Original Article

**Vaccinium myrtillus** (Bilberry) Extracts Reduce Angiogenesis In Vitro and In Vivo

Nozomu Matsunaga 1, Yuichi Chikaraishi 1, Masamitsu Shimazawa 1, Shigeru Yokota 2 and Hideaki Hara 1

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 Cγ (PLCγ). 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 – PLCγ – 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...
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 Cγ (PLCγ) 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 PLCγ (Tyr 783) and total PLCγ 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, Paeonidin 3-O-alabinopyranoside, 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 μg ml⁻¹ hydrocortisone, 50 μg ml⁻¹ gentamicin, 50 ng ml⁻¹ amphotericin B, 5 ng ml⁻¹ recombinant human basic fibroblast growth factor -B and 10 μg 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 Bioscences, Bedford, MA, USA) was coated with human fibronectin (BD Biosciences). HUVECs were collected by centrifugation, MA, USA) was coated with human fibronectin (BD Biosciences). HUVECs were collected by centrifugation, and seeded into the chamber (5 x 10⁵ cells per well). Each well was filled with HuMedia-EB2 containing 0.1% bovine serum albumin (BSA) and seeded into the chamber (5 x 10⁵ 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 x200 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 twice with 10 mM NaF in PBS, lyzed in RIPA buffer (Sigma) supplemented with protease inhibitor cocktail (Sigma), phosphatase inhibitor cocktail 1 (Sigma) and phosphatase inhibitor cocktail 2 (Sigma), and stock 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 (Nacarai 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 mgkg⁻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).
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 \(\mu\)g ml\(^{-1}\) (the highest dose in this study) was measured using an LDH cytoxicity Detection kit (Takara Bio, Tokyo, Japan).

Statistical Analysis

Data are presented as means ± SEM. Statistical comparisons were made using a one-way ANOVA followed by a Student’s \(t\)-test, paired \(t\)-test or Dunnnet’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. At 3 \(\mu\)g ml\(^{-1}\) or
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 μM) significantly inhibited VEGF-A-induced tube formation in a concentration-dependent manner (Fig. 2A–D). The highest concentration of GM6001 used (100 μM) 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 ~200% of control by VEGF-A (10 ng ml⁻¹) treatment (Fig. 3). VME (3–30 μg ml⁻¹) inhibited this proliferation in a concentration-dependent manner, its effect being significant at 3 μg 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 μg ml⁻¹) inhibited this migration in a concentration-dependent manner, its effect being significant at both 10 and 30 μg ml⁻¹ (Fig. 4B). On the other hand, VME (30 μg ml⁻¹) alone had no effect on HUVEC migration (versus control) (Fig. 4B). GM6001 (3–30 μM) 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 PLCγ**

We analyzed the effects of VME on the signaling pathways induced by VEGF-A. Activation of ERK 1/2 and PLCγ 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...
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) 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) 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) for 10 min increased the phosphorylation of Akt (p-Akt) approximately 1.5-fold and VME (30 μg/ml) significantly inhibited this increase (Fig. 5C).
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 µg ml$^{-1}$, 23.1 µM and 24.1 µM, 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 ± 0.12 U ml$^{-1}$ (n = 3) in VEGF plus VME (30 µg ml$^{-1}$)-treated medium and 0.37 ± 0.07 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 tubes (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.
These results suggest that MMP is an important factor for angiogenesis. Likewise, VME (0.3–30 μg ml⁻¹) inhibited VEGF-A-induced tube formation (Fig. 1), its effect being significant at 1–30 μg ml⁻¹. The antiangiogenic effect of VME at 3 μg 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 μg ml⁻¹) and significantly inhibited VEGF-A-induced HUVEC proliferation at concentrations of 3–30 μg 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.

| Treatments | % radical-scavenging activity | IC₅₀ (95% confidence limits) |
|------------|-----------------------------|-----------------------------|
| VME 0.3 μg ml⁻¹ | 2.7 ± 0.77 | 9.1 (7.6–11.0) μg ml⁻¹ |
| 1 | 5.1 ± 0.15 |
| 3 | 16.4 ± 1.94 |
| 10 | 51.2 ± 2.57 |
| 30 | 70.0 ± 0.18 |
| 100 | 97.1 ± 0.86 |
| NAC 3 μM | 5.6 ± 0.31 | 23.1 (21.3–25.1) μM |
| 10 | 18.0 ± 0.17 |
| 30 | 53.3 ± 1.82 |
| 100 | 90.5 ± 0.44 |
| Trolox 3 μM | 6.3 ± 0.66 | 24.1 (22.2–26.3) μM |
| 10 | 20.6 ± 0.29 |
| 30 | 61.9 ± 0.21 |
| 100 | 85.2 ± 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 ± SEM. (n = 5).

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 ± SEM (n = 9). *, P < 0.05 versus Saline (paired t-test).
Activation of the MAP kinases ERK 1/2 and/or PLCγ is important for the proliferation of HUVECs. We therefore evaluated the effect of VME on phosphorylated ERK 1/2 and PLCγ. In this study, VME inhibited the VEGF-A-induced phosphorylation of ERK 1/2, but not that of PLCγ. These results suggest that VME exert a direct inhibition downstream of PLCγ 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 from downstream of PLCγ, 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 ~60% effect being achieved at 30 μg/ml. VME (30 μg/ml), as used in this research, contains delphinidin at ~2 μM. 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 μg/ml. In the present in vivo analysis, VME at 30 μg/ml 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.

References
1. Aiello LP, Avery RL, Arrigg PG, Keyt BA, Jampel HD, Shah ST, et al. Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. New Engl J Med 1994;331:1480–7.
2. Facker TK, Reddy S, Bearellly S, Stinnett S, Fekrat S, Cooney MJ. Retrospective review of eyes with neovascular age-related macular degeneration treated with photodynamic therapy with verteporfin and intravitreal triamcinolone. Ann Acad Med Singapore 2006;35:701–5.
3. Nguyen QH, Hamed LM, Sherwood MB, Roseman RL. Neovascular glaucoma after carotid endartectomy. Ophthalmic Surg Lasers 1996;27:881–4.
4. Tamar A, Izhak H, Ahuva I, Jacob P, Jonathan S, Eli K. Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity. Nat Med 1995;1:1025–8.
5. Gospodarowicz D, Cheng J, Lirette M. Bovine brain fibroblast growth factors: comparison of their abilities to support the proliferation of human and bovine vascular endothelial cells. J Cell Biol 1983;97:1677–85.
6. Napoleon F, Keith H, Lyn J, David WL. Molecular and biological properties of the vascular endothelial growth factor family proteins. Endocr Rev 1992;13:18–32.
7. Galardy RE, Grobeleny D, Foellmer HG, Fernandez LA. Inhibition of angiogenesis by the matrix metalloproteinase inhibitor N-(3R,2S-hydroxamidocarboxymethyl)-4-methylpentanoyl)-L-tryptophan methylamid. Cancer Res 1994;54:4715–8.
8. Koike T, Gooden MD, Sadoun E, Reed MJ. Inhibited angiogenesis in aging: a role for TIMP-2. J Gerontal A Biol Sci Med Sci 2003;58:B798–805.
9. Nakajima JI, Tanaka I, Seo S, Yamazaki M, Saito K. LC/PDA/ESI-MS profiling and radical scavenging activity of anthocyanins in various berries. J Biomed Biotechnol 2004;5:241–7.
10. Baj A, Bombardelli E, Gabetta A, Martinelli EM. Qualitative and quantitative evaluation of Vaccinium myrtillus anthocyanins by high resolution gas chromatography and high performance liquid chromatography. J Chromatogr 1983;279:365–72.
11. Morazzoni P, Bombard ED. Vaccium myrtillus. Fitoterapia 1996;67:3–29.
12. Morazzoni P, Magistretti MJ. Activity of Myrtocyanan®, an anthocyanoside complex from Vaccinium myrtillus (VMA), on platelet aggregation and adhesiveness. Fitoterapia 1990;61:13–21.
13. Lieti A, Cristoni A, Picci M. Studies on Vaccinium myrtillus anthocyanosides. Arzneim Forsch/Drug Res 1976;26:829–32.
14. Colantuoni A, Bertuglia S, Magistretti MJ, Donato L. Effects of Vaccinium myrtillus anthocyanosides on arterial vasomotion. Arzneim Forsch/Drug Res 1991;41:905–9.
15. Perosini M, Guidi G, Chielini S, Siravo D. Diabetic and hypertensive retinopathy therapy with Vaccinium myrtillus anthocyanosides (Tengens) double blind placebo-controlled clinical trial. Am Ophthalm J Ocul 1987;113:1173–90.
16. Roy S, Khanna S, Alessio HM, Vider J, Bagchi D, Bagchi M, Sen CK. Anti-angiogenic property of edible berries. Free Radic Res 2002;36:1023–31.
17. Kim KS, Hong YK, Joe YA, Lee Y, Shin JY, Park HE, et al. Anti-angiogenic activity of the recombinant kringle domain of urokinase and its specific entry into endothelial cells. J Biol Chem 2003;278:11449–56.
18. Smith LE, Wesolowski E, McLellan A, Kostyk SK, D’Amato R, Sullivan R, et al. Oxygen-induced retinopathy in the mouse. *Invest Ophthalmol Vis Sci* 1994;35:101–11.
19. Shirwaikar A, Shirwaikar A, Rajendran K, Punitha IS. In *vitro* antioxidant studies on the benzyl tetra isoquinoline alkaloid berberine. *Biol Pharm Bull* 2006;29:1906–10.
20. Wu LW, Mayo LD, Dunbar JD, Kessler KM, Baerwald MR, Jaffe EA, et al. Utilization of distinct signaling pathways by receptors for vascular endothelial cell growth factor and other mitogens in the induction of endothelial cell proliferation. *J Biol Chem* 2000;275:5096–103.
21. Yasushi F, Kenneth W. Akt mediates cytoprotection of endothelial cells by vascular endothelial growth factor in an anchorage-dependent manner. *J Biol Chem*. 1999;274:16349–54.
22. Shiojima I, Walsh K. Role of Akt signaling in vascular homeostasis and angiogenesis. *Circ Res* 2002;90:1243–50.
23. Coker ML, Doscher MA, Thomas CV, Galis ZS, Spinale FG. Matrix metalloproteinase synthesis and expression in isolated LV myocyte preparations. *Am J Physiol* 1999;277:H777–87.
24. Unemori EN, Ferrara N, Bauer EA, Amento EP. Vascular endothelial growth factor induces interstitial collagenase expression in human endothelial cells. *J Cell Physiol* 1992;153:557–62.
25. Friehs I, Margossian RE, Moran AM, Cao DH, Moses MA, del Nido PJ. Vascular endothelial growth factor delays onset of failure in pressure-overload hypertrophy through matrix metalloproteinase activation and angiogenesis. *Basic Res Cardiol* 2006;101:204–13.
26. Lee CZ, Xu B, Hashimoto T, McCulloch CE, Yang GY, Young WL. Doxycycline suppresses cerebral matrix metalloproteinase-9 and angiogenesis induced by focal hyperstimulation of vascular endothelial growth factor in a mouse model. *Stroke* 2004;35:1715–19.
27. Atalay M, Gordillo G, Roy S, Rovin B, Bagchi D, Bagchi M, et al. Anti-angiogenic property of edible berry in a model of hemangioma. *FEBS Lett* 2003;544:252–7.
28. Lin MT, Yen ML, Lin CY, Kuo ML. Inhibition of vascular endothelial growth factor-induced angiogenesis by resveratrol through interruption of Src-dependent vascular endothelial cadherin tyrosine phosphorylation. *Mol Pharmacol* 2003;64:1029–36.
29. Ushio-Fukai M, Tang Y, Fukai T, Dikalov SI, Ma Y, Fujimoto M, et al. Novel role of gp91(phox)-containing NAD(P)H oxidase in vascular endothelial growth factor-induced signaling and angiogenesis. *Circ Res* 2002;91:1160–7.
30. Ali N, Yoshizumi M, Fujita Y, Izawa Y, Kanematsu Y, Ishizawa K, et al. A novel Src kinase inhibitor, M475271, inhibits VEGF-induced human umbilical vein endothelial cell proliferation and migration. *J Pharmacol Sci* 2005;98:130–41.
31. Sylvie L, Blanchette M, Michaud-Levesque J, Lafleur R, Durocher Y, Moghrabi A, et al. Delphinidin, a dietary anthocyanidin, inhibits vascular endothelial growth factor receptor-2 phosphorylation. *Carcinogenesis* 2006;27:989–96.
32. Ellis EA, Grant MB, Murray FT, Wachowski MB, Guberski DL, Kublis PS, et al. Increased NADH oxidase activity in retina of the BBZ/Wor diabetic rat. *Free Radic Biol Med* 1998;3:111–20.
33. Obrosova IG, Minchenko AG, Marinescu V, Fathallah L, Kennedy A, Stockert CM, et al. Antioxidants attenuate early up regulation of retinal vascular endothelial growth factor in streptozotocin-diabetic rats. *Diabetologia* 2004;44:1102–10.
34. Chade RA, Bentley MD, Zhu X, Martin RP, Niemeyer S, Betriz AA, et al. Antioxidant intervention prevents renal neovascularization in hypercholesterolemic pigs. *J Am Soc Nephrol* 2004;15:1816–25.