Identification of Campesterol from Chrysanthemum coronarium L. and its Antiangiogenic Activities

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Campesterol, a plant sterol in nature, is known to have cholesterol lowering and anticarcinogenic effects. Since angiogenesis is essential for cancer, it was surmised that an antiangiogenic effect may be involved in the anticancer action of this compound. This study investigated the effect of campesterol on basic fibroblast growth factor (bFGF)-induced angiogenesis in vitro in human umbilical vein endothelial cells (HUVECs) and an in vivo chorioallantoic membrane (CAM) model. Campesterol isolated from an ethylacetate fraction of Chrysanthemum coronarium L. showed a weak cytotoxicity in non-proliferating HUVECs. Within the non-cytotoxic concentration range, campesterol significantly inhibited the bFGF-induced proliferation and tube formation of HUVECs in a concentration-dependent manner, while it did not affect the motility of HUVECs. Furthermore, campesterol effectively disrupted the bFGF-induced neovascularization in chick chorioallantoic membrane (CAM) in vivo. Taken together, these results support a potential antiangiogenic action of campesterol via an inhibition of endothelial cell proliferation and capillary differentiation. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords: campesterol; Chrysanthemum coronarium L.; bFGF; HUVECs; CAM; angiogenesis.

Introduction

Campesterol is one of the most common plant sterols in nature along with β-sitosterol and stigmasterol (Rao and Janzeic, 1992; Ikeda et al., 2006). Abundant in seeds, nuts, cereals, beans, legumes and vegetable oils (Ostlund et al., 2002; Phillips et al., 2005), campesterol is similar in structure to cholesterol and similarly metabolized by intestinal bacteria (Ikeda et al., 2006). Due to structural similarity to cholesterol, plant sterols including campesterol have cholesterol-lowering effects (Ling and Jones, 1995; Jones et al., 1997; Plat and Mensink, 2001; Tikkanen, 2005). Plant sterols also have many other biological functions, such as anticarcinogenic (Li et al., 2001), antiinflammatory, antibacterial and antifungal activities (Padmaja et al., 1993). There is accumulating evidence that campesterol exhibits chemopreventive effects against many cancers, including prostate (McCann et al., 2005), lung (Schabath et al., 2005) and breast (Awad et al., 2000) cancers.

An anticarcinogenic activity of campesterol has been reported, although the mechanism by which campesterol affects vascular endothelial cells has not been elucidated. Thus, the effect of campesterol isolated from Chrysanthemum coronarium L. on basic fibroblast growth factor (bFGF) stimulated angiogenesis in HUVECs and chick chorioallantoic membrane (CAM) was studied, since bFGF is a typical angiogenesis promoting factor (Huh et al., 2005; Lee et al., 2006) employed in angiogenesis assays.

Materials and Methods

Materials. Chrysanthemum coronarium L. was bought from Garak Vegetable Market, Seoul. Recombinant human basic fibroblast growth factor (bFGF) was purchased from R&D Systems (Minneapolis, MN). M199, fetal bovine serum (FBS) and antibiotic-antimycotic were bought from Gibco (Grand Island, NY). 2,3-Bis [2-methoxy-4-nitro-5-sulfo]-2H-tetrazolium-5-carboxanilide (XTT), heparin and gelatin were purchased from Sigma Chemical (St Louis, MO). Theermanox coverslips were from Nunc (Naperville, IL) and the growth factor reduced-Matrigel was from Becton Dickinson (San Jose, CA). 48-well microchemotaxis chambers and polyester membrane (12 μm pores) were bought from Neuro Probe, Inc. (Cabin John, MD).

Isolation of campesterol. The fresh aerial parts of Chrysanthemum coronarium L. (80 kg) were extracted with
80% aqueous methanol (40 L × 3), and the liquid was concentrated in vacuo. The extracts were partitioned with H₂O (2 L), EtOAc (2 L × 3) and n-BuOH (2 L × 3). The concentrated EtOAc fraction (CCE, 168 g) was subjected to silica gel (SiO₂) column chromatography (CC) (1500 g, Φ 8 × 20 cm) and eluted with a gradient of n-hexane–EtOAc (7:1→6:1→5:1→4:1→3:1→2:1→1:1, v/v, 1 L of each), resulting in 12 fractions (CCE1→CCE12). Fraction CCE6 [12.4 g, Ve/Vt (elution volume/total volume) 0.40–0.52] was subjected to SiO₂ CC (100 g, n-hexane–EtOAc = 4:1). The chemical structure of compound 1 was identified by IR, ¹H-NMR, ¹³C-NMR and MS.

**Cell culture.** Human umbilical vein endothelial cells (HUVECs) were isolated from fresh human umbilical cord veins according to a published protocol (Jaffe et al., 1973) and cultured in M199 supplemented with 20% heat-inactivated FBS, 3 ng/mL bFGF, 5 units/mL heparin and 100 units/mL antibiotic-antimycotic in 0.1% gelatin coated flasks. HUVECs were used in passage 3. Cells were seeded onto 0.1% gelatin coated 96-well plates and incubated in a humidified incubator for 24 h. The optical density was measured using a microplate reader (Molecular Devices Co.) at 450 nm.

**Results**

**Identification of compound 1 isolated from Chrysanthemum coronarium L.**

Compound 1 isolated from *Chrysanthemum coronarium* L. was a white amorphous powder. [α]ᵢD = −33° (c 1.0, CHCl₃); IR (CaF₂ window in CHCl₃, νmax 3400, 1640, 1050, 845, 830, 802 cm⁻¹; mp 157–158°C; ¹H-NMR (400 MHz, pyridine-d₅) δ 3.34 (1H, brd, J = 5.2 Hz, H-6), 3.51 (1H, brdd, J = 7.3, 7.3 Hz, H-3), 2.28 (1H, d, J = 7.3 Hz, H-4), 1.01 (3H, s, H-19), 0.92 (1H, d, J = 6.6 Hz, H-21), 0.84 (3H, d, J = 7.2 Hz, H-27), 0.81 (3H, d, J = 7.2 Hz, H-26), 0.80 (3H, d, J = 7.0 Hz, H-28), 0.68 (3H, s, H-18), 13C-NMR (100 MHz, pyridine-d₅) δ 40.7 (C-5), 121.7 (C-6), 71.8 (C-3), 56.9 (C-14), 56.1 (C-17), 50.1 (C-9), 45.8 (C-21), 42.3 (C-4), 42.3 (C-13), 40.0 (C-12), 37.3 (C-1), 36.5 (C-10), 36.1 (C-25), 33.9 (C-22), 31.9 (C-7), 31.6 (C-20), 29.2 (C-8), 28.2 (C-2), 26.1 (C-16), 24.4 (C-28), 23.1 (C-15), 21.1 (C-23), 20.0 (C-18), 19.6 (C-11), 19.4 (C-9), 18.9 (C-21), 19.0 (C-27), 18.8 (C-26), 11.8 (C-19); EIMS m/z [rel int. (%)]: 400 [M⁺] (40), 385 (78), 382 (13), 367 (97), 315 (32), 289 (13), 213 (100). Based on the above data, the bFGF stimulated proliferation assay. HUVECs (5 × 10⁴) were seeded onto 0.1% gelatin coated 96-well plates and incubated in a humidified incubator for 24 h. The effect of campesterol on the viable cell number was assessed by XTT assay (Jost et al., 1992). Briefly, HUVECs (1 × 10⁴) were seeded onto 0.1% gelatin coated 96-well plates and incubated in a humidified incubator for 24 h. Cells were washed with PBS (pH 7.4) and various concentrations (10, 20, 30, 40 and 50 μg/mL) campesterol were added to the cells in the absence of serum. After incubation at 37 °C in a humidified incubator for 24 h, a XTT working solution was added to each well. The optical density was measured using a microplate reader (Molecular Devices Co.) at 450 nm.

**bFGF stimulated tube formation assay.** The in vitro differentiation assay of HUVECs on Matrigel into capillary-like tubes was performed as described by Grant et al. (1989). HUVECs (2 × 10⁴) were seeded onto Matrigel-coated 24-well plates and campesterol (1, 5, 10 and 20 μg/mL) was added in M199 with 1% FBS, 10 ng/mL bFGF and 5 units/mL heparin. After 24 h, randomly chosen fields were photographed under an Axiovert S 100 light microscope (Carl Zeiss, Inc., USA) at ×40 magnification and the formed tubes area was quantified by a NIH Scion image program.

**bFGF stimulated CAM assay.** In vivo angiogenic activity was assayed using CAMs as described previously (Marks et al., 2002). Campesterol (10 and 20 μg/egg) with bFGF (100 ng) was loaded onto 1/4 piece of thermoxon disks (Nunc, Naperville, IL). The dried thermoxon disk was applied to the CAMs of a 10-day-old embryo. After 48 h incubation, a fat emulsion was injected under the CAMs for better visualization of the blood vessels and the number of newly formed blood vessels was counted. The experiment was repeated twice and 15 eggs were used for each group.

**Statistical analysis.** All data are presented as mean ± SD. The statistically significant differences between control and campesterol groups were calculated by the Student’s t-test.

**RESULTS**

**Identification of compound 1 isolated from Chrysanthemum coronarium L.**

Compound 1 isolated from *Chrysanthemum coronarium* L., was a white amorphous powder. 

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compound 1 was finally identified as 24(R)-methylcholesta-5-en-3β-ol (campesterol) by the comparison of several physical and spectral data with the literature (Kircher and Rosenstein, 1974) as shown in Fig. 1.

Effect of campesterol on cell viability of non-proliferating HUVECs

The cytotoxic effect of campesterol on non-proliferating HUVECs, XTT assay was first examined with the XTT assay. Under a serum-free condition, 50 μg/mL of campesterol decreased the cell viability up to about 56% of control (IC50 of over 50 μg/mL) as shown in Fig. 2. Thus, campesterol caused weak cytotoxicity at concentrations below 20 μg/mL. Thus, all subsequent experiments were performed at nontoxic concentrations of equal or below 20 μg/mL.

Effect of campesterol on bFGF-stimulated proliferation of HUVECs

The effect of campesterol on endothelial cell proliferation was investigated using HUVECs stimulated by 10 ng/mL bFGF for 48 h. As shown in Fig. 3, campesterol inhibited the proliferation of HUVECs in a concentration-dependent manner at a non-cytotoxic range of 20 μg/mL and less.

Effect of campesterol on bFGF-stimulated migration of HUVECs

Chemotactic migration was performed using a Boyden chamber containing a polycarbonate membrane coated with 0.1% gelatin. As shown in Fig. 4, few cells were found in the untreated control in the absence of bFGF in the lower chamber, whereas many migrated cells were detected in the positive control. However, campesterol did not affect the migration of bFGF-stimulated HUVECs.
Effect of campesterol on bFGF-stimulated capillary-like tube formation of HUVECs

When HUVECs were plated on the Matrigel with bFGF (10 ng/mL), the cells formed a capillary-like network within 24 h. As shown in Fig. 5, campesterol dramatically decreased the total tube formation in each of four randomly chosen fields in a concentration-dependent manner.

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DISCUSSION

There is now considerable evidence from in vitro and in vivo animal studies to suggest an inhibitory effect of plant sterols on tumorigenesis (Ovesna et al., 2004). Dietary plant sterols were shown to inhibit both the growth of some kind of tumor cells and their metastasis in vivo and in vitro (Awad et al., 2000; Janezic and Rao, 1992; Raicht et al., 1980). One of the components of plant sterols with anticancer effects is campesterol (Rao and Janezic, 1992; Ikeda et al., 2006). However, the effects of campesterol on angiogenesis and mechanisms have not been investigated thoroughly.

Angiogenesis is the formation of new capillary blood vessels from pre-existing vessels. It is a tightly regulated process including normal reproductive function, embryonic development and wound healing (Folkman, 1971; Zetter, 1998). However, aberrant angiogenesis sterol significantly disrupted bFGF-induced angiogenesis compared with the positive control.
is a crucial factor in the pathogenesis of numerous diseases including rheumatoid arthritis, diabetic retinopathy and cancer (Carmeliet and Jain, 2000; Bodolay et al., 2002; Lee et al., 1998). Angiogenesis involves multi-step processes including endothelial cell proliferation, migration and differentiation, degradation of the extracellular matrix, tube formation and sprouting of new capillary branches (Folkman, 1995). Because each step of these angiogenesis processes usually serves as a potential target for the therapeutic intervention of the angiogenesis, antiangiogenic or proangiogenic effect of any compound is usually evaluated at each individual step of the angiogenic process.

In the present study, compound 1 was isolated from *Chrysanthemum coronarium* L. through activity based fractionation and identified as campesterol. Thereafter, the effects of campesterol on the endothelial cell proliferation, migration and tube-like formation in HUVECs stimulated by bFGF were investigated. Campesterol showed weak cytotoxicity against HUVECs. However, within non-cytotoxic concentrations (less than 20 μg/mL), campesterol inhibited bFGF-induced proliferation of HUVECs in a concentration-dependent manner. These data imply that campesterol can suppress the bFGF-induced pathological angiogenesis in HUVECs. Campesterol also dramatically suppressed capillary-like tube formation, whereas the inhibitory effect of campesterol on the migration of HUVECs stimulated by bFGF was not significant. These data suggest campesterol may inhibit the differentiation of HUVECs rather than the motility of HUVECs. Its in vivo antiangiogenic activity was confirmed using the CAM assay, which is a widely adopted in vivo model for studying angiogenesis. Campesterol significantly disrupted the development of new embryonic blood vessels without affecting the pre-existing vasculature. However, no toxicity was recognized in any of the chick embryos at doses of 10–20 μg/embryo. These findings indicate that campesterol may be a potent angiogenesis inhibitor with no toxicity.

In summary, campesterol inhibited endothelial cell proliferation and differentiation in vitro as well as neovascularization in CAM in vivo with no toxicity. These results strongly suggest that campesterol could be an antiangiogenic candidate for the prevention and treatment of angiogenesis related diseases.

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