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European Journal of Pharmacology 512 (2005) 105–115

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Antiangiogenic activity of β-eudesmol in vitro and in vivo

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Received 21 December 2004; received in revised form 16 February 2005; accepted 22 February 2005
Available online 29 March 2005

Abstract

Abnormal angiogenesis is implicated in various diseases including cancer and diabetic retinopathy. In this study, we examined the effect of β-eudesmol, a sesquiterpenoid alcohol isolated from *Atractylodes lancea* rhizome, on angiogenesis in vitro and in vivo. Proliferation of porcine brain microvascular endothelial cells and human umbilical vein endothelial cells (HUVEC) was inhibited by β-eudesmol (50–100 μM). It also inhibited the HUVEC migration stimulated by basic fibroblast growth factor (bFGF) and the tube formation by HUVEC in Matrigel. β-eudesmol (100 μM) blocked the phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 induced by bFGF or vascular endothelial growth factor. Furthermore, β-eudesmol significantly inhibited angiogenesis in subcutaneously implanted Matrigel plugs in mice and in adjuvant-induced granuloma in mice. These results indicate that β-eudesmol inhibits angiogenesis, at least in part, through the blockade of the ERK signaling pathway. We considered that β-eudesmol may aid the development of drugs to treat angiogenic diseases.

Keywords: Angiogenesis; Endothelial cell; Growth; Migration; Tube formation; MAP kinase

1. Introduction

Angiogenesis is a fundamental process by which new capillaries are formed from pre-existing vasculature. It is essential to organogenesis during embryonic development or tissue regeneration during wound healing (Liekens et al., 2001). Angiogenesis is a complex process that includes degradation of extracellular matrix, migration and proliferation of endothelial cells, tube formation, and sprouting of new capillary branches (Folkman and Shing, 1992). Each of these processes is tightly regulated by a net balance between positive and negative angiogenic factors under physiological conditions (under reviews, see Liekens et al., 2001; Papetti and Herman, 2002). Yet, once the net balance is tipped toward an angiogenic state, unregulated angiogenesis occurs and causes various diseases, such as cancer, diabetic retinopathy, and rheumatoid arthritis (Folkman, 1995; Carmeliet and Jain, 2000). In fact, angiogenesis and vascular proliferation are particularly important in the progression of malignant gliomas and are used as indicators of the degree of malignancy (Puduvalli, 2004). Therefore, the blocking of angiogenesis is a promising strategy to prevent these diseases.

We have previously reported that an extract of *Atractylodes lancea* rhizome inhibited angiogenesis in adjuvant-induced air pouch granuloma in mice, an in vivo model of chronic inflammation (Kimura et al., 1991b). The rhizome of *A. lancea* contains sesquiterpenoid alcohols, such as β-eudesmol, hinesol, and elemol. β-eudesmol is known to have various unique effects on the nervous system. For example, we have shown that β-eudesmol acts as a channel blocker for nicotinic acetylcholine receptors at the neuromuscular junction (Kimura et al., 1991a,c). In addition,

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doi:10.1016/j.ejphar.2005.02.035

Obara et al. (2002) have recently reported that β-eudesmol induces neurite outgrowth in rat pheochromocytoma PC12 cells via an increase in the intracellular Ca^{2+} concentration. However, the direct effects of β-eudesmol on angiogenesis have not yet been tested. In the present study, we investigated the potential antiangiogenic activities of β-eudesmol in cerebral and peripheral vascular endothelial cells in culture. We also examined the effect of β-eudesmol on angiogenesis in vivo, using the Matrigel plug assay and the adjuvant-induced inflammation model. Furthermore, we investigated whether β-eudesmol affects endothelial cell functions through modification of mitogen-activated protein (MAP) kinase activities.

2. Materials and methods

2.1. Materials

All reagents used were purchased from Sigma (St. Louis, MO, U.S.A.), unless otherwise indicated. β-eudesmol was given by Dr. M. Yoshizaki (Herbal garden, Toyama Medical and Pharmaceutical University, Japan). Recombinant human basic fibroblast growth factor (bFGF) and recombinant human vascular endothelial growth factor (VEGF) were purchased from Pepro Tech EC (London, U.K.). A rabbit anti-p44/p42 MAP kinase antibody, a rabbit anti-phospho-p44/p42 MAP kinase antibody, a rabbit anti-phospho-p38 MAP kinase antibody, and a rabbit anti-phospho-Akt (Ser 473) antibody were purchased from Cell Signaling Technology (Beverly, MA, U.S.A.). A rabbit anti-p38 MAP kinase antibody and a mouse anti-Akt antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). LY294002 was purchased from Calbiochem-Novabiochem (San Diego, CA, U.S.A.).

2.2. Isolation of porcine brain microvessels

We used previously described procedures (Gaillard et al., 2001; Tsuneki et al., 2004) for the isolation of porcine brain microvascular endothelial cells (PBMEC). In brief, fresh porcine brain was obtained from a slaughterhouse (Toyama Meat Center, Toyama, Japan). Gray matter in the cerebrocortical regions was isolated and then homogenized in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS). Microvessel fragments were trapped on 150-μm nylon meshes and digested with collagenase type III (Gibco BRL, Life Technologies, Rockville, MD, U.S.A.), trypsin (Worthington Biochemical, Lakewood, NJ, U.S.A.), and DNase I (Worthington Biochemical) in 10% FBS-containing DMEM for 1 h at 37 °C. After removal of debris using a 200-μm nylon mesh, the microvessel fractions were resuspended in FBS with 10% dimethyl sulfoxide (DMSO) and stored in liquid nitrogen until use.

2.3. Culture of endothelial cells

Porcine cerebrocortical microvessels were seeded in plastic flasks coated with collagen type 1 (BD Biosciences, Bedford, MA, U.S.A.) and fibronectin (Roche, Mannheim, Germany) and allowed to adhere for 5 h in 10% FBS-containing DMEM. The medium was then replaced with a growth medium consisting of a 1:1 mixture of 10% FBS-containing DMEM and 10% FBS-containing rat-astrocyte-conditioned medium (Gaillard et al., 2001) supplemented with heparin (100 unit/ml). The cells growing out from microvessel fragments, mostly endothelial cells, were cultured at 37 °C in 10% CO_2. The primary cultured endothelial cells were harvested at 70% confluence (day 4 or 5 after seeding of microvessels) with trypsin-EDTA solution (Sigma, T4299, 5 U/ml) for 2 min. All of the obtained cells were endothelial cells (passage 1), as previously reported (Tsuneki et al., 2004).

Human dermal microvascular endothelial cells (HDMEC) were purchased from Cell Systems Corp. (Kirkland, WA, U.S.A.), and human umbilical vein endothelial cells (HUVEC) were from Clonetics (Cambrex Bio Science, Walkersville, MD, U.S.A.). The respective cells were cultured in CS-C medium (Cell Systems Corp.) containing 10% FBS and in endothelial cell basal medium-2 (EBM-2, CC-3162, Clonetics) containing 5% FBS, according to the supplier's recommendation.

2.4. Proliferation assay

The endothelial cells were plated at 1.5×10^4 cells per 35-mm-diameter dish coated with collagen type 1 and incubated for 2 h at 37 °C, to allow the cells to adhere. Then, the reagents to be tested, such as β-eudesmol, thalidomide, and PD98059, were added to growth medium (the 1:1 mixture of 10% FBS-containing DMEM and astrocyte-conditioned medium for PBMEC, 10% FBS-containing CS-C for HDMEC, 5% FBS-containing EBM-2 for HUVEC). The medium was exchanged every other day. For counting the cell numbers, all the cells were detached from dishes by treating with trypsin-EDTA solution (Sigma, T4299), and viable cells were distinguished with the use of Trypan blue dye. The number of viable cells was counted using the image analysis software Win Roof (Mitani, Fukui, Japan). For comparison, the growth of rat aortic smooth muscle cells (3×10^4 cells per 24-well plates) and rat astrocytes (1.5×10^4 cells per 35-mm-diameter dish) was also assessed by counting cell numbers. The primary cultured rat smooth muscle cells and the rat cerebrocortical astrocytes (passage 1) were prepared as previously described (Kimura et al., 1992; Gaillard et al., 2001).

2.5. DNA synthesis assay

PBMEC (5×10^3 cells) were cultured in 96-well plates for 24 h, followed by 48 h of serum starvation. Then, the

cells were incubated with growth medium (the 1:1 mixture of 10% FBS-containing DMEM and 10% FBS-containing astrocyte-conditioned medium) containing either 0.1% DMSO (positive control) or β-eudesmol (10–100 μM) for 8 h. As a negative control, cells were incubated with 10% FBS-containing DMEM (in the absence of astrocyte-conditioned medium). 5-Bromo-2′-deoxyuridine (BrdU, 10 μM) was subsequently added, and the cells were further incubated for 4 h. The DNA synthesis assay based on BrdU incorporation was performed by colorimetric analysis with a multi-plate reader (NJ-2300, System Instruments, Tokyo, Japan) and an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (Cell Proliferation ELISA kit, Roche, Mannheim, Germany).

2.6. Endothelial cell migration assay

HUVEC migration was assayed as described previously (Tang et al., 1997; Kim et al., 2003). Confluent cells in 35-mm-diameter dishes were damaged by scraping with a sterile pipette tip (500 μm in diameter). The cultures were washed twice with phosphate-buffered saline to remove cellular debris. The migration of HUVEC was induced by bFGF (30 ng/ml) in 1% FBS-containing EBM-2 medium with 0.1% DMSO (positive control) or β-eudesmol (10–100 μM) at 37 °C under 5% CO_2. As a negative control, cells were incubated in 1% FBS-containing EBM-2 medium with 0.1% DMSO (vehicle) in the absence of bFGF. After 18 h, the cells that had migrated into the denuded area were photographed with a CCD camera, and their numbers were counted.

2.7. Cell adhesion assay

HUVEC adhesion was assayed as described previously (Wood et al., 2002). Culture dishes 35 mm in diameter were coated with fibronectin solution (20 μg/ml) for 2 h. HUVEC were treated with β-eudesmol (50 and 100 μM) or vehicle alone (0.1% DMSO) in 5% FBS-containing EBM-2 medium in plastic flasks. After 24 h, cells were harvested and counted. Then, 5×10^4 viable cells per dish were incubated at 37 °C for 90 min in the precoated dishes. Non-attached cells were removed by washing with phosphate-buffered saline. The number of attached cells was determined as described for the proliferation assay.

2.8. Tube formation assay

The tube formation assays were performed on 24-well plates coated with 300 μl of Matrigel basement membrane matrix (BD Biosciences) per well and polymerized at 37 °C for 30 min. HUVEC were suspended in 5% FBS-containing EBM-2 medium. Cells were plated on Matrigel at a density of 8×10^4 cells per well, and β-eudesmol was added to the culture medium. After 18 h, four fields were randomly selected from each culture and photographed with a CCD camera. Tube length was measured on the digitized photograph as pixel length, using image analysis software (Win Roof).

2.9. Western blotting

Phosphorylation of MAP kinases (p38, p42 and p44) and Akt was analyzed by Western blotting as previously described (Sasaoka et al., 1996). In experiments to analyze MAP kinase phosphorylation, PBMEC and HUVEC were serum-starved for 48 h at 60–70% confluence, and then the cells were treated with β-eudesmol for 3 h, and stimulated with bFGF or VEGF for 10 min. In experiments to analyze Akt phosphorylation, HUVEC were serum-starved for 24 h at confluence, and then the cells were treated with β-eudesmol for 3 h, and stimulated with bFGF for 30 min. Subsequently, the cells were solubilized and centrifuged. Proteins in the supernatant were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. The membranes were treated with the antibodies specified and then incubated with horseradish peroxidase-conjugated secondary antibody (donkey anti-rabbit immunoglobulin or sheep anti-mouse immunoglobulin, Amersham-Pharmacia Biotech, Piscataway, NJ, U.S.A.). Blots were detected using ECL enhanced chemiluminescence kit (Amersham). The density of protein bands was quantified with Fluor-S Multi-Imager (Bio-Rad, Hercules, CA, U.S.A.). The relative phosphorylation of MAP kinase is presented as the ratio of the amount of phosphorylated MAP kinase to that of total MAP kinase in each sample.

2.10. Matrigel plug assay

The in vivo Matrigel angiogenesis model described by Passaniti et al. (1992) was used with some modifications. Briefly, cold Matrigel (400 μl, Phenol-Red free, BD Bioscience) was mixed with bFGF (600 ng/ml), heparin (25 units/ml), and the reagent to be tested (β-eudesmol or thalidomide at a final concentration of 300 μM). In vehicle controls, 0.3% DMSO was used instead of the test reagent. Matrigel mixture was injected subcutaneously into the flank of 6-week-old male mice C57BL/6J (Japan SLC, Hamamatsu, Japan). Seven days after injection, the animals were killed under halothane anesthesia, and the Matrigel pellets were collected. The pellets were then minced and solubilized with Cell Recovery solution (BD Bioscience). The amount of hemoglobin was determined using Drabkin's solution according to the manufacturer's instructions (Sigma).

2.11. Adjuvant-induced pouch granuloma angiogenesis

Male ddY mice (Japan SLC) were housed in a temperature- and light-controlled environment and were allowed

free access to food and water. Air pouch granuloma in mice (6 weeks old, 30–34 g) was prepared by the injection of Freund's complete adjuvant, as previously reported (Kobayashi et al., 1998). Either β-eudesmol or hydrocortisone was suspended in normal saline containing 1% Avicel (Asahi Chemical, Tokyo, Japan) and injected intraperitoneally 2 h after the adjuvant injection, then once a day for 4 days. Mice were anesthetized with ether, injected with carmine solution (Merck, Darmstadt, Germany) containing gelatin, and killed under ether anesthesia. Then, the granuloma tissues were isolated and solubilized. The carmine content, an index of newly formed blood vessels in pouch granuloma (Kobayashi et al., 1998), was determined by measuring the optical intensity at 490 nm.

All procedures shown above were approved by the Toyama Medical and Pharmaceutical University Animal Research Committee, and according to the Guidelines for Animal Experiments established by the Japanese Pharmacological Society.

2.12. Data analysis

To calculate the 50% inhibitory concentrations (IC_{50}) of β-eudesmol, concentration–inhibition curves for β-eudesmol (antagonist) were fitted by nonlinear regression to the equation: $Y = Y_{max} - Y_{max}/[1+(IC_{50}/X)^{nH}]$, where Y is the fraction of the remaining response, X is the antagonist concentration, and nH is the Hill coefficient (not shown). Y_{min} was constrained to 0, and Y_{max} was constrained to 1 for the antagonist efficacy curves. In the proliferation assay, Y_{min} was defined as the number of cells plated onto culture dishes at day 0, and Y_{max} was defined as the number of cells in vehicle controls at each day. In the migration assay, Y_{min} and Y_{max} were defined as the number of migrated cells in the negative and positive control, respectively. In the tube formation assay, data are expressed as percentages of the mean total tube length in vehicle controls, and the values were directly used for this analysis. Curve fitting was analyzed using Prism software (GraphPad Software, Inc., San Diego, CA, U.S.A.).

The significance of differences between two groups was assessed by *t*-test, and the differences between multiple groups were assessed by one-way analysis of variance (ANOVA), followed by the Scheffé's multiple range test. Values of P less than 0.05 were considered to be significant.

3. Results

3.1. Inhibitory effect of β-eudesmol on endothelial cell proliferation

The number of PBMEC (passage 1) in dishes increased time dependently throughout the observation period, from day 4 to day 10 in culture (Fig. 1A). When PBMEC were treated with β-eudesmol (50 and 100 μM), the time-dependent increase in cell number was significantly suppressed. On day 10, the IC_{50} value was 53.3 μM (95% confidence intervals (CI), 48.9–58.1 μM). In Fig. 1B, the concentration–response relationship was examined on day 6 in culture. Cell numbers in vehicle controls (treated with

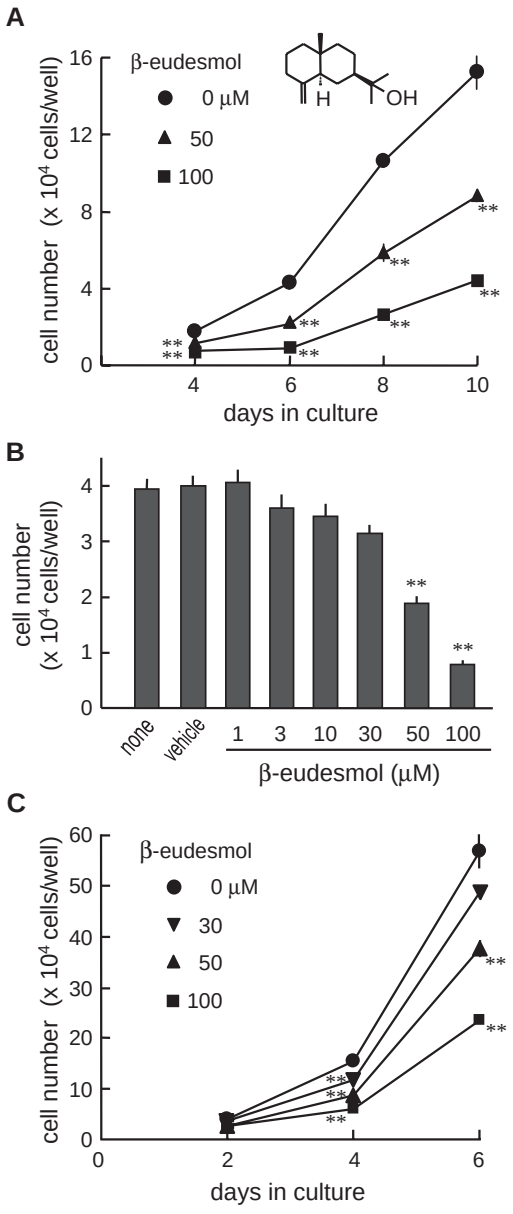

Fig. 1. Inhibitory effect of β-eudesmol on the proliferation of microvascular endothelial cells. Porcine cerebrocortical microvascular endothelial cells (PBMEC) and human dermal microvascular endothelial cells (HDMEC) were plated at 1.5×10^4 cells per dish in the absence and presence of β-eudesmol (1–100 μM). (A) Time-dependent increase in the number of PBMEC in the presence of β-eudesmol at the indicated concentrations. The inset shows the chemical structure of β-eudesmol. (B) Concentration-dependent inhibition by β-eudesmol of proliferation, assessed by the number of PBMEC on day 6 in culture. As controls, PBMEC were treated without (none) or with 0.1% DMSO (vehicle). (C) Time-dependent increase in the number of HDMEC in the presence of β-eudesmol at the indicated concentrations. Data are means ± S.E.M., $n = 4$–5 per group. $^{**}P < 0.01$ vs. vehicle control.

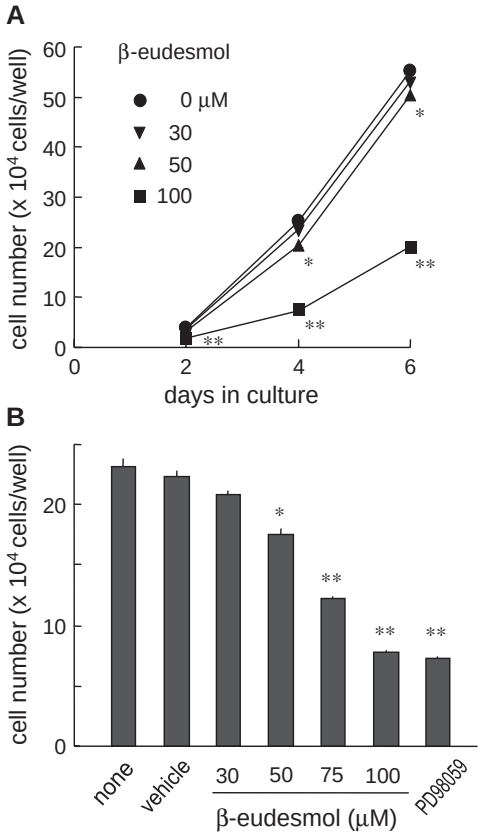

Fig. 2. Inhibitory effect of β-eudesmol on the proliferation of human umbilical endothelial cells (HUVEC). HUVEC (1.5×10^4 cells per dish) were cultured in the absence and presence of β-eudesmol (30–100 μM). (A) Time-dependent increase in the number of HUVEC in the presence of β-eudesmol at the indicated concentrations. (B) Concentration-dependent inhibition by β-eudesmol of proliferation, assessed by the number of HUVEC on day 4 in culture. As negative controls, HUVEC were treated without (none) or with 0.1% DMSO (vehicle). As a positive control, cells were treated with PD98059 (20 μM). Data are means ± S.E.M., $n=4$ per group. *$P<0.05$ and **$P<0.01$ vs. vehicle control.

0.1% DMSO) were not different from those in non-treated controls. Cell numbers tended to decrease in the presence of β-eudesmol at concentrations between 3 and 30 μM, and significantly decreased at higher concentrations (50 and 100 μM). We also examined the effect of β-eudesmol on the proliferation of another type of microvascular endothelial cell, HDMEC (Fig. 1C). Cell numbers significantly decreased in the presence of β-eudesmol (50 and 100 μM), compared with those in vehicle controls, both on day 4 and day 6 in culture. The IC_{50} of β-eudesmol determined on day 6 was 76.8 μM (95% CI, 70.6–83.5 μM).

The number of HUVEC increased time dependently throughout the observation period, from day 2 to day 6 in culture (Fig. 2A). Cell numbers significantly decreased in the presence of β-eudesmol (50 and 100 μM) on day 4 and day 6, compared with those in the absence of β-eudesmol. In Fig. 2B, the effect of β-eudesmol was investigated on day 4 in culture. The vehicle (0.1% DMSO) treatment did not affect HUVEC proliferation. β-eudesmol (50–100 μM) significantly inhibited the proliferation of HUVEC in a concentration-dependent manner. The IC_{50} was 75.7 μM (95% CI, 73.4–78.1 μM). As a positive control, we examined the effect of PD98059, an inhibitor of MAP kinase kinase (MEK, an upstream regulator of extracellular signal-regulated kinase [ERK]), on HUVEC proliferation. PD98059 (20 μM) strongly inhibited cell proliferation on day 4 in culture. The extent of inhibition by β-eudesmol (100 μM) was comparable to that induced by PD98059 (20 μM). We also used a well-established antiangiogenic compound, thalidomide (Liekens et al., 2001), as a positive control. It has been reported that thalidomide (10–30 μM) inhibits HUVEC proliferation in 10% FBS-containing M199 medium (Vacca et al., 2003), whereas the proliferation of HUVEC grown in large-vessel endothelial cell-basal medium with a cell growth supplement is unaffected by thalidomide (194 μM) (Dredge et al., 2002). Under the present conditions, thalidomide (10–200 μM) had no effect on the proliferation of HUVEC (data not shown).

We next investigated whether β-eudesmol affects the proliferation of vascular smooth muscle cells. The number of rat aortic smooth muscle cells increased time dependently and nearly reached confluence on day 6 under the present culture conditions (Fig. 3). The number of smooth muscle cells in the presence of β-eudesmol (50 and 100 μM) was not significantly different from that in the absence of β-eudesmol. Thus, β-eudesmol had no effect on the proliferation activity of the smooth muscle cells at these concentrations. When PD98059 (20 μM) was applied as a positive control, the number of smooth muscle cells did not differ from that in the control dishes up to day 4 in culture, and the significant differences were observed only in the confluent state (Fig. 3).

We further examined the effect of β-eudesmol on the proliferation of astrocytes isolated from rat cerebral cortex. Astrocytes plated at 1.5×10^4 cells per 35-mm-diameter dish

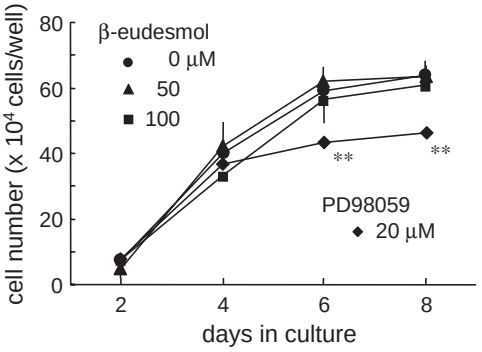

Fig. 3. No effect of β-eudesmol on the proliferation of rat aortic smooth muscle cells. The smooth muscle cells (3×10^4 cells per well) were cultured in 10% FBS-containing DMEM. Graph shows time-dependent increase in the number of smooth muscle cells in the presence of β-eudesmol at the indicated concentrations. As a positive control, the cells were treated with PD98059 (20 μM). All medium used contained 0.1% DMSO. Data are means ± S.E.M., $n=6$ per group. **$P<0.01$ vs. vehicle control.

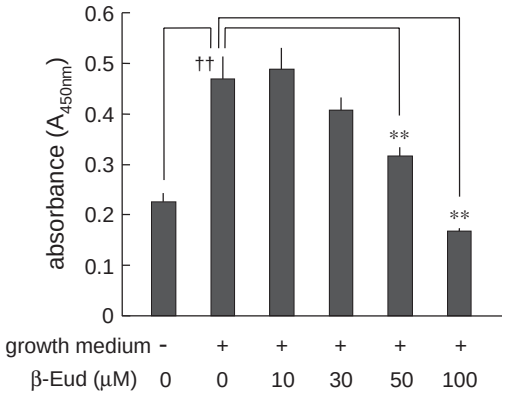

Fig. 4. β-eudesmol suppressed the DNA synthesis stimulated by growth medium in PBMEC. The PBMEC (5×10^3 cells per well) were serum-starved and then incubated with growth medium (the 1:1 mixture of 10% FBS-containing DMEM and 10% FBS-containing rat-astrocyte-conditioned medium) including either 0.1% DMSO (positive control) or β-eudesmol (β-Eud, 10–100 μM) for 8 h. As a negative control, cells were incubated with 10% FBS-containing DMEM, instead of the growth medium. Then, BrdU incorporation was measured, as an index of DNA synthesis activity. Data are means ± S.E.M., $n=6$ per group. $^{††}P<0.01$ vs. vehicle control. $^{**}P<0.01$ vs. positive control.

grew to 60% confluency for 6 days. Cell numbers in vehicle controls (0.1% DMSO: $46.3 \times 10^3 \pm 0.8 \times 10^3$, $n=4$) did not significantly differ from those in the presence of β-eudesmol (50 μM: $47.0 \times 10^3 \pm 1.1 \times 10^3$, $n=4$; 100 μM: $44.0 \times 10^3 \pm 2.2 \times 10^3$, $n=4$).

3.2. No cytotoxic effect of β-eudesmol on endothelial cells

PBMEC were incubated with β-eudesmol (50 and 100 μM) in the growth medium for 6 days, and then the cells were harvested and treated with Trypan blue to calculate the numbers of viable cells and dead cells. The proportion of viable cells was 90.1 ± 0.9% ($n=6$) in vehicle controls (treated with 0.1% DMSO), whereas that in the presence of 50 and 100 μM β-eudesmol was 91.7 ± 2.7% ($n=6$) and 92.1 ± 3.3% ($n=6$), respectively. These results indicate that β-eudesmol did not affect the proportion of viable endothelial cells.

3.3. Suppression of DNA synthesis in endothelial cells by β-eudesmol

The influence of β-eudesmol on DNA synthesis stimulated with growth medium was investigated in PBMEC. BrdU incorporation was measured as an index of DNA synthesis. BrdU incorporation was significantly increased by the growth medium, compared with the basal level obtained by treating with 10% FBS-containing DMEM (in the absence of astrocyte-conditioned medium) (Fig. 4). In the presence of β-eudesmol (50 and 100 μM), the growth medium-induced increase in BrdU incorporation was significantly reduced. In particular, β-eudesmol at 100 μM decreased BrdU incorporation to the basal level.

3.4. Inhibitory effect of β-eudesmol on endothelial cell migration

The migration of HUVEC was observed using a denudation injury model in confluent cell cultures. Scrape-damaged HUVEC monolayers were incubated with bFGF (30 ng/ml) in the absence or presence of β-eudesmol (10–100 μM) for 18 h. The migration of HUVEC into the denuded area was promoted by stimulation with bFGF, compared with non-stimulated control (Fig. 5A), resulting in a 5.9-fold increase in cell numbers within the denuded area (Fig. 5B). β-eudesmol (50 and 100 μM) significantly inhibited the bFGF-stimulated migration of HUVEC in a concentration-dependent manner (Fig. 5A,B). The IC_{50} of β-eudesmol was 43.3 μM (95% CI, 38.5–48.7 μM). Under

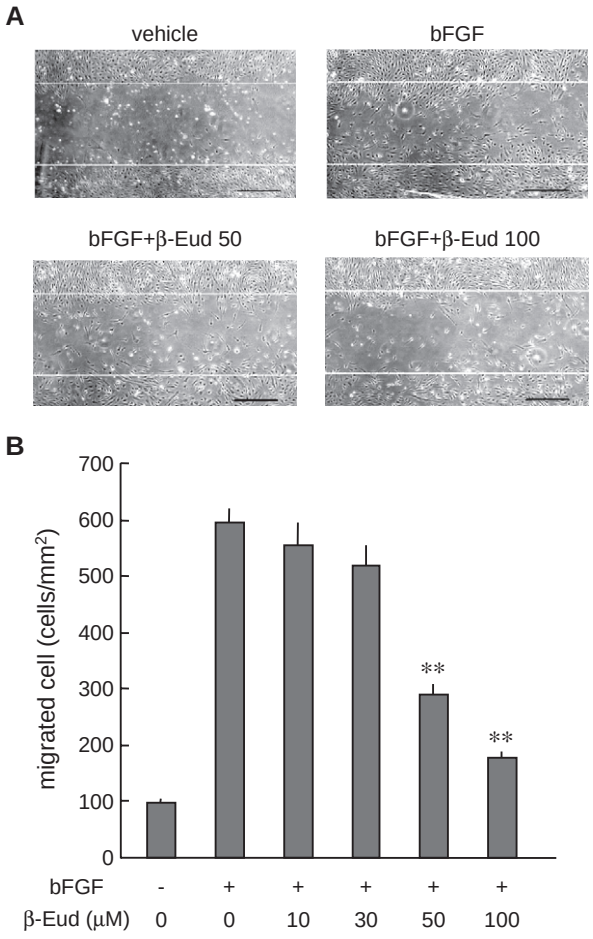

Fig. 5. Inhibitory effect of β-eudesmol on the bFGF-stimulated migration of HUVEC. The HUVEC was stimulated with bFGF (30 ng/ml) with either 0.1% DMSO (positive control) or β-eudesmol (10–100 μM) for 18 h. As a negative control, cells were incubated with 0.1% DMSO (vehicle) alone. (A) Photomicrographs showing HUVEC migration into the denuded area in the absence and presence of β-eudesmol (β-Eud, 50 and 100 μM). The zone between two lines indicates the area occupied by the initial wound. Scale bars=250 μm. The data are representative of six independent experiments. (B) The number of migrated cells in the absence and presence of β-eudesmol. Data are means ± S.E.M., $n=6$ per group. $^{**}P<0.01$ vs. the number of migrated cells in the positive control.

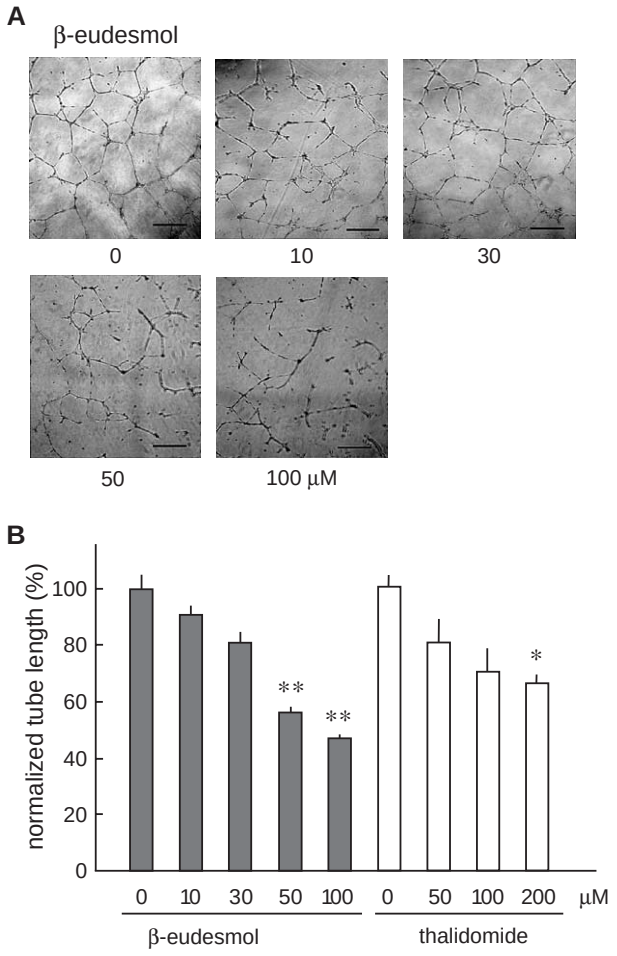

Fig. 6. Inhibitory effect of β-eudesmol on tube formation by HUVEC. HUVEC (8×10^4 cells per well) were seeded on a Matrigel-coated plate with or without β-eudesmol (10–100 μM). As a positive control, HUVEC were treated with thalidomide (50–200 μM). All medium used contained 0.1% DMSO (vehicle). (A) Typical images of tubules in the absence and presence of β-eudesmol at the indicated concentrations. Scale bars=250 μm. (B) Influence of β-eudesmol and thalidomide on the tube formation by HUVEC in Matrigel. Total tube length was measured on the digitized photograph as pixel length, and each value was normalized to the mean total length in the vehicle control and then averaged. Data are means ± S.E.M., n=4–6 per group. *P<0.05 and **P<0.01 vs. each vehicle control.

the present conditions, thalidomide (10–100 μM) had no effect on the migration of HUVEC (data not shown).

3.5. Little effect of β-eudesmol on endothelial cell adhesion to extracellular matrix molecule

The adhesion of HUVEC to culture dishes coated with fibronectin was observed, as described previously (Wood et al., 2002). In fact, no significant influence of β-eudesmol (50 and 100 μM) on cell attachment was observed: the number of attached cells was $46.8 \times 10^3 \pm 5.4 \times 10^3$ (n=6) in vehicle controls, whereas the number was $40.8 \times 10^3 \pm 6.2 \times 10^3$ (n=6) and $41.5 \times 10^3 \pm 2.1 \times 10^3$ (n=6) in the presence of β-eudesmol (50 and 100 μM), respectively.

3.6. Inhibitory effect of β-eudesmol on tube formation by endothelial cells

HUVEC that were plated on Matrigel formed a tubular network within 18 h, as evidenced by morphological changes (Fig. 6A). Treatment with β-eudesmol (50 and 100 μM) strongly prevented tube formation, as shown in Fig. 6A. In fact, the total length of the tubules was significantly decreased by this treatment (Fig. 6B): β-eudesmol at 100 μM decreased the mean total tube length by 53% of the vehicle control. The IC_{50} was 81.1 μM (95% CI, 66.2–99.5 μM). As a positive control, the effect of thalidomide was examined on tube formation by HUVEC

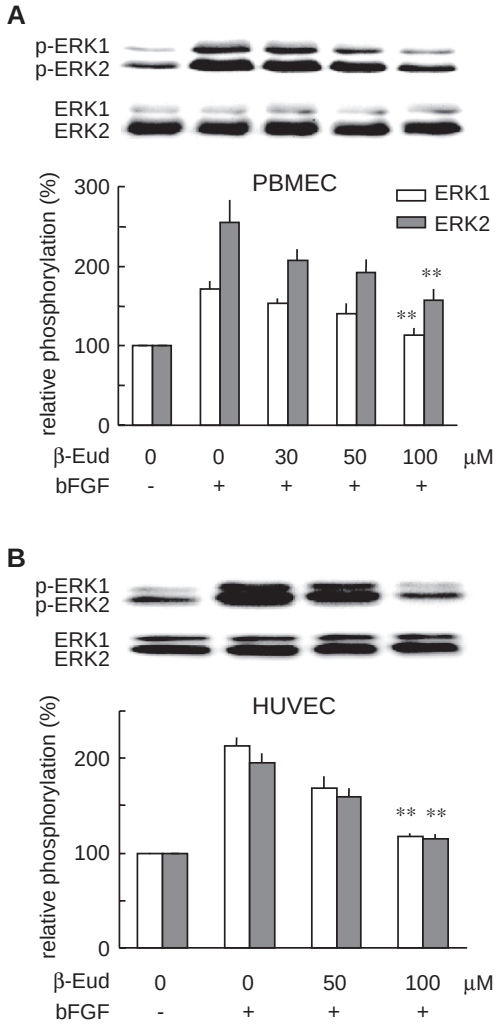

Fig. 7. β-eudesmol blocks bFGF-induced ERK1/2 activation in endothelial cells. PBMEC and HUVEC were preincubated with β-eudesmol (β-Eud, 0–100 μM) for 3 h and then stimulated with bFGF (30 ng/ml) for 10 min. (A,B) The phosphorylation and expression of ERK1/2 in PBMEC (A) and HUVEC (B) were analyzed by Western blotting. Blots shown in panels A and B are representative of six and four independent experiments, respectively. The histograms represent the relative level of ERK1/2 phosphorylation, as determined by densitometric analysis. Data are means ± S.E.M., n=4–6 per group. **P<0.01 vs. phosphorylated ERK levels increased by bFGF in the absence of β-eudesmol (positive control).

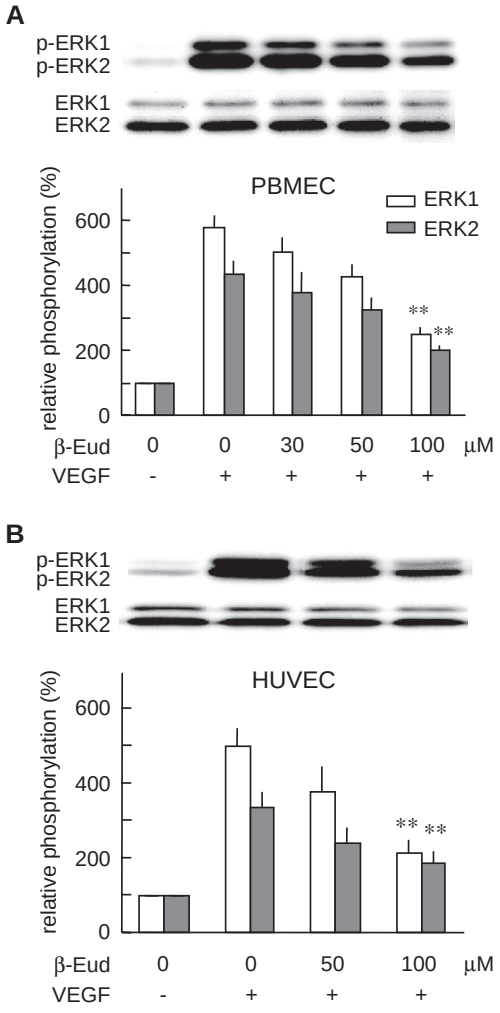

Fig. 8. β-eudesmol blocks VEGF-induced ERK1/2 activation in endothelial cells. PBMEC and HUVEC were preincubated with β-eudesmol (β-Eud, 0–100 μM) for 3 h and then stimulated with VEGF (10 ng/ml) for 10 min. (A,B) The phosphorylation and expression of ERK1/2 in PBMEC (A) and HUVEC (B) were analyzed by Western blotting. Blots shown are representative of four independent experiments. The histograms represent the relative intensity of ERK1/2 phosphorylation, as determined by densitometric analysis. Data are means ± S.E.M., $n=4$ per group. $^{**}P<0.01$ vs. phosphorylated ERK levels increased by VEGF in the absence of β-eudesmol (positive control).

in Matrigel. Thalidomide (50–200 μM) reduced tube formation in a concentration-dependent manner (Fig. 6B). The magnitude of the reduction produced by thalidomide was lower than that produced by β-eudesmol, because thalidomide reduced tube formation by only 34% at the maximal concentration used (200 μM).

3.7. Inhibition of MAP kinase activities by β-eudesmol in endothelial cells

The influence of β-eudesmol on MAP kinase phosphorylation was investigated in serum-starved PBMEC. Both ERK1 (p44 MAP kinase) and ERK2 (p42 MAP kinase) were markedly phosphorylated 10 min after stimulation with bFGF (30 ng/ml), as a positive control. If the cells were pretreated with β-eudesmol (30–100 μM) for 3 h, the bFGF stimulation of ERK1/2 phosphorylation was blocked, while total ERK1/2 expression was not affected (Fig. 7A). A significant reduction of the relative phosphorylation levels of ERK1 and ERK2 was obtained in the presence of β-eudesmol (100 μM). Similarly, in the serum-starved HUVEC, β-eudesmol (100 μM) significantly reduced the relative phosphorylation levels of ERK1/2 stimulated by bFGF (30 ng/ml), without affecting ERK1/2 expression (Fig. 7B).

The phosphorylation of ERK1 and ERK2 was induced by VEGF (10 ng/ml) in PBMEC, as another positive control. Pretreatment with β-eudesmol (30–100 μM) for 3 h blocked ERK1/2 phosphorylation, while total ERK1/2 expression was not affected (Fig. 8A). A significant reduction in the relative phosphorylation levels of ERK1 and ERK2 was observed in the presence of β-eudesmol (100 μM). A similar blockade of VEGF-stimulated ERK1/2 phosphorylation was observed in HUVEC (Fig. 8B), and the blocking effect of β-eudesmol (100 μM) was comparable to that of PD98059 (20 μM) (data not shown). In the rat aortic smooth muscle cells, however, neither β-eudesmol (100 μM) nor PD98059 (20 μM) affected the relative phosphorylation levels of ERK1/2 stimulated by bFGF (30 ng/ml) (data not shown).

Phosphorylation of p38 MAP kinase was also induced by VEGF (10 ng/ml) in serum-starved HUVEC. β-eudesmol (50 and 100 μM) had no effect on VEGF-induced phosphorylation (data not shown).

Phosphorylation of Akt (also known as protein kinase B) was observed after treatment with bFGF (30 ng/ml) for 30 min in confluent, serum-starved HUVEC. Pretreatment with β-eudesmol (30, 50 and 100 μM) for 3 h did not affect bFGF-induced Akt phosphorylation, whereas Akt phosphor-

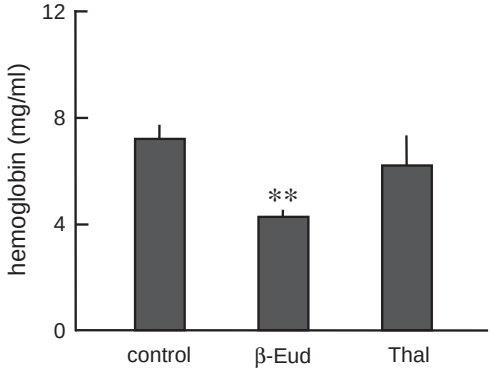

Fig. 9. Inhibitory effect of β-eudesmol on angiogenesis in subcutaneously implanted Matrigel plugs in mice. β-eudesmol (β-Eud), thalidomide (Thal), or vehicle (DMSO) was added to a mixture containing bFGF, heparin, and Matrigel. This was then injected into C57BL/6J mice. Seven days after injection, the hemoglobin content in the Matrigel pellets was measured to estimate the degree of neovascularization. Data are means ± S.E.M. The numbers for vehicle controls, β-Eud and Thal were 8, 9, and 4, respectively. $^{**}P<0.01$ vs. vehicle controls.

Table 1
Effects of β-eudesmol and hydrocortisone on angiogenesis in adjuvant-induced granuloma in mice

Drug	Dose (μmol/kg)	*n*	Carmine content (mg)	Inhibition (%)
Control	0	4	0.399 ± 0.020	
hydrocortisone	9.3	4	0.264 ± 0.023[a]	33.8
	18.5	4	0.164 ± 0.028[a]	58.9
Control	0	3	0.366 ± 0.027	
β-eudesmol	0.45	3	0.297 ± 0.035	18.9
	0.90	3	0.200 ± 0.028[a]	45.4

The carmine content is an index of newly formed blood vessels in pouch granuloma. Data are means ± S.E.M. *n* = number of data. The extent of inhibition is expressed as a percentage of the carmine content in each control.

[a] $P < 0.01$ vs. control.

ylation was completely blocked by an inhibitor of phosphoinositide 3-kinase, LY294002 (10 μM) (data not shown).

3.8. Antiangiogenic effects of β-eudesmol in vivo

The effect of β-eudesmol on angiogenesis in vivo was examined in the Matrigel plug assay. Seven days after subcutaneous injection of Matrigel (400 μl) containing bFGF and heparin into mice, the hemoglobin content in the Matrigel pellets was measured to assess the degree of vascularization. As shown in Fig. 9, β-eudesmol significantly reduced the hemoglobin content from control levels when added to the Matrigel at a final concentration of 300 μM, whereas thalidomide (300 μM) failed to affect vascularization. These results demonstrated that β-eudesmol is more potent than thalidomide in inhibiting vascularization in Matrigel pellets implanted in mice.

Angiogenesis in vivo was observed in adjuvant-induced granuloma in mice, by measuring the carmine content. When hydrocortisone, an anti-inflammatory drug, was intraperitoneally injected (9.3 and 18.5 μmol/kg, once a day for 5 days) as a positive control, the carmine content in the granuloma tissues was significantly reduced (Table 1). Intraperitoneal injection of β-eudesmol (0.90 μmol/kg) also caused a significant reduction in the carmine content, comparable to that obtained with hydrocortisone at the doses used.

4. Discussion

Most vascular endothelial cells are quiescent under physiological conditions but are switched to an angiogenic phenotype by both increased production of positive angiogenic factors and decreased production of negative regulators (Folkman, 1995; Carmeliet and Jain, 2000). Abnormal angiogenesis is implicated in various diseases including brain tumors and other cancers, so that several antiangiogenic agents are currently being investigated in clinical trials for cancer therapy (Liekens et al., 2001; Bikfalvi and Bicknell, 2002; Puduvalli, 2004). The present study shows, for the first time to our knowledge, that β-eudesmol is a novel potent antiangiogenic agent and acts by influencing the proliferation, migration, and differentiation of endothelial cells.

Since endothelial cells from different sites of the vasculature exhibit different properties (Thorin et al., 1997), the effect of β-eudesmol was examined in three types of endothelial cells in the present study, i.e. PBMEC derived from cerebral microvessels, HDMEC derived from peripheral microvessels, and HUVEC derived from peripheral veins. Here, we demonstrated that β-eudesmol inhibited the proliferation of these endothelial cells. These inhibitory effects are likely to be mediated by the suppression of DNA synthesis, because β-eudesmol inhibited growth medium-stimulated DNA synthesis in PBMEC. Furthermore, β-eudesmol exhibited inhibitory effects on bFGF-stimulated HUVEC migration and on tube formation by HUVEC. These versatile actions of β-eudesmol are not necessarily surprising, given that several endogenous growth factors, such as bFGF and VEGF, can individually stimulate multiple processes of angiogenesis (Liekens et al., 2001). It is therefore anticipated that β-eudesmol shows a broad spectrum of antiangiogenic effects by blocking growth factor signaling in endothelial cells.

MAP kinase signaling plays crucial roles in the regulation of angiogenesis, e.g. stimulation of endothelial cell proliferation, migration (Klemke et al., 1997), tube formation (Maru et al., 1998), and expression of matrix metalloproteinase-9 (Gum et al., 1997). We found that both bFGF-induced and VEGF-induced activation of ERK1/2 were blocked by β-eudesmol in endothelial cells, whereas β-eudesmol had no effect on the activation of p38 MAP kinase. The concentrations needed for inhibition of ERKs were similar to those for inhibiting endothelial cell proliferation, migration, and tube formation. Therefore, the β-eudesmol-induced inhibition of angiogenesis may be, at least in part, due to blockade of the ERK signaling pathway.

The concentrations of PD98059 needed to inhibit ERK signaling are known to differ among different cell types. In this study, we observed that PD98059 (20 μM) was less effective against proliferation and ERK phosphorylation in rat aortic smooth muscle cells than in endothelial cells. Because β-eudesmol (100 μM) did not affect these parameters in the smooth muscle cells, and because β-eudesmol (100 μM) had no effect on the proliferation of rat astrocytes, we consider that the sensitivity of β-eudesmol to ERK signaling also depends on the cell type used. Although β-eudesmol appears to cause fairly selective blockade of endothelial cell function, β-eudesmol is not completely specific to endothelial cells, since we have observed that β-eudesmol (50–100 μM) inhibits the proliferation of SGC-7901 and HeLa tumor cells (unpublished data). It has also been shown that β-eudesmol at higher concentrations (100–150 μM) reduces the proliferation of rat pheochromocytoma PC12 cells, followed by promotion of neurite outgrowth

(Obara et al., 2002). In this study, we could not exclude the possibility that β-eudesmol indirectly affects ERK signaling through an interaction with some protein kinases and/or phosphatases. Further investigations are required to determine the site of action of β-eudesmol and to reveal the precise mechanism by which it inhibits endothelial cell function in the angiogenic processes.

The rhizome of *A. lancea* has been reported to inhibit angiogenesis in adjuvant-induced granuloma in mice (Kimura et al., 1991b). Using the same model, we demonstrated that β-eudesmol, a component of this rhizome, potently inhibited granuloma angiogenesis. Interestingly, β-eudesmol (0.90 μmol/kg) and hydrocortisone (9.3 μmol/kg) inhibited in vivo angiogenesis almost equally, indicating that the potency of β-eudesmol was about 10 times higher than that of hydrocortisone at these doses. Moreover, we observed that β-eudesmol significantly inhibited angiogenesis in subcutaneously implanted Matrigel plugs in mice. These results strongly suggest that β-eudesmol has a direct effect on angiogenesis in vivo.

In conclusion, we demonstrated that β-eudesmol inhibits angiogenesis both in vitro and in vivo. It should be noted that β-eudesmol was more potent than thalidomide, a well-established antiangiogenic compound, in inhibiting in vitro cell proliferation, migration, tube formation, and in vivo Matrigel angiogenesis. We therefore consider that β-eudesmol may aid the development of therapeutic drugs for angiogenic diseases.

Acknowledgments

We thank M. Terasawa and T. Komada for their technical assistance. This work was supported by Smoking Research Foundation (Japan) (to I.K.) and by Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan (14771274 to H.T, 15590074 to S.K.).

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