Title

α-Tocopheryl succinate stabilizes the structure of tumor vessels by inhibiting angiopoietin-2 expression

Authors

Susumu Hama[a]*, Yuriko Okamura[a], Kazuho Kamei[a], Saki Nagao[a], Mari Hayashi[a], Shizuka Maeda[a], Kenji Fukuzawa[b], Kentaro Kogure[c]

[a]Department of Biophysical Chemistry, Kyoto Pharmaceutical University, Yamashina-ku, Kyoto, Japan

[b]Faculty of Pharmacy, Yasuda Women's University, Hiroshima, Japan.

[c]Institute of Biomedical Sciences, Tokushima University Graduate School, Tokushima, Japan.

*Corresponding author: Susumu Hama

Department of Biophysical Chemistry, Kyoto Pharmaceutical University.

5 Nakauchi-cho, Misasagi, Yamashina-ku, Kyoto, 607-8414, Japan.

E-mail: hama@mb.kyoto-phu.ac.jp

TEL: +81-75-595-4664; FAX: +81-75-595-4762

Abstract

α-Tocopheryl succinate (TS) is a tocopherol derivative and has multifaceted anti-cancer effects; TS not only causes cancer cell-specific apoptosis but also inhibits tumor angiogenesis. Although TS has the potential to be used as a well-tolerated anti-angiogenic drug, it is still unclear which step of the angiogenic process is inhibited by TS. Here, we show that TS inhibits the expression of angiopoietin (Ang)-2, which induces destabilization of vascular structure in the initial steps of the angiogenic process. In mouse melanoma cells, TS treatment decreased mRNA and extracellular protein levels of Ang-2; however, the mRNA level of Ang-1, which stabilizes the vascular structure, remained unchanged. Furthermore, aorta ring and Matrigel plug angiogenesis assays indicated that the conditioned medium from TS-treated cells (CM-TS) inhibited neovascularization and blood leakage from the existing blood vessels, respectively. Following immunohistochemical staining of the vessels treated with CM-TS, imaging studies showed that the vascular endothelial cells were highly packed with pericytes. In conclusion, we found that TS inhibits Ang-2 expression and, consequently, stabilizes the vascular structure during the initial step of tumor angiogenesis.

Keywords: Tocopheryl succinate; Angiogenesis; Angiopoietin; Vascular stabilization

1. Introduction

α-Tocopheryl succinate (TS) is a succinate ester of α-tocopherol whose a hydroxyl moiety showing an antioxidative activity on chroman ring is masked (Fig. 1A) [1]. Although TS has no antioxidative effects, TS induces several physiological effects, including the induction of cancer cell-specific apoptosis and inhibition of tumor angiogenesis [2,3]. Furthermore, TS inhibits proteins involved in the efflux of anti-cancer drugs [4], thereby help to overcome chemotherapy resistance. As TS is hydrolyzed to α-tocopherol and succinic acid by esterase *in vivo*, TS is considered safer than other anti-cancer drugs. Given the multifaceted anti-cancer effects, TS may have a potential clinical use [1].

Although extensively studied, [5], little is known about the mechanism by which TS induces anti-angiogenic effects. According to a proposed mechanism, TS inhibits the expression of vascular endothelial growth factor (VEGF) [6]; the decrease in VEGF level leads to the inhibition of growth, migration, and extension of endothelial cells, followed by the inhibition of angiogenesis [7,8]. The process of angiogenesis is divided into three steps [7,8]. The first step is the dissociation of pericytes from the endothelial cells, which is mediated by angiopoietin-2 (Ang-2). The second step is the growth, migration, and extension of endothelial cells, which is mediated by VEGF and fibroblast growth factor (FGF). The third step involves angiopoietin-1 (Ang-1)-mediated vessel maturation, where the endothelial cells are covered with pericytes. However, it is not clear whether TS inhibits only the

second step of the angiogenic process. Ang-1 and Ang-2 belong to the angiopoietin superfamily of proteins, which are secreted at the angiogenic site, and are vascular remodeling molecules that have the opposite angiogenic effects [9]. These proteins bind to Tie2 receptor on the vascular endothelial cells. However, Ang-2 inactivates Tie2 signaling, resulting in the promotion of angiogenesis via the destabilization of the vascular structure. In contrast, Ang-1 activates Tie2 signaling, resulting in the inhibition of angiogenesis via stabilization of the vascular structure [9]. In tumor tissues, the level of Ang-2 is higher than that of Ang-1 [10], leading to increased vascular permeability, followed by the formation of the tumor microenvironment, which is involved in tumor malignancy and the chemotherapy resistance [11]. Although Ang-2 is an attractive targeting molecule for cancer therapy, there are few Ang-2 inhibitors that can directly act on cancer cells expressing Ang-2.

In this study, we investigated the effect of TS on Ang2 expression in melanoma cells and tried to identify the mechanism by which TS exerts anti-angiogenic effects.

2. Materials and methods

2.1. Cell culture and treatment with tocopherols

The mouse melanoma cell line B16-F1 and the human melanoma cell line A375 were obtained from DS Pharma Biomedical Co., Ltd. (Osaka, Japan). B16-F1 and A375 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 37°C, 21% O_2, and 5% CO_2 under humidified conditions. α-tocopherol (α-T) and α-tocopheryl succinate (TS) were purchased from Sigma-Aldrich Co. (MO, USA). γ-Tocotrienol (γ-T3) and δ-Tocotrienol (δ-T3) were purchased from ChomaDex Inc. (CA, USA) and Santa Cruz Biotechnology, Inc., respectively. For treating cells, these tocopherols were dissolved in ethanol, and diluted in DMEM without FBS to a desired concentration; 0.1% (v/v) ethanol in DMEM without FBS was used as a control.

2.2. Cell viability assay

B16-F1 cells were seeded at a density of 3×10^3 cells per well in Corning® CellBIND® 96-well plate (NY, USA) and were cultured for 24 h. After the removal of the culture medium, cells were washed with phosphate buffer saline (PBS) (-), cells were treated with TS for 48 h. Cell viability was determined using the Premix WST-1 Cell Proliferation Assay System (TAKARA BIO INC., Shiga, Japan) as described previously [12].

2.3. Real-time PCR

B16-F1 cells or A375 cells were seeded in a 60 mm dish at a density of 1×10^5 cells and were cultured for 24 h. The culture medium was then removed, and cells were washed with PBS (-); thereafter, the cells were treated with TS, α-T, γ-T, and δ-T at the concentration of 20 μM. After incubation for 24 or 48 h, the total RNA in these cells was isolated using the RNeasy Mini Kit (Qiagen, CA, USA), and cDNA was synthesized using oligo dT primers (Life Technologies Co., CA, USA) and the PrimeScript Reverse Transcriptase kit (Takara Bio Inc., Shiga, Japan). Real-time PCR was then performed using the ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and SYBR-Green with the following specific primers: mouse VEGF-A (forward: 5'-ATCTTCAAGCCGTCCTGTGT-3', reverse: 5'-GCATTCACATCTGCTGTGCT-3'), mouse Ang-1 (forward: 5'-AGGCTTTCCAGAATCTGTTGAAGA-3', reverse: 5'-TTGGGCAGTGATGTATTGTTAGC-3'), mouse Ang-2 (forward: 5'-CCGCTACGTGCTTAAGATCC-3', reverse: 5'-ATTGTCCGAATCCTTTGTGC-3'), mouse GAPDH (forward: 5'-GAGGACCAGGTTGTCTCCTG-3', reverse: 5'-ATGTAGGCCATGAGGTCCAC-3'), human Ang-2 (forward: 5'-TCCGACCAGCAGATTTCTAAACAT-3', reverse: 5'-AGTGCACTGGGCTTAAGTCTTTG-3'), human β-actin (forward: 5'-CACTCTTCCAGCCTTCCTTCC-3', reverse: 5'-CGTACAGGTCTTTGCGGATGTC-3') as reported previously [13]. Mouse GAPDH and human β-actin mRNA were used as internal controls. Relative mRNA expression was determined using the

$2^{-\Delta\Delta CT}$ method.

2.4. Dot blotting

1×10^5 B16-F1 cells were seeded in a 60 mm dish and cultured for 24 h. The culture medium was then removed, and cells were washed with PBS (-); thereafter, cells were treated with 20 μM TS. After 24 h, the medium was removed and cells were incubated in fresh DMEM without FBS for 48 h. The supernatant was collected and concentrated using Amicon® Ultra Centrifugal Filters (3 K) (Merck Millipore Ltd. Carraigtohill, Ireland). Protein concentration was determined using the BCA protein assay kit (Thermo Fisher Scientific, Inc. MA, USA). The same amount of sample that was used to determine the protein concentration was applied on the nitrocellulose membrane. Thereafter, the membrane was incubated with 5% skimmed milk for 1 h. After washing, the membrane was incubated with a rabbit anti-mouse angiopoietin-2 polyclonal antibody (Chemicon International, Inc. CA, USA), and then with HRP-labeled anti-rabbit antibody. The dots were visualized with ECL Prime Western Blotting detection reagent (GE Healthcare), and densitometric analysis was performed using the ImageJ software.

2.5. Aorta ring assay and immunohistochemical imaging

Male balb/c mice were obtained from SHIMIZU Laboratory Supplies Co., Ltd. (Kyoto, Japan).

All animal experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Kyoto Pharmaceutical University.

The aorta ring assay was performed as described previously [14] with minor modification. Briefly, the surrounding fat tissue was cleaned, and the aorta was sliced into 5 mm rings. The aorta rings were cultured on growth factor reduced Matrigel (BD, NJ, USA) with conditioned medium, which was prepared by concentrating the culture medium from cells treated with TS (CM-TS) or ethanol (CM-Cont). After eight days, the development of new vessels from the existing vessels was observed under the microscope; we counted the number of these new vessels.

For immunohistochemistry and imaging studies, blood vessel sections (14 μm) were prepared using the LEICA CM 1100 cryostat (Leica, Wetzlar, Germany). The section was fixed with ice-cold methanol for 20 min at −20°C, followed by washing with PBS (-) containing 1% triton X-100 (PBS-T). Then, the section was permeabilized with PBS (-) containing 0.5% trypsin, followed by blocking with 5% skimmed milk for 10 min. After washing, the section was incubated overnight with an anti-mouse CD140b antibody (Thermo Fisher Scientific, Inc.) labeled with Alexa 546 and anti-mouse CD31 antibody. To visualize the anti-mouse CD31 antibody bound to the vessels, the section was treated with Alexa 488-labeled anti-rabbit antibody for 1 h. After washing, the section was observed

under confocal laser scanning microscopy (LSM510META, Carl Zeiss Co. Ltd., Jena, Germany).

2.6. Matrigel plug angiogenesis assay

Matrigel plug angiogenesis assay was performed as described previously [15] with minor modifications. Briefly, Matrigel was mixed with the conditioned medium at a ratio of 1:1, and 500 μL of the mixture was subcutaneously administered to the mice. After 12 days, 1% evans blue solution was injected to visualize the blood vessels, followed by the perfusion with 2 mM EDTA/PBS (-). The Matrigel was observed under the microscope.

2.7. Statistical analysis

Statistical significance was determined using one-way ANOVA, followed by Tukey's honestly significant difference test. P values < 0.05 were considered significant.

3. Results and discussion

3.1. Effect of TS on Ang-1 and Ang-2 expression

It is well known that TS induces apoptosis in cancer cells [1,2]. Firstly, to determine the concentration at which TS can be used without causing cytotoxicity, we examined the effect of TS on the viability of B16-F1 mouse melanoma cells. When the cells were treated with >50 μM TS, cell viability was reduced by >30% (Fig. 1B). In contrast, the viability of cells treated within 20 μM TS was similar to the control (Fig. 1B). Furthermore, VEGF-A mRNA expression was reduced by 50% in cells treated with 20 μM TS (Fig. 1C). These results suggest that TS show the anti-angiogenic effect at a concentration of 20 μM without causing cytotoxicity. Therefore, to avoid cell apoptosis-dependent changes in Ang-1 and Ang-2 expression, we used 20 μM TS. Next, we examined the effects of TS on the Ang-1 and Ang-2 mRNA expression. As shown in Fig. 1D, Ang-2 mRNA level significantly decreased in TS-treated cells compared with control; in contrast, Ang-1 mRNA level was similar to control. Consistent with the decrease in Ang-2 mRNA level, Ang-2 protein level in CM-TS was reduced by 50% compared with CM-Cont (Fig. 1E). These results suggest that TS can inhibit the mRNA expression of only Ang-2. Furthermore, we investigated the inhibitory effect of TS on Ang-2 expression in A375 human melanoma cells. As shown in Fig. 1F, compared with control, Ang-2 mRNA level decreased in A375 cells treated with TS. These results suggest that TS inhibits Ang-2 mRNA expression in both mouse and human melanoma cells.

3.2. Structure-activity correlation in the inhibition of Ang-2 expression

In vivo, TS is hydrolyzed to α-T, which has various physiological effects based on scavenging reactive oxygen species (ROS) (Fig. 1A) [16]. It is known that γ-T3 and δ-T3 (Fig. 2A), which are natural analogs of vitamin E, have anti-angiogenic effects [17,18]. To investigate the structure-activity correlation in the inhibition of Ang-2 expression, the expression level of Ang-2 was examined in B16-F1 cells treated with α-T, γ-T3, or δ-T3 at concentrations that do not cause cytotoxicity. As shown in Fig. 2B, treatment with these analogues, which have an anti-oxidative effect, did not change the mRNA level of Ang-2. These results suggest that the succinic acid ester in TS, and not the hydroxyl moiety scavenging ROS, plays a pivotal role in the inhibition of Ang-2 expression. Thus, inhibition of Ang-2 is specific to TS among the tocopherol analogs investigated in this study, suggesting that the Ang-2 inhibition by TS does not involve an anti-oxidant effect.

3.3. Effect of TS on angiogenesis

To evaluate the effect of TS on intratumor-like angiogenesis mediated by Ang-2, aorta ring assay was performed. When the aorta was treated with the conditioned medium from cells treated with ethanol (CM-Cont), we observed the development of many new vessels from the aorta (Fig. 3A), which was inhibited when the aorta was treated with the conditioned medium from cells treated with 20 μM TS (CM-TS) (Fig. 3A). As shown in Fig. 3B, the number of new vessels generated from the

aorta was significantly reduced in the presence of CM-TS compared with CM-Cont. Considering that the Ang-2 protein level in CM-TS was reduced by 50% compared with CM-Cont (Fig. 1E), Ang-2-mediated angiogenesis via the destabilization of vascular structure might be inhibited by CM-TS.

3.4. Stabilization of vascular structure by TS

To further determine whether TS stabilizes the vascular structure via inhibition of Ang-2, we investigated blood leakage using the Matrigel plug angiogenesis assay. The skin from mice injected with Matrigel and CM-Cont, shows the presence of blood vessels stained with evans blue and blood leakage. (Fig. 4A). These results suggest that CM-Cont does not interfere with Ang-2-mediated angiogenesis in immature blood vessels. In contrast, such angiogenesis was not observed in the skin of mice injected with Matrigel and CM-TS. (Fig. 4A), suggesting that the reduced Ang-2 level in the presence of CM-TS failed to mediate vessel destabilization. It is known that blood vessels are stabilized by the pericytes covering the surrounding endothelial cells [19]. Therefore, pericytes and endothelial cells on the section from the conditioned media-treated blood vessels were stained with anti-CD140b antibody and anti-CD31 antibody, respectively. As shown in Fig. 4B, CD140b-positive region per CD-31-positive region was small in blood vessels treated with CM-Cont, and CD140b rarely colocalized with CD31. This result suggested that the pericytes were dissociated from the endothelial cells, leading to destabilization of the vascular structure. When blood vessels

were treated with CM-TS, the CD140b-positive region was large. Additionally, a high intensity yellow fluorescent signal indicating the colocalization of CD140b with CD31 was observed (Fig. 4B). This result suggested that the vascular structure was stabilized by pericytes on the endothelial cells.

In summary, we found that TS, a redox-silent tocopherol analog, inhibited the expression of Ang-2 in cancer cells, while other tocopherol analogs had no effect on Ang-2 expression. The reduced Ang-2 protein level in the conditioned media from TS-treated cancer cells resulted in the stabilization of vascular structure with pericytes-lined endothelial cells. Therefore, TS has the potential to be developed as an angiogenic inhibitor due to its ability to inhibit Ang-2 expression.

Acknowledgments

We would like to thank Editage (www.editage.com) for English language editing.

Author contributions

Concept and design: S.H., K.F., and K.K. Development of methodology: S.H., Y.O., and K.K. Data acquisition: S.H., Y.O., K.K., S.N., M.H., and S.M. Data analysis and interpretation: S.H., K.F., and K.K. Writing, review, and/or revision of the manuscript: S.H., K.F., and K.K.

Conflicts of interest

The authors declare no conflict of interest.

Funding support

This work was supported in part by JSPS KAKENHI (Grant Number 18H03540), and Kyoto Pharmaceutical University Fund for the Promotion of Scientific Research.

References

[1] S. Hama, K. Kogure, Nanoparticles consisting of tocopheryl succinate are a novel drug-delivery system with multifaceted antitumor activity, Biol. Pharm. Bull. 37 (2014) 196–200.

[2] K. Kogure, S. Hama, S. Manabe, et al., High cytotoxicity of α-tocopheryl hemisuccinate to cancer cells is due to failure of their antioxidative defense systems. Cancer Lett. 186 (2002) 151–156.

[3] L.F. Dong, E. Swettenham, J. Eliasson, et al., Vitamin E analogues inhibit angiogenesis by selective induction of apoptosis in proliferating endothelial cells: the role of oxidative stress, Cancer Res. 67 (2007) 11906–11913.

[4] X. Zhang, X. Peng, W. Yu, et al., Alpha-tocopheryl succinate enhances doxorubicin-induced apoptosis in human gastric cancer cells via promotion of doxorubicin influx and suppression of doxorubicin efflux. Cancer Lett. 307 (2011) 174–181. https://doi.org/10.1016/j.canlet.2011.04.001.

[5] Y. Zhao, J. Neuzil, K. Wu, Vitamin E analogues as mitochondria-targeting compounds: from the bench to the bedside?, Mol. Nutr. Food Res. 53 (2009) 129–139. https://doi: 10.1002/mnfr.200800045.

[6] M.P. Malafa, L.T. Neitzel, Vitamin E succinate promotes breast cancer tumor dormancy. J. Surg. Res. 93 (2000) 163–170.

[7] C. Viallard C, B. Larrivée B, Tumor angiogenesis and vascular normalization: alternative therapeutic targets, Angiogenesis 20 (2017) 409–426. https://doi: 10.1007/s10456-017-9562-9.

[8] Y. Zhao, A.A. Adjei, Targeting Angiogenesis in Cancer Therapy: Moving Beyond Vascular

Endothelial Growth Factor, Oncologist 20 (2015) 660–673. https://doi: 10.1634/theoncologist.2014–0465.

[9] R.G. Akwii, M.S. Sajib, F.T. Zahra, et al., Role of Angiopoietin-2 in Vascular Physiology and Pathophysiology, Cells 8 (2019) pii: E471. https://doi: 10.3390/cells8050471.

[10] M. Saito, J. Watanabe, T. Fujisawa, et al., Angiopoietin-1, 2 and Tie2 expressions in endometrial adenocarcinoma--the Ang2 dominant balance up-regulates tumor angiogenesis in the presence of VEGF, Eur. J. Gynaecol. Oncol. 27 (2006) 129–134.

[11] J.S. Park, I.K. Kim, S. Han, et al., Koh. Normalization of Tumor Vessels by Tie2 Activation and Ang2 Inhibition Enhances Drug Delivery and Produces a Favorable Tumor Microenvironment, Cancer Cell. 30 (2016) 953–967. https://doi: 10.1016/j.ccell.2016.10.018.

[12] S. Hama, S. Utsumi, Y. Fukuda, et al., Development of a novel drug delivery system consisting of an antitumor agent tocopheryl succinate, J. Control. Release. 161 (2012) 843–851. https://doi: 10.1016/j.jconrel.2012.05.031. Epub 2012 May 23.

[13] I. Nakamura, S. Hama, S. Itakura, et al., Lipocalin2 as a plasma marker for tumors with hypoxic regions, Sci. Rep. 4 (2014) 7235. https://doi: 10.1038/srep07235.

[14] K. Bellacen, E.C. Lewis, Aortic ring assay, J. Vis. Exp. 33 (2009) pii: 1564. https://doi: 10.3791/1564.

[15] J. Michaud-Levesque, M. Demeule, R. Béliveau, In vivo inhibition of angiogenesis by a soluble

form of melanotransferrin, Carcinogenesis 28 (2007) 280–288.

[16] C. Constantinou, A. Papas, A.I. Constantinou, Vitamin E and cancer: An insight into the anticancer activities of vitamin E isomers and analogs, Int. J. Cancer 123 (2008) 739–752. https://doi: 10.1002/ijc.23689.

[17] Y. Li, W.G. Sun, H.K. Liu, et al., γ-Tocotrienol inhibits angiogenesis of human umbilical vein endothelial cell induced by cancer cell, J. Nutr. Biochem. 22 (2011) 1127–1136. https://doi: 10.1016/j.jnutbio.2010.09.012.

[18] A. Shibata, K. Nakagawa, T. Tsuduki, et al.,α-Tocopherol suppresses antiangiogenic effect of δ-tocotrienol in human umbilical vein endothelial cells, J. Nutr. Biochem. 26 (2015) 345–350. https://doi: 10.1016/j.jnutbio.2014.11.010.

[19] I. Helfrich, D. Schadendorf, Blood vessel maturation, vascular phenotype and angiogenic potential in malignant melanoma: one step forward for overcoming anti-angiogenic drug resistance?, Mol. Oncol. 5 (2011) 137–149. https://doi: 10.1016/j.molonc.2011.01.003.

Figure legends

Fig. 1. Effects of TS on Ang-2 expression. (A) Chemical structures of TS and α-T. (B–E) B16-F1 cells were treated with TS for 48 h; (B) cell viability (TS concentration, 10–100 μM), (C) relative VEGF-A mRNA levels (TS, concentration, 20 μM), (D) relative Ang-1 and Ang-2 mRNA levels (TS, concentration, 20 μM), and (E) relative Ang-2 protein levels in the culture medium (TS, concentration, 20 μM). (F) Relative Ang-2 level in A375 cells treated with 20 μM TS for 24 h. Data are presented as mean ± SD, n = 3, **P* < 0.05, ***P* < 0.01, and ***P < 0.001 (versus control).

Fig. 2. Effects of tocopherol derivatives on Ang-2 mRNA expression in B16-F1 cells. (A) Chemical structures of γ-T3 and δ-T3. (B) Relative Ang-2 levels in the B16-F1 cells treated with 20 μM α-T, γ-T3, and δ-T3 for 48 h. Data are presented as mean ± SD, n = 3.

Fig. 3. Evaluation of angiogenesis by aorta ring assay. The aorta were cultured on growth factor reduced Matrigel with the conditioned medium (CM) for eight days. CM-Cont and CM-TS indicate concentrated culture medium from cells treated with ethanol and 20 μM TS, respectively. (A) Representative images of new blood vessels generated from the aorta. Scale bars: 200 μm. (B) The number of new blood vessels. Data are presented as mean ± SD. n = 3, **P* < 0.05 (versus control).

Fig. 4. Stabilization of vascular structure by CM-TS. (A) Representative images of Matrigel plug

angiogenesis assay at 12 days after treatment with CM-Cont and CM-TS. Scale bars: 1 mm. (B) Immunohistochemistry was performed on sections of blood vessels treated with CM-Cont and CM-TS. Images of CD140b and CD31 are shown in black and white. The red and green signals in the merged images indicate CD140b and CD31, respectively. Scale bars: 100 μm.

Figure 1

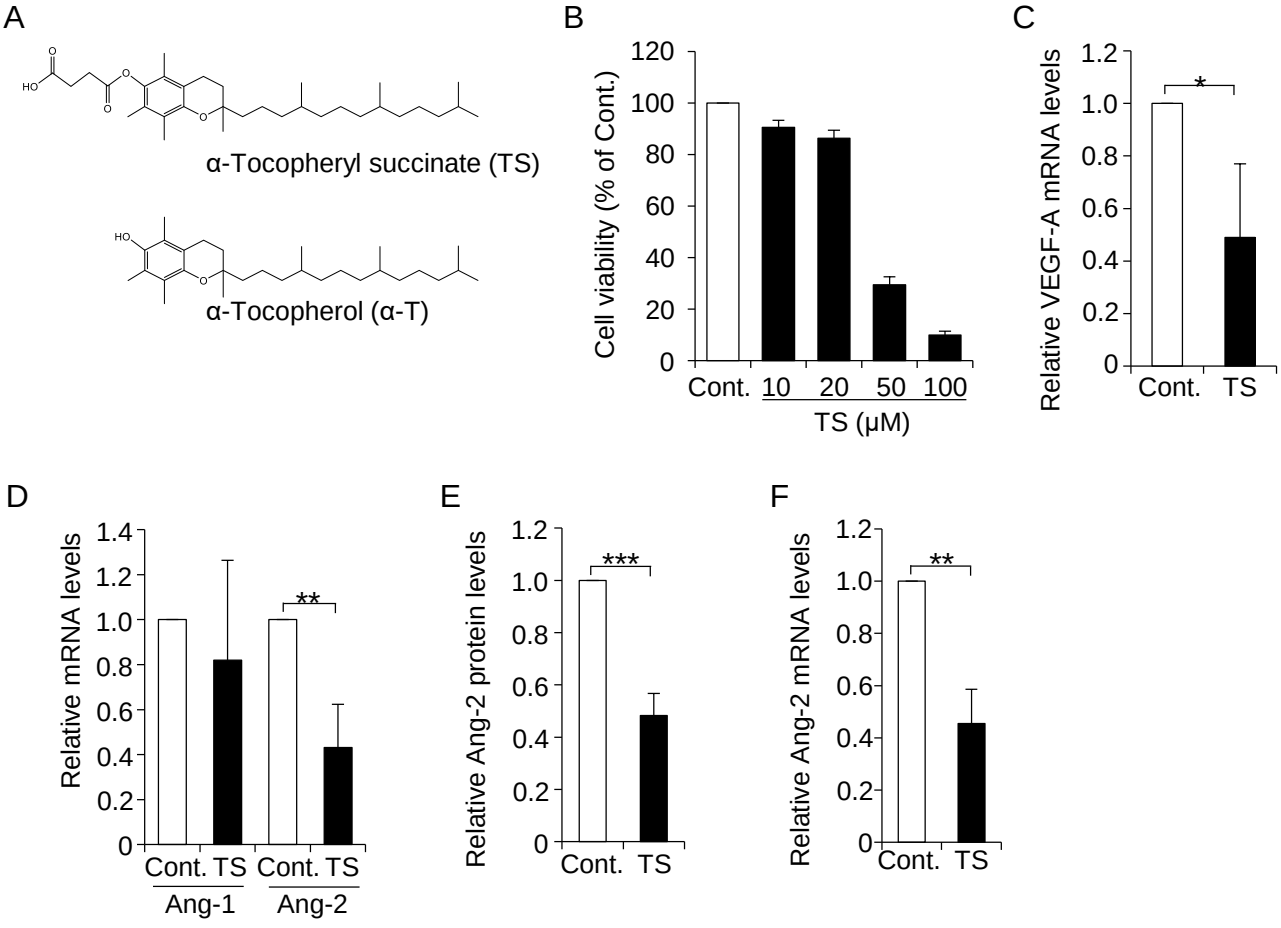
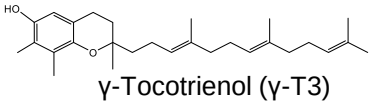
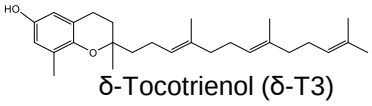

A
α-Tocopheryl succinate (TS)
α-Tocopherol (α-T)
B
Cell viability (% of Cont.)
120
100
80
60
40
20
0
Cont. 10 20 50 100
TS (μM)
C
Relative VEGF-A mRNA levels
1.2
1.0
0.8
0.6
0.4
0.2
0
*
Cont. TS
D
Relative mRNA levels
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
**
Cont. TS Cont. TS
Ang-1 Ang-2
E
Relative Ang-2 protein levels
1.2
1.0
0.8
0.6
0.4
0.2
0
***
Cont. TS
F
Relative Ang-2 mRNA levels
1.2
1.0
0.8
0.6
0.4
0.2
0
**
Cont. TS

Figure 2

A



γ-Tocotrienol (γ-T3)



δ-Tocotrienol (δ-T3)

B

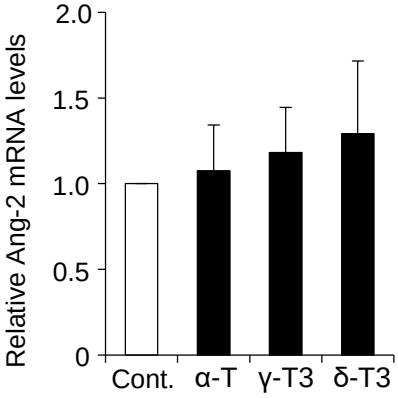
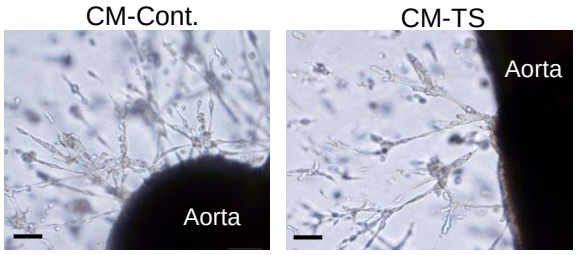


Figure 3

A


CM-Cont.
CM-TS
Aorta
Aorta

B

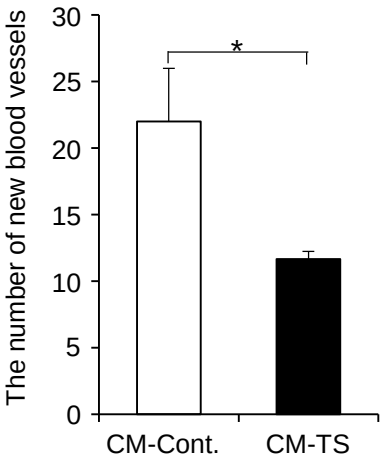

The number of new blood vessels
30
25
20
15
10
5
0
*
CM-Cont.
CM-TS

Figure 4