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Hot-water Extract of *Rubus Coreanus* Miquel Suppresses VEGF-induced Angiogenesis

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The interruption of angiogenesis using herbal extracts is now recognized as a useful approach for treating many solid tumors. To date, the best-validated antitumor approach is to target the vascular endothelial growth factor (VEGF)-induced angiogenic pathway. In the present study, we first identified the antiangiogenic activity of a hot-water extract of *Rubus coreanus* Miquel (RCMHE) *in vitro* and *ex vivo*. This extract suppressed VEGF-induced angiogenesis, the phosphorylation of extracellular regulated kinase (ERK), p38 and the activation of matrix metalloproteinases (MMPs). RCMHE also inhibited the VEGF-responsive phosphorylation of VEGFR2. These results clearly show that RCMHE may have potential therapeutic value for angiogenesis-associated human diseases through the suppression of angiogenesis and the interruption of the phosphorylation of VEGFR2.

Key words : Angiogenesis, hot-water extract of *Rubus coreanus* Miquel, VEGFR2 phosphorylation

Introduction

Many natural products have a long history of use as alternative remedies for a variety of tumors with relatively few side effects [8, 20, 27, 42, 58]. Korean black raspberry, a fruit of *Rubus coreanus* Miquel (RCM), has been used as a traditional herbal medicine for the treatment of prostate symptoms, diabetes mellitus, impotence, spermatorrhea, enuresis, and asthma [29, 38, 39, 48]. RCM extract has been reported to have antioxidant [27], anti-inflammatory [10, 38], and anti-cancer activities [25, 31]. Even though numerous biological activities of RCM have been reported, there is no evidence regarding extract's effect on angiogenesis and its underlying mechanisms.

Angiogenesis, the formation of new blood vessels from pre-existing ones, is an essential step for physiological processes such as wound healing, reproduction, and embryonic development [18], and also plays important roles in many pathological processes such as tumor growth and metastasis [11, 14]. VEGF, the most effective biological inducer of angiogenesis, is up-regulated and serves as a major angiogenic factor in the vascular development of tumors [30, 53]. Without an adequate blood supply, the tumor cannot grow larger than 1-2 mm in diameter [12]. A variety of approaches to block VEGF signaling are currently being assessed in preclinical and clinical trials [15, 23, 45], but the chronic therapeutic use of these anti-VEGF agents is limited due to side effects [28]. Therefore, anti-angiogenic therapy using natural compounds with fewer side effects is a promising strategy for tumor treatment. Recently, several plant-derived compounds have been shown to be potent inhibitors of angiogenesis [6, 9, 33, 34, 52, 54, 60].

In the present study, we found for the first time that the natural compound hot-water extract of *Rubus coreanus* Miquel (RCMHE) suppresses VEGF-induced angiogenesis by inhibiting proliferation, migration, invasion, tube formation, and vessel sprouting *in vitro* and *ex vivo*. Furthermore, we found that the VEGF-stimulated phosphorylation of ERK, p38 and VEGFR2, as well as the expression of matrix metalloproteinases (MMPs) were inhibited by RCMHE treatment. These results indicate that RCMHE is a potent anti-angiogenic drug candidate for the treatment of angiogenesis-related diseases such as tumors and diabetic retinopathy.

Materials and methods

Preparation of *Rubus coreanus* Miquel hot-water extract

RCM was harvested at 2012 in Kochang (GPS : E 126°

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39′ N 41° 33′) and Jungeup (GPS : E 126° 58′ N 47° 66′). For the sample preparation, 400 g of the dried RCM was extracted three times with 2 l of water at 100°C for one day, and then filtered with Whatman No. 1 paper, combined, and concentrated using a rotary evaporator (EYELA N-1000, Japan) at 40°C. Finally, the dried RCMHE was obtained. RCMHE was stored in a cold chamber of Lab. of Molecular Pharmacogenomics, Yonsei University at 4°C and relative humidity at 25~30%.

Reagents

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from USB Corporation (Cleveland, OH, USA). Matrigel basement membrane matrix was obtained from BD Biosciences (Bedford, MA, USA). Recombinant human VEGF was purchased from PEPRO TECH (Rocky Hill, NJ, USA). The antibodies for VEGF receptor 2, phospho-VEGF receptor 2 (Tyr 1175), phospho-p44/42 MAP kinase (Thr 202/Tyr 204), p44/42 MAP kinase, phospho-p38 MAP kinase (Thr 180/Tyr 182) and p38 MAP kinase were obtained from Cell Signaling (Beverly, MA, USA). The cell culture reagents and most other biochemical reagents were purchased from Sigma (St. Louis, MO, USA), unless otherwise specified.

Endothelial cell culture and animal maintenance

Human umbilical vein endothelial cells (HUVECs) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). HUVECs were grown in M199 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 20% (v/v) fetal bovine serum (FBS), 100 unit/ml penicillin, 100 μg/ml streptomycin, 3 ng/ml basic Fibroblast Growth Factor (Upstate Biotechnology, Lake Placid, NY, USA), and 5 unit/ml heparin, and incubated at 5% CO_2 in a 37°C incubator. HUVECs were used between passages 4-8 for all experiments. Sprague-Dawley rats (age, 7 weeks) were purchased from Orient Bio Inc. (Sungnam, Korea) and were maintained on standard chow and water *ad libitum*. These experiments were conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" adopted by the United States National Institutes of Health. The study protocols used were reviewed and approved by the Ethic Committee, Institutional Animal Care and Use Committee (IACUC) of Yonsei University Wonju Campus (IACUC Approval # YWC-131127-1).

Endothelial cell viability assay

Cell viability was evaluated using the MTT colorimetric assay. HUVECs (5×10^4 cells) were first plated in gelatinized 24-well plates and cultured overnight. Cells were then treated with 1-100 μg/ml RCMHE for 24 hr. At the end of the treatment, 100 μl of the MTT (5 mg/ml) was added to each well. After 4 hr, the residual MTT was carefully removed, and the crystals were dissolved by incubation with DMSO for 30 min. The plates were then shaken for 5 min, and the absorbance at 595 nm was measured using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The cell viability was calculated as follows: cell viability (%) = (average absorbance of the RCMHE-containing serum group/average absorbance of the blank group) ×100.

Endothelial cell proliferation assay

We determined the effects of RCMHE on the proliferation of HUVECs in response to VEGF. Endothelial cells were seeded at a density of 4×10^4 cells per well in gelatinized 12-well plates, incubated for 24 hr, and then treated with various concentrations of RCMHE in the absence or presence of VEGF (20 ng/ml). After a 48 hr incubation, cells were trypsinized and counted with a Luna™ Automated Cell counter (Logos Biosystems, Korea).

Western blot analysis

Cells were harvested and lysed with RIPA buffer containing 2 mM EDTA, 137 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1 mM Na_3VO_4, 10 mM NaF, 1 mM PMSF, 1% Triton X-100, 10% glycerol, and a protease-inhibitor cocktail. The lysates were resolved by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes (Pall Corporation, East Hills, NY, USA). The blotted membranes were then incubated with the indicated antibodies and the immunoreactive bands were visualized using a chemiluminescent substrate.

Endothelial cell migration assay

In vitro cell migration was assessed using a modified Boyden chamber assay, as described previously [51]. Transwells (24-wells, 8-μm pore size with polycarbonate membrane; Corning Costar, Cambridge, MA, USA) precoated with gelatin were used for the assay. Briefly, the lower surface of the membrane was coated with 10 μg gelatin. Next, fresh M199 medium (1% FBS) containing

VEGF was placed in the lower wells. HUVECs were plated in M199 containing 1% FBS to a final concentration of 1×10^5 cells/ml in the upper chamber of the transwell in various concentrations of RCMHE for 30 min at 37°C. After a 4 hr incubation, cells were fixed and stained with hematoxylin and eosin (H&E). Non-migrating cells on the upper surface of the membrane were removed by cotton swabs, and endothelial cell migration was measured with an optical microscope (×200) by examining the number of cells that migrated to the lower side of the membrane.

Endothelial cell invasion assay

Endothelial cell invasion assay was assessed using a Cultrex® Cell Invasion Assay Kit (Trevigen, Gaithersburg, MD. USA). The membrane of the upper invasion chamber was coated with Matrigel to prevent the migration of non-invasive cells [1]. HUVECs (5×10^4 cells) were then re-suspended in 100 μl of M199 medium (1% FBS) and seeded onto the culture inserts. The cells were then deposited into a 24-well companion plate with 600 μl of M199 medium (1% FBS) containing VEGF (20 ng/ml) and various concentrations of RCMHE. Wells containing VEGF alone served as the positive control. After 48 hr incubation, the media from the wells were withdrawn and the non-invasive cells on the upper surface of the membrane were removed by cotton swabs. The cells that had penetrated the Matrigel-coated membrane and migrated onto the lower surfaces of the membrane were stained with H&E and mounted onto microscope slides. Images of the invasive cells were captured at 100 × magnification using an optical inverted microscope. HUVEC invasion was quantified by counting the number of cells per insert.

Gelatinolytic zymography

To detect the expression of MMPs in response to VEGF in supernatant media in the presence or absence of RCMHE, we used the zymogram assay, as described previously [35]. Briefly, collected medium was centrifuged at 1,500 rpm for 5 min at 4°C to remove cellular debris. The conditioned media containing 20 μg of secreted proteins was then mixed with SDS-PAGE loading buffer without a reducing agent. Cultured media samples were then electrophoresed onto 10% SDS-PAGE copolymerized with 0.2% gelatin. Gels were washed twice for 30 min with 2.5% Triton X-100 solution, rinsed with incubation buffer (50 mM Tris-HCl buffer, pH 7.5 containing 10 mM $CaCl_2$, plus 1 μM $ZnCl_2$) and then incubated at 37°C for either 3 hr or 12 hr. MMP-2 and MMP-9 were identified following the staining of the gel in 0.25% Coomassie Brilliant Blue R250 (Sigma) and the de-staining in 7% acetic acid.

In vitro capillary-like tube formation assay

This experiment examined endothelial tube formation using a tubular morphogenesis assay. Matrigel basement membrane matrix was used as a substrate for the *in vitro* study of angiogenesis [36]. Briefly, 250 μl of growth factor-reduced Matrigel was pipetted into a 24-well culture plate and polymerized for 30 min at 37°C. HUVECs were starved for 6 hr in M199 medium containing 1% FBS, harvested, and then suspended in M199 medium containing 1% FBS. The cells were incubated with RCMHE for 30 min at room temperature and then plated onto a layer of Matrigel at a density of 2×10^5 cells per well, at which point 20 ng/ml of VEGF was added. After 20 hr cultivation at 37°C, the tubes were imaged (× 40). The area covered by the tube network was determined using an optical imaging technique in which pictures of the tubes were scanned in Adobe Photoshop and quantified using Image-Pro Plus (Media Cybermetics, Bethesda, MD, USA).

Ex vivo rat aortic sprouting assay

The three-dimensional rat aortic ring sprouting assay was used as a model for the *ex vivo* angiogenesis study [3]. Briefly, a 48-well plate was first covered with Matrigel (120 μl) and incubated for 30 min at 37°C. Subsequently, the dorsal aortas from the sacrificed Sprague-Dawley rats were dissected and cut into 1 mm long pieces using a sterile surgical blade. Afterwards, the aortic rings were placed into wells pre-coated with Matrigel and then covered with another layer of Matrigel (50 μl). After polymerization for 30 min, VEGF with or without RCMHE was then added to each well in a final volume of 600 μl of human endothelial serum-free M199 media. On day 7, the rings were photographed using a Nikon eclipse TS100 inverted microscope and the sprouted neovessels were quantified. Sprouting was measured using the following scale: 0 = no sprouting; 1 = migrated cells without sprouting; 2 = isolated sprouting; 3 = sprouting in 25-50% of the arterial ring circumference; 4 = sprouting in 50-75% of the circumference; and 5 = sprouting in 75-100% of the circumference. The assay was scored from 0 to 5 in a double-blinded manner, and each data point was quantified six times.

Statistical analysis

The results are presented as means ± standard deviations (SDs). Statistical analysis of the data was performed using the Student's t-test and one-way analysis of variance (ANOVA). $p<0.05$ was considered statistically significant.

Results

Effect of RCMHE on the viability of vascular endothelial cells

We first examined the viability of HUVECs after exposure to RCMHE. Treatment with various concentrations of RCMHE for 24 hr induced cytotoxicity in a dose-dependent manner (Fig. 1A). A significant inhibitory effect on cell viability was observed in response to RCMHE at concentrations ≥50 μg/ml. No significant cytotoxicity was observed at doses of up to 25 μg/ml RCMHE during the 24 hr cultivation period.

RCMHE treatment inhibits VEGF-induced endothelial cell proliferation

We next examined the effect of RCMHE on VEGF-induced endothelial cell proliferation by employing a cell counting assay. Proliferation of the endothelial cells in response to an angiogenic factor is an important step during angiogenesis. HUVECs were pretreated for 40 min with various concentrations of RCMHE (1-25 μg/ml) before being exposed to VEGF (20 ng/ml) for 24 hr. RCMHE inhibited VEGF-induced proliferation, with half maximal inhibition taking place at 5 μg/ml (Fig. 1B). These inhibitory effects were not due to cytotoxicity because RCMHE up to 25 μg/ml had no effect on the normal growth of HUVECs in the absence of VEGF (Fig. 1A).

RCMHE inhibits ERK, p38, and VEGFR2 phosphorylation by VEGF

In order to identify the downstream signaling pathways targeted by RCMHE, we examined the phosphorylation of ERK and p38 MAPKs, the important key signaling pathways that drive endothelial cell proliferation, migration and tube formation. The VEGF-induced phosphorylation of ERK and p38 was inhibited by RCMHE in a dose-responsive manner (Fig. 2A and B). Next, to evaluate the possibility that the anti-angiogenic effects of RCMHE were mediated through the inhibition of VEGFR2 phosphorylation, we also performed Western blot analysis. We found that RCMHE treatment inhibited the VEGF-induced phosphorylation of VEGFR2, but VEGFR2 expression was not affected by RCMHE treatment itself (Fig. 2C).

RCMHE reduces VEGF-induced endothelial cell migration

Because the migration of endothelial cells is essential for tumor angiogenesis, we next examined the ability of

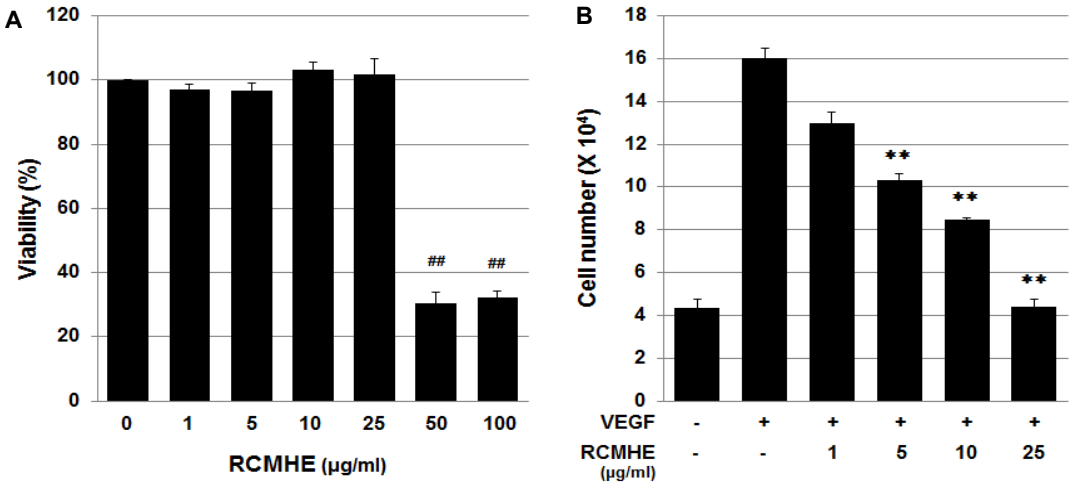

Fig. 1. Inhibitory effect of RCMHE on VEGF-induced proliferation of HUVECs. (A) HUVECs were exposed to RCMHE at various concentrations (1, 5, 10, 25, 50, and 100 μg/ml). After 24 hr, the viability (%) was determined by MTT assay. The vehicle-treated cells were used as the control (100%). (B) HUVECs were treated with various concentrations (1, 5, 10, and 25 μg/ml) of RCMHE for 40 min before exposure to VEGF (20 ng/ml). The number of proliferating cells was counted 24 hr later. Data were expressed as the mean ± SD of three independent experiments. $^{\#\#}p<0.01$ versus RCMHE-untreated group. $^{**}p<0.01$ versus VEGF alone.

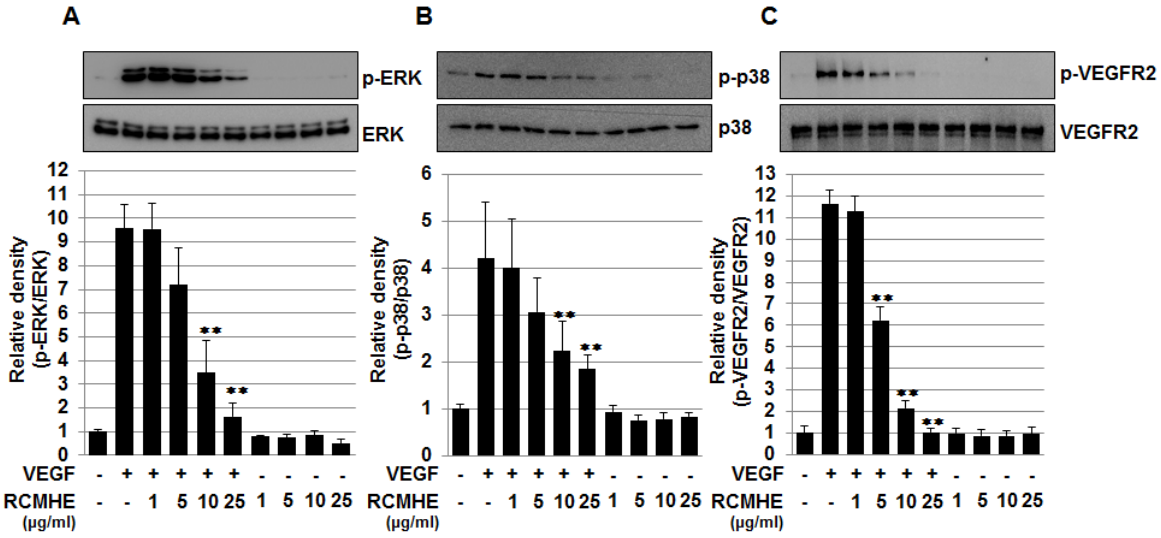

Fig. 2. Effect of RCMHE on the phosphorylation of ERK, p38 and VEGFR2 induced by VEGF. HUVECs were incubated for 40 min with various concentrations (1, 5, 10, and 25 μg/ml) of RCMHE, followed by VEGF stimulation (20 ng/ml) for 10 min. Next, cells were harvested and the levels of phosphorylated and total ERK (A), p38 (B) and VEGFR2 (C) were determined by Western blot analysis. Densitometric quantification of the Western blot data is expressed as the mean ± SD (n = 3). **p<0.01 versus VEGF alone.

RCMHE to inhibit cell motility in migration assays. In the absence of RCMHE, a large number of endothelial cells migrated to the lower side of the filter in the Transwell Chamber following stimulation with 20 ng/ml VEGF. This VEGF-induced migration of HUVECs was significantly reduced by RCMHE treatment in a dose-dependent manner (Fig. 3). However, RCMHE alone had no significant effect on the basal migration of endothelial cells.

RCMHE inhibits endothelial cell invasion and MMP expression in response to VEGF

Next, we evaluated the ability of RCMHE to inhibit the invasion of human endothelial cells using a Transwell culture plate. The number of invading cells in response to VEGF was significantly reduced in a dose-dependent manner by RCMHE treatment (Fig. 4A). An essential pattern of this invasion included degradation of the basement mem-

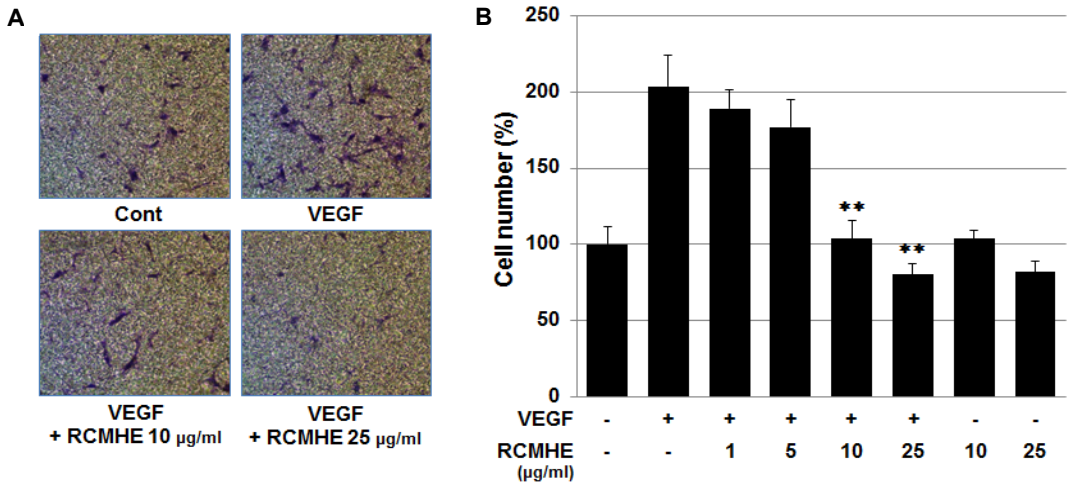

Fig. 3. Effect of RCMHE on VEGF-induced endothelial cell motility. HUVECs were pretreated for 40 min with various concentrations (1, 5, 10, and 25 μg/ml) of RCMHE before exposure to VEGF (20 ng/ml). After incubation for 4 hr, chemotactic migration was evaluated. Cells that migrated to the bottom of the filter were photographed (A) and counted (B) using optical microscopy. An *in vitro* migration assay was performed as described in the Materials and methods section. Data are expressed as the mean ± SD from triplicate experiments. **p<0.01 versus VEGF alone.

brane (BM). MMPs are inducible enzymes that degrade extracellular matrix (ECM) components, allowing cells to efficiently traverse the BM. Therefore, we performed zymogram assay to examine the effect of RCMHE on the VEGF-stimulated expression of MMP-2 and -9. Both MMP-2 and -9 can hydrolyze gelatin substrates incorporated into an SDS-PAGE gel, and gelatin hydrolysis by MMP-2 and -9 can be visualized by Coomassie Brilliant Blue R250 staining. The VEGF-stimulated activation of MMP-2 and -9 was inhibited by RCMHE treatment (Fig. 4B). In addition, we used conditioned medium with PMA-stimulated HUVECs to distinguish between different MMPs [22].

RCMHE treatment suppresses the VEGF-stimulated tube formation of HUVECs

To characterize the anti-angiogenic activity of RCMHE, we examined its ability to inhibit VEGF-induced tube formation by endothelial cells on Matrigel, a well-known angiogenesis assay. HUVECs exposed to VEGF for 20 hr showed extensive and robust tube-like structures with increasing numbers of branch points. By contrast, RCMHE treatment dose-dependently reduced both the width and length of the endothelial tubes induced by VEGF (Fig. 5).

RCMHE inhibits VEGF-stimulated vessel sprouting *ex vivo*

To further verify the anti-angiogenic effect of RCMHE *ex vivo*, we employed the rat aortic ring sprouting assay [44], a widely used *ex vivo* anti-angiogenesis assay in which growing microvessels undergo many key features of angiogenesis over a timescale similar to that observed *in vivo*. Rat aortic rings were embedded in Matrigel and fed with medium containing different concentrations of RCMHE. Next, the rings were stimulated with 20 ng/ml VEGF and the subsequent sprouting was examined by inverted microscopy. VEGF significantly stimulated vessel outgrowth when compared to the results with medium alone. However, RCMHE treatment dramatically reduced the sprout length and density induced by VEGF (Fig. 6).

Discussion

VEGF, the most important angiogenic factor in tumor growth and metastasis, has emerged as an attractive target for anti-angiogenesis treatment [16, 21]. Many anti-angiogenic approaches have been used to target VEGF including anti-VEGF antibodies, soluble VEGF receptor analogues, and small molecule VEGF inhibitors [4]. However, the chronic therapeutic use of anti-VEGF agents is limited due

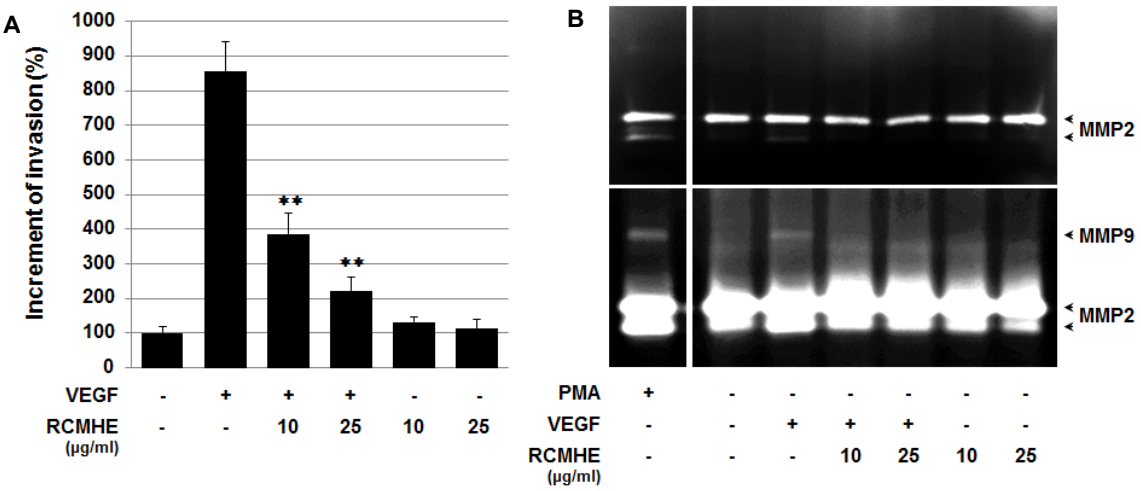

Fig. 4. Effect of RCMHE on endothelial cell invasion and MMP expression induced by VEGF. (A) Effect of RCMHE on HUVEC invasion using a Transwell plate. After incubation for 40 min with 10 or 25 μg/ml of RCMHE, HUVECs were exposed to VEGF (20 ng/ml) for 16 hr. (B) Changes in MMP-2 and MMP-9 activity were measured by gelatinolytic zymogram analysis. After incubation with 10 or 25 μg/ml of RCMHE for 40 min, the cells were treated with VEGF (20 ng/ml) for 12 hr. The cultured media were electrophoresed, and incubated at 37°C for either 3 hr (upper panel) or 12 hr (lower panel), and stained with Coomassie brilliant blue R250. The culture medium from HUVECs treated with PMA (40 ng/ml for 12 hr) was used to distinguish between different types of MMPs. Experiments were repeated three times, and the results are shown as the mean ± SD of triplicate determinations. $^{**}p<0.01$ versus VEGF alone.

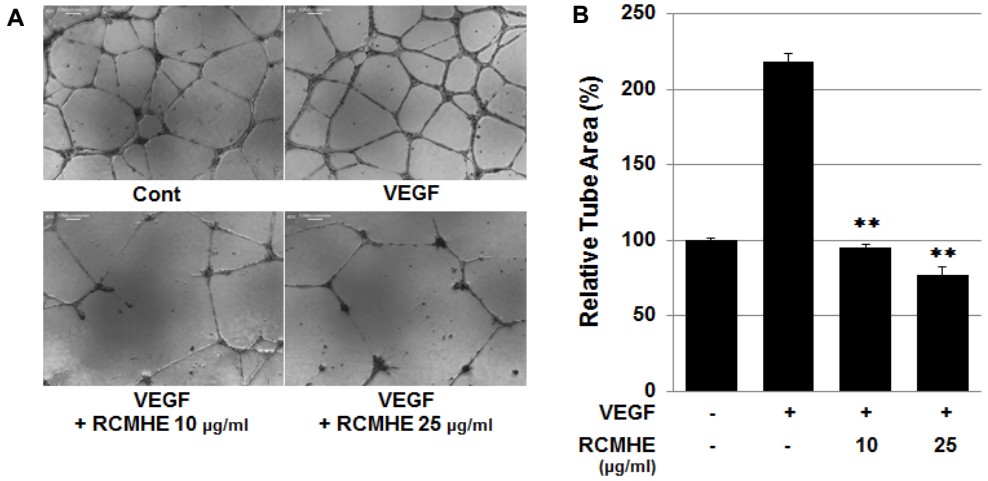

Fig. 5. Effect of RCMHE on the VEGF-induced tube formation of HUVECs. HUVECs were pre-incubated for 40 min with 10 or 25 μg/ml RCMHE, plated on Matrigel-coated plates, and then incubated in the presence or absence of 20 ng/ml VEGF. Photographs were obtained after 20 hr (×40). (A) Representative images of endothelial tube formation. (B) The area covered by the capillary-like tubes was measured using Image-Pro Plus software. Data shown are the mean ± SD of triplicate determinations. $^{**}p<0.01$ versus VEGF alone.

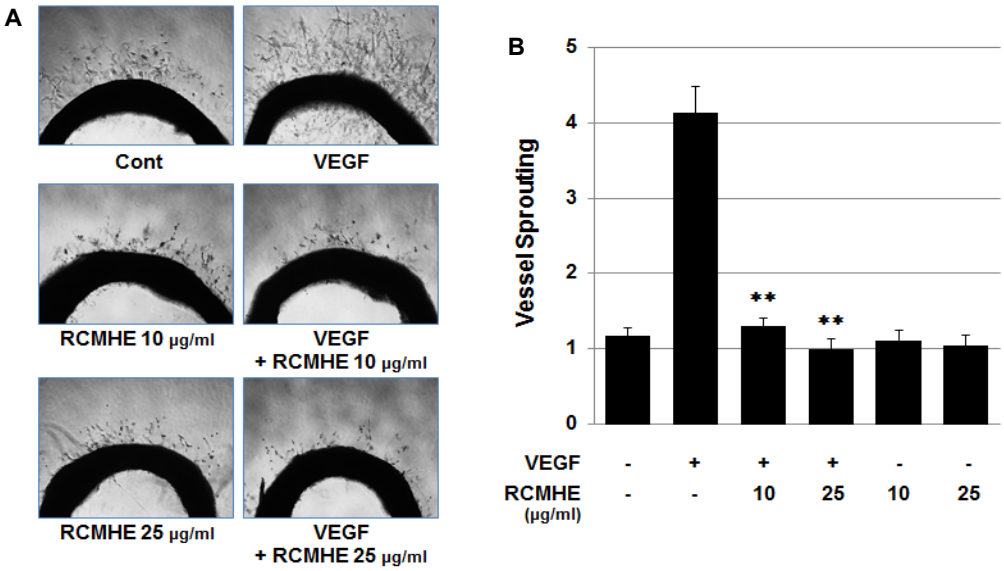

Fig. 6. Inhibitory effect of RCMHE on VEGF-induced vessel sprouting *ex vivo*. Rat aortic rings were placed in Matrigel and treated with 10 or 25 μg/ml RCMHE in the presence or absence of VEGF (20 ng/ml) and photographed by microscopy on day 7. (A) Representative images were photographed. (B) RCMHE inhibits VEGF-induced vessel sprouting. The assay was scored from 0 (least positive) to 5 (most positive), and the data shown is the mean ± SD (n=6). $^{**}p<0.01$ versus VEGF alone.

to serious side effects [28]. Therefore, there has been a renewed interest in identifying VEGF inhibitors from natural herbal sources, given the advantage of their proven safety for human use [5, 7, 13]. Additionally, certain plant-derived diets containing phytochemicals have been implicated in the prevention of cancer development and progression [2, 24, 50, 56]. The fruit of the black raspberry, RCM, was reported to have anti-inflammatory, anti-nociceptive, anti-gastropathic, anti-rheumatic, and chemopreventive effects [12, 31, 40]. However, the anti-angiogenic properties of RCMHE and its underlying mechanisms have not been studied. This study is the first to identify the anti-angiogenic activity of RCMHE using both *in vitro* and *ex vivo* models.

The binding of VEGF to KDR/VEGFR2, a primary mediator of VEGF signaling [19], results in the phosphorylation of VEGFR2, which in turn transmit signals to various intra-

cellular proteins such as p44/42 MAPK (ERK1/2) and p38 MAPK. This cascade promotes survival, induces proliferation, enhances migration and invasion, and results in tube formation in the endothelial cells, which contribute to vascular sprouting in the tumor [37, 46, 47, 51]. In the present study, we observed that RCMHE significantly suppressed the stimulatory effect of VEGF on endothelial cell proliferation (Fig. 1B), and reduced the migration and tube area of the endothelial tubes induced by VEGF (Fig. 3 and Fig. 5). Since RCMHE had no significant effect on the normal growth of endothelial cells up to concentrations of 25 μg/ml (Fig. 1A), its anti-proliferative action at low concentrations seems to reflect a specific response to the endothelial cells stimulated by VEGF. Recently, we reported that cold-water extracts of *Rubus coreanus* Miquel (RCME) inhibited angiogenesis *in vitro* and *ex vivo* (in press).

To clarify the anti-angiogenic signaling mechanisms of RCMHE, we examined its effect on the activation of ERK, p38 and the tyrosine phosphorylation of KDR/ VEGFR2 in response to VEGF. As shown in Fig. 2, the VEGF-stimulated phosphorylation of VEGFR2 was inhibited by RCMHE treatment, resulting in the down-regulation of ERK and p38 MAP kinase phosphorylation in response to VEGF in HUVECs. Thus, RCMHE may be useful as a potent angiogenesis inhibitor by inhibiting VEGFR2- mediated signaling pathways. This finding requires further study to understand the inhibitory action of RCMHE on the interactions of VEGF-KDR.

Endothelial cell invasion, the penetration of endothelial cells into new areas of the body, is also an essential event during angiogenesis. This process involves the degradation of the extracellular matrix (ECM) and the basement membrane (BM), and is achieved by the activation of extracellular endopeptidases and matrix metalloproteinases (MMPs) [55]. The degradation of the ECM by MMPs provides space into which vascular endothelial cells can migrate and form new sprouts, thus liberating key growth factors fettered within the ECM, including VEGF [41]. Several of the MMPs, most notably MMP-2 and MMP-9, have been linked to angiogenesis [43] and are up-regulated by VEGF [49, 59]. Selective inhibitors of MMP-2 and MMP-9 have been shown to block tumor and endothelial cell migration *in vitro* and tumor growth and invasion *in vivo* [32]. However, the relationship between MMPs and angiogenesis is much more complicated. In this experiment, we observed that RCMHE markedly reduces the number of invaded endothelial cells and the activation of MMP-2 and MMP-9 (Fig. 4). Similarly, RCMHE strongly suppressed the sprouting of endothelial cells in the rat aorta in response to VEGF (Fig. 6). This *ex vivo* anti-angiogenic activity of RCMHE may be explained by its inhibitory effect on the proliferation, migration, invasion, and tube formation of endothelial cells in response to VEGF through the inhibition of the tyrosine phosphorylation of VEGFR2.

In conclusion, our present study demonstrated for the first time that RCMHE inhibits VEGF-induced angiogenesis *in vitro* and *ex vivo*. Our results not only provide additional pharmacological information regarding the therapeutic efficacy of RCM, but also show its potential as a novel candidate for the development of new pharmaceutical anti-angiogenic drugs targeting the VEGF signaling pathway.

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초록 : 복분자 온수추출물의 VEGF-유도성 혈관신생 억제효과

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약초 추출물을 이용한 혈관신생 억제는 많은 고형종양을 치료하기 위한 효과적인 방안으로 인식되어 왔다. 현재까지 가장 효과적으로 종양을 억제하는 방법은 VEGF-유도성 혈관형성 경로를 목표로 하는 것이다. 본 연구에서는 처음으로 복분자 온수추출물의 혈관형성 억제효과를 *in vitro*와 *ex vivo* 실험을 통해서 확인하였다. 복분자 온수추출물은 VEGF-유도성 혈관신생을 억제할 뿐만 아니라 ERK와 p38의 인산화, MMP의 활성화를 억제하였다. 또한, 복분자 온수추출물은 VEGF에 의해서 유도된 VEGFR2 인산화를 억제하였다. 이 결과들은 복분자 온수추출물이 VEGFR2의 인산화를 저해함으로써 혈관신생을 억제하고 이것은 혈관신생과 관련된 질병을 치료하는데 좋은 소재가 될 수 있을 것으로 사료된다.