In Vivo Angiogenesis Is Suppressed by Unsaturated Vitamin E, Tocotrienol\textsuperscript{1,2}

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Abstract

Antiangiogenic therapy using drugs and food components is a recognized strategy for the prevention of various angiogenesis-mediated disorders such as tumor growth, diabetic retinopathy, and rheumatoid arthritis. Our preliminary cell culture studies, using both bovine aortic endothelial cells and human umbilical vein endothelial cells (HUVEC) on screening for food-derived antiangiogenic compounds, showed tocotrienol (T3), an unsaturated version of vitamin E, to be a potential angiogenic inhibitor. We therefore investigated the in vivo antiangiogenic properties of T3 using 2 well-characterized angiogenic models [mouse dorsal air sac (DAS) assay and the chick embryo chorioallantoic membrane (CAM) assay]. In the DAS assay, the increased neovascularization (angiogenesis index, 4.8 ± 0.6) in tumor cell–implanted mice was suppressed (angiogenesis index, 2.7 ± 0.6) by dietary supplementation of 10 mg T3-rich oil/d (equivalent to 4.4 mg T3/d). In the CAM assay, T3 (500–1000 µg/egg) inhibited new blood vessel formation on the growing CAM and increased the frequency of avascular zone (36–50%). To evaluate the antiangiogenic mechanism, we conducted cell culture studies and found that T3 significantly reduced fibroblast growth factor–induced proliferation, migration, and tube formation in HUVEC (P < 0.05), with δ-T3 having the highest activity. Western blot analysis revealed that δ-T3 suppressed the phosphorylation of phosphoinositide-dependent protein kinase (PDK) and Akt, and increased the phosphorylation of apoptosis signal-regulating kinase and p38 in fibroblast growth factor–treated HUVEC, indicating that the antiangiogenic effects of T3 are associated with changes in growth factor-dependent phosphatidylinositol-3 kinase/PDK/Akt signaling as well as induction of apoptosis in endothelial cells. Our findings suggest that T3 has potential as a therapeutic dietary supplement for preventing angiogenic disorders, and therefore future clinical study will be required to evaluate the efficacy and safety of T3. J. Nutr. 137: 1938–1943, 2007.

Introduction

Vitamin E occurs naturally in 8 different forms: α-, β-, γ-, and δ-isomers of both tocopherol (Toc)\textsuperscript{7} and tocotrienol (T3) (Fig. 1). They differ structurally in that Toc has a saturated phytyl side chain attached to its chroman ring, whereas T3 possesses an unsaturated isoprenoid side chain. Toc is abundant in common vegetable oils and nuts, and T3, a minor plant constituent, is abundant in rice bran, palm, and wheat germ (1,2).

Recent studies indicate that T3 has better antioxidative (3,4), antihypercholesterolemic (5), anticancer (6), and neuroprotective (7,8) activities than Toc. Further, the potent ability of T3 to induce cell cycle arrest (9), regulate HMG-CoA reductase (5), activate p53 and caspase-8 (10,11), suppress adhesion molecules (12), inhibit nuclear factor-κB (13), and downregulate c-Myc and telomerase (14) was reported. These studies (3–14) suggest that T3 may serve as a food component with a wide variety of health benefits.

In our own recent cell culture studies (15–17), T3 suppressed angiogenesis by inhibiting proliferation, migration, and tube formation of endothelial cells. Angiogenesis, or new blood vessel growth from an existing vasculature, has become a promising target for experimental therapies in pathological states (e.g., diabetic retinopathy, rheumatoid arthritis, and tumor growth) (18,19). Although physiological conditions (e.g., reproductive cycles and wound healing) are angiogenesis dependent (20), more than 20 angiogenesis inhibitors are registered by the National Cancer Institute, including metalloproteinase inhibitors in early phases of clinical trials in the treatment of cancer (21). Considering...
The mice were then killed with sodium pentobarbital, and their skins (ICN Biomedicals) as a vehicle. Control mice received only the vehicle once a day for 5 d by gavage using 50 mg vitamin E–stripped corn oil CO2. Human umbilical vein endothelial cells (HUVEC) were purchased from Kurabo. HUVEC were cultured in growth medium HuMedia-EB2 with 2% FBS, 10 μg/L FGF, and 0–35 μmol/L T3. After incubating for 72 h, viable cells were estimated using the water-soluble tetrazolium salt assay (25), which measures the metabolic activity of viable cells by the formation of a formazan dye. For the migration assay, confluent HUVEC were cultured in 24-well collagen type I-coated culture plates in HuMedia-EB2 medium without growth factors. After 12 h, the cells were scratched with a yellow pipette tip to obtain a wounded monolayer culture (26). After washing with PBS, the cells were cultured for 36 h in the test medium (HuMedia-EB2 with 10 μg/L FGF and 0–7 μmol/L Δ3-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 (Becton Dickinson) and incubated at 37°C for 1 h to allow solidification. Trypsin-harvested HUVEC (6 × 104 cells) were suspended in 500 μL FGF, and 0–7 μmol/L Δ3-T3. After washing with PBS, the cells were cultured for 24 h. The in vivo effects of T3 on FGF-induced proliferation, migration, and tube formation of HUVEC was investigated as follows. For the proliferation assay, HUVEC were transferred into 96-well plates (2500 cells/well) and preincubated in HuMedia-EB2 medium for 24 h. After the HUVEC were washed with PBS, the culture medium was replaced with test medium (HuMedia-EB2 supplemented with 2% FBS, 10 μg/L FGF, and 0–35 μmol/L T3). After incubating for 72 h, viable cells were estimated using the water-soluble tetrazolium salt assay (25), which measures the metabolic activity of viable cells by the formation of a formazan dye. For the migration assay, confluent HUVEC were cultured in 24-well collagen type I-coated culture plates in HuMedia-EB2 medium without growth factors. After 12 h, the cells were scratched with a yellow pipette tip to obtain a wounded monolayer culture (26). After washing with PBS, the cells were cultured for 36 h in the test medium (HuMedia-EB2 with 10 μg/L FGF and 0–7 μmol/L Δ3-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 (Becton Dickinson) and incubated at 37°C for 1 h to allow solidification. Trypsin-harvested HUVEC (6 × 104 cells) were suspended in 500 μL test medium (HuMedia-EB2 with 1% FBS, 10 μg/L FGF, and 0–7 μmol/L Δ3-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 photographed. The length of tube-structured cells was quantified using a Kurabo angiogenesis Image Analyzer (imaging software; Kurabo). The CAM assay (24) was performed to further evaluate in vivo antiangiogenic activities of T3. A pellet of ethylene-vinyl acetate copolymer 40 (Aldrich Chemical) impregnated with Δ3-T3 (0–1000 μg), α-Toc (0–1000 μg), or without vitamin E (control) was placed on the CAM surface of 5-d-old chick embryos. The embryos were incubated for 2 d at 37°C in a humidified incubator, after which an appropriate volume of 10% fetal bovine serum was injected into the chorioallantois to improve visualization of the vascular network. Inhibition of angiogenesis was determined by measuring the avascular zone in the CAM. A positive antiangiogenic response was defined as an avascular zone of ≥3 mm in diameter.

Materials and Methods

Materials. T3 was purchased from Calbiochem, Toc was obtained from Sigma, and Tocomin 50 (T3 extract from palm oil) was kindly supplied by Koyo Mercantile. Tocomin 50 was composed of 14% α-Toc (wt:wt) and 44% T3 (14% α-Toc, 24% γ-T3, and 6% Δ3-T3). The vitamin E–free AIN-76 diet (22) was from Funahashi Farm. Vitamin E–stripped corn oil and Tocomin 50 (T3 extract from palm oil) was kindly supplied by Koyo Mercantile. HUVEC were cultured in growth medium HuMedia-EB2 with 2% FBS, 10 μg/L FGF, and 0–35 μmol/L T3. After incubating for 72 h, viable cells were estimated using the water-soluble tetrazolium salt assay (25), which measures the metabolic activity of viable cells by the formation of a formazan dye. For the migration assay, confluent HUVEC were cultured in 24-well collagen type I-coated culture plates in HuMedia-EB2 medium without growth factors. After 12 h, the cells were scratched with a yellow pipette tip to obtain a wounded monolayer culture (26). After washing with PBS, the cells were cultured for 36 h in the test medium (HuMedia-EB2 with 10 μg/L FGF and 0–7 μmol/L Δ3-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 (Becton Dickinson) and incubated at 37°C for 1 h to allow solidification. Trypsin-harvested HUVEC (6 × 104 cells) were suspended in 500 μL test medium (HuMedia-EB2 with 1% FBS, 10 μg/L FGF, and 0–7 μmol/L Δ3-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 photographed. The length of tube-structured cells was quantified using a Kurabo angiogenesis Image Analyzer (imaging software; Kurabo).

Antiangiogenic mechanism of T3 evaluated by Western blot analysis. HUVEC were treated with T3 under 2 different conditions; 1) 24-h incubation with 10 μg/L FGF and 0–7 μmol/L Δ3-T3 in HuMedia-EB2 medium with 2% FBS, or 2) 10-min stimulation with 10 μg/L FGF after preincubation for 8 h in HuMedia-EB2 medium with 2% FBS and 0–7 μmol/L Δ3-T3. After that, cellular proteins were prepared from HUVEC as previously described (27), and the cellular proteins (50 μg/ well) were separated by SDS-PAGE gel electrophoresis (10–20% e-PAGE, Atto). The protein bands were transferred to polyvinylidene fluoride membrane (Amersham Pharmacia Biotech). After being blocked of nonspecific sites, the membrane was probed with primary antibodies, followed by a horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology). The detection of the antibody reactions was performed with ECL Plus blotting reagents (Amersham Pharmacia Biotech). The antibodies used were anti-Cyclin D1 and anti-β-Actin (Santa Cruz Biotechnology); anti-Phospho phosphoerinoid-side-dependent protein kinase (PDK), anti-Phospho Akt, anti-Phospho extracellular signal-regulated kinase 1/2 (ERK 1/2), anti-Phospho p38, anti-Phospho apoposis signal-regulating kinase (ASK-1), anti-Phospho glycogen synthase kinase 3 α/β (GSK3 α/β, and anti-Phospho endothelial nitric oxide synthase (eNOS) (Cell Signaling Technology); anti-Casparase 9 (Medical Biological Laboratories); anti-Casparase 3 (Neomarkers); and anti-p21 (Upstate Biotechnology).

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Statistical analysis. The data are expressed as mean ± SD unless otherwise indicated and were tested by 1-way ANOVA, followed by Newman-Keules test. For the CAM assay, Fisher’s exact probability test was performed. Differences were considered significant at $P < 0.05$.

Results

We first conducted a DAS assay to evaluate the in vivo effect of Tocomin 50 (T3-rich oil) on DLD-1 induced angiogenesis (Fig. 2). The DLD-1 chamber implanted control mice showed remarkable neovascularization from surrounding blood vessels. Vessel formation was clearly suppressed in mice given Tocomin 50 orally at a dose of 10 mg/d (1.4 mg $\alpha$-T3, 2.4 mg $\gamma$-T3, 0.6 mg $\delta$-T3, and 1.4 mg $\alpha$-Toc). Because $\alpha$-Toc (1.5 mg/d) showed no angiogenic inhibition, the antiangiogenic effect of Tocomin 50 could be mainly ascribed to T3. We further examined the in vivo effect of T3 on angiogenesis using the CAM assay (Fig. 3). T3-free pellets (ethylene-vinyl acetate copolymer) did not exhibit any antiangiogenic effects. $\delta$-T3, which was the most potent antiangiogenic T3 isomer in our previous in vitro studies (15–17), caused a suppression of embryonic angiogenesis, resulting in avascular zone. No antiangiogenic activity was found in $\alpha$-Toc-treated CAM.

Because growth factors (i.e., FGF) are closely involved in the neovascularization of DAS and CAM models as well as pathological angiogenesis (18,19), we conducted a series of in vitro studies (FGF-induced proliferation, migration, and tube formation in HUVEC) to evaluate the antiangiogenic effects and inhibitory mechanism of T3 on FGF action. All T3 isomers inhibited FGF-induced HUVEC proliferation (Fig. 4). The median ($\pm$SD) concentrations resulting in 50% inhibition (IC50) of T3 isomers were: $\delta$-T3 (5.5 ± 0.1 μmol/L), $\beta$-T3 (9.4 ± 0.2 μmol/L), $\gamma$-T3 (9.4 ± 0.2 μmol/L), and $\alpha$-T3 (13.1 ± 0.2 μmol/L). $\delta$-T3 inhibited HUVEC migration and reduced the length of endothelial tubes (Fig. 5). Western blot analysis indicated that $\delta$-T3 suppressed the phosphorylation of PDK, Akt, ERK1/2, eNOS, and GSK3 $\alpha/\beta$, as well as increased the expression of p21 and the phosphorylation of ASK-1 and p38 in FGF-treated HUVEC (Fig. 6). Furthermore, at a relatively high concentration (7 μmol/L), $\delta$-T3 cleaved procaspase-3 and procaspase-9 to their active forms and decreased Cyclin D1.

Discussion

Angiogenesis, the formation of new blood vessels, plays an important role in many pathological processes, such as the growth and metastasis of solid tumors, diabetic retinopathy, rheumatoid arthritis, and psoriasis (18,19). Because 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 antiangiogenic agents are available in foods (28–33). Even if these agents possess moderate antiangiogenic effects, daily consumption of these compounds may help to prevent angiogenic disorders. Our preliminary cell-culture studies, which screened for food-derived antiangiogenic compounds, showed T3 to be a potential angiogenic inhibitor (15–17).
We demonstrated, to our knowledge for the first time, that T3 exerts antiangiogenic effects in vivo. The antiangiogenic effects of T3 would be associated with changes in phosphatidylinositol-3 kinase (PI3K)/PDK/Akt signaling as well as apoptosis induction in endothelial cells.

One of the problems faced by angiogenesis researchers has been the difficulty in finding suitable methods for assessing the in vivo effects of angiogenic regulators (34). We chose 2 common and reliable methods for evaluating the antiangiogenic effects of T3 in the in vivo study: the DAS and CAM assays. The advantages of the DAS assay were that it is technically simple and also provides a natural environment in which to study blood vessels (23). The CAM assay is widely used in vivo to study angiogenesis (24). In the DAS assay, neovascularization of mouse subcutaneous tissues, which was triggered by DLD-1 cells, was effectively inhibited by the oral administration of Tocomin 50 (T3-rich oil) (Fig. 2). We found no negative effects in preexisting vessels treated with Tocomin 50. The results clearly suggested the notable in vivo antiangiogenic effects of T3. The calculated effective dose of T3 (150 mg/kg mouse/d) was lower than or similar to the reported dose associated with anticancer activities of T3 in murine experiments (35). We speculated 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. However, there is little possibility for a direct effect of T3 on DLD-1 cells in the DAS assay. CAM assay was therefore performed for further evaluation of in vivo antiangiogenic activity of T3. T3 treatment of CAM resulted in an inhibition of the angiogenic response (Fig. 3), which occurred in the absence of any effects on embryo viability, suggesting very little toxicity from T3 in vivo. Because 4- to 6-d-old chick embryos do not have established mature immune responses (36), the observed antiangiogenesis effects of T3 in CAM were presumably mediated by direct inhibition of endothelial cell function. Considering both the DAS and CAM results, we concluded that T3 exerts antiangiogenic effects in vivo, potentiating the usability of T3 as a therapeutic dietary supplement for preventing angiogenic disorders (e.g., the inhibition of tumor growth and metastasis by disrupting angiogenesis). In one of our animal studies, orally administrated T3 to rats was found to be distributed in their blood stream and various tissues (37). This data would be advantageous for application of T3 in the use for functional and nutraceutical purposes. Because human life would not be possible without angiogenesis (20) and the dietary supplementation of vitamin E (Toc) increases bleeding tendency.

**FIGURE 4** Effects of T3 on FGF-induced HUVEC proliferation. HUVEC were cultured with 0–35 μmol/L T3 in the presence of 10 μg/mL 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 5** Effects of δ-T3 on FGF-induced HUVEC migration and tube formation. In the migration assay (A), HUVEC monolayers were sheared, followed by exposure to a test medium containing 10 μg/mL FGF and 0–7 μmol/L δ-T3. After 36 h, the lengths of wounded cells were measured. Values are mean ± SD, n = 6. Means without a common letter differ, P < 0.05. In the tube formation assay (B), HUVEC were suspended in test medium containing 10 μg/mL FGF and 0–7 μmol/L δ-T3, and placed on the surface of Matrigel. After incubation for 18 h, the tube lengths were quantified. Values are mean ± SD, n = 4. Means without a common letter differ, P < 0.05.
(38), future therapeutic applications of T3 should be developed with minimal toxicities except during times of wound healing, inflammation, ovulation, pregnancy, ischemia, hypertension, or other diseases associated with bleeding.

How T3 mediates its antiangiogenic effects is not fully understood, although its ability to suppress the growth factor-dependent activation of PI3K/PDK/Akt signaling in neoplastic mammary cells (39) 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 (40). In contrast, various growth factors such as FGF are closely involved in neovascularization. FGF 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 (41). Considering the critical role of both PI3K/PDK/Akt signaling and antiapoptosis in growth factor–induced angiogenesis (42), we hypothesized that T3 must modulate angiogenesis through this pathway. To test this hypothesis, we conducted in vitro model studies (FGF-induced proliferation, migration, and tube formation of HUVEC) to evaluate the antiangiogenic effects and mechanisms of T3 action. We demonstrated that T3 reduced FGF-induced proliferation, migration, and tube formation in HUVEC (Figs. 4 and 5), and that these effects were related to a decrease in the intracellular protein activity associated with the PI3K/PDK/Akt pathway (Fig. 6). 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 downregulated 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 associated with changes in the PI3K/PDK/Akt signaling and apoptosis induction.

DNA chip analysis supported the inhibitory mechanism of T3 (data not shown). Although the exact intracellular sites of action targeted by T3 were unknown, we postulated that the antiangiogenic effects of T3 occur upstream of the PI3K/PDK/Akt signaling pathway at the level of the growth factor receptor (e.g., suppression of FGF receptor tyrosine phosphorylation by T3). This possibility is still suggestive, so further studies are needed to clarify the mechanism.

In our study, T3 displays greater antiangiogenic 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 (43). For this reason, the greater antiangiogenic effect of T3 may be due in part to their effective incorporation into endothelial cells.

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

**Literature Cited**


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**FIGURE 6** Western blot analysis of the intracellular proteins associated with the PI3K/PDK/Akt pathway and apoptosis. HUVEC were treated with T3 under 2 different conditions; (A) 24 h incubation with 10 μmol/L FGF and 0–7 μmol/L δ-T3, or (B) 10 min stimulation with 10 μmol/L FGF after preincubation with 0–7 μmol/L δ-T3 for 8 h. Each Western blot is a representative example of data from 3 replicate experiments.


