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γ-Tocotrienol inhibits angiogenesis of human umbilical vein endothelial cell induced by cancer cell

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Abstract

Antiangiogenic therapy mediated by food components is an established strategy for cancer chemoprevention. Growth factors play critical roles in tumor angiogenesis. A conditioned medium containing growth factors from human gastric adenocarcinoma SGC-7901 cell conditioned medium was used as an angiogenic stimulus in this study. The purpose of this study was to evaluate the inhibitory effect and possible mechanism of γ-tocotrienol on tumor angiogenesis. The results showed that γ-tocotrienol (10–40 μmol/L) significantly suppressed proliferation, migration and tube formation of human umbilical vein endothelial cells (HUVECs) induced by SGC-7901 cell conditioned medium in a dose-dependent manner. γ-Tocotrienol (800–1200 μg/egg) also inhibited new blood vessel formation on the growing chick embryo chorioallantoic membrane in a dose-dependent manner. Moreover, the inhibitory effects of γ-tocotrienol on HUVECs were correlated with inducing the apoptosis and arresting cell cycle at the G_0/G_1 phase at a dose of 40 μmol/L γ-tocotrienol. In addition, γ-tocotrienol inhibited angiogenesis in HUVECs by down-regulation of β-catenin, cyclin D1, CD44, phospho-VEGFR-2 and MMP-9. The antiangiogenic effects of γ-tocotrienol on HUVECs may be attributable to regulation of Wnt signaling by decreasing β-catenin expression. Thus, our results suggest that γ-tocotrienol has a potential chemopreventive agent via antiangiogenesis.

Keywords: γ-Tocotrienol; Angiogenesis; HUVEC; Wnt signaling

1. Introduction

Angiogenesis, the formation of new blood vessels from a preexisting vascular bed, is of fundamental importance in several pathological states, such as tumor growth, diabetic retinopathy and rheumatic arthritis [1,2]. Tumor-induced angiogenesis is a pathological condition that results from aberrant development of normal angiogenesis. Angiogenesis is especially important for the development of tumor as the delivery of blood-borne nutrients to the tumor cells, which is essential for their survival [3], and for the spread of metastatic tumor cells [4]. Furthermore, angiogenesis has been reported to have a close correlation with prognosis of solid tumors [5–7]. Due to the knowledge that a solid tumor cannot grow beyond a critical size of 1–2 mm^3 or metastasize without an adequate blood supply [8,9], tumor-associated angiogenesis can be effectively targeted as an anticancer therapeutic strategy [10–13].

Natural vitamin E is a mixture of two classes of compounds, tocopherols and tocotrienols. The differences between tocopherols and tocotrienols are that the former contains a saturated and phytyl tail, whereas the latter contains an unsaturated isoprenoid side chain. Although both tocopherols and tocotrienols have various biological effects, tocotrienols have also been reported to have an antiproliferative activity and to induce apoptosis in many kinds of malignant carcinoma cells, such as breast [14–18], colon [19,20], prostate [21] and hepatocarcinoma [22] cells. In our previous study, γ-tocotrienol induced human gastric adenocarcinoma SGC-7901 cell apoptosis through mitochondria-dependent apoptosis pathway and was associated with down-regulation of the Raf–ERK signaling pathway [23–25]. γ-Tocotrienol also induced human colon carcinoma HT-29 cell apoptosis in a dose-dependent manner [20]. Moreover, during the past decade, some evidence demonstrating the antiangiogenesis activity of tocotrienol has accumulated

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[2,26–30]. These results suggest that tocotrienol has potential use as a therapeutic dietary supplement for minimizing tumor angiogenesis. However, the inhibitory mechanism of tocotrienols on tumor-induced angiogenesis remains unclear. Thus, many approaches are needed to disclose how tocotrienol affects angiogenesis in a biological system. The objective of this study was to determine the effects of γ-tocotrienol on proliferation, migration and tube formation of human umbilical vein endothelial cells (HUVECs), the key steps of angiogenesis and the possible mechanisms of antiangiogenesis of γ-tocotrienol in HUVECs.

2. Materials and methods

2.1. Chemicals and reagents

3-[4,5-Dimethylthiazol-2,5-diphenyl tetrazolium bromide (MTT), α-tocopherol and Suramin sodium salt were purchased from Sigma Chemical (St. Louis, MO, USA). Purified γ-tocotrienol was purchased from Davos (Singapore). Cycle TEST PLUS DNA reagents were purchased from BD Biosciences. Rabbit polyclonal antibody specific to β-actin was from Sigma Chemical. Rabbit polyclonal antibodies specific to MMP (matrix metalloproteinase)-9 (G657), cyclin D1 (SP4), CD44 (BA0321), β-catenin (sc-7199) and phospho-VEGFR-2 (Tyr1054) were bought from Cell Signaling (Danvers, MA, USA), NeoMarkers (Theremo Scientific, Fremont, CA, USA), Boster (Harbin, China), Santa Cruz (Santa Cruz, CA, USA) and Millipore (Billerica, MA, USA), respectively. A nuclear and cytoplasmic protein extraction kit (P0027) was bought from Beyotime (Jiang Su, China).

2.2. Cell culture

Human gastric adenocarcinoma SGC-7901 cell line was obtained from the Cancer Institute of the Chinese Academy of Medical Science (Beijing, China). SGC-7901 cells were cultured in RPMI-1640 containing 10% fetal bovine serum (FBS) (Life Technologies, Gaithsburg, MD, USA), 2 mmol/L L-glutamin, 100 U/ml of penicillin and 100 μg/ml of streptomycin. HUVECs (Sciencell) were cultured in endothelial cell medium (ECM) supplemented with 5% FBS, 1% endothelial cell growth supplement and 1% penicillin/streptomycin solution. The cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2. Confluent HUVECs (passages 5–8) were used in the experiment.

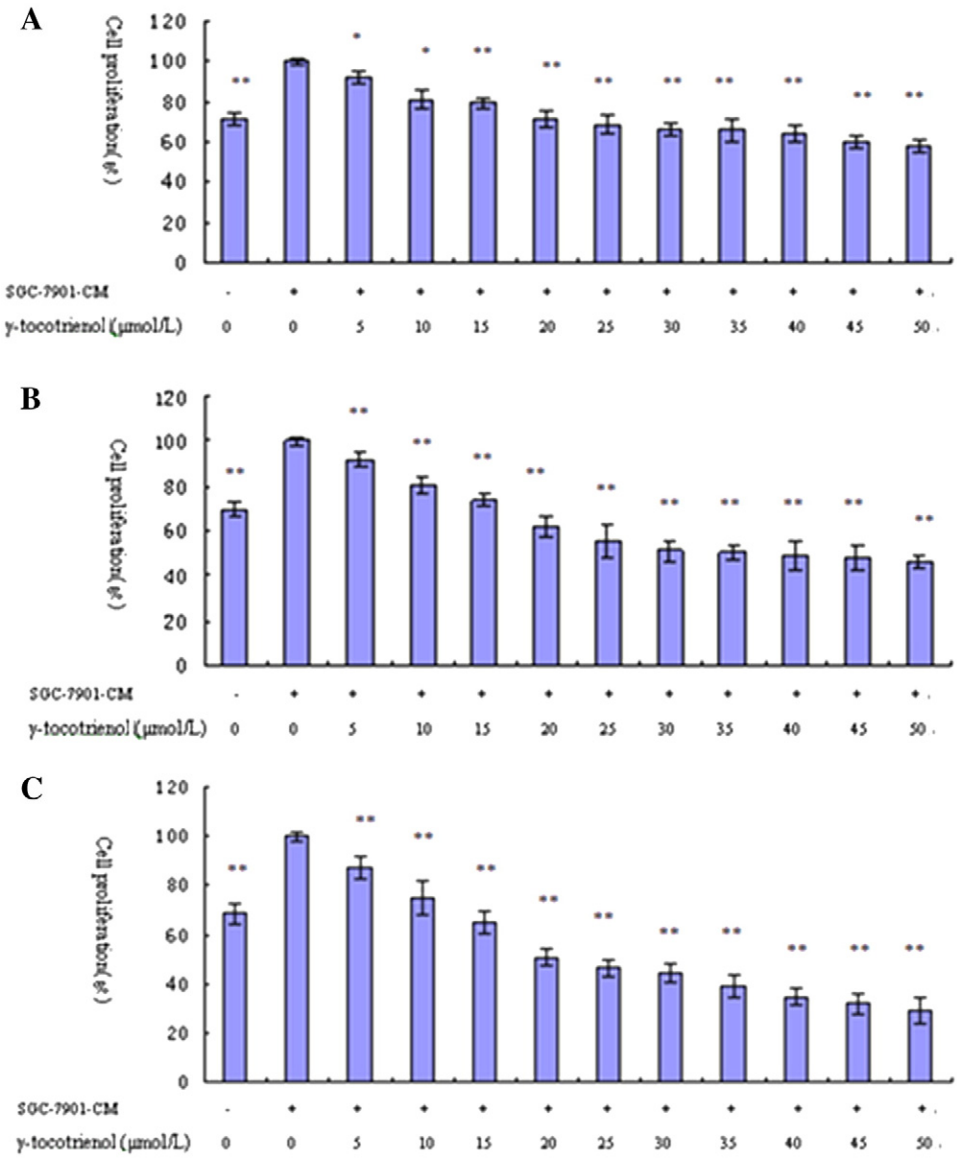

Fig. 1. Effect of γ-tocotrienol on proliferation of HUVECs induced by SGC-7901-CM. SGC-7901-CM: SGC-7901 cells (~90% confluent) were washed with serum-free RPMI-1640 and incubated in RPMI-1640 medium containing 2% FBS. After 24 h, SGC-7901-CM was collected and centrifuged at 1000 rpm for 10 min. The cell proliferative effects were determined by MTT assay. HUVECs were seeded in 96-well plates (5×10^3 cells/well) and treated with different concentrations of γ-tocotrienol for (A) 12 h, (B) 24 h and (C) 48 h. Each concentration was repeated in four wells ($n=4$). $^{*}P<.05$ and $^{**}P<.01$, compared with SGC-7901-CM-only treatment.

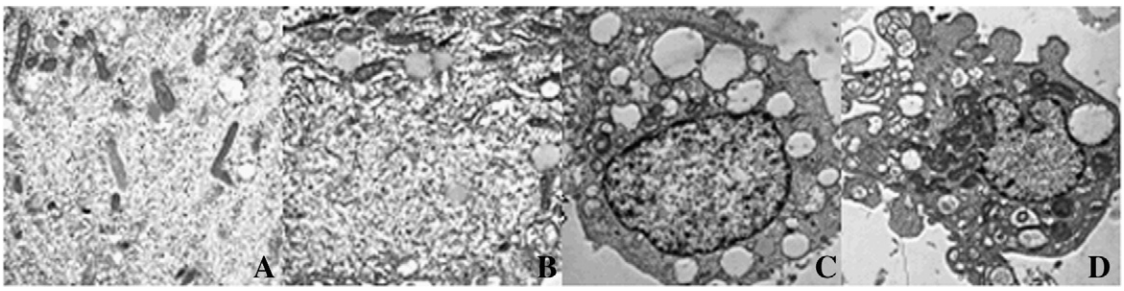

Fig. 2. Morphological changes of apoptosis were observed in SGC-7901-CM-induced HUVECs induced by γ-tocotrienol. Characteristics of apoptosis observed were chromatin condensation and margination, cell blebbing and vacuoles in HUVECs after treatment with 40 μmol/L γ-tocotrienol for 48 h (C and D), in comparison with the cell without SGC-7901-CM (A) and SGC-7901-CM-only treatment for 48 h (B).

2.3. Preparation of SGC-7901 cell conditioned medium

SGC-7901 cell conditioned medium (SGC-7901-CM) was used as an angiogenic stimulus in the experiments. SGC-7901 cells (approximately 90% confluent) were washed with serum-free RPMI-1640 and incubated in the RPMI-1640 medium containing 2% FBS. After 24 h, SGC-7901-CM was collected, centrifuged at 1000 rpm for 10 min and stored at −30°C until used.

2.4. Cell proliferation assay

The effect of γ-tocotrienol on cell proliferation was determined with the MTT assay [31,32]. Briefly, HUVECs were plated in a 96-well plate at 5×10^3 cells/well. After 24 h, cells were treated with 100 μl of culture medium (containing 1% FBS with 0–50 μmol/L γ-tocotrienol) and then 100 μl of SGC-7901-CM was added to each well. Four replicates were employed in each dose. After incubation for 12, 24 and 48 h, cell proliferation was determined. Twenty microliters of 10 mg/ml of MTT solution in phosphate-buffered saline (PBS) was added to each well and incubated for 4 h. After careful removal of the medium, 200 μl of dimethyl sulfoxide was added to each well, and the plate was then shaken until the crystals were solubilized. The absorbance was recorded on a microplate reader (Elx800 Universal Microplate Reader, Bio-Tek Instruments) at a wavelength of 570 nm.

2.5. Transmission electron microscopy

HUVECs were harvested from the negative control group, SGC-7901-CM-treated group and γ-tocotrienol-treated group (40 μmol/L, 48 h). The cells were washed with cold PBS, and then cells were fixed with 4% glutaraldehyde in PBS overnight at 4°C. After fixation with 1% OsO_4 in cacodylate buffer for 1 h at 4°C, cell pellets were dehydrated in graded ethanol solutions and embedded in Epon 812. Ultrathin sections of pellet were counterstained with acetate and lead citrate. The slides were observed under a transmission electron microscope [32].

2.6. Cell cycle analysis

HUVECs were harvested, washed three times with cool PBS, fixed with 70% cool ethanol for 2 h and stained with propidium iodide mixture (Cycle TEST PLUS DNA Reagent Kit). For each treatment, at least 2×10^4 cells were analyzed using a FACSort flow cytometer (BD Biosciences). The proportions at the G_0/G_1, S and G_2/M phases were estimated using ModFit LT analysis software.

2.7. Cell migration assay

The trans-well cell culture chambers (Corning Costar 3422, Corning, Cambridge, MA, USA) were used for cell migration assay [33]. The membrane was coated with

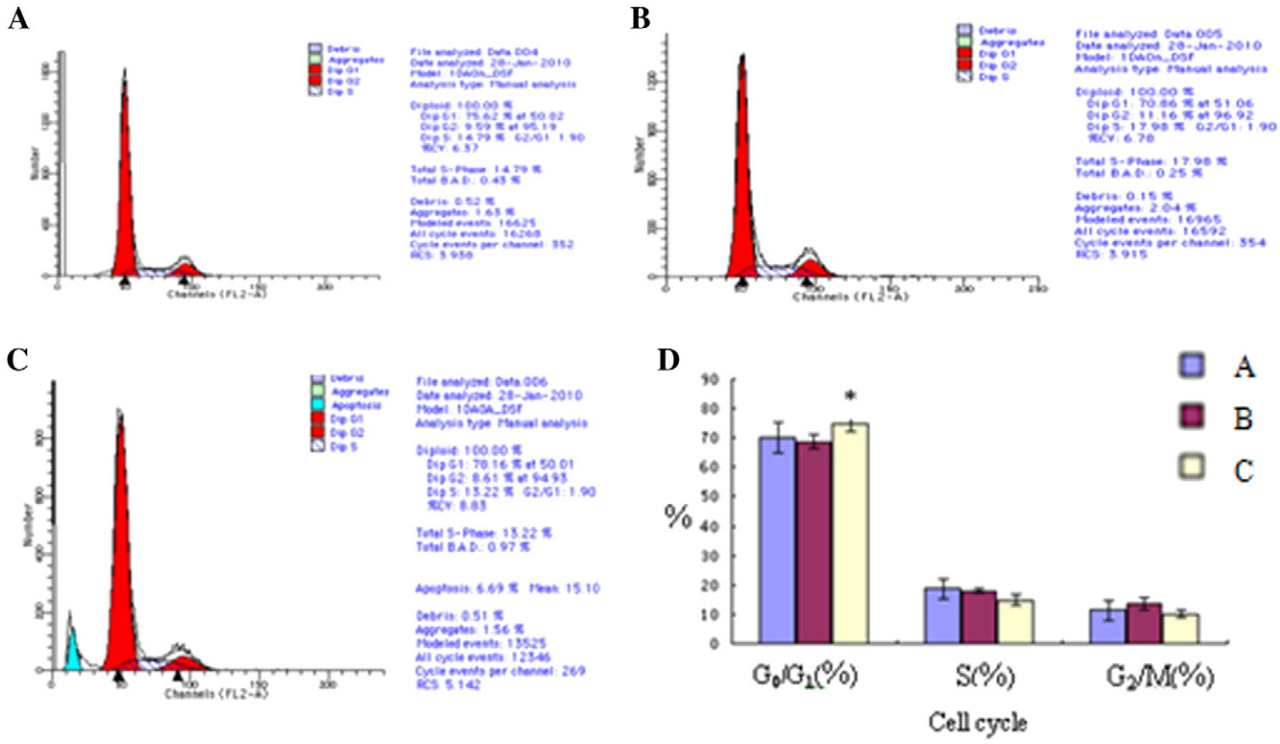

Fig. 3. Cell cycle distributions in SGC-7901-CM-induced HUVECs treated with (B) 0 μmol/L γ-tocotrienol and (C) 40 μmol/L γ-tocotrienol and (A) in cells without SGC-7901-CM were analyzed by flow cytometry. (D) Summary of results. *$P<.05$, compared with 0 μmol/L γ-tocotrienol (B).

a thin layer of ECM gel (Sigma Chemical). SGC-7901-CM was added into the lower chamber. Trypsin-harvested HUVECs (2×10^5 cells) were suspended in ECM containing 1% FBS with 0, 10, 20 and 40 μmol/L γ-tocotrienol and then added into the upper chamber. After 24 h of incubation at 37°C, the non-migrated cells were removed from the upper surface of the membrane by wiping with a cotton swab. The membrane was then fixed with methanol for 15 min and then stained with hematoxylin and eosin. The stained cells were observed through a phase-contrast microscope (Nikon Company, Japan). The number of migrated cells was counted in three randomly selected microscopic fields [34].

2.8. Tube formation assay

Culture (24-well) plates were coated with 0.3 ml of ECM gel and incubated at 37°C for 4 h for solidification [25,35]. Trypsin-harvested HUVECs were treated with γ-tocotrienol under two conditions. First, HUVECs were suspended in 0.5 ml of ECM containing 1% FBS with 0, 10, 20 or 40 μmol/L γ-tocotrienol and then were mixed with 0.5 ml of SGC-7901-CM. After incubation for 24 h, cells were fixed and photographed. Second, HUVECs were cultured in 0.5 ml of ECM (1% FBS) and 0.5 ml of SGC-7901-CM for 12 h. After incubation, cells were treated with γ-tocotrienol and incubated for another 12 h. The cells were fixed and photographed.

2.9. Chick chorioallantoic membrane (CAM) assay

α-Tocopherol and γ-tocotrienol were dissolved in soybean oil. A pellet of methylcellulose (Sigma Chemical) impregnated with 20 μl of α-tocopherol (60 g/L), γ-tocotrienol (20, 40 and 60 g/L), soybean oil (negative control) or Suramin sodium salt (positive control) was placed on the CAM surface of 7-day-old chick embryos. The embryos were incubated for 2 days at 37°C in a humidified incubator, after which the chorioallantois was fixed. Inhibition of angiogenesis was determined by the number of classes of blood vessel [36].

2.10. Immunofluorescence

HUVECs from the negative control group, SGC-7901-CM-treated group and γ-tocotrienol-treated group (20 and 40 μmol/L for 48 h) were fixed with 4% paraformaldehyde for 15 min and washed with PBS. The cells were permeabilized with 0.1% Triton X-100 for 5 min and blocked for 30 min at room temperature with 1% bovine serum albumin (BSA). The cells were incubated with anti-β-catenin antibody overnight at 4°C. Cells were rinsed in PBS, incubated with fluorescein-conjugated AffiniPure goat anti-rabbit IgG (ZF-0311, ZSGB-Bio) for 1 h at room temperature and then covered with DAPI (C1002, Beyotime). Images were analyzed using an Olympus immunofluorescence microscope (Olympus Company, Japan).

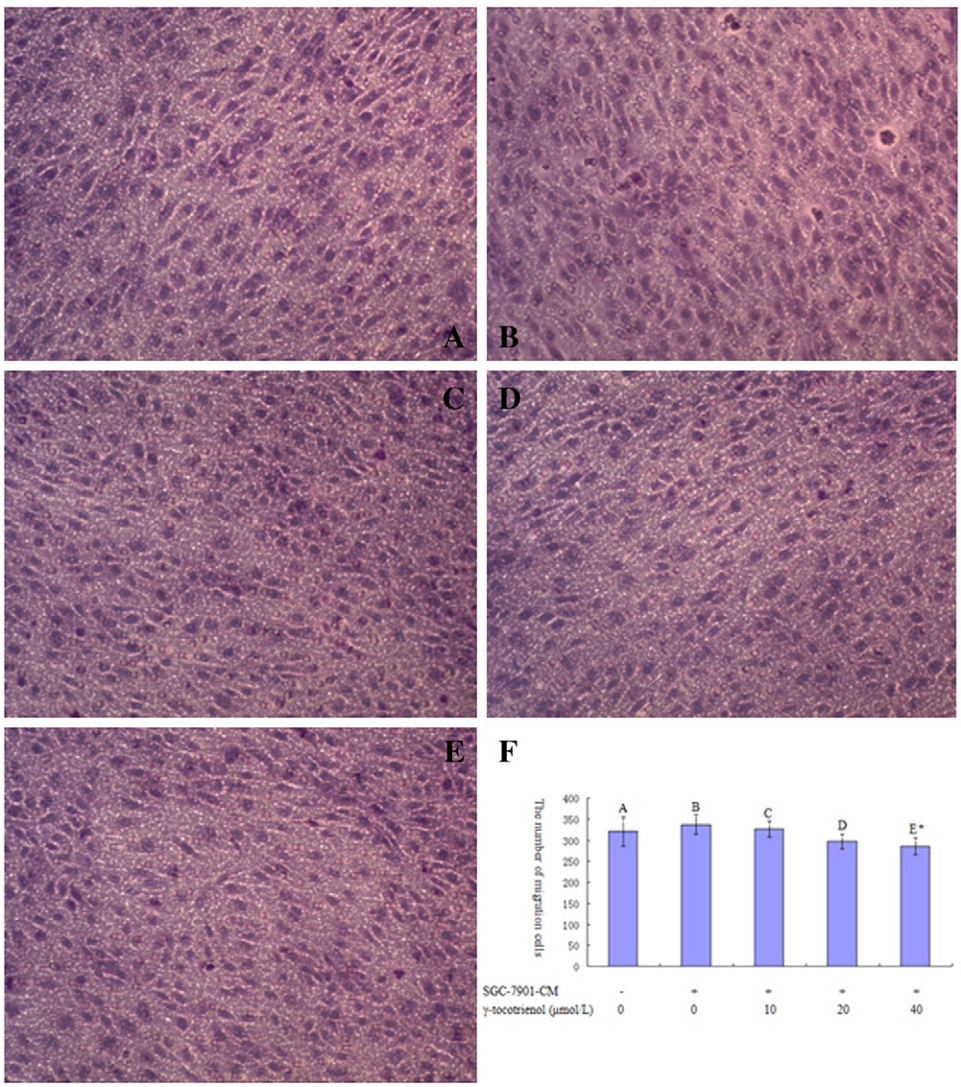

Fig. 4. Effect of γ-tocotrienol on HUVEC migration induced by SGC-7901-CM. The trans-well cell culture chambers coated with ECM gel on the surface were used for cell migration assay. SGC-7901-CM was placed in the lower chamber (except A). Trypsin-harvested HUVECs (2×10^5) were suspended in ECM containing 1% FBS and 0 (B), 10 (C), 20 (D) and 40 (E) μmol/L γ-tocotrienol, and they were then added into the upper chamber. After incubation at 37°C for 24 h, the non-migrated cells were removed from the upper surface of the membrane by wiping with a cotton swab. Cells on the lower side were stained with hematoxylin and eosin. The number of cells was randomly counted using a microscope (×200). The experiment was set up in independent triplicates ($n=3$). (F) Summary of results. $^*P<.05$, compared with 0 μmol/L γ-tocotrienol (B).

2.11. Western blot analysis

SGC-7901-CM-induced HUVECs in different treatment groups (0, 10, 20 and 40 μmol/L) were incubated for 48 h. Cellular protein was prepared from HUVECs. The protein concentrations of each sample were determined using a nucleic acid and protein analyzer (DU 640, Beckman) according to the manufacturer's instructions. For Western blotting, 50–80 μg of total protein was resolved over 10% or 12% polyacrylamide gels and transferred to a nitrocellulose membrane. The membrane was blocked in blocking buffer [1% BSA and 1% Tween-20 in 20 mM Tris-buffered saline (TBST), pH 7.6] for 1 h at 37°C in a hybridization oven (Amersham Life Science, Little Chalfont, Bucks, UK), incubated with appropriate monoclonal or polyclonal primary antibody in blocking buffer for 2 h at 37°C or overnight at 4°C. The membrane was

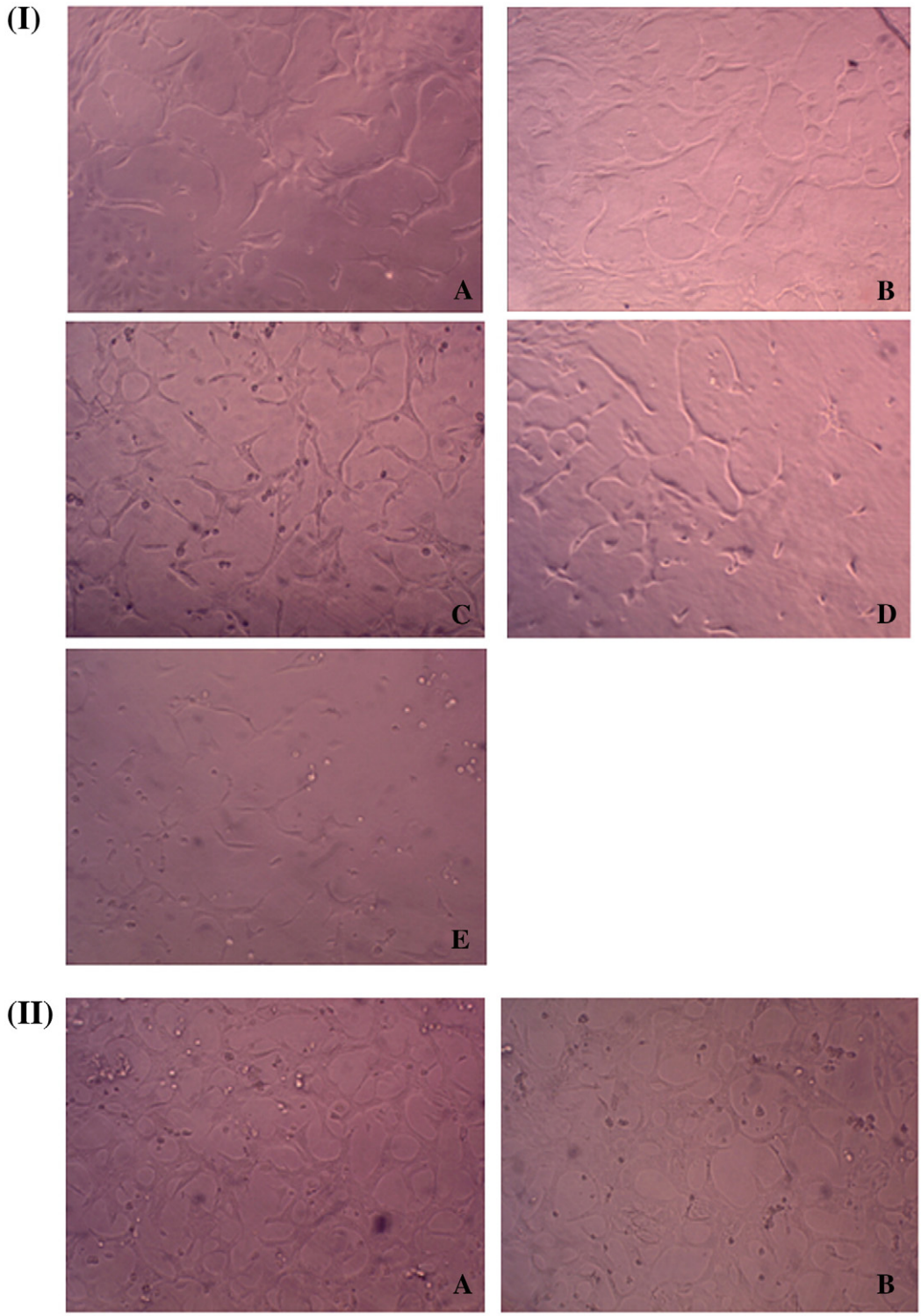

Fig. 5. Effect of γ-tocotrienol on tube formation of HUVECs induced by SGC-7901-CM. I: HUVECs (5×10^4) were suspended in 1.0 ml ECM containing 1% FBS (A); HUVECs were plated in 0.5 ml of ECM containing 1% FBS and 0 μmol/L γ-tocotrienol (B), 10 μmol/L γ-tocotrienol (C), 20 μmol/L γ-tocotrienol (D) and 40 μmol/L γ-tocotrienol (E) and were then mixed with 0.5 ml of SGC-7901-CM. After incubation for 24 h, HUVECs were fixed and photographed. II: HUVECs were cultured in 0.5 ml of ECM (1% FBS) and 0.5 ml of SGC-7901-CM (A) for 12 h. After incubation, cells were treated with 40 μmol/L γ-tocotrienol (B) and then continued to incubate for 12 h; cells were then fixed and photographed.

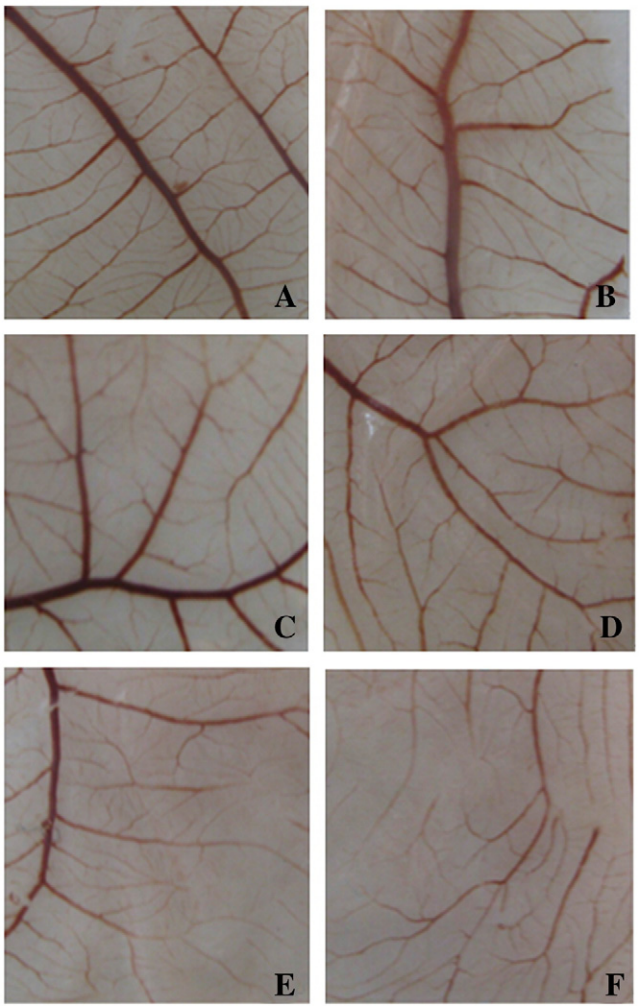

Fig. 6. Effect of γ-tocotrienol on the blood vessel formation of the CAM. A pellet of methylcellulose impregnated with α-tocopherol (1200 μg (B)), γ-tocotrienol (400 (C), 800 (D) and 1200 μg (E)), soybean oil (negative control (A)) or Suramin sodium salt (positive control (F)) was placed on the CAM surface of 7-day-old chick embryos and incubated for 2 days. The CAM was fixed and photographed. The most pyknic blood vessel was presumed as class I. Blood vessel separated from class I was called class II. The class III blood vessel separated from class II. The angiogenic response was evaluated by the number of classes of blood vessel (n=10). The results are summarized in Table 1.

washed with TBST three times (5 min each time) followed by incubation with secondary antibody at 37°C for 1 h. The membrane was washed with TBS one time. The membrane was then incubated with alkaline phosphatase until an appropriate signal level was obtained. The protein bands were detected with FluorChem Imaging System (Alpha Innotech, San Leandro, CA, USA).

Table 1
Effects of γ-tocotrienol on the formation of new blood vessels in CAM (mean±S.D., n=10)

Group	Class I	Class II	Class III
Soybean oil (A)	1.6±0.5	11.3±2.2	104.0±5.1
α-Tocopherol (B)	1.5±0.5	9.5±1.8	98.7±6.6
γ-Tocotrienol (C)	1.4±0.5	9.7±1.8	86.2±5.1**
γ-Tocotrienol (D)	1.3±0.5	8.5±1.5**	80.3±6.2**
γ-Tocotrienol (E)	1.3±0.5	7.9±1.2**	75.6±4.7**
Suramin sodium salt (F)	1.2±0.8	7.8±1.3**	41.8±3.9**

CAMs were treated with soybean oil control (A), 1200 μg/egg α-tocopherol (B), 400 μg/egg γ-tocotrienol (C), 800 μg/egg γ-tocotrienol (D), 1200 μg/egg γ-tocotrienol (E) and Suramin sodium salt as a positive control (F). After γ-tocotrienol or α-tocopherol treatment, CAM was fixed and photographed. The most pyknic blood vessel was presumed as class I. Blood vessel separated from class II was called class III. The class III blood vessel separated from class II. The angiogenic response was evaluated by the number of classes of blood vessel (n=10). **P<.01, compared with soybean oil control (A).

2.12. Statistical analysis

Data were expressed as the mean±S.D. Each experiment was performed in triplicates. Differences were analyzed for significance using one-way ANOVA with Bonferroni post hoc multiple comparisons, used to assess the difference between independent groups. Statistical significance was set at P<.05 or P<.01, and all P values were unadjusted for multiple comparisons.

3. Results

3.1. Effect of γ-tocotrienol on proliferation of HUVECs

The inhibitory effect of γ-tocotrienol on proliferation of HUVECs was examined by MTT assay. The cells were treated with various concentrations (5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 μmol/L) of γ-tocotrienol for 12, 24 and 48 h. γ-Tocotrienol significantly inhibited cell proliferation of HUVECs in a time- and dose-dependent manner (Fig. 1). The median inhibiting concentrations (IC_{50}) of γ-tocotrienol for inhibition of HUVECs were 36.76±1.28 and 23.34±1.06 μmol/L at 24 and 48 h, respectively.

3.2. Apoptosis on HUVECs induced by γ-tocotrienol

Morphological changes were observed with a transmission electron microscope in HUVECs treated with 40 μmol/L γ-tocotrienol for 48 h. The cells showed evidently characteristic changes of apoptosis, including cytoskeletal disruption, cell shrinkage, chromatin condensation and margination of nucleus, cell blebbing, formation of apoptotic body and mitochondrial denaturation, such as swelling and disappearance of mitochondrial cristate in the γ-tocotrienol-treated cells (Fig. 2C and D). In addition, cells in the control group had the clear cell organs in cytoplasm, and mitochondrial cristate was also observed clearly (Fig. 2A and B). The cell cycle distribution of HUVECs treated with γ-tocotrienol was also determined by flow cytometry. As shown in Fig. 3, SGC-7901-CM-induced HUVECs treated with 40 μmol/L γ-tocotrienol showed strong cell cycle arrest at the G_0/G_1 phase. The proportion at the G_0/G_1 phase ranged from 68.79%±2.35% in untreated cells to 75.06%±2.73% in treated cells (P<.05). A peak of apoptosis was found in HUVECs treated with 40 μmol/L γ-tocotrienol.

3.3. γ-Tocotrienol inhibited HUVEC migration

Effect of γ-tocotrienol on migration in HUVECs induced by SGC-7901-CM was determined by the trans-well cell culture chamber coated with ECM gel on the surface. The numbers of migration cells with SGC-7901-CM were 336.7±23.0, 326.0±19.1, 296.7±18.2 and 285.7±20.5 in HUVECs treated with 0, 10, 20 and 40 μmol/L γ-tocotrienol for 24 h, respectively. The number of migration cells without SGC-7901-CM was 320.7±34.7 (Fig. 4). A dose response was observed in this study.

3.4. γ-Tocotrienol inhibited HUVEC tube formation

Effect of γ-tocotrienol on tubular morphogenesis in HUVECs induced by SGC-7901-CM was examined. HUVECs incubated with SGC-7901-CM showed an increase in the lengths and reticular formation of endothelial tubes in comparison with those without SGC-7901-CM. An inhibition of tube formation was observed in SGC-7901-CM-induced HUVECs treated with γ-tocotrienol in a dose-dependent manner (Fig. 5, I). In addition, γ-tocotrienol did not affect the luminal structure (Fig. 5, II). It seems that γ-tocotrienol inhibits capillary tube

formation, but it does not affect existing capillary tubes of HUVECs induced by SGC-7901-CM on ECM gel.

3.5. Inhibition of angiogenesis by γ-tocotrienol in vivo

We further examined the effect of γ-tocotrienol on angiogenesis using CAM assay. The results are shown in Fig. 6 and Table 1. γ-Tocotrienol significantly inhibited embryonic angiogenesis of CAM, resulting in a lower number of classes of blood vessel. γ-Tocotrienol also significantly inhibited classes II and III of blood vessel of CAM in a dose-dependent manner. No antiangiogenic activity was found in the α-tocopherol-treated group.

3.6. Wnt pathway protein expression

The inhibitory mechanism of γ-tocotrienol on tumor-induced angiogenesis was further investigated by immunofluorescence and Western blot. Considering the critical role of Wnt signaling in tumor angiogenesis, the effect of γ-tocotrienol on the Wnt pathway was examined. γ-Tocotrienol down-regulated the expression of β-catenin, which is the central player in Wnt signaling. In this study, γ-tocotrienol also down-regulated the expression of total β-catenin and nuclear β-catenin in a dose-dependent manner. γ-Tocotrienol promoted the translocation of β-catenin from cell nucleus to cell membrane (Fig. 7). In addition, γ-tocotrienol inhibited the expression

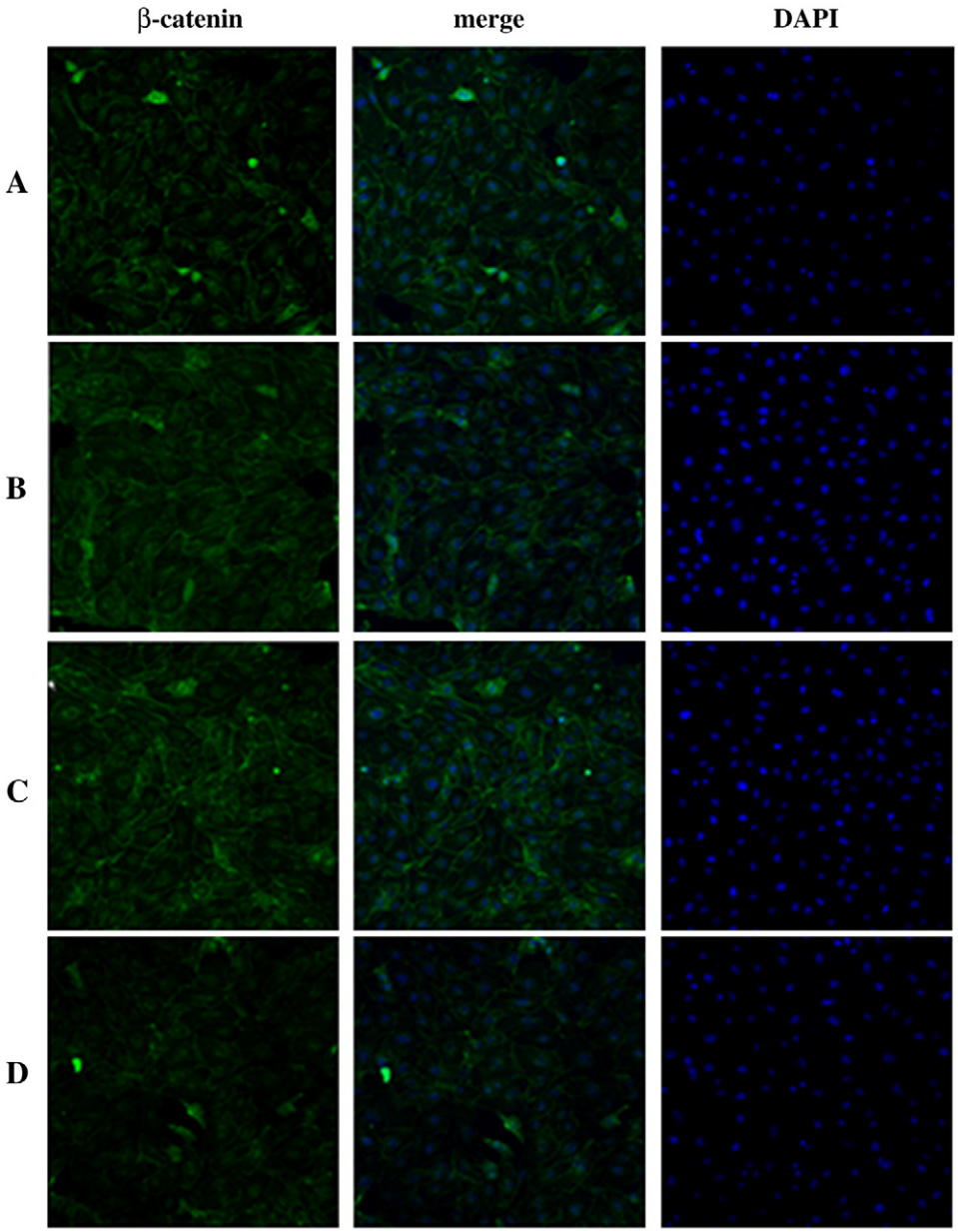

Fig. 7. Effect of γ-tocotrienol on β-catenin localization in SGC-7901- CM-induced HUVECs by immunofluorescence. HUVECs were suspended in 2.0 ml of ECM containing 1% FBS (A); HUVECs were plated in 1.0 ml of ECM (1% FBS) with 0 μmol/L γ-tocotrienol (B), 20 μmol/L γ-tocotrienol (C) and 40 μmol/L γ-tocotrienol (D) and then were mixed with 1.0 ml of SGC-7901-CM. After incubation for 48 h, cells were fixed with 4% paraformaldehyde for 15 min and washed with PBS. Then, cells were permeabilized with 0.1% Triton X-100 for 5 min and blocked for 30 min at room temperature with 1% BSA and incubated with anti-β-catenin antibody overnight at 4°C. Cells were rinsed in PBS, incubated with anti-rabbit IgG/DyLight 488 for 1 h at room temperature and then covered with DAPI mounting medium. Images were analyzed using an Olympus immunofluorescence microscope.

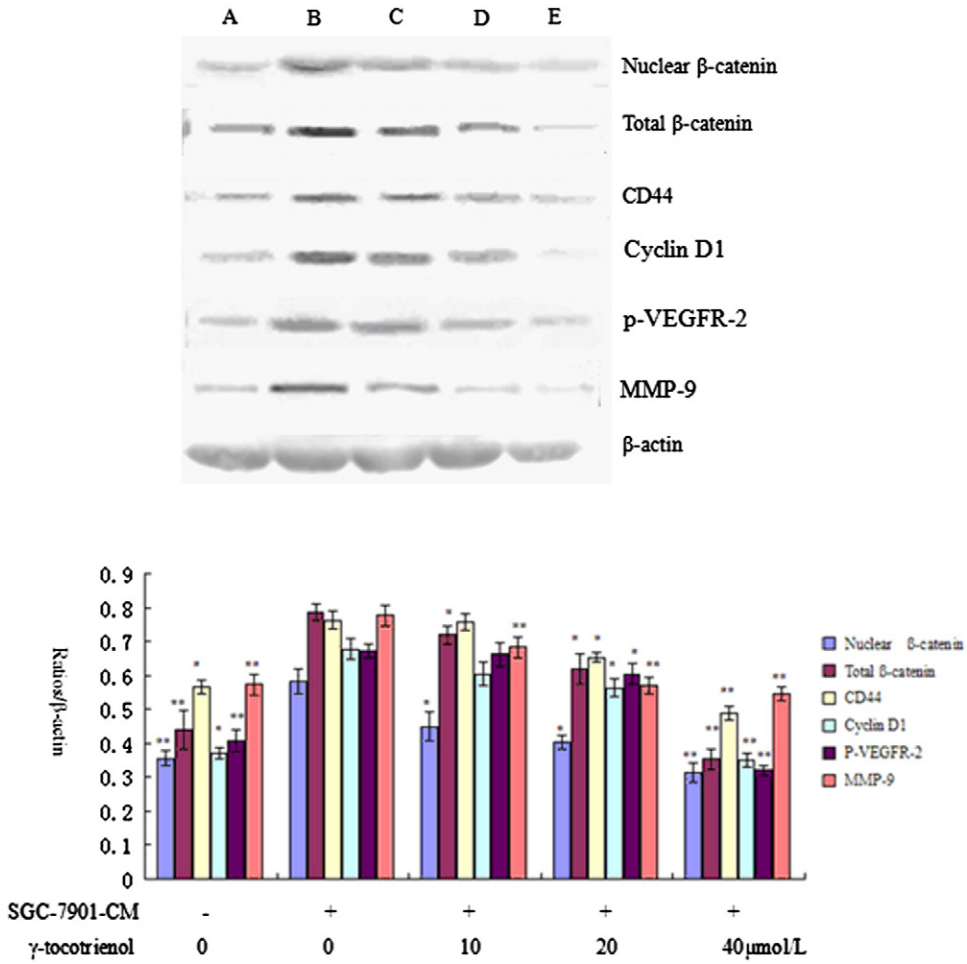

Fig. 8. Expression of β-catenin, CD44, cyclin D1, MMP-9, phospho-VEGFR-2 and β-actin in SGC-7901-CM-induced HUVECs treated with different concentrations of γ-tocotrienol for 48 h. Proteins were separated on 10% or 12% SDS-PAGE gel, transferred to nitrocellulose membrane and probed with anti-β-catenin, anti-CD44, anti-cyclin D1, anti-MMP-9, anti-phospho-VEGFR-2 or anti-β-actin. The experiment was performed in triplicates. (A) No CM negative group; (B) CM negative group; (C) CM with 10 umol/L r- tocotrienol; (D) CM with 20 umol/L r- tocotrienol; (E) CM with 40 umol/L r- tocotrienol. $^{*}P<.05$, $^{**}P<.01$. compared with SGC-7901-CM-only treatment.

of cyclin D1 and CD44, which are involved in cell cycle progression and cell migration. γ-Tocotrienol also inhibited SGC-7901-CM-induced phospho-VEGFR-2 and MMP-9 expression (Fig. 8).

4. Discussion

Epidemiological and laboratory studies have shown that the consumption of vegetables, fruits and grains is associated with a low risk of cancer [37–42]. Many natural dietary phytochemicals found in fruits, vegetables, spices and tea have been shown to be protective against cancer in various animal models [38–40,42–44]. Tocotrienols have been shown to possess biological activity besides antioxidant activity, such as anticarcinogenic properties, which are evidenced mainly *in vivo* and *in vitro* [45]. Recently, tocotrienols have also been reported to have led to the induction of apoptosis in many various cancer cells, such as rat hepatoma dRLh-84 cells, human hepatoma Hep3B cells and human breast carcinoma MCF-7 cells [22,46,47]. γ-Tocotrienol has also been reported to have antitumor effects *in vivo*. γ-Tocotrienol feeding has been demonstrated to decrease tumor weight and to prolong the survival rate of C57BL female mice transplanted with melanoma [48]. Tocotrienol mixture (38% γ-tocotrienol, 22% α-tocotrienol and 12% δ-tocotrienol) significantly suppressed liver and lung carcinogenesis in C3H/HeN male mice [49]. Furthermore, it has been reported that accumulation is critical for antitumor activity of tocotrienol *in vivo* [50]. In our previous study, γ-tocotrienol showed a significantly inhibitory effect on invasion and metastasis of SGC-7901 cells [34]. Moreover, tocotrienol at a low dose range inhibited proliferation, migration and tube formation of bovine aortic endothelial cells and HUVECs. These findings suggested that tocotrienols, but not tocopherols, have considerable potential as an angiogenesis inhibitor. In a previous study, the effects of δ-tocotrienol on antiangiogenesis would be associated with changes in phosphatidylinositol-3 kinase (PI3K)/PDK/Akt signaling and apoptosis induction in endothelial cells [28]. However, the activities of tocotrienol for tumor angiogenesis in HUVECs and the relationship among these cell signalings are not entirely understood.

Angiogenesis, the formation of new capillaries through sprouting, is the primary process responsible for tumor neovascularization. Angiogenesis normally involves a series of steps, including endothelial cell activation and breakdown of the basement membrane, followed by proliferation, migration and tube formation of the endothelial cells. This process depends on the balance between the effects of proangiogenic and antiangiogenic factors. Thus, SGC-7901-CM was used as the angiogenic stimulus. In our study, we demonstrated the inhibitory effect of γ-tocotrienol on tumor angiogenesis *in vitro* and *in vivo*. γ-Tocotrienol significantly inhibited proliferation, tube formation and migration in HUVECs. In addition, the cell cycle arrest and apoptosis induction were also observed in SGC-7901-CM-induced HUVECs. γ-Tocotrienol also inhibited the new blood vessel formation on the CAM. Moreover, δ-tocotrienol has also been reported to inhibit

the human colorectal adenocarcinoma cell medium (DLD-1-CM) and induced angiogenesis in nude mice [30]. In addition, once capillary tubes were formed, γ-tocotrienol did not affect the luminal structure, implying that γ-tocotrienol has no cytotoxicity on endothelial cells. It demonstrated that γ-tocotrienol may be safe as a candidate agent for chemoprevention of cancer.

Wnt signaling pathways play important roles in the regulation of cellular proliferation, differentiation, motility and morphogenesis [51–54]. Wnt signaling has a crosstalk with other signaling pathways regulating apoptosis, angiogenesis, invasion and metastasis [55]. In endothelial cells, Wnt signaling has been reported to increase cell proliferation [56] and to regulate migration and cell cycle progression [57]. Moreover, defects of yolk sac and placental angiogenesis in mice lacking the Frz5 gene directly demonstrate the involvement of Wnt signaling in vascular development [58]. β-Catenin is phosphorylated and subsequently ubiquitinated and degraded by the 26S proteasome system, and this degradation is initiated by phosphorylation at S45, T41, S37 and S33 at its NH_2-terminal region in normal cells [59]. Activation of Wnt signaling pathway leads to inhibition of GSK-3β activity, resulting in deregulated accumulation of cytoplasmic β-catenin that can shift to nucleus and activates transcription of a series of genes, including cyclin D1, CD44, Cox-2, c-Myc, etc., by binding with T cell factors and lymphoid-enhancing factors [60,61]. Consequently, β-catenin has been regarded as a central player in Wnt signaling. As cell adhesion molecules play a role in cell–cell and cell–extracellular matrix adhesion and interactions, CD44 is involved in the process of tumor invasion and metastases. Moreover, CD44 might also be important in angiogenesis. Evidence confirms the involvement of CD44 in endothelial cell proliferation, migration and angiogenesis *in vitro* [62]. Cyclin D1, a known key regulator of cell proliferation, plays a critical role in cell cycle progression during mid G_1 by initiating the multistep process that leads to pRb inactivation [63]. In this study, γ-tocotrienol significantly inhibited expression of β-catenin in SGC-7901-CM-induced HUVECs. In addition, γ-tocotrienol significantly inhibited the expression of total β-catenin and nuclear β-catenin in SGC-7901-CM-induced HUVECs. γ-Tocotrienol also promoted the translocation of β-catenin from cell nucleus to cell membrane. Furthermore, γ-tocotrienol down-regulated the expression of cyclin D1 and CD44 in a dose-dependent manner. It indicated that the inhibitory effects of tocotrienol in HUVECs may be related to a decrease in the intracellular PI3K/PDK/Akt (Wnt) signaling protein activity. Thus, in the future, how γ-tocotrienol adjusts Wnt and PI3K/PDK/Akt signaling should be investigated.

VEGFRs are members of the receptor tyrosine kinase superfamily [64]. VEGFR-2 is considered to be the major mediator of the mitogenic, angiogenic and permeability-enhancing effects of VEGF and hence is a major target for antiangiogenic therapies. It is well known that VEGF induces HUVEC migration and tube formation through the phosphorylation of VEGFR-2, which is expressed on endothelial cells [65–67]. In our study, γ-tocotrienol inhibited phosphorylation of VEGFR-2 in SGC-7901-CM-induced HUVECs. It has been reported that inhibiton of Wnt signaling down-regulated VEGFR-2 messenger RNA and protein [68]. It becomes tempting to suggest the γ-tocotrienol may actually down-regulate phospho-VEGFR-2 levels through its inhibitory action of Wnt signaling.

MMPs are a family of zinc-dependent proteolytic endopeptidases with extracellular matrix remodeling and degrading properties [69]. They stimulate cancer cell growth, migration, invasion, angiogenesis and metastasis [13,33]. Among MMPs, MMP-9 is considered to play an important role in invasion and metastasis of cancer cells [70–76]. As γ-tocotrienol has been shown to remarkably inhibit angiogenesis of SGC-7901-CM-induced HUVECs, the activity of MMP-9 was futher investigated in HUVECs. The results showed that γ-tocotrienol inhibited the protein expression of MMP-9 in a dose-dependent manner. Thus, γ-tocotrienol may inhibit MMP-9 activity to suppress angiogenesis in HUVECs.

In summary, γ-tocotrienol inhibited proliferation, migration and tube formation in SGC-7901-CM-induced HUVECs and the new blood vessel formation *in vivo*. This suppression may partly involve the Wnt signaling pathway by down-regulation of β-catenin, cyclin D1, CD44, p-VEGFR-2 and MMP-9. These findings suggest that γ-tocotrienol may be a potential chemopreventive agent of cancer.

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