*), to inhibit nuclear factor-κB (*22*), and to downregulate c-Myc and telomerase (*23*) have been reported. These unique effects of T3 could be

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partly explained by its absorption and metabolic fate in vivo. In humans, a key factor in determining the efficacy of T3 is gaining an understanding of its absorption and metabolism. Although the absorption mechanisms are basically the same for all vitamin E analogues, T3 is reported to be absorbed into cells or degraded to metabolites to a greater extent than Toc (*24*, *25*).

Our previous cell culture studies showed that T3 has better antiangiogenic activities than Toc, with δ-T3 being the most potent and α-Toc the least potent (*11*). These findings were in accordance with an animal experiment (*12*), that cancer neovascularization in mouse subcutaneous tissue was effectively inhibited by oral administration of T3, whereas Toc had no such effect. However, there are disagreements on the potential of α-Toc and its other isomers in prevention of tumor angiogenesis (*26*, *27*), whose differences in the angiopreventive effects of T3 and Toc have not been clarified.

On the basis of this background, we carried out a series of investigations on the specific molecular events in VEGF signaling that contribute to the antiangiogenic effect of T3. These investigations had the aim of clarification on the different inhibitory activity between T3 and Toc, and on the effects of these two compounds on VEGF-induced proliferation, migration, tube formation and signal transduction mediated by endothelial angiogenic responses. In the latter part of this study, the in vivo antiangiogenic properties of T3 and Toc were evaluated using a mouse Matrigel plug angiogenesis assay.

MATERIALS AND METHODS

Reagents, Cells, and Animals. T3 isomers (Eisai, Tokyo, Japan (*28*)) were used, and its purity was 98%. *d*-α-Tocopherol (α-Toc) was purchased from Sigma (St. Louis, MO) and WST-1 reagent from Dojindo Laboratories (Kumamoto, Japan). All other reagents were of analytical grade. Human umbilical vein endothelial cells (HUVECs) were obtained from Kurabo (Osaka, Japan), and were maintained in growth medium HuMedia-EG2 (Kurabo) at 37 °C in a humidified atmosphere of 5% CO_2. The HuMedia-EG2 medium consisted of the base medium (HuMedia-EB2) supplemented with 2% fetal bovine serum (FBS), 10 μg/L human epidermal growth factor (hEGF), 5 μg/L human basic fibroblast growth factor (hFGF), 1 mg/L hydrocortisone, 10 mg/L heparin, 50 mg/L gentamicin, and 50 μg/L anfoterin B. Confluent HUVECs at passages 5 to 8 were used in the experiments. Human colorectal adenocarcinoma cells (DLD-1) were obtained from the Cell Resource Center for Biomedical Research at Tohoku University School of Medicine (Sendai, Japan). The cells were maintained at 37 °C in RPMI-1640 medium containing 0.3 g/L L-glutamine and 2 g/L sodium bicarbonate (Sigma, St. Louis, MO), supplemented with 10% FBS, 100 kU/L penicillin (Gibco BRL, Rockville, MD), and 100 mg/L streptomycin (Gibco BRL, Rockville, MD).

Male athymic nude mice (BALB/cA Jcl-nu nu/nu, 4 weeks of age) were obtained from CLEA (Tokyo, Japan) and were housed in cages kept at 23 °C with a 12 h light/dark cycle in pathogen-free conditions. The mice were acclimatized with laboratory rodent chow (CL-2; CLEA, Tokyo, Japan) and water for 1 week prior to the study. Approximate composition of CL-2 (g/kg diet): carbohydrate, 482; protein, 244; fat, 60; fiber, 43; moisture, 86; ash, 86.

Preparation of Test Medium for Cell Culture Studies. Test medium (HuMedia-EB2 containing 1−2% FBS) with or without supplementation of 10 μg/L VEGF was prepared for the following cell culture studies. Vitamin E was dissolved in ethanol, and the solution was diluted to final concentrations of 0−20 μM in the test medium. The concentration of ethanol never exceeded 0.1% (v/v).

Proliferation Assay. HUVECs (2000−2500 cells/well) were preincubated in HuMedia-EG2 medium in 96-well plates for 24 h. After the HUVECs were washed with PBS, the medium was replaced with the test medium. After incubation for 24−72 h, the number of viable cells was estimated using the water-soluble tetrazolium salt (WST-1) assay (*29*). WST-1 is a tetrazolium salt that is converted into the soluble formazan salt by succinate-tetrazolium reductase of the respiratory chain of active mitochondria of proliferating viable cells. Briefly, 10 μL of WST-1 solution was added to each well and incubated at 37 °C for 3 h, and the absorbance (450/655 nm) of the cultured medium was then measured using a microplate reader (Model 550, Bio-Rad, Tokyo, Japan).

Migration Assay. Migration was evaluated by a modified Boyden chamber assay (*30*) (Becton Dickinson, Bedford, MA). The chamber consisted of 3 μm pore size membrane culture inserts coated with fibronectin. Trypsin-harvested HUVECs (1×10^5 cells) were suspended in 500 μL of the test medium and incubated for 30 min at room temperature. The suspension was then added to the top of the chamber, and vitamin E-free test medium was placed in the lower chamber. After incubation of the whole chamber for 22 h, nonmigrated cells were removed from the upper surface of the membrane by wiping with a cotton swab. The membrane was then fixed with 4% paraformaldehyde, and the cells that had migrated to the undersurface of the membrane were stained with toluidine blue. The number of migrated cells was counted in 5−6 randomly selected microscopic fields and expressed as a pixel value using Adobe Photoshop (Adobe Systems, Mountain View, CA).

Tube Formation Assay. A 24-well culture plate was coated with 350 μL of Matrigel (Becton Dickinson, Bedford, MA), and incubated for 1 h to allow solidification. Trypsin-harvested HUVECs (5×10^4 cells) were suspended in 500 μL of the test medium. The suspension was placed onto the surface of the Matrigel and incubated for 18 h. The cells were fixed in 4% paraformaldehyde, photographed and the lengths and areas of tube-structured cells then quantified using a Kurabo Angiogenesis Imaging Analyzer (imaging software; Kurabo, Osaka, Japan).

Hoechst Staining. To examine nuclear morphological changes, HUVEC nuclei were stained with a dye (Hoechst 33258; Sigma, St. Louis, MO). HUVECs (5×10^5 cells) were precultured for 24 h in HuMedia-EG2 medium. After washed with PBS, the culture medium was replaced and HUVECs were incubated with test medium for 24 h. After that, HUVECs were washed with PBS, harvested, fixed with 4% paraformaldehyde, rinsed with PBS, and incubated with 0.1 g/L Hoechst 33258 in PBS for 10 min at room temperature, respectively. The cells were observed under UV illumination with an IX-FLA fluorescence microscope (Olympus, Tokyo, Japan). Apoptosis was expressed as the percentage of fragmented Hoechst-positive nuclei relative to the total of Hoechst-positive nuclei.

Cellular Uptake of T3. Cellular uptake of T3 in HUVECs was measured by high performance liquid chromatography (HPLC) with fluorescence detection (*31*). In brief, subconfluent HUVECs were cultured in VEGF-free test medium containing each T3 isomer (1 μM, closer to the concentrations found in vivo, and the concentration hardly affect the HUVEC proliferation), or in VEGF-free test medium containing all T3 isomers. After 24 h incubation, the cells were washed with PBS, suspended in 2 mL of water, and then subjected to sonication for 90 s. A 1 mL aliquot of 6% ethanolic pyrogallol and 1 mL of 1 μM ethanolic 2,2,5,7,8-pentamethyl-6-hydroxychroman (internal standard) were mixed with the cell suspension. The sample mixture was added with 0.2 mL of 60% aqueous KOH and incubated at 70 °C for 30 min. After the cooling process, 1.5 mL of water and 5 mL of hexane were added for extraction. The samples were centrifuged at 1000*g* for 5 min, and the upper hexane layer was collected and dried. The residue was dissolved in 100 μL of hexane, and a portion of the aliquot (50 μL) was subjected to HPLC. Separation was performed at 35 °C using a silica column (ZORBAX Rx-SIL, 4.6 × 250 mm; Agilent, Palo Alto, CA). A mixture of hexane/1,4-dioxane/2-propanol (988:10:2) was used as the mobile phase at a flow rate of 1.0 mL/min. The T3 isomers were detected and determined by an RF-10AXL FLD detector (excitation 294 nm, emission 326 nm; Shimadzu, Kyoto, Japan).

Western Blot Analysis. HUVECs were grown in 100 mm dishes to 80−90% confluency in HuMedia-EG2 medium, washed with PBS and treated with vitamin E under two different conditions: (1) 24 h incubation in the test medium or (2) 5 min stimulation with VEGF after preincubation for 6 h in VEGF-free test medium. After these treatments, cellular proteins were prepared from the HUVECs as described previously (*12*), and then were separated by SDS−PAGE gel electrophoresis (10−20% e-PAGEL, 50 μg/well; Atto, Tokyo, Japan). The protein bands were then transferred to polyvinylidine fluoride membranes, which were then blocked overnight at 4 °C in blocking buffer (10% BSA in TBS containing 0.1% Tween-20). After blocking of nonspecific sites, the membranes were incubated with antibodies including anti-caspase-3, anti-caspase-9, anti-phospho Akt, anti-phospho extracellular signal-regulated kinase 1/2 (ERK 1/2),

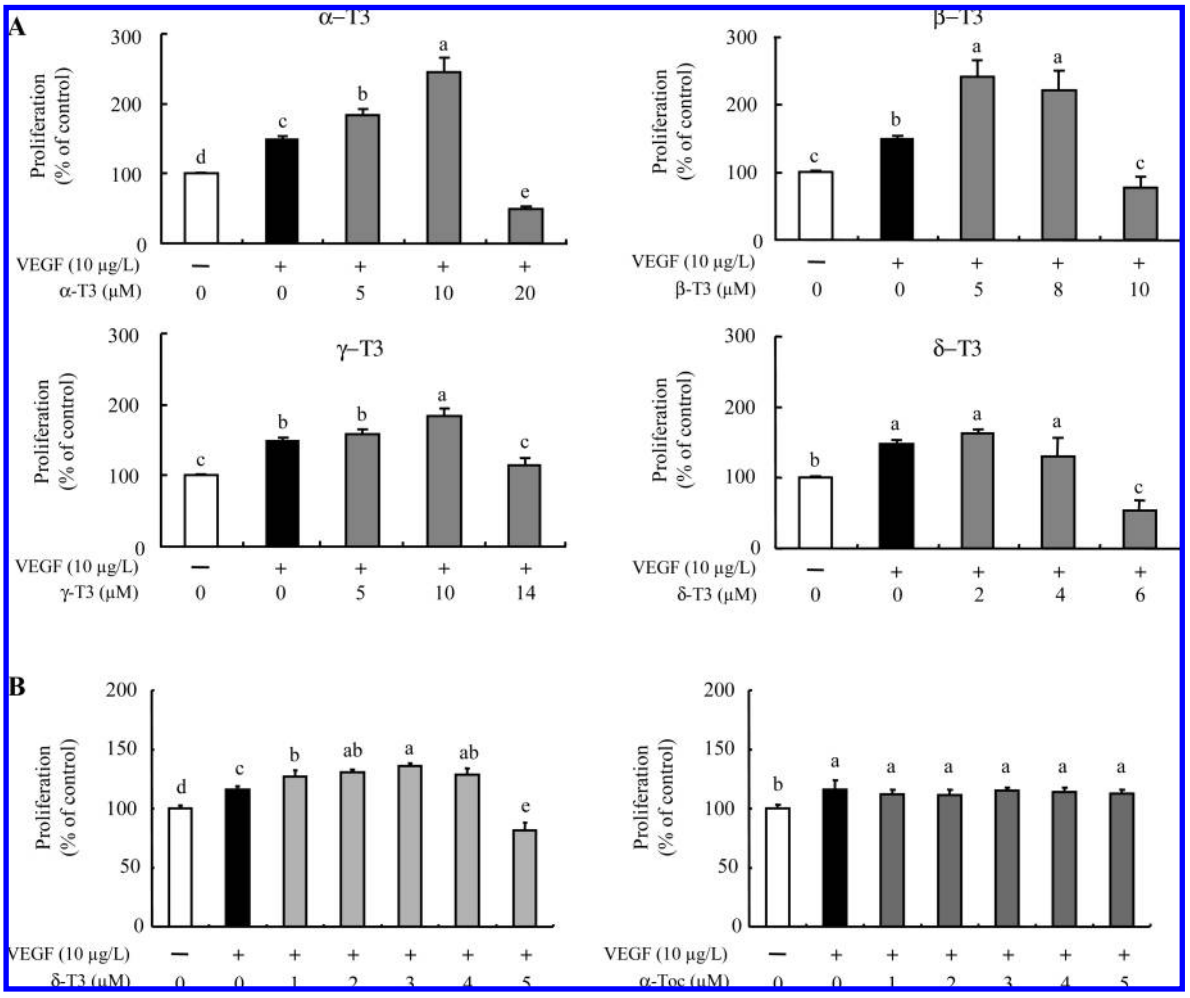

Figure 1. Effects of T3 on VEGF-induced HUVEC proliferation. (**A**) HUVECs (2000 cells/well) were preincubated in HuMedia-EG2 medium, followed by the medium being replaced with 100 μL of test medium (2% FBS HuMedia-EB2 containing 0−20 μM T3 and 10 μg/L VEGF) and incubation for a further 72 h. (**B**) HUVECs (2500 cells/well) were incubated for 24 h in medium containing VEGF and either δ-T3 or α-Toc at concentrations from 0 to 5 μM. The viable cells were then assessed by WST-1. Values are mean ± SD, $n = 6$. Means without a common letter differ, $P < 0.05$.

anti-phospho phosphatase and tensin homologue deleted on chromosome 10 (PTEN), anti-phospho VEGFR-2, anti-phospho p38, anti-phospho apoptosis signal-regulating kinase (ASK1), and anti-β-actin (Cell signaling, MA). The membranes were washed with TBS (containing 0.1% Tween-20), and incubated for 30 min at room temperature with horseradish peroxidase-conjugated secondary antibody, respectively. Detection of the antibody reactions was performed with ECL Plus Western blotting reagents (Amersham Pharmacia Biotech, Piscataway, NJ).

Measurement of Caspase Activity. Subconfluent HUVECs were cultured for 0−24 h in the test medium. The cells were then washed with PBS and lysed in 1 mL of lysis buffer containing 0.1% Triton X-100, 50 mM HEPES, 5 mM EDTA, 150 mM NaCl, 1 mM PMSF, 10 mg/L pepstatin A, and 10 mg/L aporotinin. The lysates were kept on ice for 10 min, scraped from the dishes, and sonicated for 30 s to extract cell contents. These extracts were centrifuged at 10000*g* for 15 min, and the supernatants were collected and concentrated using a centrifugal filter device. Protein concentrations in the obtained samples were measured by the Bradford protein assay (Bio-Rad Laboratories, Watford, Herts, U.K.). The proteins were use in preparation of cell lysates (60 μg protein/50 μL of lysis buffer). A 50 μL aliquot of buffer (10 mM DTT, 100 mM HEPES, and 5 mM EDTA) and a 5 μL aliquot of 4 mM caspase substrate (Ac-DEVD-pNA for caspase-3, Ac-IETD-pNA for caspase-8, or Ac-LEHD-pNA for caspase-9) were added to the lysates, followed by incubation at 37 °C for 2.5 h. At the end of incubation, caspase activity was measured using a microplate reader at the wavelength of 405 nm.

Matrigel Plug Angiogenesis Assay. The in vivo Matrigel plug angiogenesis assay was performed as described previously by Liu et al. (*32*). DLD-1 cells were suspended in a serum- and phenol red-free RPMI-1640 medium (Gibco BRL Rockville, MD). Aliquots of the cell suspension (1×10^6 cells/0.1 mL) were mixed with 0.2 mL of phenol red-free Matrigel containing 50 ng of VEGF and 0−30 μg of δ-T3 or α-Toc, and the mixture was injected into the flanks of nude mice using a 21 gauge needle. The mice were allowed free access to the laboratory rodent chow (CL-2; CLEA, Tokyo, Japan) and water for 11 days. After that, the Matrigel plugs were removed and photographed 11 days, and the hemoglobin (Hb) contents were measured using a test kit (Hemoglobin test, Wako, Osaka, Japan). These protocols were reviewed by the Committee on the Ethics of Animal Experiments of Tohoku University, and this experiment was carried out in accordance with the Animal Experiment Guidelines of Tohoku University.

Statistical Analysis. The data are expressed as the mean ± SD. Statistical analysis was performed using ANOVA, followed by Bonferoni/Dunn test test for multiple comparisons of the different groups. Differences were considered significant at P values < 0.05.

RESULTS

Suppressive Effect of T3, but Not α-Toc, on VEGF-Induced Angiogenesis in Vitro. We first examined the effect of T3 on VEGF-induced proliferation of endothelial cells. HUVECs incubated with VEGF for 72 h showed an increase in proliferation compared with cultures without VEGF (**Figure 1A**). All T3 isomers suppressed the proliferation, and the inhibitory effect tended to be as δ- > β- > γ- > α-T3. A similar tendency (δ- > γ- > β- > α-T3) was observed for T3 uptake by the HUVECs

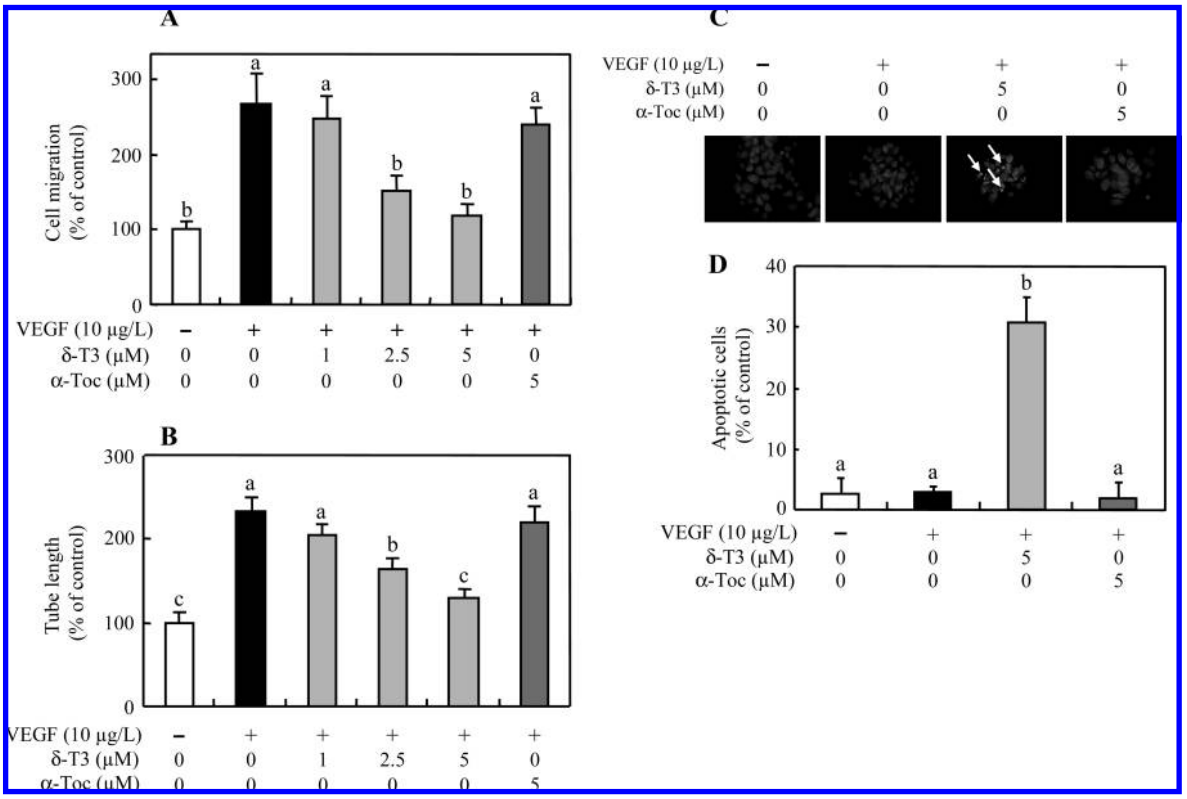

Figure 2. (**A**) Effects of δ-T3 and α-Toc on VEGF-induced HUVEC migration. The migration assays were carried out in a modified Boyden chamber consisting of a cell culture insert membrane (3 μm pore membrane coated with fibronectin). HUVECs (1.0×10^5 cells) were suspended in 500 μL of HuMedia-EB2 medium containing 1% FBS and either δ-T3 or α-Toc, and were then added to the top chamber. The lower chamber was filled with 750 μL of HuMedia-EB2 medium containing 1% FBS and 10 μg/L VEGF. The whole chamber was incubated for 22 h, and the number of cells that had migrated to the lower side of the filter was quantified. Values are mean ± SD ($n = 4$). Means without a common letter differ, $P < 0.05$. (**B**) Effects of δ-T3 and α-Toc on VEGF-induced HUVEC tube formation. HUVECs (5×10^4 cells) were suspended in 500 μL of test medium (1% FBS HuMedia-EB2 containing 10 μg/L VEGF and either δ-T3 or α-Toc). Following incubation of the cell suspension on the Matrigel plate for 18 h, tube lengths were quantified by photography using an optical microscopy. Values are mean ± SD, $n = 4$. Means without a common letter differ, $P < 0.05$. (**C**) Effect of δ-T3 and α-Toc on apoptosis in VEGF-treated HUVECs. Apoptosis of HUVECs was evaluated after 24 h of δ-T3 or α-Toc (5 μM) treatment. Apoptosis was visualized by nuclear fragmentation (Hoechst staining). Nuclei stained with Hoechst 33258 were identified using a fluorescence microscope. The images shown refer to representative experiments. Apoptosis was expressed as the percentage of fragmented Hoechst-positive nuclei relative to the total of Hoechst-positive nuclei (**D**). The pictures are a representative example of data from three replicate experiments. Results represent the average ± SD of three replicate experiments. Means without a common letter differ, $P < 0.05$.

Table 1. T3 Incorporation for HUVEC

	nmol/mg protein of HUVEC[a]	
	treatment of each T3 isomer[b]	treatment of all T3 isomers[c]
α-T3	6.0 ± 0.5 a	6.1 ± 1.1 a
β-T3	7.4 ± 1.2 a	7.3 ± 0.7 a
γ-T3	7.6 ± 0.3 b	7.7 ± 0.7 b
δ-T3	7.9 ± 0.8 b	7.9 ± 0.7 b

[a] Values are mean ± SD (n=4). Means without a common letter differ, $P < 0.05$. [b] Subconfluent HUVECs were cultured in VEGF-free test medium containing 1 μM of each T3 isomer for 24 h. [c] Subconfluent HUVECs were cultured in VEGF-free test medium containing all T3 isomers (1 μM) for 24 h.

(**Table 1**), although δ-T3 and γ-T3 (also β-T3 and α-T3) were not statistically different from each other. We used 1 μM T3 in **Table 1**, and tested more high concentrations (5−20 μM) in **Figure 1A**. So, two studies (**Table 1** and **Figure 1A**) were not easily compared, but it is likely that the greater proliferation inhibitory potency of δ-T3 may be due, in part, to its more effective incorporation into HUVECs. On the basic of these results, δ-T3 over the concentration range of 1−5 μM was chosen for the subsequent cell culture studies that evaluated the difference in angiopreventive potential between T3 and Toc and investigated the intracellular antiangiogenic mechanism.

In the next series of investigations, we compared the effects of δ-T3 and α-Toc on HUVEC proliferation, migration, and tube formation. In the proliferation assay (**Figure 1B**), δ-T3 (5 μM) suppressed VEGF-induced HUVEC proliferation. In the migration assay (**Figure 2A**), VEGF-treated HUVEC were allowed to migrate across the fibronectin-coated membrane insert. δ-T3 suppressed VEGF-induced migration with 50% inhibition at the concentration of 2.0 μM. In the tube formation assay (**Figure 2B**), in which HUVECs were placed on a Matrigel coated plate in the presence of VEGF, an increase in the length of tubelike structures was observed. The VEGF-induced tube formation was suppressed by δ-T3 treatment in a dose-dependent manner, with 50% inhibition at the concentration of 2.8 μM. In contrast, α-Toc did not exhibit any effects on VEGF-induced HUVEC proliferation, migration or tube formation, even at a high concentration of 50 μM (data not shown). These results suggest that δ-T3 is the more bioactive compound than α-Toc in term of the angiogenesis inhibitor.

Antiangiogenic Mechanism of δ-T3. In the proliferation assay (**Figure 1A**,**B**), when the HUVECs were treated with δ-T3 (5 μM), cell viability was decreased to some extent. To evaluate whether apoptosis was induced by δ-T3, Hoechst 33258 staining assay was performed. As we had anticipated, nuclear condensation and brightness were observed in Hoechst

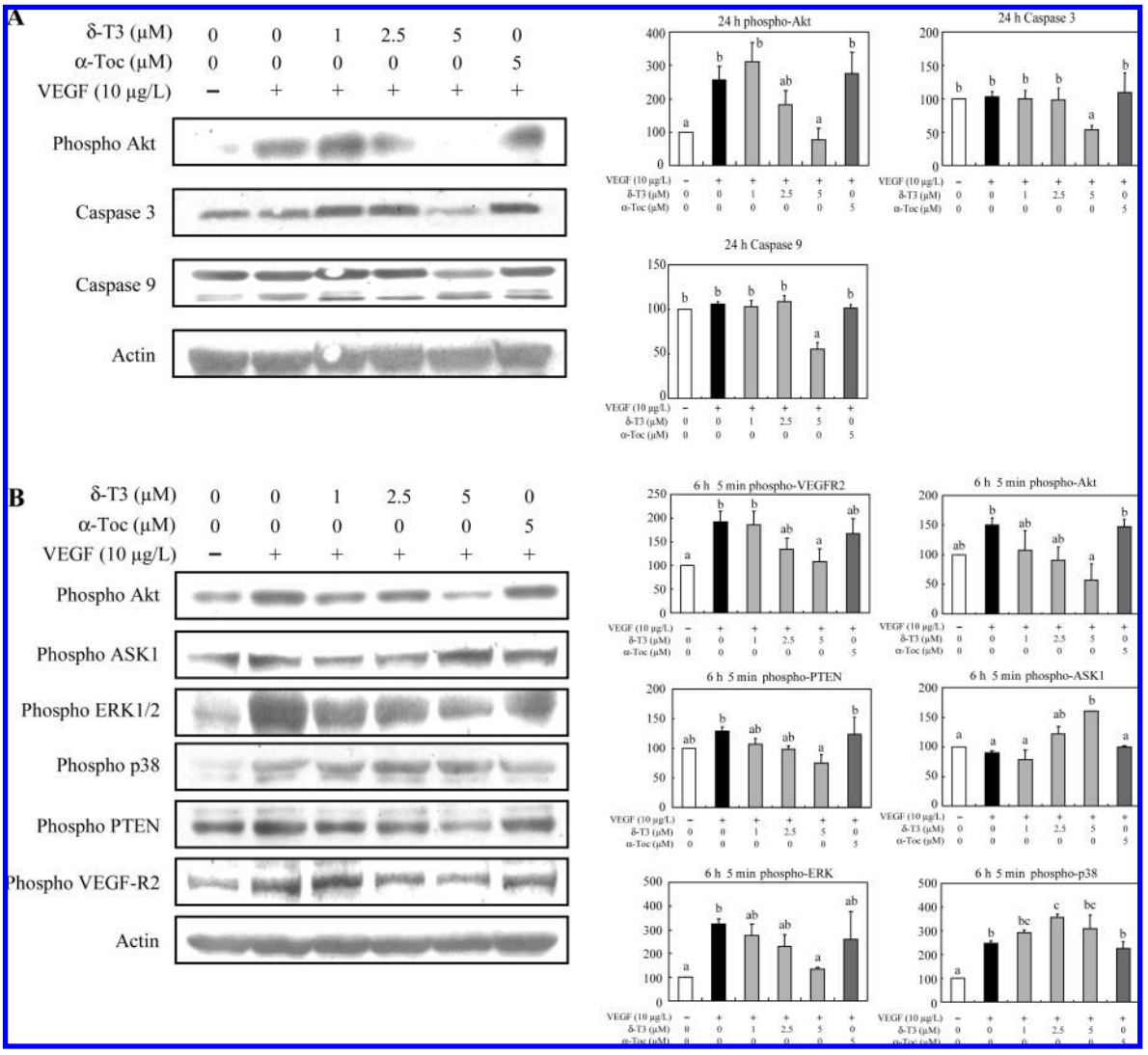

Figure 3. Western blot analysis of the intracellular proteins associated with the PI3K/PDK/Akt pathway and apoptosis. HUVECs were treated with δ-T3 or α-Toc under two different conditions: (**A**) 24 h incubation with 10 μg/L VEGF and 0−5 μM δ-T3 or α-Toc, or (**B**) 5 min stimulation with 10 μg/L VEGF after preincubation with 0−5 μM δ-T3 or α-Toc for 6 h. Pro-caspases-3 and -9 (35 kDa and 47 kDa, respectively) were cleaved to the active forms after 24 h of treatment, with the decrease in pro-caspase inactive forms always being associated with a corresponding increase in the cleaved active forms. Each Western blot is a representative example of data from 3 replicate experiments. Band intensities were estimated by densitometric scans, and graphs of the intensities were made. Results represent the average ± SD of three replicate experiments. Means without a common letter differ, $P < 0.05$.

33258-stained cells treated with δ-T3 (**Figure 2C**,**D**). In contrast, α-Toc was ineffective for inducing apoptosis in HUVECs. These distinguishable effects of T3 and Toc on cell apoptosis are in agreement with previous reports on mouse mammary epithelial cells (*33*) and human breast cancer cells (*34*). Our findings suggest that the apoptotic effects on endothelial cells induced by δ-T3 may contribute, in part, to its antiangiogenic activity (**Figures 1** and **2**).

Next, we evaluated the inhibitory mechanism of δ-T3 on VEGF-induced angiogenesis by Western blot analysis. HUVECs were treated with or without δ-T3 for 24 h in the presence of VEGF, and the effect of δ-T3 on the PI3K/PDK/Akt pathway was examined. Without δ-T3, VEGF induced activation of the PI3K/PDK/Akt pathway by phosphorylation of Akt (**Figure 3A**). In cultures with δ-T3, the phosphorylation of Akt was inhibited. At that time, δ-T3 did not affect the level of nonphosphorylation of Akt (data not shown). δ-T3 at a high dose (5 μM) cleaved procaspases-3 and -9 to their active forms. To more clarify the mechanism, HUVECs were treated with or without δ-T3 for 6 h, and stimulated with VEGF for 5 min. The stimulation with VEGF resulted in activation of Akt, PTEN, and ERK 1/2, and these changes were reduced to basal (nonstimulated) levels by δ-T3 treatment (**Figure 3B**). In addition, δ-T3 dose-dependently increased the phosphorylation of stress response proteins, such as ASK1 and p38 mitogen-activated protein kinase, and inhibited phosphorylation of VEGFR-2 (**Figure 3B**). These results suggest that the antiangiogenic activity of δ-T3 is attributable to regulation of PI3K/PDK/Akt signaling as well as induction of apoptosis, and that this effect occurs at the level of the VEGFR-2 receptor. In contrast, α-Toc had no effect on the PI3K/PDK/Akt pathway, which is a possible reason for its lower antiangiogenic activity.

In accordance with the results of apoptosis and Western blotting assays (**Figures 2** and **3**), caspase activations by δ-T3 were observed (**Figure 4**). Incubation of HUVECs for 12−24 h with 5 μM δ-T3 in the presence of VEGF caused a significant increase in caspase-3, -8, and -9 activities. However, the α-Toc did not cause the effect up to 50 μM (data not shown). Therefore, it is likely that caspase-dependent mechanisms play a role in the proapoptotic and antiangiogenic activities of δ-T3.

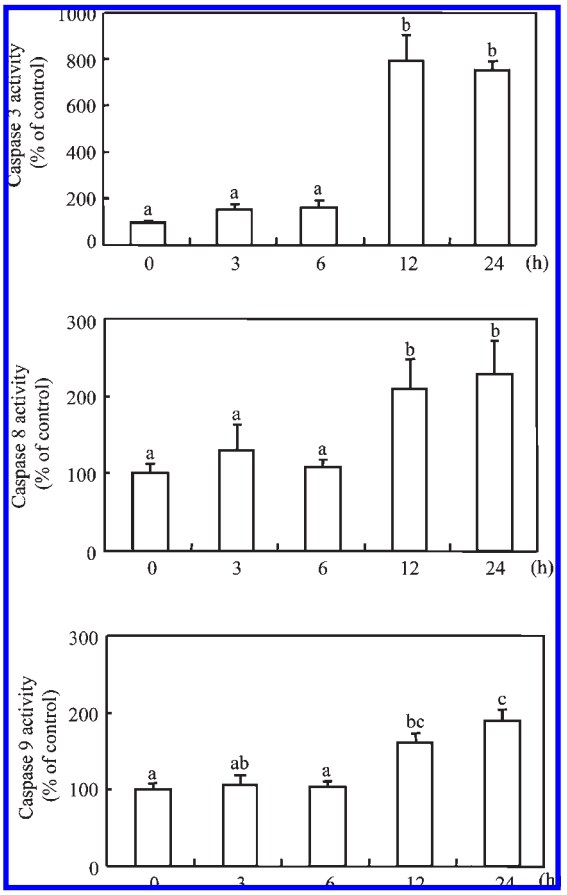

Figure 4. Effect of δ-T3 treatment on caspase activity. HUVECs treated with 5 μM δ-T3 for 0, 3, 6, 12, and 24 h. Caspase-3, -8, and -9 enzymatic activity was evaluated by a calorimetric assay that measured the release of pNA from Ac-DEVD-pNA, Ac-IETD-pNA, and Ac-LEHD-pNA substrates, respectively. The data are expressed as mean ± SD; $n = 3$. Means without a common letter differ, $P < 0.05$.

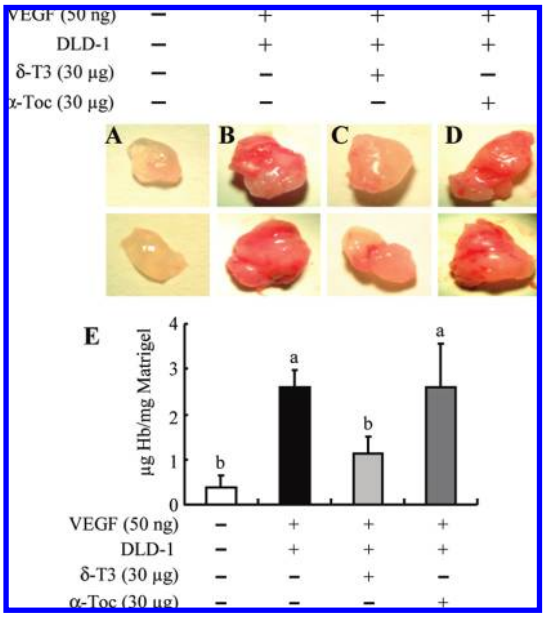

Figure 5. Effect of δ-T3 on DLD-1 induced vessel formation in the Matrigel plug assay. DLD-1 cells were suspended in serum free RPMI-1640 medium. Aliquots of the cell suspension (1×10^6 cells/0.1 mL) were mixed with 0.2 mL of Matrigel and 50 ng of VEGF, with or without 30 μg of δ-T3 or α-Toc, and the mixtures were then injected into the flanks of nude mice. The Matrigel plugs were removed 11 days after implantation. (**A**) Matrigel with medium only; (**B**) Matrigel containing DLD-1 and 50 ng VEGF; (**C**) Matrigel containing DLD-1, 50 ng of VEGF and 30 μg of δ-T3; (**D**) Matrigel containing DLD-1, 50 ng of VEGF and 30 μg of α-Toc. The hemoglobin content of the Matrigels was measured (**E**). Values are mean ± SD ($n = 6$). Means without a common letter differ, $P < 0.05$.

Inhibition of DLD-1-Induced Angiogenesis by δ-T3, but Not α-Toc in Nude Mice. Finally, in vivo tumor angiogenesis, a Matrigel plug angiogenesis assay was conducted. DLD-1 cells were mixed with Matrigel, and injected into the dorsal region of nude mice. After 11-day implantation, neovascularization was significantly developed in DLD-1-Matrigel implanted control mice, and the Hb content in the Matrigel plug was judged as 2.6 ± 0.17 μg Hb/mg Matrigel when that of mice with Matrigel alone was 0.38 ± 0.28 μg Hb/mg Matrigel (**Figure 5**). Suppression of vessel formation was clearly observed (1.1 ± 0.14 μg Hb/mg Matrigel) in mice implanted with δ-T3 (30 μg), whereas α-Toc did not inhibit angiogenesis (2.6 ± 0.39 μg Hb/mg Matrigel). These results clearly demonstrated that δ-T3 has superior antiangiogenic activities to α-Toc in vivo.

DISCUSSION

The goal of this study was to investigate the difference in angiopreventive potential between T3 and Toc. We demonstrated conclusively that δ-T3 was superior for inhibiting in vitro angiogenesis by evaluating VEGF-induced proliferation, migration, and tube formation of endothelial cells, and also for suppressing in vivo angiogenesis by assessing DLD-1-induced vessel formation in a mouse Matrigel plug assay.

The present data confirm and extend our previous investigations (*11*, *12*), which demonstrated that T3 inhibited proliferation of both bovine aortic endothelial cells and HUVECs, with δ-T3 having the highest inhibitory activity. However, the relationship between the incorporation of T3 into endothelial cells and inhibition of proliferation was not investigated. In this study, as we anticipated, the inhibitory potency of the isomers varied in the order δ- > β- > γ- > α-T3 (**Figure 1A**). This ranking order was similar to the incorporation of each T3 into HUVECs (**Table 1**). The same tendency was reported for inhibition of telomerase or DNA polymerase λ by T3 (*23*, *35*). Structurally, δ-T3 lacks the 5- and 7-methyl groups attached onto its chroman ring, so it is allowed to pass easily through cell membranes. The better incorporation of δ-T3 into HUVECs would be the reason for its greater proliferation inhibitory effect. On the other hand, α-, β-, and γ-T3 promoted cell proliferation when its concentration was under 10 μM for α-T3, 8 μM for β-T3, and 10 μM for γ-T3, even though they inhibited the proliferation at 20 μM for α-T3, 10 μM for β-T3, and 14 μM for γ-T3. Therefore, T3 seems to have biphasic effect. T3 at low concentration (e.g., 5−10 μM for α-T3) may cause generation of reactive oxygen species in HUVECs, and thus stimulate cell proliferation.

Angiogenesis is known to involve a series of steps including endothelial cell activation and breakdown of the basement membrane, followed by migration, proliferation, and tube formation of the cells (*36*). The series can be initiated by tumor cells releasing molecules, such as VEGF, that activate angiogenic gene expression in endothelial cells and enhance vascular permeability (*37*). Accordingly, in this study, we evaluated and compared the effects of δ-T3 and α-Toc on the key steps of VEGF-induced angiogenesis, namely, endothelial proliferation, migration and tube formation. We showed that δ-T3 suppressed the proliferation and that δ-T3 remarkably inhibited migration and tube formation in HUVECs (**Figures 1** and **2**). The amount of δ-T3

required for suppressing cell proliferation (5 μM) was higher than that required for suppressing either cell migration (2 μM) or tube formation (2.8 μM). These findings suggest that the main antiangiogenic effect of δ-T3 would be the suppression of migration and tube formation in HUVECs. Another related antiangiogenic effect was the apoptotic induction by δ-T3 at concentrations >5 μM (**Figure 2C**,**D**). Besides δ-T3, many antiangiogenic agents, including AZD2171 or luteolin, can inhibit cell migration and tube formation or can induce apoptosis in endothelial cells, by targeting specific molecular events such as growth factor receptor signaling (*38*, *39*). Now, there is increasing evidence that inhibition of migration and tube formation as well as induction of endothelial cells-selective apoptosis would be highly important for angioprevention (*40*). In contrast to T3, α-Toc even at high dose (50 μM) did not change any angiogenic-related events (data not shown). Overall, our results demonstrate that δ-T3 has considerably more potent antiangiogenic effects than α-Toc even if difference of cellular uptake of δ-T3 and α-Toc was considered. This implies that the three unsaturated double bonds in the side chain and three methyl groups on the chromanol ring of δ-T3 are important for the antiangiogenic effect.

Recently, the potent abilities of T3 to induce cell cycle arrest (*17*, *41*), to regulate HMG-CoA reductase (*18*), to activate p53 (*19*) and caspase-8 (*20*), to suppress adhesion molecules (*21*) and to inhibit nuclear factor-κB (NF-κB) (*22*) have been reported. However, the antiangiogenic mechanisms of T3 are not yet fully understood, although we and some researchers have evidenced the possibility for the growth factor-dependent activation of PI3K/PDK/Akt signaling (*12*, *42*, *43*). The PI3K/PDK/Akt pathway has been shown to be activated by various growth factors, such as insulin, platelet-derived growth factor and interleukins, as well as the proangiogenic factor VEGF (*44*). Constitutive active PI3K induces angiogenesis, and there is evidence that inhibition of PI3K/PDK/Akt signaling interferes with angiogenesis in endothelial cells (*45*). PI3K/PDK/Akt signaling also mediates VEGF expression in cancer cells, indicating its involvement in the angiogenic process (*46*). In PI3K/PDK/Akt signaling pathway, the active PDK phosphorylates and activates Akt. The activated Akt promotes cell survival, and protects against apoptosis through multiple mechanisms that include suppressing caspase-3 and caspase-9 activation, promoting production of Bcl-xL, repressing the activity of the proapoptotic factor BAD, and suppressing the production of the Fas ligand (*47*). On the other hand, Akt is negatively regulated by PTEN tumor suppressor (*48*). Therefore, it appears that PDK, Akt and PTEN are associated with several stages of the angiogenesis by phosphorylating or dephosphorylating multiple downstream substrates. When PTEN is dephosphorylated, its phosphatase activity is upregulated, leading to suppression of the PI3K/PDK/Akt pathway. In the present study, stimulation of HUVECs with VEGF caused significant phosphorylation of Akt and PTEN, resulting in the activation of PI3K/PDK/Akt signaling in these cells (**Figure 3**). Treatment with δ-T3 markedly decreased the intracellular levels of activated Akt and PTEN. These findings suggest that the antiangiogenic effect of δ-T3 (inhibition of proliferation, migration, and tube formation) is mediated by reduction of VEGF-induced PI3K/PDK/Akt activity in endothelial cells. Additional evidence for the possibility is that δ-T3 inactivated signals downstream of PI3K/PDK/Akt, such as ERK 1/2, which are involved in cell proliferation and survival (*49*). In addition, δ-T3 enhanced phosphorylation of ASK1 and p38, a pathway to cause apoptosis in endothelial cells (*50*). On the other hand, p38 MAPK signaling is known to be able to lead to a mitogenic response (*51*). However, as mentioned above, it is also known that activation of ASK1 and/or suppression of Akt can induce p38 activation, which results in apoptosis through signals involving mitochondrial cell death pathway. In this study, we found activation of ASK1 and p38 as well as suppression of Akt by δ-T3. These changes tend to lead a stress-induced proapoptotic reaction, but not a mitogenic response. It is therefore likely that a high dose of δ-T3 (>5 μM), by blocking Akt, not only inhibits downstream survival signals but also enhances the ASK1 and p38 pathway, thereby eliciting an apoptotic effect in endothelial cells. This is supported by our finding that δ-T3 affected caspase activation (**Figure 4**) and induced apoptosis, as observed as condensation of chromatin (**Figure 2C**,**D**). In contrast, α-Toc had no effect on the PI3K/PDK/Akt pathway, which may account for its lower antiangiogenic activity.

VEGFR-2 is located in the cell membrane and acts as the key protein in VEGF signal transduction. Some studies suggested that breast cancer cells themselves express VEGFR-2 on epithelial and stromal cells, leading to speculations that tumor-produced VEGF has additional biological functions, perhaps promoting the proliferation and survival of tumor cells (*52*). Therefore, the regulation of VEGFR-2 activation may be a possible molecular target of an antiangiogenic compound (*53*). In this study, δ-T3 almost completely inhibited VEGF-induced VEGFR-2 phosphorylation (**Figure 3**), indicating that the antiangiogenic effect of δ-T3 may occur upstream of the PI3K/PDK/Akt signaling pathway at the level of VEGFR-2. Additional studies on the detailed molecular mechanisms and precise molecular target of the antiangiogenic action of T3 are the subject of ongoing investigation.

Finally, to evaluate the effect of δ-T3 or α-Toc on in vivo angiogenesis, we conducted a Matrigel plug assay using nude mice. This assay allows the quantitative analysis of antiangiogenic activity (*54*). As shown in **Figure 5**, δ-T3, but not α-Toc, significantly inhibited in vivo tumor angiogenesis. This could be due to the inhibitory effects of δ-T3 on endothelial signaling of DLD-1 releasing molecules such as VEGF. It is also possible that the antiangiogenic effect of δ-T3 is not due solely to its direct action on endothelial cells, but may be a consequence of effects on endothelial cells and other cell types such as macrophages and leukocytes. A recent study showed that T3 inhibits NF-κB action, which determines transcription of angiogenic genes (*22*). Therefore, T3 may control tumor angiogenesis by inhibiting the activities of both endothelial and tumor cells.

Screening for compounds with antiangiogenic property is currently an active area of research. Curcumin (*55*), flavonoids (*56*), selenium (*57*), *N*-acetylcysteine (*58*), vitamin D3 (*59*) and several unsaturated and conjugated fatty acids (*60*−*62*) have all been shown to inhibit angiogenesis in vitro and/or in vivo. In the present study, the antiangiogenic effect of δ-T3 was considerably stronger than that of α-Toc, suggesting the importance of unsaturated structure to antiangiogenic effect.

In this study, because the small amount (2.5−5 μM) of δ-T3 performed an antiangiogenic effect, it seems likely that antiangiogenic result will be expected when T3 is taken orally. While the use of T3 in foods and dietary supplements is likely to be beneficial, the safety of T3 must be confirmed in long-term studies and clinical trials, similarly to Toc.

ABBREVIATIONS USED

ASK1, apoptosis signal-regulating kinase; DLD-1, human colorectal adenocarcinoma cell; EGF, epidermal growth factor; ERK 1/2, extracellular signal-regulated kinase 1/2; FBS, fetal bovine serum; FGF, fibroblast growth factor; HUVEC, human umbilical vein endothelial cell; MAPK, mitogen-activated protein

kinase; NF-κB, nuclear factor-κB; PDK, phosphoinositide-dependent protein kinase; PI3K, phosphatidylinositol-3 kinase; PTEN, phosphatase and tensin homologue deleted on chromosome 10; T3, tocotrienol; Toc, tocopherol; VEGF, vascular endothelial growth factor; WST-1, water-soluble tetrazolium salt.

LITERATURE CITED

(1) Folkman, J.; Klagsbrun, M. Angiogenic factors. *Science* **1987**, *235*, 442–447.

(2) Tonini, T.; Rossi, F.; Claudio, P. P. Molecular basis of angiogenesis and cancer. *Oncogene* **2003**, *22*, 6549–6556.

(3) Folkman, J. Influence of geometry on growth of normal and malignant cells. *Adv. Pathobiol.* **1976**, *4*, 12–18.

(4) Ferrara, N.; Gerber, H. P. The role of vascular endothelial growth factor in angiogenesis. *Acta Haematol.* **2001**, *106*, 148–156.

(5) Kerbel, R.; Folkman, J. Clinical translation of angiogenesis inhibitors. *Nat. Rev. Cancer* **2002**, *2*, 727–739.

(6) Cross, M. J.; Dixelius, J.; Matsumoto, T.; Claesson-Welsh, L. VEGF-receptor signal transduction. *Trends Biochem. Sci.* **2003**, *28*, 488–494.

(7) Sun, M.; Wang, G.; Paciga, J. E.; Feldman, R. I.; Yuan, Z. Q.; Ma, X. L.; Shelley, S. A.; Jove, R.; Tsichlis, P. N.; Nicosia, S. V.; Cheng, J. Q. AKT1/PKBα kinase is frequently elevated in human cancers and its constitutive activation is required for oncogenic transformation in NIH3T3 cells. *Am. J. Pathol.* **2001**, *159*, 431–437.

(8) Kim, K. J.; Li, B.; Winer, J.; Armanini, M.; Gillett, N.; Philips, H. S.; Ferrara, N. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. *Nature* **1993**, *362*, 841–844.

(9) Hanrahan, E. O.; Heymach, J. V. Vascular endothelial growth factor receptor tyrosine kinase inhibitors vandetanib (ZD6474) and AZD2171 in lung cancer. *Clin. Cancer Res.* **2007**, *134*, 617–622.

(10) Scott, E. N.; Meinhardt, G.; Jacques, C.; Laurent, D.; Thomas, A. L. Vatalanib: the clinical development of a tyrosine kinase inhibitor of angiogenesis in solid tumours. *Expert Opin. Invest. Drugs* **2007**, *16*, 367–379.

(11) Inokuchi, H.; Hirokane, H.; Tsuzuki, T.; Nakagawa, K.; Igarashi, M.; Miyazawa, T. Anti-angiogenic activity of tocotrienol. *Biosci., Biotechnol., Biochem.* **2003**, *67*, 1623–1627.

(12) Nakagawa, K.; Shibata, A.; Yamashita, S.; Tsuzuki, T.; Kariya, J.; Oikawa, S.; Miyazawa, T. In vivo angiogenesis is suppressed by unsaturated vitamin E, tocotrienol. *J. Nutr.* **2007**, *137*, 1938–1943.

(13) Serbinova, E.; Kagan, V.; Han, D.; Packer, L. Free radical recycling and intramembrane mobility in the antioxidant properties of α-tocopherol and α-tocotrienol. *Free Radical Biol. Med.* **1991**, *10*, 263–275.

(14) Mutalib, M. S. A.; Khaza'ai, H.; Wahle, K. W. J. Palm-tocotrienol rich fraction (TRF) is a more effective inhibitor of LDL oxidation and endothelial cell lipid peroxidation than alpha-tocopherol in vitro. *Food Res. Int.* **2003**, *36*, 405–413.

(15) Qureshi, A. A.; Sami, S. A.; Salser, W. A.; Khan, F. A. Dose-dependent suppression of serum cholesterol by tocotrienol-rich fraction (TRF25) of rice bran in hypercholesterolemic humans. *Atherosclerosis* **2002**, *161*, 199–207.

(16) Khanna, S.; Roy, S.; Ryu, H.; Bahadduri, P.; Swaan, P. W.; Ratan, R. R.; Sen, C. K. Molecular basis of vitamin E action: tocotrienol modulates 12-lipoxygenase, a key mediator of glutamate-induced neurodegeneration. *J. Biol. Chem.* **2003**, *278*, 43508–43515.

(17) Wada, S.; Satomi, Y.; Murakoshi, M.; Noguchi, N.; Yoshikawa, T.; Nishino, H. Tumor suppressive effects of tocotrienol in vivo and in vitro. *Cancer Lett.* **2005**, *229*, 181–191.

(18) Parker, R. A.; Pearce, B. C.; Clark, R. W.; Gordon, D. A.; Wright, J. J. Tocotrienols regulate cholesterol production in mammalian cells by post-transcriptional suppression of 3-hydroxy-3-methylglutaryl-coenzyme A reductase. *J. Biol. Chem.* **1993**, *268*, 11230–11238.

(19) Agarwal, M. K.; Agarwal, M. L.; Athar, M.; Gupta, S. Tocotrienol-rich fraction of palm oil activates p53, modulates Bax/Bcl2 ratio and induces apoptosis independent of cell cycle association. *Cell Cycle* **2004**, *3*, 205–211.

(20) Shah, S.; Sylvester, P. W. Tocotrienol-induced caspase-8 activation is unrelated to death receptor apoptotic signaling in neoplastic mammary epithelial cells. *Exp. Biol. Med.* (*Maywood*) **2004**, *229*, 745–755.

(21) Theriault, A.; Chao, J. T.; Gapor, A. Tocotrienol is the most effective vitamin E for reducing endothelial expression of adhesion molecules and adhesion to monocytes. *Atherosclerosis* **2002**, *160*, 21–30.

(22) Ahn, K. S.; Sethi, G.; Krishnan, K.; Aggarwal, B. B. γ-Tocotrienol inhibits nuclear factor-κB signaling pathway through inhibition of receptor-interacting protein and TAK1 leading to suppression of antiapoptotic gene products and potentiation of apoptosis. *J. Biol. Chem.* **2007**, *282*, 809–820.

(23) Eitsuka, T.; Nakagawa, K.; Miyazawa, T. Down-regulation of telomerase activity in DLD-1 human colorectal adenocarcinoma cells by tocotrienol. *Biochem. Biophys. Res. Commun.* **2006**, *348*, 170–175.

(24) Birringer, M.; Pfluger, P.; Kluth, D.; Landes, N.; Brigelius-Flohe, R. Identities and differences in the metabolism of tocotrienols and tocopherols in HepG2 cells. *J. Nutr.* **2002**, *132*, 3113–3118.

(25) Yap, S. P.; Yuen, K. H.; Lim, A. B. Influence of route of administration on the absorption and disposition of α-, γ- and δ-tocotrienols in rats. *J. Pharm. Pharmacol.* **2003**, *55*, 53–58.

(26) Schindler, R.; Mentlein, R. Flavonoids and vitamin E reduce the release of the angiogenic peptide vascular endothelial growth factor from human tumor cells. *J. Nutr.* **2006**, *136*, 1477–1482.

(27) Navarra, T.; Del, Turco, S.; Papa, A.; Battaglia, D.; Lazzerini, G.; Basta, G. Lack of effect of α-tocopherol on in vitro angiogenesis. *Microvasc. Res.* **2006**, *72*, 12–19.

(28) Saito, H.; Kiyose, C.; Yoshimura, H.; Ueda, T.; Kondo, K.; Igarashi, O. γ-Tocotrienol, a vitamin E homolog, is a natriuretic hormone precursor. *J. Lipid Res.* **2003**, *44*, 1530–1535.

(29) Ishiyama, M.; Tominaga, H.; Shiga, M.; Sasamoto, K.; Ohkura, Y.; Ueno, K. A combined assay of cell viability and in vitro cytotoxicity with a highly water-soluble tetrazolium salt, neutral red and crystal violet. *Biol. Pharm. Bull.* **1996**, *19*, 1518–1520.

(30) Albini, A.; Iwamoto, Y.; Kleinman, H. K.; Martin, G. R.; Aaronson, S. A.; Kozlowski, J. M.; McEwan, R. N. A rapid in vitro assay for quantitating the invasive potential of tumor cells. *Cancer Res.* **1987**, *47*, 3239–3245.

(31) Sookwong, P.; Nakagawa, K.; Murata, K.; Kojima, Y.; Miyazawa, T. Quantitation of tocotrienol and tocopherol in various rice brans. *J. Agric. Food Chem.* **2007**, *55*, 461–466.

(32) Liu, L. Z.; Fang, J.; Zhou, Q.; Hu, X.; Shi, X.; Jiang, B. H. Apigenin inhibits expression of vascular endothelial growth factor and angiogenesis in human lung cancer cells: implication of chemoprevention of lung cancer. *Mol. Pharmacol.* **2005**, *68*, 635–643.

(33) McIntyre, B. S.; Briski, K. P.; Gapor, A.; Sylvester, P. W. Antiproliferative and apoptotic effects of tocopherols and tocotrienols on preneoplastic and neoplastic mouse mammary epithelial cells. *Proc. Soc. Exp. Biol. Med.* **2000**, *224*, 292–301.

(34) Yu, W.; Simmons-Menchaca, M.; Gapor, A.; Sanders, B. G.; Kline, K. Induction of apoptosis in human breast cancer cells by tocopherols and tocotrienols. *Nutr. Cancer* **1999**, *33*, 26–32.

(35) Mizushina, Y.; Nakagawa, K.; Shibata, A.; Awata, Y.; Kuriyama, I.; Shimazaki, N.; Koiwai, O.; Uchiyama, Y.; Sakaguchi, K.; Miyazawa, T.; Yoshida, H. Inhibitory effect of tocotrienol on eukaryotic DNA polymerase l and angiogenesis. *Biochem. Biophys. Res. Commun.* **2006**, *339*, 949–955.

(36) Folkman, J.; Shing, Y. Angiogenesis. *J. Biol. Chem.* **1992**, *267*, 10931–10934.

(37) Ferrara, N.; Houck, K.; Jakeman, L.; Leung, D. W. Molecular and biological properties of the vascular endothelial growth factor family of proteins. *Endocr. Rev.* **1992**, *13*, 18–32.

(38) Wedge, S. R.; Kendrew, J.; Hennequin, L. F.; Valentine, P. J.; Barry, S. T.; Brave, S. R.; Smith, N. R.; James, N. H.; Dukes, M.; Curwen, J. O.; Chester, R.; Jackson, J. A.; Boffey, S. J.; Kilburn, L. L.; Barnett, S.; Richmond, G. H.; Wadsworth, P. F.; Walker, M.; Bigley, A. L.; Taylor, S. T.; Cooper, L.; Beck, S.; Jürgensmeier, J. M.; Ogilvie, D. J. AZD2171: a highly potent, orally bioavailable, vascular endothelial growth factor receptor-2 tyrosine kinase inhibitor for the treatment of cancer. *Cancer Res.* **2005**, *65*, 4389–4400.

(39) Bagli, E.; Stefaniotou, M.; Morbidelli, L.; Ziche, M.; Psillas, K.; Murphy, C.; Fotsis, T. Luteolin inhibits vascular endothelial growth

factor-induced angiogenesis; inhibition of endothelial cell survival and proliferation by targeting phosphatidylinositol 3′-kinase activity. *Cancer Res*. **2004**, *64*, 7936–7946.

(40) Dong, L. F.; Swettenham, E.; Eliasson, J.; Wang, X. F.; Gold, M.; Medunic, Y.; Stantic, M.; Low, P.; Prochazka, L.; Witting, P. K.; Turanek, J.; Akporiaye, E. T.; Ralph, S. J.; Neuzil, J. Vitamin E analogues inhibit angiogenesis by selective induction of apoptosis in proliferating endothelial cells: the role of oxidative stress. *Cancer Res*. **2007**, *67*, 11906–11913.

(41) Srivastava, J. K.; Gupta, S. Tocotrienol-rich fraction of palm oil induces cell cycle arrest and apoptosis selectively in human prostate cancer cells. *Biochem. Biophys. Res. Commun*. **2006**, *346*, 447–453.

(42) Shah, S. J.; Sylvester, P. W. γ-Tocotrienol inhibits neoplastic mammary epithelial cell proliferation by decreasing Akt and nuclear factor κB activity. *Exp. Biol. Med*. **2005**, *230*, 235–241.

(43) Samant, G. V.; Sylvester, P. W. γ-Tocotrienol inhibits ErbB3-dependent PI3K/Akt mitogenic signalling in neoplastic mammary epithelial cells. *Cell Prolif*. **2006**, *39*, 563–574.

(44) Talapatra, S.; Thompson, C. B. Growth factor signaling in cell survival: implications for cancer treatment. *J. Pharmacol. Exp. Ther*. **2001**, *298*, 873–878.

(45) Jiang, B. H.; Zheng, J. Z.; Aoki, M.; Vogt, P. K. Phosphatidylinositol 3-kinase signaling mediates angiogenesis and expression of vascular endothelial growth factor in endothelial cells. *Proc. Natl. Acad. Sci. U.S.A*. **2000**, *97*, 1749–1753.

(46) Blancher, C.; Moore, J. W.; Robertson, N.; Harris, A. L. Effects of ras and von Hippel-Lindau (VHL) gene mutations on hypoxia-inducible factor (HIF)-1α, HIF-2α, and vascular endothelial growth factor expression and their regulation by the phosphatidylinositol 3′-kinase/Akt signaling pathway. *Cancer Res*. **2001**, *61*, 7349–7355.

(47) Yu, Q.; Kovacs, C.; Yue, F. Y.; Ostrowski, M. A. The role of the p38 mitogen-activated protein kinase, extracellular signal-regulated kinase, and phosphoinositide-3-OH kinase signal transduction pathways in CD40 ligand-induced dendritic cell activation and expansion of virus-specific CD8+ T cell memory responses. *J. Immunol*. **2004**, *172*, 6047–6056.

(48) Kandel, E. S.; Hay, N. The regulation and activities of the multifunctional serine/threonine kinase Akt/PKB. *Exp. Cell Res*. **1999**, *253*, 210–229.

(49) Meier, F.; Schittek, B.; Busch, S.; Garbe, C.; Smalley, K.; Satyamoorthy, K.; Li, G.; Herlyn, M. The RAS/RAF/MEK/ERK and PI3K/AKT signaling pathways present molecular targets for the effective treatment of advanced melanoma. *Front. Biosci*. **2005**, *10*, 2986–3001.

(50) Ichijo, H.; Nishida, E.; Irie, K.; Dijke, P.; Saitoh, M.; Moriguchi, T.; Takagi, M.; Matsumoto, K.; Miyazono, K.; Gotoh, Y. Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. *Science* **1997**, *275*, 90–94.

(51) Yamaguchi, T.; Chattopadhyay, N.; Kifor, O.; Sanders, J. L.; Brown, E. M. Activation of p42/44 and p38 mitogen-activated protein kinases by extracellular calcium-sensing receptor agonists induces mitogenic responses in the mouse osteoblastic MC3T3-E1 cell line. *Biochem. Biophys. Res. Commun*. **2000**, *279*, 363–368.

(52) Ferrara, N.; Gerber, H. P.; LeCouter, J. The biology of VEGF and its receptors. *Nat. Med*. **2003**, *9*, 669–676.

(53) Schlaeppi, J. M.; Wood, J. M. Targeting vascular endothelial growth factor (VEGF) for anticancer therapy, by anti-VEGF neutralizing monoclonal antibodies and VEGF receptor tyrosine-kinase inhibitors. *Cancer Metastasis Rev*. **1999**, *18*, 473–481.

(54) Fang, J.; Zhou, Q.; Liu, L. Z.; Xia, C.; Hu, X.; Shi, X.; Jiang, B. H. Apigenin inhibits tumor angiogenesis through decreasing HIF-1α and VEGF expression. *Carcinogenesis* **2007**, *28*, 858–864.

(55) Thaloor, D.; Singh, A. K.; Sidhu, G. S.; Prasad, P. V.; Kleinman, H. K.; Maheshwari, R. K. Inhibition of angiogenic differentiation of human umbilical vein endothelial cells by curcumin. *Cell Growth Differ*. **1998**, *9*, 305–312.

(56) Cao, Y.; Cao, R. Angiogenesis inhibited by drinking tea. *Nature* **1999**, *398*, 381.

(57) Jiang, C.; Jiang, W.; Ip, C.; Ganther, H.; Lu, J. Selenium-induced inhibition of angiogenesis in mammary cancer at chemopreventive levels of intake. *Mol. Carcinog*. **1999**, *26*, 213–225.

(58) Cai, T.; Fassina, G.; Morini, M.; Aluigi, M. G.; Masiello, L.; Fontanini, G.; D'Agostini, F.; De, Flora, S.; Noonan, D. M.; Albini, A. N-acetylcysteine inhibits endothelial cell invasion and angiogenesis. *Lab. Invest*. **1999**, *79*, 1151–1159.

(59) Mantell, D. J.; Owens, P. E.; Bundred, N. J.; Mawer, E. B.; Canfield, A. E. 1α, 25-dihydroxyvitamin D3 inhibits angiogenesis in vitro and in vivo. *Circ. Res*. **2000**, *87*, 214–220.

(60) Masso-Welch, P. A.; Zangani, D.; Ip, C.; Vaughan, M. M.; Shoemaker, S.; Ramirez, R. A.; Ip, M. M. Inhibition of angiogenesis by the cancer chemopreventive agent conjugated linoleic acid. *Cancer Res*. **2002**, *62*, 4383–4389.

(61) Kim, H. J.; Vosseler, C. A.; Weber, P. C.; Erl, W. Docosahexaenoic acid induces apoptosis in proliferating human endothelial cells. *J. Cell Physiol*. **2005**, *204*, 881–888.

(62) Tsuzuki, T.; Shibata, A.; Kawakami, Y.; Nakagawa, K.; Miyazawa, T. Conjugated eicosapentaenoic acid inhibits vascular endothelial growth factor-induced angiogenesis by suppressing the migration of human umbilical vein endothelial cells. *J. Nutr*. **2007**, *137*, 641–646.

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