

Original Article

Vaccinium myrtillus* (Bilberry) Extracts Reduce Angiogenesis In Vitro and In Vivo*Nozomu Matsunaga¹, Yuichi Chikaraishi¹, Masamitsu Shimazawa¹, Shigeru Yokota² and Hideaki Hara¹**

¹Department of Biofunctional Evaluation, Molecular Pharmacology, Gifu Pharmaceutical University, 5-6-1 Mitahora-higashi, Gifu 502-8585 and ²Wakasa Seikatsu Co. Ltd, 22 Naginataboko-cho, Shijo-Karasuma, Shimogyo-ku, Kyoto 600-8008, Japan

Vaccinium myrtillus (Bilberry) extracts (VME) were tested for effects on angiogenesis *in vitro* and *in vivo*. VME (0.3–30 µg ml⁻¹) and GM6001 (0.1–100 µM; a matrix metalloproteinase inhibitor) concentration-dependently inhibited both tube formation and migration of human umbilical vein endothelial cells (HUVECs) induced by vascular endothelial growth factor-A (VEGF-A). In addition, VME inhibited VEGF-A-induced proliferation of HUVECs. VME inhibited VEGF-A-induced phosphorylations of extracellular signal-regulated kinase 1/2 (ERK 1/2) and serine/threonine protein kinase family protein kinase B (Akt), but not that of phospholipase C γ (PLC γ). In an *in vivo* assay, intravitreal administration of VME inhibited the formation of neovascular tufts during oxygen-induced retinopathy in mice. Thus, VME inhibited angiogenesis both *in vitro* and *in vivo*, presumably by inhibiting the phosphorylations of ERK 1/2 and Akt. These findings indicate that VME may be effective against retinal diseases involving angiogenesis, providing it can reach the retina after its administration. Further investigations will be needed to clarify the major angiogenesis-modulating constituent(s) of VME.

Keywords: angiogenesis – VEGF – Bilberry (*Vaccinium myrtillus*) extraction – GM6001 – MMP – antioxidant – ERK 1/2 – PLC γ – Akt

Introduction

Angiogenesis is the process by which blood vessels are formed from pre-existing ones. In adults, physiological angiogenesis is observed only at restricted sites, such as the endometrium and ovarian follicle, and it is normally transient. However, abnormal angiogenesis causes many ocular diseases, such as diabetic retinopathy (1), age-related macular degeneration (2) and neovascular glaucoma (3). Previous studies have revealed that angiogenesis is explicitly increased by several growth

factors, such as VEGF (4), basic fibroblast growth factor (5) and platelet-derived growth factor (6).

Galardy *et al.* (7) reported that a carcinoma extract implanted in the rat cornea can be used to stimulate angiogenesis from the vessels of the limbus, and also that continuous administration of GM6001, a broad-spectrum matrix metalloproteinase (MMP) inhibitor, reduced both the vessel number and vessel area. More recently, Koike *et al.* (8) found that GM6001 decreases tubulogenesis in microvascular endothelial cells from young humans. These findings suggest that MMP plays a pivotal role in angiogenesis, and that MMP inhibitors may be effective angiostatic agents.

Vaccinium myrtillus (Bilberry), a member of the Ericaceous family, can be found in the mountains and forests of Europe and North America. *Vaccinium myrtillus* extracts (VME) containing 15 different

For reprints and all correspondence: Professor H. Hara, PhD, Department of Biofunctional Evaluation, Molecular Pharmacology, Gifu Pharmaceutical University, 5-6-1 Mitahora-higashi, Gifu 502-8585, Japan. Tel: +81-58-237-8596; Fax: +81-58-237-8596; E-mail: hidehara@gifu-pu.ac.jp

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anthocyanins (9,10) have been shown to possess potent antioxidant properties (9), stabilize collagen fibers and promote collagen biosynthesis (11) and inhibit platelet aggregation (12). Animal studies have demonstrated VME to be of benefit in improving vascular tone, blood flow and vasoprotection (13,14). When administered to healthy subjects or to patients with visual disorders, VME (either alone or in combination with β -carotene and vitamin E) induces a significant improvement in night vision, a quicker adaptation to darkness and a more rapid restoration of visual acuity following exposure to a flash of bright light (11). Hence, bilberries (or VME) have been utilized as a popular edible aid or supplement for asthenopia and improved visual function. Furthermore, an extract of *V. myrtillus* fruits (a low concentration of anthocyanosides in a highly purified extract) has been reported to induce significant improvements in ophthalmoscopic and angiographic images in diabetic or hypertensive patients (15), but it has remained unclear whether it inhibits angiogenesis.

Roy *et al.* (16) noted that in the human keratinocytes cell-line HaCaT, VEGF expression is decreased by a variety of berry seeds, such as bilberry, raspberry, strawberry, blueberry and optiberry (a blend of wild blueberry, strawberry, cranberry and raspberry seeds, and elderberry and wild bilberry samples). They also observed that optiberry inhibits the tube formation among human microvascular endothelial cells induced by basement proteins from mouse tumors. These findings suggest that certain berry seeds have inhibitory actions against angiogenesis, although, the precise mechanism remains unclear. We therefore examined the *in vitro* effects of VME on the angiogenesis (tube formation, and cell proliferation and migration) and phosphorylation of extracellular signal-regulated kinase 1/2 (ERK 1/2), phospholipase C γ (PLC γ) and serine/threonine protein kinase family protein kinase B (Akt) that are induced by vascular endothelial growth factor-A (VEGF-A). We also evaluated the *in vivo* effects of VME on oxygen-induced retinopathy in mice.

Methods

Reagents

GM6001, *N*-[(2S)-2-(methoxycarbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide, and VEGF-A were purchased from Sigma (St. Louis, MO, USA) and Kurabo (Osaka, Japan), respectively. The oxygen-scavenger *N*-acetyl-L-cysteine (NAC) and Trolox, a soluble vitamin E derivative, were purchased from Wako (Osaka, Japan) and Sigma, respectively. Antibodies against phosphorylated ERK 1/2 (Thr 202/Tyr 204), total ERK 1/2, phosphorylated PLC γ (Tyr 783) and total PLC γ were purchased from Cell Signaling Technology (Beverly, MA, USA). An antibody against β -actin was

purchased from Sigma. VME were purchased from Fushimi Chemical Co., Ltd (Kyoto, Japan). It was extracted in accordance to a method as previously described by Nakajima *et al.* (9). Briefly, VME were extracted from commercially available paste frozen fruits of bilberry using ethanol, filtrated and concentrated. Then ethanol extracts of bilberry were applied to column chromatography, removed ethanol and freeze-dried to powder. Fifteen kinds of anthocyanin components of VME were ascertained by use of high-pressure liquid chromatography. VME were containing 25% anthocyanin (conversion of anthocyanin into delphinidin), and 15 kinds of anthocyanin were Delphinidin 3-O-galactopyranoside, Delphinidin 3-O-glucopyranoside, Cyanidin 3-O-galactopyranoside, Delphinidin 3-O-alabinopyranoside, Cyanidin 3-O-glucopyranoside, Petunidin 3-O-galactopyranoside, Cyanidin 3-O-alabinopyranoside, Petunidin 3-O-glucopyranoside, Paeonidin 3-O-galactopyranoside, Petunidin 3-O-alabinopyranoside, Paeonidin 3-O-glucopyranoside, Malvidin 3-O-glucopyranoside, Paeonidin 3-O-alabinopyranoside, Malvidin 3-O-galactopyranoside and Malvidin 3-O-alabinopyranoside, respectively.

Animals

C57BL/6 mice were obtained from Japan SLC (Hamamatsu, Japan). All mice were handled according to the ARVO statement for the Use of Animals in Ophthalmic and Vision Research, and the experiments were approved and monitored by the Institutional Animal Care and Use Committee of Gifu Pharmaceutical University.

Cell Culture

Human umbilical vein endothelial cells (HUVECs, Kurabo) were cultured in a growth medium (HuMedia-EG2; Kurabo) at 37°C in a humidified atmosphere of 5% CO₂ in air. The HuMedia-EG2 medium consists of a base medium (HuMedia-EB2, Kurabo) supplemented with 2% fetal bovine serum (FBS), 10 ng ml⁻¹ recombinant human epidermal growth factor, 1 μ g ml⁻¹ hydrocortisone, 50 μ g ml⁻¹ gentamicin, 50 ng ml⁻¹ amphotericin B, 5 ng ml⁻¹ recombinant human basic fibroblast growth factor -B and 10 μ g ml⁻¹ heparin.

Tube Formation Assay

An angiogenesis assay kit (Kurabo) was used according to the manufacturer's instructions. Briefly, HUVECs co-cultured with fibroblasts were cultivated in the presence or absence of various concentrations of test drugs plus VEGF-A (10 ng ml⁻¹). After 11 days, cells were fixed in 70% ethanol. The cells were incubated with diluted primary antibody (mouse anti-human CD31, 1:4000) for 1 h at 37°C, and with the secondary antibody (goat anti-mouse IgG alkaline phosphatase-conjugated

antibody, 1:500) for 1 h at 37°C, and visualization was achieved using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium. Images were obtained from five different fields (5.5 mm² per field) for each well, and tube area, length, joints and paths were quantified using Angiogenesis Image Analyzer Ver.2 (Kurabo).

Cell Proliferation Assay

HUVECs were seeded into 96-well plates at a density 2000 cells per well at 37°C for 12 h, and preincubated in HuMedia-EB2 containing 2% FBS at 37°C for 6 h. The HUVECs were incubated for 48 h in fresh medium containing VEGF-A (10 ng ml⁻¹) with or without various concentrations of test drugs, and then incubated for a further 48 h in the same (fresh) medium. After incubation, the viable cell numbers were measured by means of a WST-8 assay. Briefly, 10 µl of CCK-8 (Dojindo, Kumamoto, Japan) was added to each well, incubated at 37°C for 3 h and the absorbance measured at 492 nm (reference wave, 660 nm).

Cell Migration Assay

Cell migration was evaluated using a modified Boyden chamber assay (17). The microporous membrane (8 µm) of 24-well cell-culture inserts (BD Biosciences, Bedford, MA, USA) was coated with human fibronectin (BD Biosciences). HUVECs were collected by centrifugation, resuspended in HuMedia-EB2 containing 0.1% bovine serum albumin (BSA) and seeded into the chamber (5 × 10⁴ cells per well). Each well was filled with HuMedia-EB2 containing 0.1% BSA and VEGF-A (10 ng ml⁻¹) with or without test drugs, and the chamber was incubated at 37°C for 4 h in 5% CO₂. Any migrated cells on the upper surface of the membrane were removed by scrubbing with a cotton swab. Migrated cells on the lower surface of the membrane were fixed in Diff-Quik Fixative (Sismex, Kobe, Japan) and stained using hematoxylin. The migrated cells were then counted in five fields (for each membrane) under a microscope at ×200 magnification and the average number per field was calculated.

Immunoblotting

Subconfluent HUVECs were incubated in HuMedia-EB2 containing 2% FBS for 6 h at 37°C in a 5% CO₂ atmosphere. Then, the medium was changed to Dulbecco's modified Eagle medium containing 25 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (Invitrogen, Grand Island, NY, USA) and either 2% FBS or 0.5% FBS (for Akt detection), and incubation allowed to proceed for a further 1 or 18 h, respectively, at 37°C. Next, the medium was changed to fresh medium (constituents as above) containing VEGF-A (10 ng ml⁻¹)

concomitantly with or without VME (30 µg ml⁻¹), and incubation continued for 5 or 10 min (we performed pilot study for time course of changes in phosphorylated – ERK 1/2 and Akt after VEGF treatment, and they were the highest at 5 and 10 min after that, respectively). The HUVECs were washed two times with 10 mM NaF in PBS, lysed in RIPA buffer (Sigma) supplemented with protease inhibitor cocktail (Sigma), phosphatase inhibitor cocktail 1 (Sigma) and phosphatase inhibitor cocktail 2 (Sigma), and stocked at –80°C. Equal amounts of each sample were electrophoresed on 7.5% SDS-PAGE gel, then transferred to polyvinylidene difluoride membranes. After blocking with Blocking One-P (Nacalai tesque, Kyoto, Japan) for 30 min, the membranes were incubated with one of the following, as the primary antibody: anti-phosphorylated ERK 1/2, anti-total ERK 1/2, anti-phosphorylated PLCγ, anti-total PLCγ, anti-phosphorylated Akt, anti-total Akt or anti β-actin antibody. After this incubation, the membrane was incubated with secondary antibody: HRP conjugated goat anti-rabbit or -mouse IgG (Pierce Biotechnology, Rockford). The immunoreactive bands were visualized using Super Signal[®] West Femto Maximum Sensitivity Substrate (Pierce Biotechnology) and measured using GelPro (Media Cybernetics, Silver Spring, MD).

Oxygen-induced Retinopathy in Mice

Oxygen-induced retinopathy was induced in newborn mice as previously described by Smith *et al.* (18) Briefly, on post-natal day 7 (P7) mice were placed along with their dam into a custom-built chamber in which the partial pressure of oxygen was maintained at 75%. Mice were maintained in 75% oxygen for up to 5 days (P12), after which they were transferred back to their cage in room air. VME (300 ng per eye) or saline was intravitreally injected on P12. Mice were anesthetized by intraperitoneal administration of sodium pentobarbital salt (Dainippon sumitomo pharma, Osaka, Japan) at 50 mg kg⁻¹. Through a median sternotomy, the left ventricle of the heart was identified and perfused with FITC dextran (20 mg per animal). Then, the eyes were enucleated and placed in 4% paraformaldehyde. Under a dissecting microscope, the retina was removed, flat-mounted by radical cutting and covered with a coverslip after a few drops of VECTASHIELD[®] mounting median (Vector Laboratories, Burlingame, CA) had been placed on the slide. Images of flat-mounted retinas were acquired via a fluorescence microscope (BX50; OLYMPUS, Tokyo, Japan) using a high-resolution charged-coupled device camera (DP30BW; OLYMPUS, Tokyo, Japan). The areas of neovascular tufts in the retina were measured using imaging software (Metamorph; Universal Imaging Corp., Downingtown, PA).

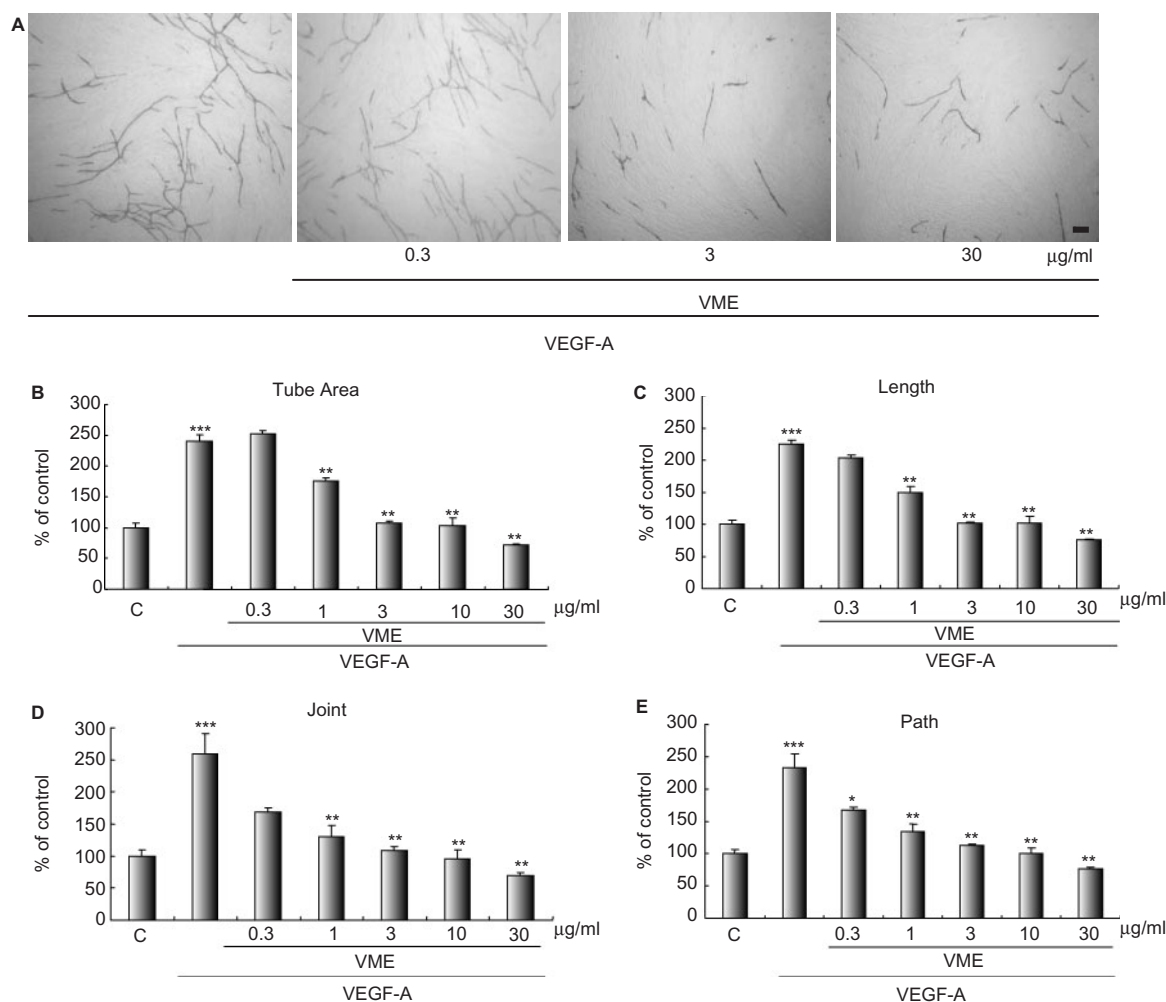


Figure 1. VME inhibited tube formation induced by VEGF-A. Representative photographs of tube formation (A). Scale bar = 100 μm . HUVECs were co-cultured with human fibroblasts, as described in Methods section, and incubated for 11 days with or without the indicated concentrations of VME, with the concomitant addition of VEGF-A (10 ng ml^{-1}). Tube formation was observed in five randomly chosen fields, and tube area (B), length (C), joints (D) and paths (E) were measured using an Angiogenesis Image Analyzer. Data are shown as mean \pm SEM ($n = 3-6$). C: Control. *, $P < 0.05$; **, $P < 0.01$ versus VEGF-A (Dunnett's multiple-comparison test). ***, $P < 0.01$ versus Control (Student's t -test).

Measurement of 2,2-diphenyl-1-picrylhydrazyl Radical-scavenging Activity

Radical-scavenging activity was measured by means of a 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (19). VME, NAC and Trolox were dissolved and diluted in ethanol at various concentrations and then 0.025 mg ml^{-1} DPPH in ethanol was added, and the whole left to stand for 30 min at room temperature. This was followed by measurement of the absorbance of the resulting solution at 517 nm using a spectrophotometer.

Measurement of Lactate Dehydrogenase Activity

Lactate dehydrogenase (LDH) activity in the culture medium containing VEGF with or without VME at 30 $\mu\text{g ml}^{-1}$ (the highest dose in this study) was measured using an LDH cytotoxicity Detection kit (Takara Bio, Tokyo, Japan).

Statistical Analysis

Data are presented as means \pm SEM. Statistical comparisons were made using a one-way ANOVA followed by a Student's t -test, paired t -test or Dunnett's multiple-comparison test. A value of $P < 0.05$ was considered to indicate statistical significance.

Results

VME Inhibited VEGF-A-induced Tube Formation in HUVEC Co-cultured with Fibroblast

Representative images of tube formation induced by VEGF-A with or without VME are shown in Fig. 1A. VME (0.3–30 $\mu\text{g ml}^{-1}$) concentration-dependently inhibited tube formation and quantitative analysis showed that VME at 1–30 $\mu\text{g ml}^{-1}$ significantly inhibited tube area, length, joints and paths (Fig. 1B–E). At 3 $\mu\text{g ml}^{-1}$ or

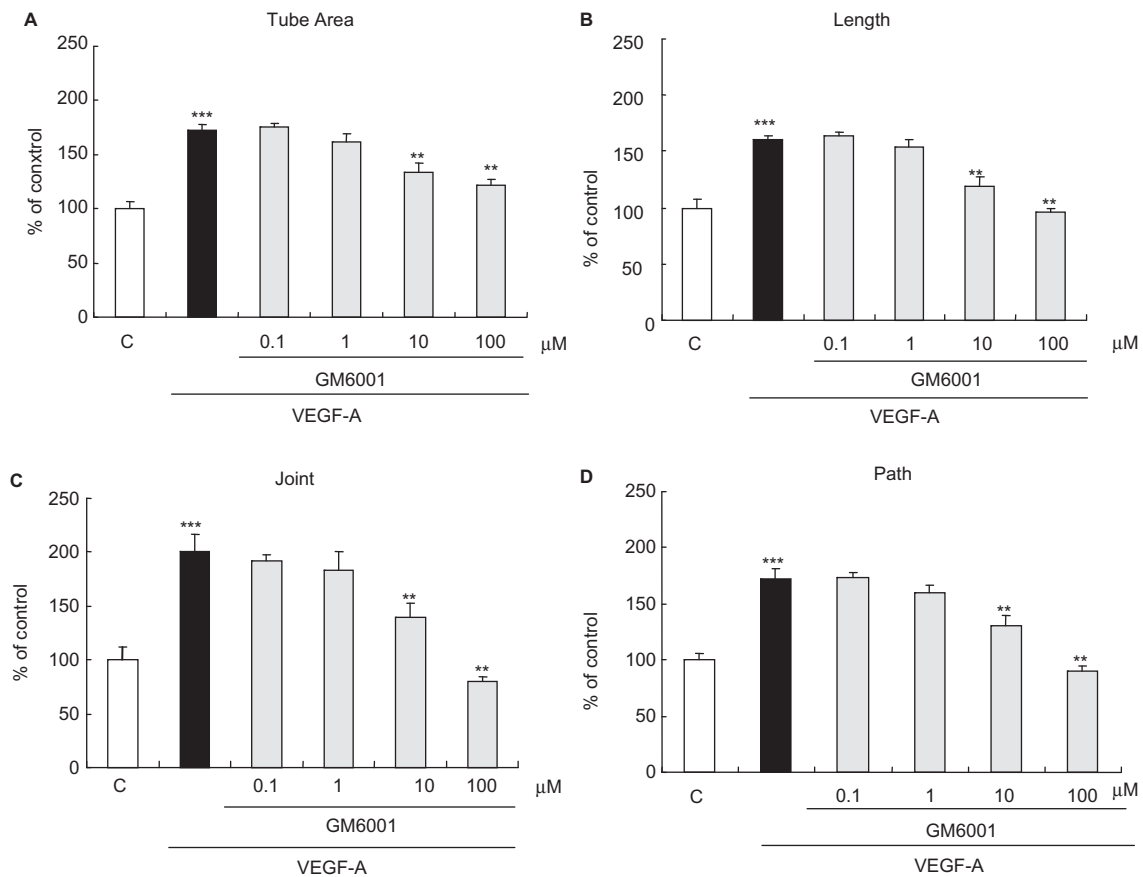


Figure 2. GM6001 inhibited tube formation induced by VEGF-A. HUVECs were co-cultured with human fibroblasts, as described in Methods section, and incubated for 11 days with or without the indicated concentrations of GM6001, with the concomitant addition of VEGF-A (10 ng ml^{-1}). Tube formation was observed in five randomly chosen fields, and tube area (A), length (B), joints (C) and paths (D) were measured using an Angiogenesis Image Analyzer. Data are shown as mean \pm SEM ($n = 4-8$). C: Control. **, $P < 0.01$ versus VEGF-A (Dunnett's multiple-comparison test). ***, $P < 0.01$ versus Control (Student's t -test).

more, VME reduced all four parameters to the non-treated control level (Fig. 1B–E).

GM6001 Inhibited VEGF-A-induced Tube Formation in HUVEC Co-cultured with Fibroblast

GM6001 ($10-100 \mu\text{M}$) significantly inhibited VEGF-A-induced tube formation in a concentration-dependent manner (Fig. 2A–D). The highest concentration of GM6001 used ($100 \mu\text{M}$) reduced tube formation to the non-treated control level (Fig. 2A–D).

VME Inhibited VEGF-A-induced HUVEC Proliferation

Cell proliferation in HUVECs was increased to $\sim 200\%$ of control by VEGF-A (10 ng ml^{-1}) treatment (Fig. 3). VME ($3-30 \mu\text{g ml}^{-1}$) inhibited this proliferation in a concentration-dependent manner, its effect being significant at $3 \mu\text{g ml}^{-1}$ or more (Fig. 3A). On the other hand, VME alone (without VEGF-A) had little or no effect on basal proliferation (Fig. 3A).

VME and GM6001 Inhibited VEGF-A-induced HUVEC Migration

Cell migration in HUVECs was increased to 190% of control by VEGF-A (10 ng ml^{-1}) treatment (Fig. 4). VME ($3-30 \mu\text{g ml}^{-1}$) inhibited this migration in a concentration-dependent manner, its effect being significant at both 10 and $30 \mu\text{g ml}^{-1}$ (Fig. 4B). On the other hand, VME ($30 \mu\text{g ml}^{-1}$) alone had no effect on HUVEC migration (versus control) (Fig. 4B). GM6001 ($3-30 \mu\text{M}$) significantly inhibited the HUVEC migration induced by VEGF-A (10 ng ml^{-1}).

VME Inhibited VEGF-A-induced Phosphorylation of ERK 1/2, but not that of PLC γ

We analyzed the effects of VME on the signaling pathways induced by VEGF-A. Activation of ERK 1/2 and PLC γ has been reported to be involved in VEGF-induced proliferation (20). Treatment with VEGF-A (10 ng ml^{-1}) for 5 min increased the phosphorylation of

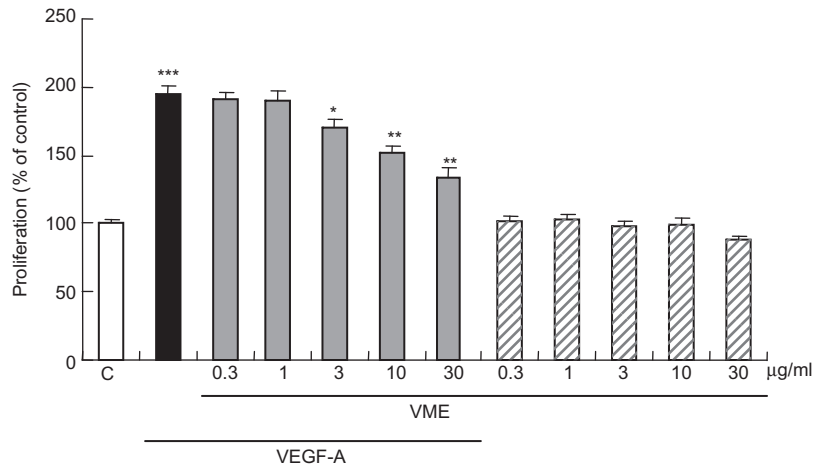


Figure 3. VME inhibited proliferation of HUVECs induced by VEGF-A. HUVECs were cultured in 96-well plates at a density of 2000 cells per well, then incubated for a total of 96 h at 37°C in 5% CO₂. HUVECs were supplemented with or without VEGF-A (10 ng ml⁻¹) plus various concentrations of test drugs, and measurements were made by WST-8 assay. Data are shown as mean ± SEM (*n* = 5–8). C: Control. *, *P* < 0.05; **, *P* < 0.01 versus VEGF-A (Dunnett's multiple-comparison test). ***, *P* < 0.01 versus Control (Student's *t*-test).

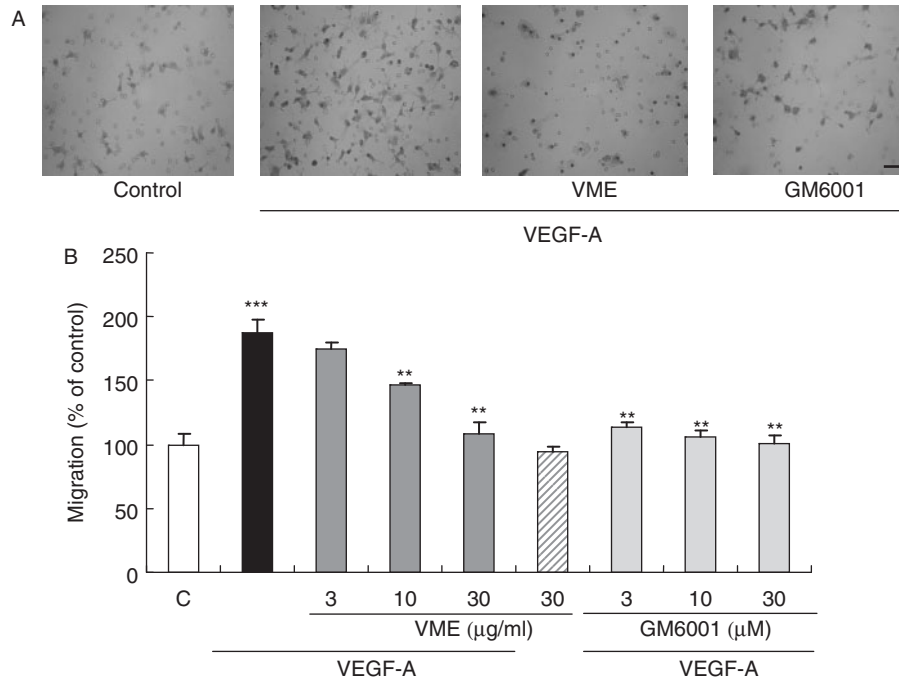


Figure 4. VME and GM6001 inhibited migration induced by VEGF-A. Representative photographs showing effects of VME and GM6001 on HUVEC migration (A). Scale bar = 50 µm. The migrated cells were counted in five fields for each membrane (see Methods section) (B). Data are shown as mean ± SEM (*n* = 5 or 6). C: Control. **, *P* < 0.01 versus VEGF-A (Dunnett's multiple-comparison test). ***, *P* < 0.01 versus Control (Student's *t*-test).

ERK 1/2 (p-ERK 1/2) approximately 2.5-fold and the phosphorylation of PLCγ (p-PLCγ) approximately 5.2-fold (Fig. 5A and B). VME (30 µg ml⁻¹) significantly inhibited the VEGF-A-induced increase in p-ERK 1/2 (Fig. 5A), but not that in p-PLCγ (Fig. 5B). VME (30 µg ml⁻¹) had no effects on either p-ERK 1/2 or p-PLCγ (Fig. 5A and B).

VME Inhibited VEGF-A-induced Phosphorylation of Akt

Activation of Akt is known to be an important step in the VEGF-induced migration of HUVECs (21,22). Treatment with VEGF-A (10 ng ml⁻¹) for 10 min increased the phosphorylation of Akt (p-Akt) approximately 1.5-fold and VME (30 µg ml⁻¹) significantly inhibited this increase (Fig. 5C).

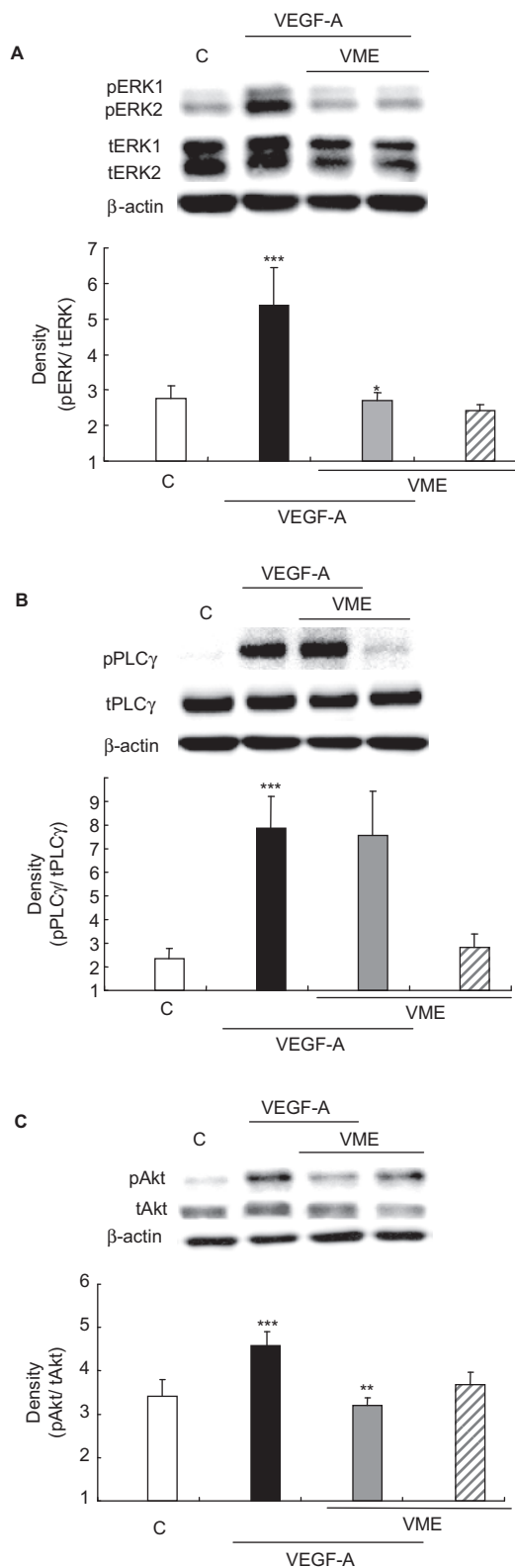


Figure 5. VME inhibited phosphorylations of ERK 1/2 and Akt induced by VEGF-A, but not that of PLC γ . Effects of VME ($30 \mu\text{g ml}^{-1}$) on VEGF-A (10 ng ml^{-1})-induced ERK 1/2 (A), PLC γ (B) and Akt (C) phosphorylations. Data are shown as mean \pm SEM ($n = 4-7$). C: Control; *, $P < 0.05$; **, $P < 0.01$ versus VEGF-A (Student's t -test). ***, $P < 0.05$ versus Control (Student's t -test).

VME Inhibited Angiogenesis during Oxygen-induced Retinopathy in Mice

The excessive neovascularization observed in flat-mounted retinal sections obtained from mice after prolonged exposure to 75% oxygen was estimated by analysis of the vascular tufts. Representative images of such neovascularization in mice treated with or without VME are shown in Figs 6A and B. Intravitreal administration of VME (300 ng per eye) significantly inhibited the area of the neovascular tufts (versus vehicle) (Fig. 6C).

VME and Anti-oxidants Exhibited Radical-scavenging Activity against DPPH Radical

The radical-scavenging activity of VME was compared with those of NAC and Trolox using a DPPH assay. As shown in Table 1, VME, NAC and Trolox concentration-dependently exhibited radical scavenging ability against DPPH radical, the IC_{50} values being $9.1 \mu\text{g ml}^{-1}$, $23.1 \mu\text{M}$ and $24.1 \mu\text{M}$, respectively.

LDH Activity in HUVEC Culture Medium was not Significantly Increased by Treatment of VME

We measured LDH activity in culture medium to examine cytotoxicity of VME. LDH activity was $0.54 \pm 0.12 \text{ U ml}^{-1}$ ($n = 3$) in VEGF plus VME ($30 \mu\text{g ml}^{-1}$)-treated medium and $0.37 \pm 0.07 \text{ U ml}^{-1}$ ($n = 6$) in VEGF-treated medium, and those activity of LDH were not significantly different.

Discussion

In the present study, we found that VME inhibited angiogenesis both *in vitro* and *in vivo*, and our results suggest that its effect may be due in part to reductions in cell proliferation and migration through inhibition of both p-ERK 1/2 and p-Akt.

Angiogenesis is a multi-step process, and VEGF promotes many of the events necessary for angiogenesis, such as proliferation and migration of endothelial cells, remodeling of extracellular matrix and formation of capillary tubules (19). Extracellular-matrix degradation is critical during angiogenesis, which requires proteolysis of endothelial cells as well as synthesis of new matrix components. Degradation of matrix components is mediated by specific proteases called MMP, which are produced by endothelial cells, fibroblasts, vascular smooth muscle cells and reportedly also by myocytes (23,24). Furthermore, *in vitro* and *in vivo* studies have shown that MMP inhibitors (GM6001 and TIMP1, a tissue inhibitor of metalloproteinase-1) decrease the angiogenesis induced by VEGF (7,25,26). In the present study, GM6001 inhibited VEGF-A-induced tube formation in HUVECs co-cultured with human fibroblast cells

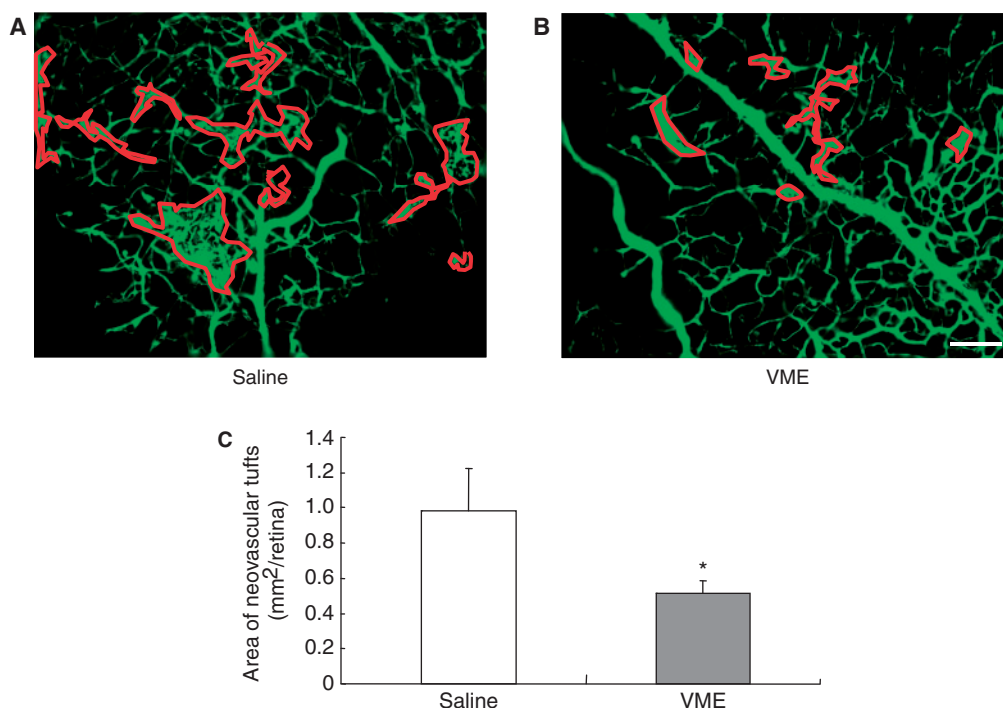


Figure 6. VME inhibited neovascular tufts on oxygen-induced retinopathy in mice. Retinal flat mounts were examined by FITC-dextran angiography. Representative photographs of retina from saline-treated eye (A) and VME-treated eye (B). Scale bar = 100 μm . Areas of neovascular tufts in saline- and VME-treated eyes (C). Each column and bar represents mean \pm SEM ($n = 9$). *, $P < 0.05$ versus Saline (paired t -test).

Table 1. Radical-scavenging activities of VME, NAC and Trolox

Treatments	% radical-scavenging activity	IC ₅₀ (95% confidence limits)
VME 0.3 $\mu\text{g ml}^{-1}$	2.7 \pm 0.77	9.1 (7.6–11.0) $\mu\text{g ml}^{-1}$
1	5.1 \pm 0.15	
3	16.4 \pm 1.94	
10	51.2 \pm 2.57	
30	70.0 \pm 0.18	
100	97.1 \pm 0.86	
NAC 3 μM	5.6 \pm 0.31	23.1 (21.3–25.1) μM
10	18.0 \pm 0.17	
30	53.3 \pm 1.82	
100	90.5 \pm 0.44	
Trolox 3 μM	6.3 \pm 0.66	24.1 (22.2–26.3) μM
10	20.6 \pm 0.29	
30	61.9 \pm 0.21	
100	85.2 \pm 0.86	

VME, NAC, and Trolox were incubated with DPPH for 30 min, and the absorbance at 517 nm due to DPPH radical was determined. Data are shown as mean \pm SEM. ($n = 5$).

(Fig. 2). These results suggest that MMP is an important factor for angiogenesis.

Likewise, VME (0.3–30 $\mu\text{g ml}^{-1}$) inhibited VEGF-A-induced tube formation (Fig. 1), its effect being significant at 1–30 $\mu\text{g ml}^{-1}$. The antiangiogenic effect of

VME at 3 $\mu\text{g ml}^{-1}$ was almost equal to that of GM6001 at 100 μM . It has been reported that a number of berries, including bilberry, inhibit angiogenesis *in vivo* (27) as well as VEGF expression in human keratinocytes *in vitro* (16). However, our finding is the first report demonstrating that VME can inhibit VEGF-A-induced angiogenesis. When we evaluated the effects of VME on the proliferation and migration of HUVECs, we found that VME significantly inhibited VEGF-A-induced HUVEC proliferation, although VME alone had no effect.

GM6001 strongly inhibited VEGF-A-induced HUVEC migration. This result indicates that the inhibitory effect of GM6001 on tube formation is mediated by a reduction in cell migration through a suppression of MMP activity. According to Lin *et al.* (28), an antioxidant substance, NAC, inhibits the VEGF-A-induced migration of HUVECs and its effect is mediated by an inhibition of the Src (cytoplasmic protein tyrosine kinase) signal pathway. Furthermore, Ushio-Fukai *et al.* (29) reported that VEGF-induced endothelial cell signaling and angiogenesis is tightly controlled by the reduction/oxidation environment. Here, VME displayed radical-scavenging activity (IC₅₀ = 9.1 $\mu\text{g ml}^{-1}$) and significantly inhibited VEGF-A-induced HUVEC proliferation at concentrations of 3–30 $\mu\text{g ml}^{-1}$. Many berry species, including bilberry, contain a lot of anthocyanins, which possess antioxidant activities. Therefore, the inhibitory action of VME on cell migration may be due in part to its antioxidant effect.

Activation of the MAP kinases ERK 1/2 and/or PLC γ is important for the proliferation of HUVECs. We therefore evaluated the effect of VME on phosphorylated ERK 1/2 and PLC γ . In this study, VME inhibited the VEGF-A-induced phosphorylation of ERK 1/2, but not that of PLC γ . These results suggest that VME exert a direct inhibition downstream of PLC γ and upstream of ERK 1/2 in the signaling cascade induced by VEGF-A. Since activation of Akt is known to be important for the migration of HUVECs, we also evaluated the effect of VME on phosphorylated Akt. VME inhibited the phosphorylation of Akt induced by VEGF-A. Ali *et al.* (30) indicated that PD98059, an ERK 1/2 inhibitor, inhibits the VEGF-A-induced proliferation of HUVECs, but not migration, and LY294002, an Akt inhibitor, inhibits both proliferation and migration. They concluded that phosphorylation of ERK 1/2 induced proliferation of HUVECs, and phosphorylation of Akt induced both proliferation and migration of HUVECs. These findings suggest that VME reduce the VEGF-A-induced proliferation through inhibiting direct and/or upstream of ERK 1/2 from downstream of PLC γ , and the VEGF-A-induced proliferation and migration through inhibiting direct and/or upstream of Akt. However, further studies are needed to clarify the precise molecular targets for VME.

Recently, Sylvie *et al.* (31) reported that delphinidin, a kind of anthocyanidin (non-glycosylated form of anthocyanin), inhibits the VEGF-induced phosphorylation of ERK 1/2, its half maximal effect being achieved at 11.8 μM . In the present study, VME inhibited VEGF-A-induced phosphorylation of ERK 1/2, an $\sim 60\%$ effect being achieved at 30 $\mu\text{g ml}^{-1}$. VME (30 $\mu\text{g ml}^{-1}$), as used in this research, contains delphinidin at $\sim 2 \mu\text{M}$. Thus, we consider that the inhibitory effect of VME on the VEGF-A-induced phosphorylation of ERK 1/2 may be mediated by delphinidin and/or other constituents. Further studies will be needed to identify the effective constituents of VME.

In our *in vivo* study, we examined the effect of VME on angiogenesis using a murine oxygen-induced retinopathy model. Intravitreal administration of VME (300 ng per eye) significantly inhibited the area of neovascular tufts. We chose that concentration of VME, because the concentration reached in the vitreous body was an estimated 30 $\mu\text{g ml}^{-1}$. In the present *in vitro* analysis, VME at 30 $\mu\text{g ml}^{-1}$ inhibited the tube formation, HUVEC proliferation and migration and phosphorylations of ERK 1/2 and Akt induced by VEGF-A. Taken together, the above observations suggest that VME inhibit angiogenesis *in vitro* and *in vivo* within the same range of concentrations. Furthermore, recent research demonstrated that oxidative stress is associated with increased production of VEGF under *in vitro* conditions, and believed to be an upregulation of VEGF expression during diabetes (32–34). Collectively, these reports and

our data suggest that the antioxidative effects of VME may lead to an inhibition of VEGF expression, and that by this mechanism VME may inhibit VEGF-induced angiogenesis in the retina.

In conclusion, our findings indicate that VME inhibits VEGF-induced angiogenesis, and that this effect is mediated by inhibition of both cell proliferation and migration. Further experiments will be needed to clarify the major antiangiogenic constituents of VME.

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