

## Anti-angiogenic Potential of Tocotrienol *in vitro*

T. Miyazawa<sup>1\*</sup>, H. Inokuchi<sup>1</sup>, H. Hirokane<sup>1</sup>, T. Tsuzuki<sup>1</sup>, K. Nakagawa<sup>1</sup>, and M. Igarashi<sup>2</sup>

<sup>1</sup>Laboratory of Food and Biodynamic Chemistry, Graduate School of Life Science and Agriculture, Tohoku University, Sendai 981-8555, Japan; fax: 81-22-717-8905; E-mail: miyazawa@biochem.tohoku.ac.jp

<sup>2</sup>Sugiyama Human Nutrition Research Center, Sugiyama Jogakuen University, Nagoya 464-8662, Japan

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**Abstract**—Modulation of angiogenesis is now a recognized strategy for the prevention of various angiogenesis-mediated disorders. We investigated, using well-characterized *in vitro* systems, the anti-angiogenic property of vitamin E compounds, with particular emphasis on tocotrienol, a natural analog of tocopherol. Tocotrienol, but not tocopherol, inhibited the proliferation of bovine aortic endothelial cells in dose dependent manner at half-maximal concentrations in the low micromolar range. Tocotrienol also significantly inhibited the formation of networks of elongated endothelial cells within 3D collagen gels. From these results, we suggest that tocotrienol is a potential candidate for the development of useful therapeutic agents or preventive food factors for tumor angiogenesis.

**Key words:** angiogenesis, tocotrienol, tocopherol

The physiological activity of vitamin E has been recognized to be associated with its well-defined antioxidant property, especially against lipid peroxidation in biological membranes [1, 2]. Among the vitamin E group,  $\alpha$ -tocopherol is considered to be the most active form *in vivo* [3]. However, a recent study suggested that tocotrienol may be a better antioxidant than tocopherol [1]. Moreover, tocotrienol has been reported to have an anti-tumor effect [4], indicating that tocotrienol may serve as an effective agent in the prevention and/or treatment of cancer.

On the other hand, focusing on cancer prevention, anti-angiogenic therapy is now a recognized new strategy [5]. Since oxidative stress has been implicated in angiogenesis [6], we guess that vitamin E may prevent angiogenesis. Angiogenesis, the process of forming new blood vessels from an existing vascular bed, normally involves a series of steps, which include endothelial cell activation, breakdown of the basement membrane, migration, proliferation, and tube formation of the endothelial cell [7]. Thus, the purpose of this study was to obtain direct evidence on the effects of vitamin E, especially for tocotrienol, on proliferation, migration, and tube formation of bovine aortic endothelial cells (BAEC), the key steps of angiogenesis.

## MATERIALS AND METHODS

**Chemicals.**  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -tocotrienols were kindly supplied by Fuji Chemical Industry Co. Ltd. (Japan), while  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -tocopherols were donated by Eisai Co. (Japan). Bovine aortic endothelial cells (BAEC) were purchased from Dainippon Pharmaceutical Co. Ltd. (Japan). Minimum essential medium Eagle (MEM) was obtained from Sigma (USA). Vitrogen collagen was from Cohesion (USA). Collagen-coated culture dish and plates were procured from Asahi Techno Glass Co. Ltd. (Japan). All the other reagents used were of analytical grade.

**Cell culture.** BAEC were cultured on 60 mm collagen-coated culture dishes in 5 ml MEM containing 10% (v/v) fetal bovine serum (FBS), 100 units/ml penicillin G, and 100  $\mu$ g/ml streptomycin. BAEC were used between 8 and 12 population doublings. Stock solutions of vitamin E homologs were prepared in ethanol. Immediately before use, the stock solutions were diluted in culture medium to give a final ethanol concentration of 0.1% (v/v). Control cultures received vehicle alone (ethanol) in every experiment.

**Proliferation assay.** BAEC were seeded at densities of  $2 \cdot 10^3$  cells/well in 100  $\mu$ l MEM containing 10% FBS on 96-well collagen-coated culture plates. After incubation at 37°C in a 5% CO<sub>2</sub> incubator for 24 h, the cells were

\* To whom correspondence should be addressed.

replaced in 100  $\mu$ l fresh MEM containing 2% FBS and various concentrations of tocotrienol or tocopherol. Twenty-four hours later, 10  $\mu$ l of WST-1 (benzene [3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolyl]-1,3-disulfonate) solution was added to each well for evaluating cell proliferation [8]. After 3 h incubation, the plates were measured by a microplate reader (Model 550, Bio Rad, Japan) with a wavelength of 450 nm and a reference wavelength of 655 nm.

**Wounding migration assay.** Wounding migration assay was performed as follows [9]; BAEC were grown to confluence in 12-well collagen-coated culture plates. Then the plates were incubated in serum free MEM to avoid the effect of cell growth (to inhibit the cell proliferation to observe the effects of migration rather than cell proliferation on wound closure). After 24 h, the endothelial monolayers were wounded using a pipette tip. The media and dislodged cells were aspirated, and the plates were washed three times with phosphate buffered saline. The wounded cells were incubated in the test medium (MEM + various concentrations of vitamin E). After 24 h incubation, each well was observed by optical microscopy, and the cellular migration was determined by measuring the widths of the wounds.

**Tube formation assay.** Tube formation was assessed using the three-dimensional culture method [10]. BAEC ( $1 \cdot 10^5$  cells/well) were preincubated for 24 h in a 1.5 ml MEM containing 10% FBS on 12-well collagen-coated culture plates. After removal of the medium, 0.5 ml of collagen gel solution (consisting of a mixture of eight volumes of Vitrogen collagen, one volume of 10 times concentrated MEM, and one volume of 0.1 M NaOH) was overlaid. Fresh MEM (100  $\mu$ l) containing 2% FBS and various concentrations of sample were added to each well and incubated. After 72 h, cells were examined for morphological changes and photographed. The lengths of the tubes were measured with Adobe Photoshop version 5.5 (Adobe Systems Incorporated, USA).

## RESULTS

**Inhibitory effect of tocotrienols on BAEC proliferation.** Among the tocotrienol group, we tested  $\alpha$ -tocotrienol first.  $\alpha$ -Tocotrienol at relatively low concentrations (1–30  $\mu$ M) had no effect on the viability of BAEC, which was assessed by WST-1. But a sudden decrease in the viable cell numbers was confirmed when 30–45  $\mu$ M of  $\alpha$ -tocotrienol was used to treat BAEC. The other tocotrienol isomers ( $\beta$ ,  $\gamma$ , and  $\delta$ ) all inhibited the cell proliferation in a manner similar to  $\alpha$ -tocotrienol, but the inhibitory potency of each tocotrienol isomer varied markedly. The median inhibition concentration for  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -tocotrienol was calculated as 38.0, 6.3, 11.2, and 5.1  $\mu$ M, respectively. Since tocotrienol effectively inhibited the growth of

endothelial cells, we next investigated the inhibitory effect of tocotrienol on BAEC migration and tube formation as described below.

**Tocotrienols inhibit tube formation, but not migration.** In the migration assays, tocotrienols at a physiological concentration of below 100  $\mu$ M had virtually no effect on the migration of BAEC, with the exception of high dose (100  $\mu$ M)  $\delta$ -tocotrienol that caused significant suppression of migration. In contrast, when tocotrienol was applied to the tube formation assay, all isomers (1–30  $\mu$ M) significantly reduced the width and the length of endothelial tubes. The rank order of the most effective tocotrienol isomers acting as inhibitors of tube formation was as follows:  $\delta$ - >  $\beta$ - >  $\gamma$ - >  $\alpha$ -tocotrienol. It is important to note that the inhibition of BAEC tube formation by tocotrienol occurs at lower concentrations than the concentrations needed to inhibit the growth of the cells; i.e.,  $\alpha$ -tocotrienol at 15  $\mu$ M showed about 60% inhibition of tube formation, but that concentration of  $\alpha$ -tocotrienol could not inhibit the endothelial cell growth.

**Tocopherol does not inhibit angiogenesis.** In contrast with tocotrienol,  $\alpha$ -,  $\beta$ -, and  $\gamma$ -tocopherol failed to inhibit the proliferation, migration, and tube formation of BAEC, even though 100  $\mu$ M of these substances were used to treat BAEC. An exception,  $\delta$ -tocopherol at 100  $\mu$ M significantly suppressed BAEC proliferation and tube formation.

## DISCUSSION

Since angiogenesis is an essential process for tumor growth, inhibition of the process has a good chance of preventing cancer from becoming metastatic [11]. Foods have a possibility for containing anti-angiogenic factors [12–14]. Even though such factors possess a slight anti-angiogenic effect, these compounds, if taken daily, may prevent cancers from inducing new blood vessel growth. Several studies have reported that dietary exposure to vitamin E has potential health benefit in preventing cancer [15], but the actual mechanisms are not understood. These considerations led us to hypothesize that the inhibition of tumor growth by vitamin E is mediated in part by of its anti-angiogenic activity. Hence, in the present study we tested this hypothesis *in vitro*.

Our study showed that tocotrienol prevented the proliferation and tube formation of BAEC. In contrast, tocopherol presented a weak efficiency. This finding indicates that the anti-angiogenic effect of tocotrienol is higher than that of tocopherol. Structurally, tocopherol and tocotrienol are distinguished by their side chains. It has been reported that tocotrienols, with their unsaturated side chain, allows them to pass through the cellular membrane in a more efficient manner and faster rate compared to the all saturated tocopherols [2]. Hence,

tocotrienols revealed higher anti-angiogenic effect, presumably due to their effective incorporation in BAEC. On the other hand, recent studies have reported that HMG-CoA reductase inhibitors (i.e., statins) interfere with angiogenic processes both *in vitro* and *in vivo* [16, 17]. Since tocotrienol, but not tocopherol, is known to inhibit HMG-CoA reductase [18], it is possible that the inhibitory effect of tocotrienol on angiogenesis may be attributable in part to reduction in the activity of this enzyme.

Oxygen radicals are induced under various pathologic conditions associated with neovascularization. Shono et al. [6] reported that exposure to H<sub>2</sub>O<sub>2</sub> enhances tubular morphogenesis of human microvascular endothelial cells. Thus, oxidative stress would be an angiogenesis inducer that is possibly mediated by the activation of nuclear factor κB and by interleukin-8. Therefore, the anti-angiogenic effect of tocotrienols may be associated with their antioxidant activities. Recently, Tang and Meydani [19] investigated the ability of α-tocopherol to inhibit tube formation *in vitro* using a model in which human microvascular endothelial cells were exposed to a constant physiologically low level of H<sub>2</sub>O<sub>2</sub>. Their results demonstrated clearly that incorporating 40 μM α-tocopherol in the culture media significantly reduced interleukin-8 production and angiogenesis. These experiments did not, however, investigate whether tocotrienol had similar or potentially greater effects.

Today, much work on screening of potentially angiogenic compounds has been done. The dietary constituents—selenium [20], N-acetylcysteine [21], vitamin D3 [22], curcumin [13], flavonoids [12, 14], and several fatty acids (i.e., eicosapentaenoic acid) [23]—have all been shown to inhibit angiogenesis *in vitro* and/or *in vivo*. As shown in our report, tocotrienol would represent a member of a new class of dietary-derived anti-angiogenic compounds. Such angiogenic inhibitors derived from natural products have great advantage; that is, they are proven non-toxic at physiological doses, can be given orally, and can be easily manufactured.

In conclusion, tocotrienol significantly inhibited the proliferation and tube formation of BAEC in a dose-dependent manner. Among the vitamin E group, only tocotrienol exhibited the novel anti-angiogenic effect in an *in vitro* system. However, the inhibitory mechanisms of tocotrienol *in vitro* are suggestive, further studies are needed to gain a full understanding of the effects of tocotrienol on angiogenesis.

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