BIOCHEMICAL PHARMACOLOGY 76 (2008) 330–339

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Tumor anti-angiogenic effect and mechanism of action of δ-tocotrienol

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ARTICLE INFO

Article history:
Received 2 April 2008
Accepted 16 May 2008

Keywords:
Anti-angiogenesis
Endothelial cells
Tocotrienol
Tumor
Vitamin E

ABSTRACT

Anti-angiogenic therapy mediated by drugs and food components is an established strategy for cancer prevention. Our previous cell-culture studies identified a food-derived anti-angiogenic compound, tocotrienol (T3, an unsaturated vitamin E), as a potential angiogenic inhibitor. Among T3 isomers, δ-T3 is considered as the most potent compound. The purpose of this study was therefore to evaluate the inhibitory effect of δ-T3 on tumor angiogenesis. As growth factors (e.g., vascular endothelial growth factor and fibroblast growth factor) play critical roles in tumor angiogenesis, a conditioned medium rich in these growth factors from human colorectal adenocarcinoma cells (DLD-1-CM) was used as an angiogenic stimulus. δ-T3 (2.5–5 μM) significantly suppressed DLD-1-CM-induced tube formation, migration, and adhesion on human umbilical vein endothelial cells. These effects were partly associated with reactive oxygen species generation by δ-T3. Western blot analysis revealed that the anti-angiogenic effect of δ-T3 is attributable to regulation of growth factor-dependent phosphatidylinositol-3 kinase (PI3K)/phosphoinositide-dependent protein kinase (PDK)/Akt signaling as well as to induction stress response in endothelial cells. Moreover, we conducted an in vivo mouse Matrigel plug angiogenesis assay, and found that δ-T3 (10–20 μg) exhibits dose-dependent inhibition of DLD-1-induced vessel formation. These results suggest that T3 has potential use as a therapeutic dietary supplement for minimizing tumor angiogenesis.

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Abbreviations: ASK-1, apoptosis signal-regulating kinase; CM, conditioned medium; DAB, 3,3′-diaminobenzidine; DCDHF, 2,7-dichlorodihydrofluorescein; DHF, 2,7-dichlorofluorescein; DLD-1, human colorectal adenocarcinoma cells; EGF, epidermal growth factor; eNOS, endothelial nitric oxide synthase; ERK 1/2, extracellular signal-regulated kinase 1/2; FBS, fetal bovine serum; FGF, fibroblast growth factor; FPP, farnesylpyrophosphate; GGPP, geranylgeranylpyrophosphate; GSK3 α/β, glycogen synthase kinase 3 α/β; H&E, hematoxylin and eosin; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; HUVEC, human umbilical vein endothelial cells; PDK, phosphoinositide-dependent protein kinase; PECAM-1, platelet endothelial cell adhesion molecule-1; PI3K, phosphatidylinositol-3 kinase; PIP2, phosphatidylinositol (4,5)-bisphosphate; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; PTEN, phosphatase and tensin homologue deleted on chromosome 10; ROS, reactive oxygen species; Toc, tocopherol; TOS, tocopheryl succinate; T3, tocotrienol; VCAM-1, vascular cell adhesion molecular-1; VEGF, vascular endothelial growth factor; VEGFR-2, VEGF Receptor 2.

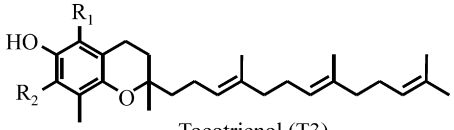
0006-2952/$ – see front matter
doi:10.1016/j.bcp.2008.05.017

1. Introduction

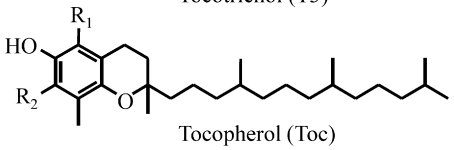
Vitamin E occurs naturally in eight different forms: α-, β-, γ- and δ-isomers of both tocopherol (Toc) and tocotrienol (T3) (Fig. 1). The two differ structurally in that Toc has a saturated phytyl side chain attached to its chroman ring, while T3 possesses an unsaturated isoprenoid side chain. Humans and animals are unable to synthesize vitamin E and therefore must obtain the isomers from plant sources. Toc is abundant in common vegetable oils and nuts, while T3, a minor plant constituent, is abundant in rice bran, palm, and wheat germ [1,2].

A major physiological activity of vitamin E is its well-defined anti-oxidative action and protective effect against lipid peroxidation in biological membranes [3], 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 anti-oxidative [4], anti-hypercholesterolemic [5], and neuroprotective [6] activities that differs somewhat from those of Toc. Further, the potent abilities of T3 to induce cell cycle arrest [7], to regulate HMG-CoA reductase [8], to activate p53 and caspase-8 [9,10], to suppress adhesion molecules [11], to inhibit nuclear factor-κB [12], and to down-regulate c-Myc and telomerase [13] have been reported. These unique effects of T3 could be partly explained by its absorption and metabolic fate in vivo. Although the absorption mechanisms are basically the same for all vitamin E analogs, T3 is reported to be absorbed into cells or degraded to metabolites to a greater extent than Toc [14,15].

Besides above properties, several lines of evidences support the beneficial effect of T3 on inhibiting tumor development [16]. For instance, when mammary tumors are induced by 7,12-dimethylbenz(α)anthracene, T3 treated mice show a remarkable elongation in tumor latency, while Toc has no effect [17]. Various factors have been implicated in such anti-cancer action of T3, including decrease of oxidative stress and modulation of cell signaling pathways in endothelial cells. Nevertheless, the in vivo potency and exact intracellular mechanisms for the anti-cancer properties of T3 remain poorly understood.



Tocotrienol (T3)



Tocopherol (Toc)

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

Fig. 1 – Chemical structure of T3. T3 has an unsaturated isoprenoid tail, which differs from Toc bearing a saturated phytyl side chain.

On the other hand, our previous studies show a new function of T3 as an inhibitor of angiogenesis [18–20]. Angiogenesis is the formation of new blood vessels from pre-existing endothelium, and is closely involved in cancer progression [21]. In angiogenic process, endothelial cells secrete proteases, migrate through the extracellular matrix, proliferate, and differentiate [22]. The final step is the formation of newly fused blood vessels with vascular smooth muscle cells, leading to blood flow into the tumors. Angiogenesis starts with tumor cells releasing specific molecules (e.g., vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and epidermal growth factor (EGF)) that activate angiogenic gene expression in endothelial cells and enhance vascular permeability [23]. Therefore, it is of high interest whether T3 suppress cancers through its suppressive effect on tumor angiogenesis.

The purpose of this study was to obtain direct evidence for the effect of T3 on tumor angiogenesis in vitro and in vivo. The in vitro anti-angiogenic property of T3 was investigated by using tumor cell culture medium containing certain growth factors as angiogenic stimuli. The in vivo evaluation was performed by mouse Matrigel plug angiogenesis assay. Because our previous cell culture studies [18] showed that δ-T3 is the most effective anti-angiogenic compound among T3 isomers, δ-T3 was investigated in this study.

2. Materials and methods

2.1. *Reagents, cells, and animals*

δ-T3 (Eisai, Tokyo, Japan [24]) was used, and its purity was 98%. WST-1 reagent was from Dojindo Laboratories (Kumamoto, Japan). All other reagents were of analytical grade. Human colorectal adenocarcinoma cells (DLD-1) were obtained from Cell Resource Center for Biomedical Research at Tohoku University School of Medicine (Sendai, Japan). The cells were maintained in RPMI-1640 medium (containing 0.3 g/L L-glutamine, and 2.0 g/L sodium bicarbonate) (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Dainippon Pharmaceutical, Osaka, Japan), 100 kU/L penicillin (Gibco BRL Rockville, MD, USA), and 100 mg/L streptomycin (Gibco) at 37 °C in a humidified atmosphere of 5% CO_2. Human umbilical vein endothelial cells (HUVEC) (Kurabo, Osaka, Japan) were cultured in the base medium (HuMedia-EB2) supplemented with 2% FBS, 10 μg/L human epidermal growth factor (EGF), 5 μg/L human basic fibroblast growth factor (FGF), 1 mg/L hydrocortisone, 10 mg/L heparin, 50 mg/L gentamicin, and 50 μg/L anfoterin B (Kurabo). Confluent HUVEC (passage 5–8) were used in the experiments. Male athymic nude mice (BALB/cA Jcl-nu nu/nu, 4 weeks old) 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 condition. They were acclimatized with MF Standard Rodent Chow (Oriental Yeast, Tokyo, Japan) and distilled water (free access) for 1 week.

2.2. *Preparation of DLD-1 conditioned medium*

DLD-1 (approximately 90% confluent) were rinsed with serum-free RPMI-1640 medium and incubated in the RPMI-1640

medium (containing 1% FBS) for 24 h in a 100 mm dish. The conditioned medium (DLD-1-CM) was collected, centrifuged at 700 × *g* for 10 min, and the supernatant was stored at −30 °C until used as an angiogenic stimulus.

2.3. Preparation of δ-T3 solution for cell culture experiments

Stock solution of δ-T3 was prepared in ethanol at a concentration of 20 μM. For cell culture experiments (as described below), the solution was diluted to final concentrations of 0–5 μM in test medium. The concentration of ethanol never exceeded 0.1% (v/v).

2.4. Tube formation assay

Culture plates (24-well) were coated with 350 μL of Matrigel (Becton Dickinson, Bedford, MA, USA) and incubated at 37 °C for 1 h for solidification. Trypsin-harvested HUVEC were treated with δ-T3 under two different protocols. In the first protocol, HUVEC (5×10^4 cells) were suspended in 500 μL of test medium (HuMedia-EB2 containing 1% FBS and δ-T3), and then were mixed with 500 μL of DLD-1-CM. The cell suspension was placed on the surface of the Matrigel and was incubated for 18 h. In the second protocol, HUVEC (5×10^4 cells) in 500 μL of test medium (HuMedia-EB2 containing 1% FBS) and 500 μL of DLD-1-CM were cultured in the Matrigel plate for 6 h. After cultivation, the forming rudimentary capillary network was treated with δ-T3 and incubated at 37 °C for 12 h. Cells in both protocols were fixed with 4% paraformaldehyde (Wako, Osaka, Japan) and photographed. The lengths of tube-structured cells were quantified using angiogenesis imaging software (Kurabo). It is noted that the Matrigel used in this study contained small amounts of growth factors, and caused no angiogenic action under present experimental conditions.

2.5. Proliferation assay

Proliferation was evaluated by WST-1 assay [25]. WST-1 is a tetrazolium salt that is converted into the soluble formazan salt by succinate-tetrazolium reductase in the respiratory chain of active mitochondria of proliferating viable cells. The amount of formazan produced is directly proportional to the number of viable cells. HUVEC (2500 cells/well) were pre-incubated in HuMedia-EG2 medium in 96-well plates for 24 h, and the medium was then changed to 100 μL of test medium (HuMedia-EB2 containing 1% FBS and δ-T3). 100 μL of DLD-1-CM was added to each well. After incubation for 12 h, 10 μL of WST-1 solution was added to each well and incubated at 37 °C for 3 h. Cell proliferation was determined by measuring the absorbance (450/655 nm) of the medium using a microplate reader (Model 550, Bio-Rad Laboratories, Inc., Hercules, CA, USA).

2.6. Migration assay

Migration assays were performed in the modified Boyden chamber [26] consisting of a cell culture insert membrane (3 μm pore size, Becton Dickinson) seated in each well of a 24-well plate. The membrane was coated with thin layer of fibronectin, laminin, or collagen I. Trypsin-harvested HUVEC (1.5×10^5 cells) were suspended in 500 μL of HuMedia-EB2 medium containing 1% FBS and δ-T3, and were added to the upper chamber. The lower chamber contained 750 μL of DLD-1-CM. After the whole chamber was incubated for 22 h, the non-migrated 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 migrated to the undersurface of the membrane were stained with toluidine blue (Sigma). The number of migrated cells was counted in randomly selected microscopic 5–6 fields, and expressed as a pixel value by using Adobe Photoshop (Adobe Systems, Mountain View, CA, USA).

2.7. Cell adhesion assay

Cell adhesion assay was performed in a 96-well plate precoated with fibronectin (Becton Dickinson). The wells were hydrated with DLD-1-CM at 37 °C for 30 min. Trypsin-harvested HUVEC were suspended in DLD-1-CM containing δ-T3, and then were incubated at 37 °C for 2 h. The resultant cell suspension was added into each well (1.5×10^4 cells/well). After incubation for 1 h, the medium was aspirated, and the non-adherent cells were discarded by washing with PBS. After adherent cells were fixed with 4% paraformaldehyde and stained with toluidine blue, the stain was extracted by 1% SDS in PBS. Cell adhesion was evaluated by measuring the absorbance (595 nm) of the stain extract.

2.8. Evaluation of reactive oxygen species

The generation of intracellular reactive oxygen species (ROS) was evaluated using the fluorescent dye 2,7-dichlorodihydrofluorescein (DCDHF) diacetate (Cayman Chemicals, Ann Arbor, MI, USA) [27]. ROS in cells causes oxidation of DCDHF diacetate, yielding the fluorescent product 2,7-dichlorofluorescein (DHF). Confluent HUVEC were cultured in 100 μL of test medium (HuMedia-EB2 containing 1% FBS and δ-T3) in 96-well plates for 3 h. Then, the medium was changed to DLD-1-CM containing 10 μM DCDHF diacetate, followed by incubation for 20 min. The cells were washed with Hanks' Balanced Salt Solution, and fluorescence intensity was determined using a GENios Plus Multi-Detection Microplate Reader with enhanced fluorescence (Tecan Inc., Research Triangle Park, NC, Switzerland) at the excitation wavelength of 485 nm and the emission wavelength of 535 nm.

2.9. Western blot analysis

Confluent HUVEC were cultivated in 10 mL of test medium (HuMedia-EB2 containing δ-T3) in 100 mm dishes. After 6 h cultivation to incorporate enough δ-T3 into cells and to evaluate more obvious change of signal transduction, the medium was changed to DLD-1-CM, and the incubation was performed for 10 min. Then, cellular proteins were prepared from HUVEC as previously described [18], and the cellular proteins (50 μg/well) were separated by SDS-PAGE gel electrophoresis (10–20% e-PAGEL, Atto, Tokyo, Japan). The protein bands were transferred to polyvinylidine fluoride membrane

(Amersham Pharmacia Biotech, NJ, USA). After being blocked of nonspecific sites, the membrane was probed with primary antibodies, followed by a horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology, Beverly, MA, USA). The detection of the antibody reactions was performed with ECL Plus Western blotting reagents (Amersham Pharmacia Biotech). The antibodies used were anti-phospho phosphoinositide-dependent protein kinase (PDK), anti-phospho Akt, anti-phospho extracellular signal-regulated kinase 1/2 (ERK 1/2), anti-phospho phosphatase and tensin homologue deleted on chromosome 10 (PTEN), anti-phospho VEGF Receptor 2 (VEGFR-2), anti-phospho p38, anti-phospho apoptosis signal-regulating kinase (ASK-1), anti-phospho glycogen synthase kinase 3 α/β (GSK3 α/β), anti-phospho endothelial nitric oxide synthase (eNOS), and anti-β-actin. All antibodies were purchased from Cell Signaling Technology.

2.10. *Effect of isoprenoid intermediates on anti-tube formation property of δ-T3*

Effect of isoprenoid intermediates such as farnesyl- and geranylgeranylpyrophosphate (FPP and GGPP, respectively, Sigma) on anti-tube formation property of δ-T3 was evaluated by Angiogenesis kit (Kurabo). Briefly, HUVEC co-cultured with fibroblasts were cultivated in the presence or absence of VEGF (10 μg/L), FPP (10 μM), GGPP (10 μM), and δ-T3 (3 μM). After 11 days, cells were fixed in 70% ethanol, and then visualized with van Willebrand factor antibody. Tube length was quantified using angiogenesis imaging software (Kurabo).

2.11. *Matrigel plug angiogenesis assay*

In vivo Matrigel plug angiogenesis assay was performed as previously described by Liu et al. [28]. DLD-1 cells were suspended in a serum- and phenol red-free RPMI-1640 medium (Gibco). Aliquots of the cell suspension (2×10^6 cells/0.1 mL) were mixed with 0.1 mL of phenol red-free Matrigel (containing 0–20 μg δ-T3) (Becton Dickinson), and the mixtures were subsequently injected into flanks of nude mice using a 21-gauge needle. After the implantation, mice were allowed free access to MF Standard Rodent Chow (Oriental Yeast) and distilled water for 14 days. Then, the Matrigel plugs were removed, and were subjected to the measurement of hemoglobin content using a kit (Hemoglobin test, Wako) and immunohistochemical staining as described below. This experiment was conformed to the policies and procedures detailed in the Animal Experiment Guidelines of Tohoku University.

2.12. *Immunohistochemical analysis*

The Matrigel plugs were fixed with a solution of 4% paraformaldehyde in PBS, rinsed with 70% ethanol, and embedded in paraffin. Five-μm-thick sections of the Matrigel were stained with hematoxylin and eosin (H&E). Immunohistochemical staining of CD31/platelet endothelial cell adhesion molecule-1 (PECAM-1)-positive endothelial cells was performed according to the following procedures. Five-μm-thick sections were washed with TBS, subjected to microwave for antigen activation for 10 min, and incubated in 3% methanolic hydrogen peroxide for 15 min. After being washed with TBS, the sections were blocked with serum-free Protein Block (Dako, Carpenteria, CA, USA) at room temperature for 10 min, and incubated with a 1:500 dilution of a goat anti-mouse CD31/PECAM-1 monoclonal antibody (Santa Cruz Biotechnology, CA, USA). The samples were incubated overnight in a humid chamber at 4 °C, washed with TBS, and incubated with anti-goat secondary IgG (Nichirei, Tokyo, Japan) for 30 min. After being washed with TBS, the sample was incubated with 3,3′-diaminobenzidine (DAB)/H_2O_2 for the detection of CD31/PECAM-1-positive endothelial cells. Sections incubated with normal goat IgG instead of the primary antibody (Santa Cruz) were used as the negative control.

2.13. *Statistical analysis*

The data are expressed as mean ± S.D. We performed statistical analysis using 1-way ANOVA, followed by Newman–Keules test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. *Suppressive effect of T3 on tumor angiogenesis in vitro*

The effect of δ-T3 on tubular morphogenesis of endothelial cells was first examined. HUVEC incubated with DLD-1-CM showed an increase in the lengths of endothelial tubes compared with those cultured without DLD-1-CM (Fig. 2A). δ-T3 showed suppression of the DLD-1-CM-induced tube formation in a dose-dependent manner. On the other hand, as shown in Fig. 2B, once capillary tubes were formed, δ-T3 did not affect the luminal structure. These contrastive results (Fig. 2A and B) suggest that δ-T3 inhibits capillary tube organization but does not affect existing capillary tubes by HUVEC on Matrigel, implying that δ-T3 has no cytotoxity on endothelial cells. Next, the effect of δ-T3 on proliferation and migration of HUVEC was examined, as these properties are closely related to tubular morphogenesis. In the proliferation assay (Fig. 2C), DLD-1-CM treated HUVEC showed an induction in cell proliferation. Although δ-T3 slightly promoted cell proliferation when its concentration was under 3 μM, it inhibited the proliferation at 5 μM. In the migration assay (Fig. 3), DLD-1-CM treated HUVEC were allowed to migrate across the membrane insert coated with fibronectin, collagen I, or laminin. δ-T3 suppressed the DLD-1-CM-induced migration in a dose-dependent manner, especially the cell migration on fibronectin. As shown in Fig. 4, when HUVEC were treated with DLD-1-CM and δ-T3 for the relatively short period (2–3 h), such cells did not adhere to the plate coated with fibronectin, and slight increase of intracellular ROS was observed.

3.2. *Anti-angiogenic mechanism of T3 by Western blot analysis*

We next evaluated the inhibitory mechanism of δ-T3 on tumor-induced angiogenesis in vitro by Western blot analysis. Considering the critical role of phosphatidylinositol-3 kinase

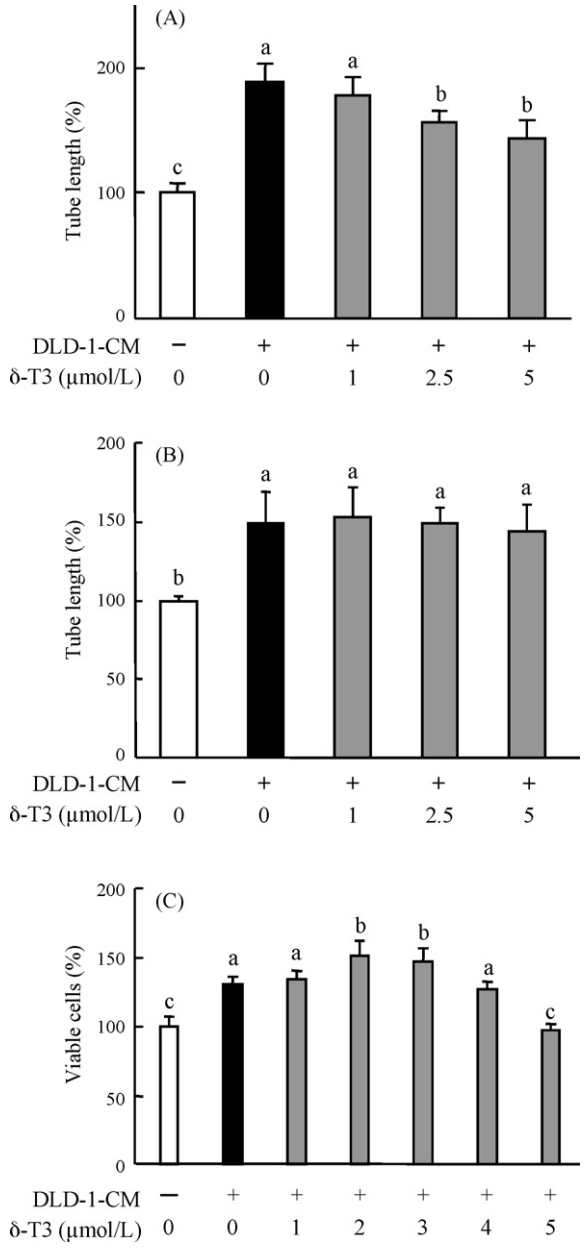


Fig. 2 – Effect of δ-T3 on DLD-1-CM induced HUVEC tube formation (A, B) and proliferation (C). (A) HUVEC (5×10^4 cells) were suspended in 500 μL of test medium (HuMedia-EB2 containing 1% FBS and δ-T3), and then were mixed with 500 μL of DLD-1-CM. The cell suspension was incubated on Matrigel plate for 18 h. (B) T3-untreated HUVEC (5×10^4 cells) in test medium (HuMedia-EB2 containing 1% FBS) and DLD-1-CM were cultured on Matrigel plate. After 6 h of cultivation, the forming rudimentary capillary network was treated with δ-T3, and was incubated for 12 h. (C) HUVEC (2500 cells/well) were preincubated in HuMedia-EG2 medium, and the medium was then changed to 100 μL of test medium (HuMedia-EB2 containing 1% FBS and δ-T3). 100 μL of DLD-1-CM was added to each well, and incubation was performed for 12 h. Values are mean ± S.D. ($n = 6$ for tube formation assay; $n = 4$ for proliferation assay). Means without a common letter differ, $P < 0.05$.

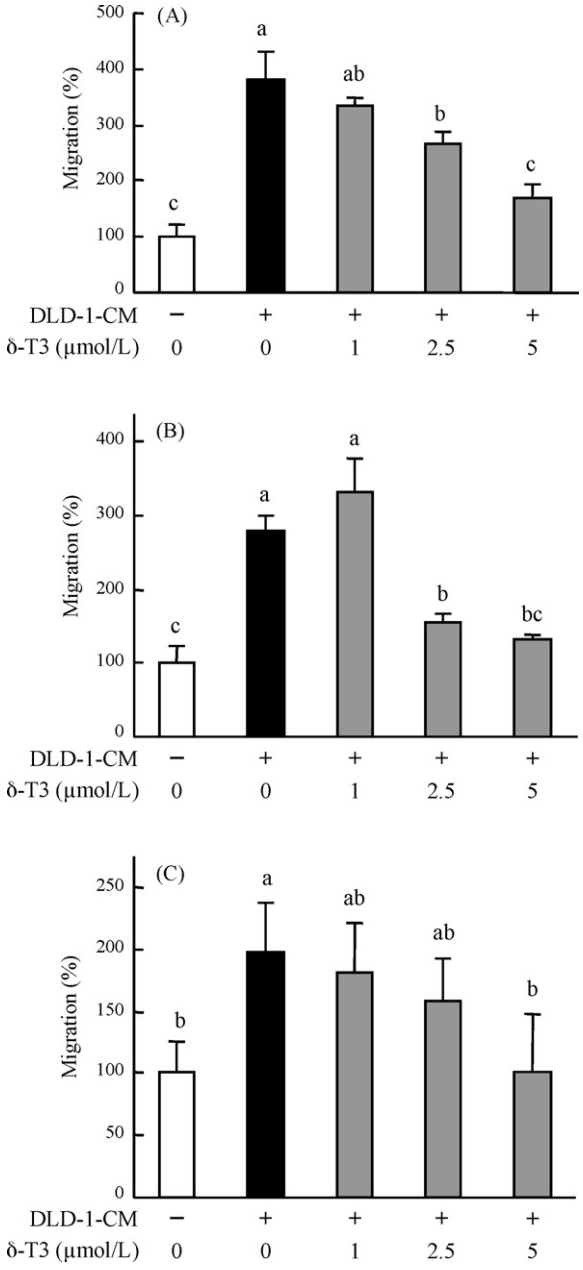


Fig. 3 – Effect of δ-T3 on DLD-1-CM induced HUVEC migration. Migration assay was performed in a modified Boyden chamber consisting of a cell culture insert membrane. The 3 μm pore membrane was coated with collagen I (A), fibronectin (B), or laminin (C). HUVEC (1.5×10^5 cells) were suspended in 500 μL of HuMedia-EB2 medium containing 1% FBS and δ-T3, and were added to the top chamber. The lower chamber contained 750 μL of DLD-1-CM. The whole chamber was incubated for 22 h. Values are mean ± S.D. ($n = 4$). Means without a common letter differ, $P < 0.05$.

(PI3K)/PDK/Akt signaling in tumor angiogenesis [29], the effect of δ-T3 on the PI3K/PDK/Akt pathway was examined. In the culture without δ-T3, DLD-1-CM induced the activation (phosphorylation) of PI3K/PDK/Akt pathway proteins such as PDK, Akt and PTEN (Fig. 5A). In culture with addition of δ-T3,

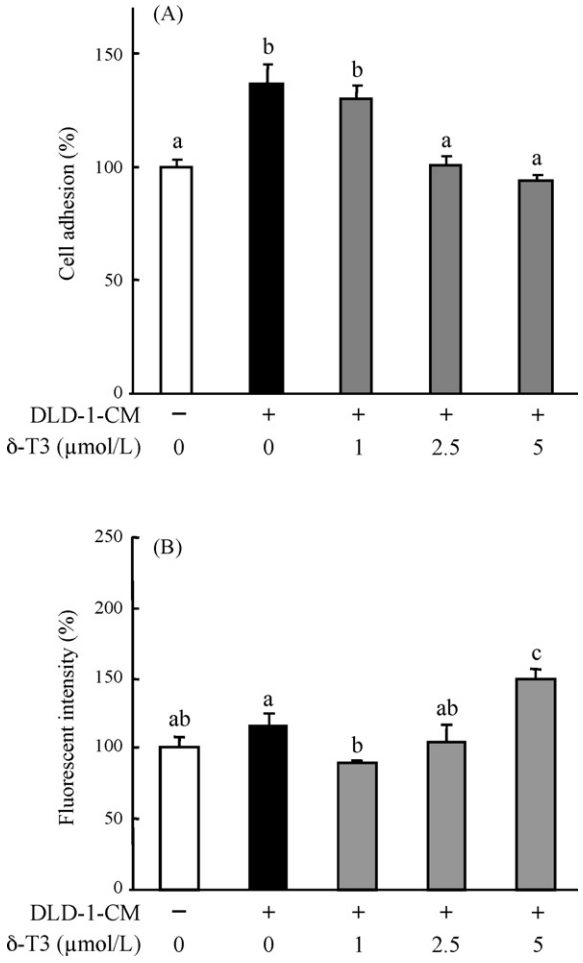


Fig. 4 – Effect of δ-T3 on DLD-1-CM induced HUVEC adhesion (A) and ROS generation (B). (A) HUVEC were suspended in DLD-1-CM containing δ-T3 and incubated for 2 h. The resultant cell suspension was added into each well (15,000 cells/well) of fibronectin coated plate, and incubation was performed for 1 h. (B) Confluent HUVEC were cultured in a test medium (HuMedia-EB2 containing 1% FBS and δ-T3) for 3 h. Then, the medium was changed to DLD-1-CM containing 10 μM DCDHF diacetate, followed by incubation for 20 min. Values are mean ± S.D. (n = 6). Means without a common letter differ, $P < 0.05$.

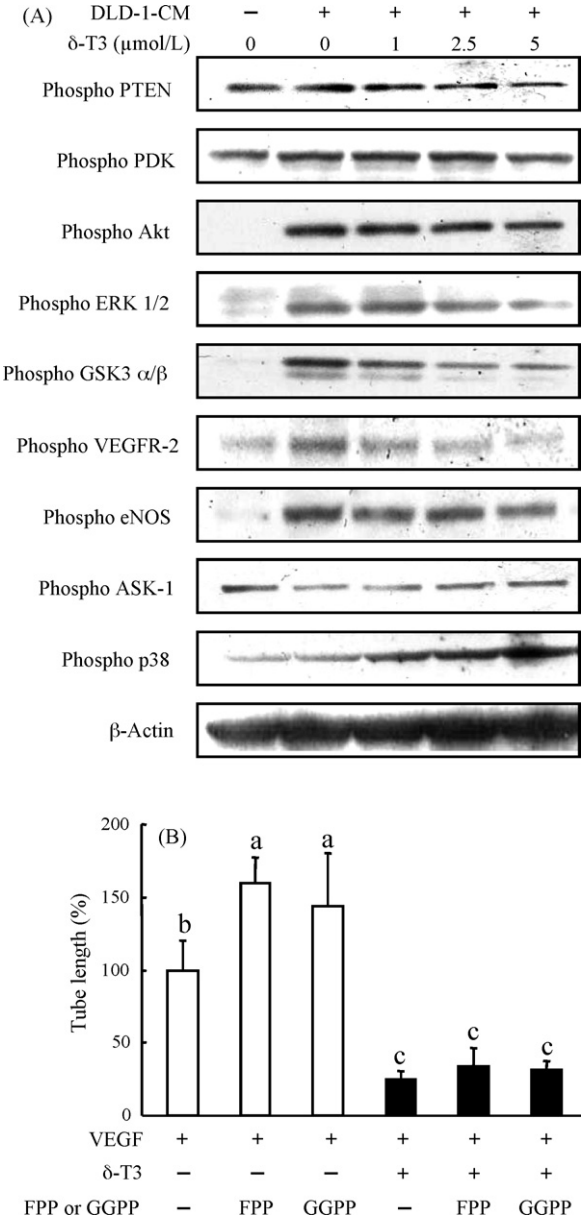


Fig. 5 – Western blot analysis of the intracellular protein associated with the PI3K/PDK/Akt pathway (A). HUVEC were treated with 0–5 μM δ-T3 for 6 h, and were then stimulated with DLD-1-CM for 10 min. Each Western blot is a representative example of data from 3 replicate experiments. Effects of FPP and GGPP on anti-tube formation property of δ-T3 (B). HUVEC co-cultured with fibroblasts were cultivated in the presence or absence of VEGF (10 μg/L), FPP (10 μM), GGPP (10 μM), and δ-T3 (3 μM) for 11 days. Values are mean ± S.D. (n = 4). Means without a common letter differ, $P < 0.05$.

inhibition of phosphorylation of PDK, Akt and PTEN was confirmed. We next investigated the effect of δ-T3 on signals downstream of PI3K/PDK/Akt. Stimulation of HUVEC with DLD-1-CM resulted in activation of eNOS, GSK3 α/β and ERK 1/2, and the changes were reduced to basal (non-stimulated) levels by δ-T3. In addition, δ-T3 increased the phosphorylation of stress response proteins, such as ASK-1 and p38 mitogen-activated protein kinase. Moreover, δ-T3 inhibited the DLD-1-CM-induced phosphorylation of VEGFR-2. At that time, δ-T3 did not affect the expression of non-phosphorylation of these phosphorylated proteins (data not shown). On the other hand, T3 was reported to inhibit 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase activity [8]. HMG-CoA reductase inhibitors (i.e., statins) were known to interfere with angiogenesis by inhibiting FPP and GGPP synthesis in endothelial cells [30]. Because FPP and GGPP did not cancel the anti-tube formation property of δ-T3 (Fig. 5B), anti-angiogenic effect of δ-T3 would be mainly mediated by regulation of PI3K/PDK/Akt signaling in endothelial cells, but not by reduction of HMG-CoA reductase activity.

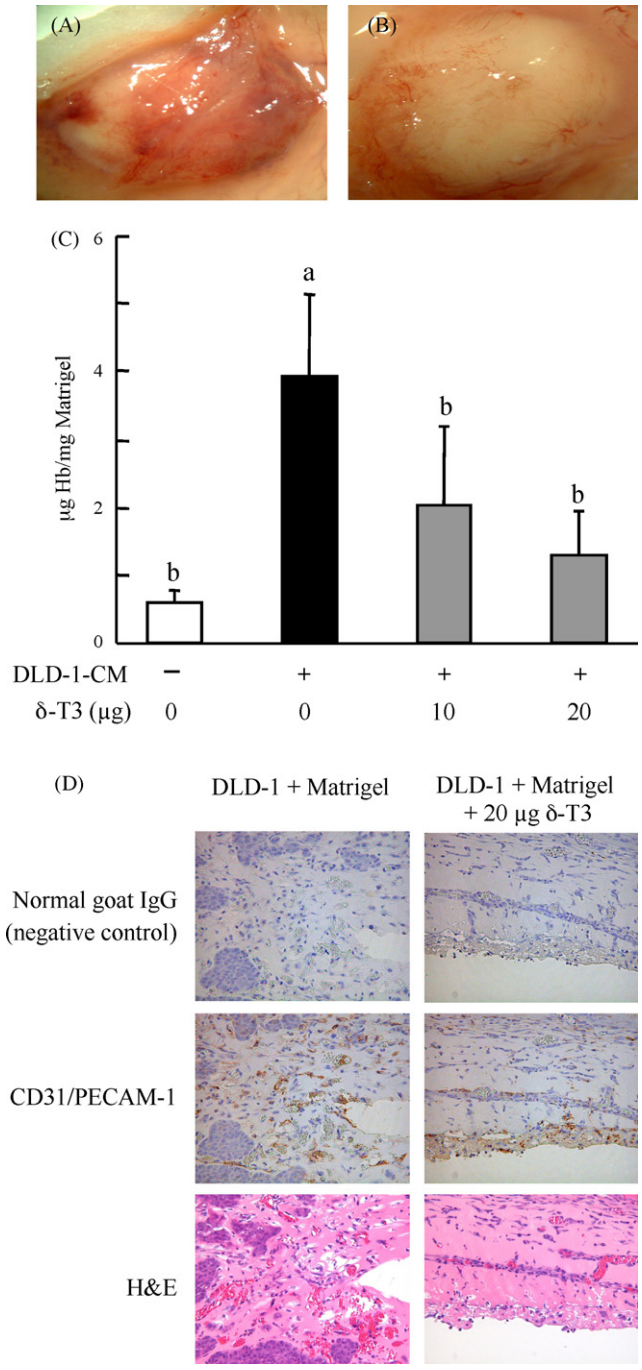


Fig. 6 – Effect of δ-T3 on DLD-1 induced vessel formation in the Matrigel plug assay. DLD-1 cells were suspended in serum free RPMI-1640 medium. Aliquots of the cell suspension (2×10^6 cells/0.1 mL) were mixed with 0.1 mL of Matrigel (containing 0–20 μg δ-T3), and the mixture was injected into flanks of nude mice. The Matrigel plugs were removed 14 days after the implantation ((A) Matrigel containing DLD-1; (B) Matrigel containing DLD-1 and 20 μg δ-T3). Matrigels were subjected to the measurement of hemoglobin content (C) and histological analysis (D). Values are mean ± S.D. ($n = 6$). Means without a common letter differ, $P < 0.05$.

3.3. *Inhibition of DLD-1-induced angiogenesis by T3 in nude mice*

Finally, to investigate whether δ-T3 inhibits in vivo tumor angiogenesis, a Matrigel plug angiogenesis assay was conducted. The DLD-1-Matrigel implanted control mice appeared to show significant neovascularization (as judged by Hb content in Matrigel plug, 3.9 ± 1.2 μg Hb/mg Matrigel) (Fig. 6), compared with mice injected with Matrigel alone (0.6 ± 0.2 μg Hb/mg Matrigel). The suppression of vessel formation in mice implanted with the DLD-1-Matrigel containing δ-T3 (20 μg) was clearly observed (1.3 ± 0.6 μg Hb/mg Matrigel). Histological analysis of the DLD-1-Matrigel plug of control mice indicated an obvious angiogenic response. The CD31/PECAM-1 positive endothelial cells and the red blood cells dyed by H&E were clearly present, indicating that endothelial cells had infiltrated the DLD-1-Matrigel. In contrast, the DLD-1-Matrigel containing δ-T3 showed a low number of both CD31/PECAM-1-positive and erythroid cells, indicating a potent anti-angiogenic effect of δ-T3 in vivo.

4. Discussion

Some anti-angiogenic drugs are now in clinical trials involving patients with a wide variety of cancers [31], and some of them are showing considerable promise for the treatment. It has been documented that some anti-angiogenic agents are available from natural sources [32–34]. Our previous cell-culture studies therefore aimed at screening for natural source-derived anti-angiogenic compounds, and we found δ-T3 as one such potential compound [18–20]. Accordingly, the goal of this study was to directly test the effect of δ-T3 on tumor angiogenesis. Since growth factors (e.g., VEGF, FGF, and EGF) are generated from a variety of tumors that are closely associated with the induction and maintenance of neovasculature in cancer [35], a DLD-1-CM (containing many of these growth factors [36]) was used as the angiogenic stimulus. In our results, we conclusively demonstrated the inhibitory effect of δ-T3 on tumor angiogenesis in vitro and in vivo.

At the cellular level, δ-T3 almost completely suppressed the stimulatory effect of DLD-1-CM on endothelial cell tube formation (Fig. 2) and migration (Fig. 3). These effects appeared to be related to inhibition of HUVEC adhesion and partly associated with ROS generation in HUVEC (Fig. 4). Like δ-T3, Lan-Feng et al. [37] reported that vitamin E analogues (e.g., α-tocopheryl succinate (α-TOS)) inhibit angiogenesis via apoptosis with generating ROS. α-TOS is nontoxic to arrested endothelial cells, but it can cause apoptosis in proliferating endothelial cells. Therefore, apoptosis of proliferating cells by vitamin E analogues would be from the accumulation of relatively high levels of ROS, whereas the level of ROS generated in the arrested cells may be low due to the difference in its cellular systems or in its resistance mechanism to ROS. Therefore, like α-TOS, δ-T3 may cause apoptosis in proliferating endothelial cells at 5 μM. Now, we are confirming such apoptotic effect of δ-T3 in HUVEC (data not shown).

Several endothelial signaling pathways, particularly PI3K/PDK/Akt pathway, are involved in tumor angiogenesis [29,38–44]. It has been reported that, in cancer patients, PI3K/PDK/Akt

signaling is elevated in tumors and is correlated with tumor progression [40]. PI3K is a lipid kinase that generates both phosphatidylinositol (3,4,5)-trisphosphate (PIP3) as a second messenger, and PDK is activated by binding to PIP3 [42]. The activated PDK then phosphorylates and consequently activates Akt [42]). Activated Akt has been shown to phosphorylate various proteins associated with endothelial cell survival and proliferation [43]. Inactivation of Akt is regulated via two phosphatases, PTEN and PP2A by inhibiting the activation of PDK and regulating negatively Akt via dephosphorylation, respectively [44]. In the present study, stimulation of HUVEC with DLD-1-CM caused significant phosphorylation of PDK, Akt, and PTEN, indicating activation of PI3K/PDK/Akt signaling in HUVEC (Fig. 5A). Treatment with δ-T3 markedly decreased the intracellular levels of activated PDK, Akt, and PTEN. These findings suggest that the anti-angiogenic effect of δ-T3, at least in part, is mediated by reduction of PI3K/PDK/Akt activity in endothelial cells. Another evidence to support our suggestion is that δ-T3 inactivated signals downstream of PI3K/PDK/Akt, such as eNOS, GSK3α/β and ERK 1/2 which all are involved in cell proliferation and survival. In addition, δ-T3 enhanced the phosphorylation of ASK-1 and p38, which are closely involved in stress response [45,46]. Therefore, δ-T3 blocks PI3K/PDK/Akt signals by not only inactivating downstream survival signals but also by enhancing the ASK-1 and p38 pathway, thus inhibiting angiogenic responses in endothelial cells. On the other hand, induction of p38 MAPK signaling is known to be able to lead to a mitogenic response [47]. However, as mentioned above, it is also known that activation of ASK-1 and/or suppression of Akt can induce p38 activation, which result in apoptosis through signals involving mitochondrial cell death pathway. In this study, we found activation of ASK-1 and p38 as well as suppression of Akt by δ-T3. It is therefore likely that these changes tend to lead a stress-induced proapoptotic reaction, but not a mitogenic response. Considering Fig. 5B, the anti-angiogenic effect of δ-T3 would not be related to the ability of δ-T3 to reduce HMG-CoA reductase activity.

It is well-known that VEGFR-2 is a principal receptor for VEGF signaling. Upon ligand binding, VEGFR-2 undergoes autophosphorylation and becomes activated [48]. Signaling from VEGFR-2 is necessary for the performance of VEGF-stimulated proliferation, chemotaxis, as well as the survival of endothelial cells. Blocking the kinase activity of VEGFR-2 is a possible mechanism for anti-angiogenic compounds [49]. In this study, because δ-T3 almost inhibited DLD-1-CM-induced VEGFR-2 phosphorylation, the anti-angiogenic effect of δ-T3 may occur upstream of the PI3K/PDK/Akt signaling pathway at the level of VEGFR-2.

To evaluate the effect of δ-T3 on in vivo tumor angiogenesis, we conducted Matrigel plug assay using nude mice. Numerous studies reported the usability of the Matrigel plug assay to assess the in vivo efficacy of inhibitors for tumor-associated angiogenesis [50,51]. As shown in Fig. 6, we demonstrated that δ-T3 significantly inhibits in vivo tumor angiogenesis as evaluated by Hb content in Matrigel plug. Because immunohistochemical analysis of DLD-1-Matrigel plug containing δ-T3 showed inhibition of endothelial cell invasion and neovessel formation, these observations may be due to the inhibitory effects of δ-T3 on endothelial signaling of pro-angiogenic factors, such as VEGF. It is also possible that the in vivo anti-angiogenic effect of δ-T3 is not due only to its direct action on endothelial cells, but also to the consequent effects on both endothelial cells and other cell types such as macrophages, leukocytes, and tumor cells.

Although δ-T3 is a natural product, questions on its safety and toxicity must be addressed. Several preclinical studies, including our previous study, have shown no T3-related critical weight loss or adverse events in animals [52,53]. T3 is absorbed through the intestine [54], and is distributed into the blood stream of humans, suggesting that T3 is bioavailable to exert its biological effects. Studies of orally administration of T3 (12–40 mg/day) to rats for 3 months suggested that T3 reached a concentration of 15–50 μmol/kg in aorta (data not shown). In the present study, the concentrations of δ-T3 (2.5–5 μM) were sufficient to inhibit in vitro angiogenic steps of HUVEC. It is thus tempting to speculate that the inclusion of T3 in diets may have anticancer effect through angiogenesis inhibition. To further evaluate this speculation, we are now conducting Matrigel plug assay on animal model orally administered T3. On the other hand, currently there are considerable works being undertaken to screen potential anti-angiogenic compounds. Dietary constituents including epigallocatechin gallate [55], capsaicin [56], apigenin [51], and conjugated fatty acids [33] have been shown to inhibit angiogenesis in vitro and/or in vivo. Based on the reported in vitro data [33,51,55,56], anti-angiogenic potential of δ-T3 is equal to or more than that of these dietary constituents.

In conclusion, we demonstrated that δ-T3 even at low concentration inhibits tumor angiogenesis, and that the inhibitory effect is mainly mediated by regulation of the PI3K/PDK/Akt pathway and VEGFR-2 activity in endothelial cells. In case of relatively high dose (>5 μM), δ-T3 not only blocks Akt and inhibits downstream survival signals, but also enhances the ASK1 and p38 pathway, thereby eliciting an apoptotic effect in endothelial cells. We propose that δ-T3 is a promising anticancer agent or an adjuvant for minimizing tumor angiogenesis, which warrants its testing in other models of cancer with a realistic prospect of its use in human therapy.

Acknowledgments

This work was supported by a Grant-in-Aid from the Bio-oriented Technology Research Advancement Center of the National Agricultural and Biological Research Organization, Japan. The financial support was provided by Project M Co. Ltd. (Sendai, Japan).

Conflict of interest

None declared.

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