Original Article

Anti-angiogenic function of tocotrienol

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Angiogenesis means the formation of new blood vessels from preexisting vascular, is of fundamental importance in several pathological states such as tumor growth, rheumatoid arthritis, and diabetic retinopathy. Angiogenesis involves a set of steps, including activation and movement of endothelial cells and tube formation. Control of these steps by drugs or dietary food components is a hopeful approach for the prevention of angiogenic disorders. Based on these backgrounds, we searched the anti-angiogenic food components. As a result, we found that tocotrienol (T3), especially δ, β, and γ-T3 has the potent anti-angiogenic activity in vitro and in vivo experiments. T3, which is rich in rice bran and palm oil, inhibited growth factor-induced proliferation, migration and tube formation in human umbilical vein endothelial cells. T3 showed inhibition of tumor cell-induced angiogenesis in mouse dorsal air sac (DOS) assay. These results indicated that T3 is a potent anti-angiogenesis compound. Tocopherol (Toc) did not inhibit angiogenesis. The anti-angiogenic mechanism of T3 and Toc was evaluated by western blotting. T3 inhibited activation of growth factor-induced extracellular signal-regulated kinase, Akt (protein kinase B), and endothelial nitric oxide synthase (eNOS), which are located downstream of the various growth factor receptors. T3 suppressed phosphorylation of vascular endothelial growth factor (VEGF) receptor 2. These effects were dose-dependent manner. Anti-angiogenic mechanism of T3 mediates inhibition of growth factor induced survival, migration and angiogenesis signals. These findings suggested that T3 may have potential for preventing angiogenic disorders in humans.

Key Words: anti-angiogenesis, tocotrienol, vitamin E, HUVEC, PI3K

INTRODUCTION

Vitamin E occurs naturally in eight different forms: α-, β-, γ- and δ-isomers of both tocopherol (Toc) and tocotrienol (T3). 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 (Fig. 1). 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] Recent studies have shown that T3 has better anti-oxidative,[2] anti-hypercholesterolemic,[3] and anti-cancer[4] activities than Toc. Furthermore, the potent ability of T3 to induce cell cycle arrest, regulate HMG-CoA reductase,[3] suppress adhesion molecules,[5] inhibit nuclear factor-κB,[6] and down-regulate c-Myc and telomerase[7] has been reported. These studies suggest that T3 may serve as a food component with a wide variety of health benefits.

As a new function of T3, we recently found its inhibitory effect on angiogenesis by inhibiting proliferation, migration, and tube formation of endothelial cells in vitro.[8] Since angiogenesis is of fundamental importance in pathological states (e.g., diabetic retinopathy, rheumatic arthritis, and tumor growth), we hypothesize that T3 is potentially useful as a functional component for prevention of these angiogenesis-mediated disorders.

To validate our hypothesis, we investigated the *in vivo* anti-angiogenic properties of T3 using two well-characterized angiogenic systems (mouse dorsal air sac assay (DAS). The anti-angiogenic mechanism of T3 was evaluated in cell-culture by Western blot analysis. We demonstrated that T3 is a potent inhibitor of angiogenesis *in vivo*.[9] T3 regulated phosphatidylinositol 3-kinase (PI3K)/phosophoinositide-dependent protein kinase (PDK)/Akt signaling and induced apoptosis in the endothelial cells, which would be responsible as its anti-angiogenic effect.

MATERIALS AND METHODS

T3 was purchased from Calbiochem (San Diego, CA, USA). Toc was obtained from Sigma (St. Louis, MO, USA). Tocomin 50 was kindly supplied by Koyo Mercantile Co., Ltd. (Tokyo, Japan). Tocomin 50 is composed of 14% α-Toc and 44% T3 (14% α-T3, 24% γ-T3, and 6 % δ-T3).

In the mouse dorsal air sac (DAS) assay, DLD-1 human colon carcinoma cells (from the Cell Resource Center for Biomedical Research at Tohoku University, Sendai, Japan) were suspended in PBS at a concentration of 1 x 10^8 cells/mL, and 0.2 mL of this suspension was injected into a chamber ring (Millipore Co., MA, USA).

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This chamber was implanted into a dorsal air sac produced in a 5-week-old male ICR mouse (CLEA Japan, Inc. Tokyo, Japan). The treated mice were fed Tocomin 50 (2.5 and 10 mg) or α-Toc (1.5 mg) once a day for 5 days by gavage using 50 mg vitamin E-stripped corn oil (ICN Biomedicals, Aurora, OH, USA) as a vehicle. Angiogenic response was assessed by counting newly formed capillary vessels within the area attached to the chamber.

The *in vitro* effect of T3 on growth factor-induced proliferation, migration, and tube formation of human umbilical vein endothelial cell (HUVEC) was investigated as follows.

For the proliferation assay, the HUVEC were transferred into 96-well plates (2500 cells/well) and preincubated in HuMedia-EG2 medium for 24 h. The culture medium was replaced with test medium (HuMedia-EB2 supplemented with 2% FBS, 10 ng/mL fibroblast growth factor (FGF) or vascular endothelial growth factor (VEGF), and 0-35 μmol/L T3). After incubation for 72 h, viable cells were estimated using the WST-1 assay.

For the migration assay, confluent HUVEC were cultured in 24-well collagen type I-coated culture plates in HuMedia-EG2 medium without growth factors. After 12 h, the cells were scratched with a yellow pipette tip to obtain a wounded monolayer culture.[10] After washing with PBS, the cells were cultured for 36 h in HuMedia-EB2 medium with 10 ng/mL FGF and 0-7 μmol/L δ-T3. Cell migration was recorded as the lengths of wounded cells using a phase contrast inverted microscope.

In the tube formation assay, culture plates (24-well) were coated with 350 μL of Matrigel (Collaborative Research, Bedford, MA, USA). HUVEC (6×10^4 cells) were suspended in 500 μL of test medium (HuMedia-EB2 with 1% FBS, 10 ng/mL FGF, and 0-7 μmol/L δ-T3). The cell suspension was placed on the surface of Matrigel, and was incubated for 18 h. After that, the cells were fixed, and then were photographed. The length of tube-structured cells was quantified using a KURABO angiogenesis Image Analyzer (imaging software; Kurabo, Osaka, Japan).

For western blot analysis, HUVEC were treated with T3 under two different conditions; (A) 24 h incubation with 10 ng/mL FGF and 0-7 μmol/L δ-T3 in HuMedia-EB2 medium with 2% FBS, (B) 10 min stimulation with 10 ng/mL FGF after pre-incubation for 8 h in HuMedia-EB2 medium with 2% FBS and 0-7 μmol/L δ-T3 or (C) 10 min stimulation with 10 ng/mL VEGF after pre-incubation for 6 h in HuMedia-EB2 medium with 2% FBS and 0-5 μmol/L δ-T3 or α-Toc. HUVEC were lysed in 1 mL lysis buffer. The cellular proteins (50 μg/well) were separated by SDS-PAGE. The protein bands were transferred to polyvinylidine fluoride membrane. The membrane was probed with primary antibodies, followed by a horseradish peroxidase-conjugated secondary antibody. The detection was performed with ECL Plus Western blotting reagents (Amersham Pharmacia Biotech, NJ, USA). The antibodies used were anti-Cyclin D1 and anti-β-Actin (Santa Cruz Biotechnology, CA, USA); anti-phospho PDK, anti-phospho Akt, anti-phospho ERK 1/2, anti-phospho p38, anti-phospho ASK-1, anti-phospho VEGF-R2, anti-Phospho GSK3 α/β and anti-phospho eNOS (Cell signaling Technology Inc, Beverly, MA, USA); anti-Caspase 9 (Medical Biological Laboratories, Nagoya, Japan); anti-Caspase 3 (Neomarkers, CA, USA); and anti-p21 (Upstate Biotechnology, NY, USA).

The data were expressed as mean ± SD. We performed statistical analysis using a 1-way ANOVA, followed by a Dunnett's test for multiple comparisons among several groups. Difference of $p < 0.05$ versus control was considered statistically significant.

RESULTS

Fig. 1 shows the in vivo effect of Tocomin 50 (T3-rich oil) on DLD-1 induced angiogenesis in the DAS assay. The DLD-1 chamber implanted control mice showed significant neovascularization (angiogenesis index 4.8 ± 0.6) from surrounding blood vessels. Vessel formation was clearly suppressed (angiogenesis index 2.7 ± 0.6) in mice given Tocomin 50 orally at a dose of 10 mg/day (1.4 mg α-T3, 2.4 mg γ-T3, 0.6 mg δ-T3, and 1.4 mg α-Toc). Since α-Toc (1.5 mg/day) showed no angiogenic inhibition, the anti-angiogenic effect of Tocomin 50 could be mainly ascribed to T3. This result indicates that T3 acts as an anti-angiogenic compound *in vivo*.

Since growth factors are closely involved in the neovascularization of DAS model as well as pathological angiogenesis, we conducted a series of *in vitro* studies (growth factor-induced proliferation, migration, and tube formation in HUVEC) to evaluate the anti-angiogenic effects and inhibitory mechanism of T3. As shown in Fig. 2, all T3 isomers tested at the micromolar range (5-20 μmol/L) significantly inhibited HUVEC proliferation in a

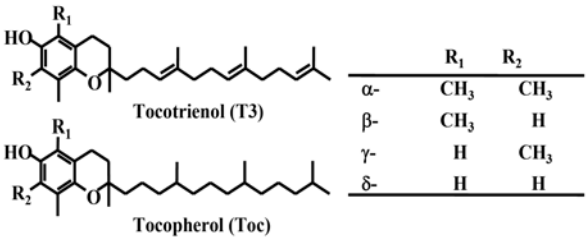

Figure 1. Chemical structure of T3 and Toc. T3 has an unsaturated isoprenoid tail, which differs from Toc bearing a saturated phytyl side-chain.

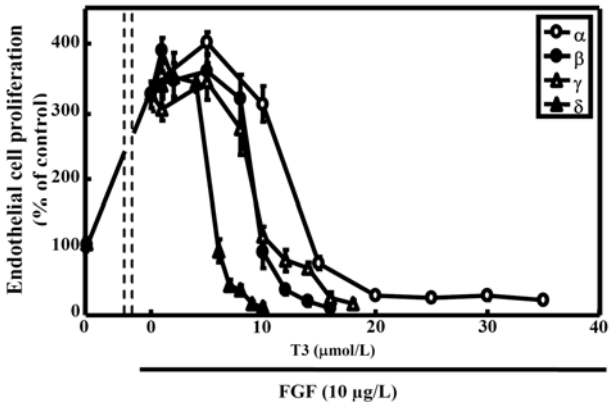

Figure 2. Effects of T3 on FGF-induced HUVEC proliferation. HUVEC were cultured with 0–35 μmol/L T3 in the presence of 10 μg/L FGF for 72 h. Viable cells were estimated using the water-soluble tetrazolium salt (WST-1) assay. Values are mean ± SD, $n = 6$. Means without a common letter differ, $p < 0.05$.

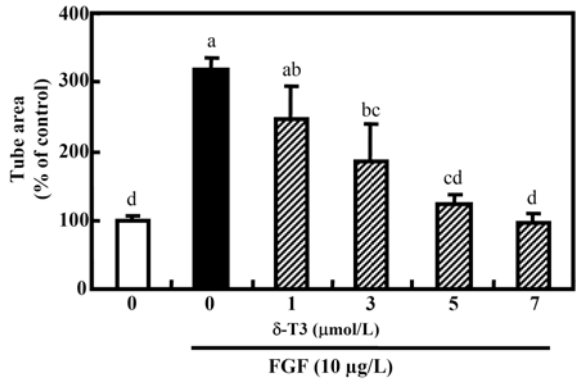

Figure 3. Effects of δ-T3 on FGF-induced HUVEC tube formation. In the tube formation assay, HUVEC were suspended in test medium containing 10 μg/L FGF and 0-7 μmol/L δ-T3. The cell suspension was placed on the surface of Matrigel, and incubated for 18 h. The areas of tube-structured cells were quantified. Values are expressed as mean±SD (n=4). Means without a common letter differ, $p < 0.05$. T3, tocotrienol.

dose-dependent manner. The inhibitory potency of T3 isomers had the following order: δ- >β- > γ- > α-T3. δ-T3 at concentrations of 1-7 μmol/L was able to inhibit wound closure of HUVEC in the migration assay, and reduce the length of endothelial tubes in the tube formation assay (Fig. 3). Western blot analysis showed that δ-T3 suppressed the phosphorylation of PDK, Akt, ERK1/2, eNOS, and GSK3 α/β in a dose-dependent manner, as well as increased the phosphorylation of ASK-1 and p38 in FGF-treated HUVEC. Furthermore, at a relatively high concentration (7 μmol/L), δ-T3 cleaved procaspase-3 and -9 to their active forms and decreased Cyclin D1 expression (Fig. 3). Pretreatment of HUVECs with δ-T3 inhibited the VEGF induced tyrosine phosphorylation in dosedependent manner (data not shown). These results suggest that the anti-angiogenic effects of T3 are due to regulation of PI3K/PDK/Akt signaling as well as induction of endothelial cell apoptosis.

DISCUSSION

Angiogenesis, the formation of new blood vessels, plays an important role in many pathological processes, such as growth and metastasis of solid tumors, diabetic retinopathy, and rheumatoid arthritis.[11] Since angiogenic processes are involved in endothelial cell proliferation, migration, and tube formation, the modulation of these processes serves as a good strategy for preventing angiogenesis-mediated disorders. It has been documented that some anti-angiogenic agents are available in foods.[12,13] Even if these agents possess moderate anti-angiogenic effects, daily consumption of these compounds may result in help for preventing angiogenic disorders. Our preliminary cell-culture studies screening for food-derived anti-angiogenic compound showed T3 to be a potential angiogenic inhibitor. This study, we have demonstrated for the first time that T3 exerts anti-angiogenic effects in vivo. The anti-angiogenic effects of T3 would be mediated by regulation of PI3K/PDK/Akt signaling as well as apoptosis induction in endothelial cells.

First, we evaluated the anti-angiogenic effects of T3 in in vivo study, the DAS assay. In the DAS assay, neovascularization of the mouse subcutaneous tissues, which was triggered by DLD-1 cells, was effectively inhibited by the oral administration of Tocomin 50 (T3-rich oil) (data not shown). No negative effects were observed in preexisting vessels treated with Tocomin 50. These results clearly suggested the notable in vivo anti-angiogenic effects of T3. We speculate that a substantial amount of T3 would be absorbed from mouse intestine and distributed to the tissues surrounding the DLD-1 chamber, where T3 can inhibit the vascularization of the cells bearing growth factor receptors, such as endothelial cells, smooth muscle cells, and the others responsible for neovascularization. T3 exerts anti-angiogenic effects in vivo, potentiating the usability of T3 as a therapeutic dietary supplement for preventing angiogenic disorders. In one of our animal studies, orally administrated T3 to rats was found to be distributed in blood stream and various tissues.[14] This data would be advantageous for application of T3 in the use for functional and nutraceutical purposes.

How T3 mediates its anti-angiogenic effects is not fully understood, although its ability to suppress the growth factor-dependent activation of PI3K/PDK/Akt signaling in neoplastic mammary cells has been reported. PI3K is a lipid signaling kinase that activates PDK, leading to activation of Akt, which in turn phosphorylates various intracellular substrates associated with cell proliferation and apoptosis. On the other hand, various growth factors are closely involved in neovascularization. FGF and VEGF induces endothelial cells to secrete proteases and plasminogen activators that degrade the vessel basement membrane, leading to cell invasion into the surrounding matrix and the formation of new vessels. Considering the critical role of both PI3K/PDK/Akt signaling and anti-apoptosis in growth factor-induced angiogenesis, we hypothesized that T3 must modulate angiogenesis through this pathway. We conducted in vitro studies to evaluate the anti-angiogenic effects and mechanisms of T3 action. We demonstrated that T3 significantly reduced growth factor-induced proliferation, migration, and tube formation in HUVEC (Fig. 2), and that these effects were related to a decrease in the intracellular protein activity associated with the PI3K/PDK/Akt pathway (Fig. 3). Our studies also showed that T3 at a relatively high dose (7 μmol/L) affected some proteins such as ASK-1 and p38 associated with apoptosis. In addition, T3 down-regulated p21 and Cyclin D1 which are involved in cell cycle progression and arrest. Taken together, these results strongly suggest that angiogenesis inhibition by T3 is mediated by regulation of the PI3K/PDK/Akt pathway and apoptosis induction. Although the exact intracellular sites of action targeted by T3 were unknown, we postulated that the anti-angiogenic effects of T3 occur upstream of the PI3K/PDK/Akt signaling pathway at the level of the growth factor-receptor. Next, we demonstrated that δ-T3 partially inhibit VEGF-induced VEGFR-2 phosphorylation at 5 μmol/L. But, α-tocopherol did not have this effect (data not shown). Some recent studies have demonstrated 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. These results point out the

possibility that T3 inhibit angiogenesis via regulation of growth factor receptor on cell surface.

In this study, T3 displays significantly greater anti-angiogenic activity than Toc in vivo and in vitro. Structurally, T3 and Toc can be distinguished by their side chains, and it has been reported that the unsaturated side chain of T3 allows it to pass through cell membranes more efficiently and at a faster rate than the saturated phytyl side-chain of Toc.[15] For this reason, the greater anti-angiogenic effect of T3 may be due in part to their effective incorporation into endothelial cells.

Dietary constituents including vitamin D[12] and conjugated fatty acids[13] have been shown to inhibit angiogenesis in vitro and/or in vivo. As shown in the present study, T3 represents a member of a new class of diet-derived anti-angiogenic compounds.

In conclusion, we demonstrated that T3 inhibits the angiogenic response in vivo and that this inhibitory effect is mediated by regulation of the PI3K/PDK/Akt pathway as well as by apoptosis induction in endothelial cells. Therefore, T3 has potential use as a functional compound for prevention of angiogenic disorders.

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AUTHOR DISCLOSURES

Teruo Miyazawa, Akira Shibata, Kiyotaka Nakagawa and Tsuyoshi Tsuzuki, no conflicts of interest.

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