Communication

Anti-angiogenic Activity of Tocotrienol

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The anti-angiogenic property of vitamin E compounds, with particular emphasis on tocotrienol, has been investigated in vitro. Tocotrienol, but not tocopherol, inhibited both the proliferation and tube formation of bovine aortic endothelial cells, with δ-tocotrienol appearing the highest activity. Also, δ-tocotrienol reduced the vascular endothelial growth factor-stimulated tube formation by human umbilical vein endothelial cells. Our findings suggest that tocotrienol has potential use as a therapeutic dietary supplement for minimizing tumor angiogenesis.

Key words: angiogenesis inhibitor; cancer; tocopherol; tocotrienol; vitamin E

Vitamin E occurs in nature as at least eight different isoforms that include α-, β-, γ-, and δ-isomers of both tocopherol and tocotrienol (Fig. 1). Humans and animals are unable to synthesize vitamin E and therefore must obtain the isomers from plant sources. Tocopherol is abundantly present in staple foods such as nuts and common vegetable oils, whereas tocotrienol is only a minor constituent of plants with high levels occurring in palm oil, cereal grains, and rice bran.

A major physiological activity of vitamin E is its well-defined antioxidant action and protective effect against lipid peroxidation in biological membranes, with α-tocopherol having the highest biological activity among the vitamin E isoforms. However, a recent study suggested that tocotrienol may be a better antioxidant than tocopherol as it was found to inhibit 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity and thereby reduce cholesterol synthesis. Two other studies reported tocotrienol also had potential anti-thrombotic and anti-tumor effects. Taken together, these findings indicate tocotrienol may be an effective agent in the prevention and/or treatment of cardiovascular disease and cancer.

Anti-angiogenic therapy has recently become established as a strategy for cancer prevention. As oxidative stress is an important factor in angiogenesis, it is possible that established antioxidants such as vitamin E may minimize the formation of new blood vessels from existing vascular beds. This process normally involves a series of steps including endothelial cell activation and breakdown of the basement membrane, followed by migration, proliferation, and tube formation of the endothelial cells. The purpose of this study was to determine the effects of vitamin E, especially tocotrienol, on the in vitro angiogenesis by measuring endothelial cell proliferation and tube formation.

Bovine aortic endothelial cells (BAEC) were obtained from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). Cells were cultured on 60-mm collagen-coated culture dishes in 5 ml of minimum essential medium eagle (MEM; Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% CO2. The cells were used after 8 to 12 doublings of the population. Stock solutions of vitamin E homologues (Fuji Chemical Industry Co., Ltd., Toyama, Japan) dissolved in ethanol were prepared. Immediately before use, the stock solutions were diluted in culture medium to give a final ethanol concentration of 0.1% (v/v). Control cul-

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Abbreviations: BAEC, bovine aortic endothelial cells; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cells; MEM, minimum essential medium eagle; VEGF, vascular endothelial growth factor
tures received vehicle alone (ethanol) in every experiment.

Proliferation of BAEC was assessed by the WST-1 method. Briefly, the cells were seeded onto 96-well collagen coated culture plates at a density of $2 \times 10^5$ cells/well in 100 μl of MEM containing 10% FBS. After incubation for 24 h at 37°C, the cells were placed in 100 μl of fresh MEM containing 2% FBS with a range of concentrations of either tocopherol or tocotrienol. Twenty-four hours later, 10 μl of WST-1 solution was added to each well in order to evaluate cell proliferation. After 3 h of incubation, cell proliferation was measured using a microplate reader (Model 550, Bio-Rad, Tokyo, Japan) at a wavelength of 450 nm and a reference wavelength of 655 nm. Tube formation was assessed using a three-dimensional culture method in which BAEC (1 × 10^5 cells/well) were pre-incubated for 24 h on 12-well collagen coated culture plates in 1.5 ml MEM containing 10% FBS. After removal of the medium, the cells were overlaid with 0.5 ml of collagen gel solution consisting of 8 volumes of Vitrogen collagen, 1 volume of ten-times concentrated MEM and 1 volume of 0.1 M NaOH. A 1.5-ml portion of fresh MEM containing 2% FBS and various concentrations of vitamin E isomers were added to each well followed by incubation for 72 h. The cells were then examined for morphological changes and photographed with the length of tube formation measured with the Adobe Photoshop version 5.5 (Adobe Systems Inc., USA).

In this study, before the evaluation of anti-angiogenic activity, the effects of tocotrienol and tocopherol on BAEC proliferation was tested first. Tocotrienol in the low micromolar range inhibited all BAEC proliferation that was assessed by WST-1 (Fig. 2), but the inhibitory potency of each tocotrienol isomer varied markedly as the following order; $\delta$- > β- > γ- > α-tocotrienol. On the other hand, tocopherol did not affect BAEC proliferation even though increasing their concentration at 100 μM (data not shown). These results suggested that tocotrienol may be a more bioactive compound than tocopherol, and that tocotrienol has a potential for acting as an angiogenesis inhibitor. Such quite different effects of tocotrienol and tocopherol on cell proliferation is consistent with previous reports using mouse mammary epithelial cells and human breast cancer cells. According to the inhibitory effect and mechanism of vitamin E on cell proliferation, further studies would be needed. From these results, vitamin E in the concentration range of 1–30 μM for tocotrienol and of 1–100 μM for tocopherol was chosen for the next tube formation assay to evaluate their anti-angiogenic properties.

Potting tocotrienol (1–30 μM) through the tube formation assay, we confirmed that all the isomers significantly reduced the width and the length of endothelial tubes of BAEC (Fig. 3). The ranked order of this inhibitory effect was $\delta$- > β- > γ- > α-tocotrienol. It is important to note that the inhibition of BAEC tube formation by tocotrienol occurred at a lower concentration than that required for inhibiting the cell growth. For example, 15 μM α-tocotrienol resulted in an approximately 60% inhibition of tube formation (Fig. 3), but did not inhibit cell growth (Fig. 2). In contrast with the findings with tocotrienol, α-, β-, and γ-tocopherol failed to inhibit tube formation of BAEC at physiological concentrations of below 100 μM (data not shown). However, $\delta$-tocopherol at a concentration of 100 μM was associated with minor suppression of BAEC tube formation. These findings (Figs. 2, 3) indicated that the anti-angiogenic effect of tocotrienol is higher than that of tocopherol. Structurally, tocopherol and tocotrienol are distinguished by their side chains and it has been demonstrated that the unsaturated side chain of tocotrienol allows it to pass through cell membranes more efficiently and at a faster rate than the saturated side-chain of tocopherols. It is possible therefore that the greater anti-angiogenic effect of tocotrienols may be due, in part, to their effective incorporation into BAEC.

Since, in this study, $\delta$-tocotrienol would be a most potent anti-angiogenic compound among vitamin E, we then investigated the effects of the compound on the vascular endothelial growth factor (VEGF)-stimulated tube formation of human umbilical vein endothelial cells (HUVEC), by using an Angiogenesis kit (Kurabo Industries, Ltd., Osaka, Japan). The assay procedure was done according to the manufacturer’s instructions. When HUVEC co-cultivated with fibroblasts were cultured in the presence of 10 ng/ml of VEGF for 11 days, the increases of the tube-like structure was confirmed (Fig. 4). Simultane-
Effects of Tocotrienol on Tube Formation of BAEC.

Cells were cultured between collagen gel layers in the absence (control) or presence of $\alpha$-($\square$), $\beta$-($\Delta$), $\gamma$-($\triangledown$) or $\delta$-tocotrienol ($\diamond$) at various concentrations. After 72 h, morphological changes were photographed (A), and the length of tube-structured cells was determined (B). Data expressed as mean ± SD; n = 3. *Cells were died, and therefore the tube length could not be measured. T3, tocotrienol.

Currently there is considerable work being undertaken to screen potentially anti-angiogenic compounds. The dietary constituents curcumin, flavonoids, selenium, N-acetylcysteine, vitamin D3 and several fatty acids including eicosapentaenoic acid have all been shown to inhibit angiogenesis in vitro and/or in vivo. As shown in this study, tocotrienol may represent a member of a new class of dietary-derived anti-angiogenic compounds. Angiogenic inhibitors derived from natural products...
Fig. 4. Effects of δ-Tocotrienol on the VEGF-Stimulated Tube Formation of HUVEC.

HUVEC co-cultured with fibroblasts were incubated in medium containing VEGF (10 ng/ml) with several concentrations of δ-tocotrienol. After 11 days, the cells were made visible by CD31 antibody and photographed (A), and the length of the tube was measured (B). Data expressed as mean ± SD; n = 4. *Cells were died, and therefore the tube length could not be measured. T3, tocotrienol.

have an advantage in that they are non-toxic at physiological doses, can be given orally, and can be easily obtained or manufactured.

In conclusion, tocotrienol significantly inhibited the proliferation and tube formation of BAEC as well as VEGF-induced tube formation by HUVEC. Among the vitamin E group, only tocotrienol had a significant anti-angiogenic effect.

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1627


