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The Anti-angiogenic Potential of a *Phellodendron amurense* Hot Water Extract *in Vitro* and *ex Vivo*

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Blocking new blood-vessel formation (angiogenesis) is now recognized as a useful approach to the therapeutic treatment of many solid tumors. The best validated approach to date is to target the vascular endothelial growth-factor (VEGF) pathway, a key regulator of angiogenesis. Many natural products and extracts that contain a variety of chemopreventive compounds have been shown to suppress the development of malignancies through their anti-angiogenic properties. *Phellodendron amurense*, which is widely used in Korean traditional medicine, has been shown to possess antitumor, antimicrobial, and anti-inflammatory properties, among others. The present study investigated the effects of *P. amurense* hot-water extract (PAHWE) on angiogenesis, a key process in tumor growth, invasion, and metastasis. To investigate PAHWE's anti-angiogenic properties, this study's authors performed an analysis of angiogenesis and endothelial-cell proliferation, migration, invasion, and tube formation, as well as zymogram assays and the rat aortic ring-sprouting assay. PAHWE inhibited cell growth, mobility, and vessel formation in response to VEGF *in vitro* and *ex vivo*. Furthermore, it reduced VEGF-induced intracellular signaling events, such as the activation of matrix metalloproteinases (MMPs) -2 and -9. These results indicate that PAHWE's anti-angiogenic properties might lead to the development of potential drugs for treating angiogenesis-associated diseases such as cancer.

Key words : Angiogenesis, cancer, *Phellodendron amurense h*ot water extract, vascular endothelial growth factor

Introduction

Angiogenesis, the formation of new blood vessels from existing ones, has been identified as a hallmark of tumor growth and metastasis [11] and is a tightly regulated process including proliferation, migration, invasion, and tube formation of endothelial cells [7, 8], and there is extensive evidence suggesting that anti-angiogenic therapy might be a promising anticancer therapeutic strategy [46]. A variety of growth factors can stimulate angiogenesis both *in vitro* and *in vivo* [12]. VEGF is the most commonly expressed known angiogenic factor [9]. To date, only bevacizumab, a humanized monoclonal antibody targeting VEGF, has been approved by the U.S. Food and Drug Administration (FDA) for the treatment of certain types of cancer, including colon, lung, kidney, and brain cancer [32, 33]. However, adverse effects of bevacizumab have been reported, such as hypertension, and there are a number of potential problems that warrant caution in human clinical trials [3, 45]. Recently, several plant-derived compounds have been shown to be potent inhibitors of angiogenesis both *in vitro* and *vivo* [19, 29, 50]. Therefore, herbal medicine with few side effects is a promising strategy for anti-angiogenic tumor therapy.

P. amurense has multiple pharmacological activities and has been used to treat inflammation, gastroenteritis, abdominal pain, diarrhea and psychosomatic diseases, and is shown to exhibit minimal toxicity to normal tissues [25, 28, 39, 53]. *P. amurense* extract containing isoquinoline alkaloids, phenolic compounds and flavone glycosides has been reported to inhibit proliferation of prostate and lung cancer cells, and its antiproliferative effects are comparable with those of berberine, which is a well-known chemopreventative agent [15, 20, 34, 36]. In addition, *P. amurense* ex-

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tract prevents the development and progression of prostate tumors [16, 28], and inhibits pancreatic cancer cell growth [17] and tumorigenesis in mouse skin, and induces apoptotic cell death in human squamous carcinoma and melanoma cells [26]. Thus, it may be inferred that *P. amurense* extract is a potent anticancer agent for various cancers; however, there have been no scientific reports identifying the anti-angiogenic activity of *P. amurense* extracts.

We have demonstrated that the *P. amurense* hot water extract (PAHWE) efficiently inhibits the VEGF-induced angiogenic activity of human umbilical vein endothelial cells (HUVECs) and reduces new vessel formation from the rat aorta.

Materials and Methods

This experiment followed the guidelines provided in the "Guide for the Care and Use of Laboratory Animals" adopted by the United States National Institutes of Health. The research protocols used in this study were reviewed and approved by the Ethics Committee and the Institutional Animal Care and Use Committee (IACUC) of Yonsei University Wonju Campus (IACUC Approval # YWC-131127-1).

Preparation of *P. amurense* hot water extract

P. amurense was cultivated according to the good agricultural practices method of the Korea Rural Development Administration and harvested in 2009 in Eumseong (GPS: E 128° 62′ N 36° 56′). For sample preparation, the dried *P. amurense* 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 PAHWE was obtained and dissolved in distilled water at different concentrations.

Reagents

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from the USB Corporation (Cleveland, OH, USA). Matrigel basement membrane matrix was obtained from BD Biosciences (Bedford, MA, USA). $hVEGF_{165}$, recombinant human $VEGF_{165}$ with rat cross reactivity, was purchased from PEPRO TECH (Rocky Hill, NJ, USA). The cell culture reagents and most other biochemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise specified.

Cell culture and animal maintenance

HUVECs were purchased from American Type Culture Collection (ATCC®, Manassas, VA, USA). HUVECs at early passages (passages 6-10) were used in all experiments and were cultured on gelatin-coated plates in M199 medium (GIBCO®, Grand Island, NY, USA) supplemented with 20% fetal bovine serum (FBS) (GIBCO®, Gaithersburg, MD, USA), 3 ng/ml basic fibroblast growth factor (KOMABIOTECH, Seoul, Korea), 100 unit/ml penicillin, 100 mg/ml streptomycin (GIBCO®, Grand Island, NY, USA), and 5 unit/ml heparin (Sigma-Aldrich, St. Louis, MO, USA) and incubated at 37°C in 5% CO_2 in air. Seven-week-old Sprague-Dawley rats were purchased from Orient Co. (Sungnam, Korea) and were maintained on standard chow and water *ad libitum*.

Cell viability assay

An MTT assay was conducted to assess HUVEC cell viability after PAHWE treatments. Briefly, HUVECs were cultured at a concentration of 5×10^4 cells/well on gelatin-coated 24-well plates in M199 media (20% FBS). After 24 hr, the medium of each well was replaced with fresh M199 media containing 1% FBS, supplemented with PAHWE (1-100 μg/ml). The cells were incubated for 24 hr, and then washed out using phosphate buffered saline (PBS). Subsequently, 1 ml of serum free M199 media containing 0.5 mg/ml of MTT dye was provided to the cells and incubated for 4 hr. After incubation, the medium was carefully removed from the plate, and dimethyl sulfoxide (DMSO) was added to solubilize the formazan produced from MTT by viable cells. Absorbance was measured at 595 nm using a microplate reader (Molecular Devices, Sunnyvale, CA).

Cell proliferation assay

HUVECs (4×10^4 cells/well) were seeded in M199 medium containing 20% FBS in gelatinized 12-well plates and incubated for 24 hr. The cells were starved in M199 medium containing 1% FBS for 6 hr, and then grown with PAHWE at final concentrations of 1-25 μg/ml in the presence or absence of VEGF (20 ng/ml). After 48 hr of incubation, cell proliferation was determined by an MTT assay or counted with a Luna™ Automated Cell Counter (Logos Biosystems, Korea).

Migration and invasion assay

Cell migration and invasion were assayed in modified Boyden chambers as previously described with some modifications [31]. Briefly, for the migration assay, cell culture inserts with membrane filters with a 3-μm pore size (Transwell®, Corning Costar) were coated with 10 μg of gelatin on the lower-surface. The cell suspensions were incubated for 30 min at 37°C in M199 medium (1% FBS) containing various concentrations of PAHWE and then seeded into the inserts at 5×10^4 cells (100 μl)/insert. M199 medium (1% FBS, 600 μl) containing VEGF (20 ng/ml) was added into the chamber as a chemoattractant. The plate was incubated for 4 hr at 37°C in a humidified atmosphere of 5% CO_2. The invasion assay was performed in the same way except that the insert was used directly with Matrigel (3 μl, Matrigel® Matrix, BD Biosciences) coating. The number of migrated or invaded cells on the under-surface of the insert was counted in eight fields of each membrane under an inverted optical microscope (x200), and the average number of cells in each field was calculated. The data were expressed as percent invasion or migration compared with the control.

Zymogram assay

The activity of matrix metalloproteinases was determined by gelatin zymography. Briefly, after cells were treated with VEGF for 12 hr, cultured medium was centrifuged at 1500 rpm for 5 min at 4°C to remove cellular debris. Supernatants containing 20 μg of secreted proteins were then mixed with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer without reducing agent. Protein samples were then loaded onto 10% SDS-PAGE copolymerized with 0.2% gelatin and subjected to electrophoresis. The 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 overnight. The gels were stained with 0.25% Coomassie brilliant blue R250 (Sigma-Aldrich) and destained with 7% acetic acid. In addition, we used conditioned medium with PMA (40 ng/ml)-stimulated HUVECs to distinguish between different matrix metalloproteinases (MMPs).

Tube formation assay

Matrigel was used to assess the tube formation potential of PAHWE. In brief, each well of 24-well plates was coated with 250 μl of Matrigel. After a 30 min incubation at 37°C in 5% CO_2 to form a matrix, HUVECs preincubated with PAHWE for 40 min were seeded onto the gel at the concentration of 2×10^5 cells/well in M199 supplemented with 1% FBS, different concentrations of PAHWE (10 and 25 μg/ml), and VEGF (20 ng/ml) for 20 hr. To evaluate tube- ike structure formation, cells were photographed with an inverted microscope and the average branch areas were measured using Image-Pro Plus (Media Cybermetics, Bethesda, MD).

Rat aortic ring assay

To investigate the anti-angiogenic activity of PAHWE, its inhibitory effect on neovessel formation was evaluated using an *ex vivo* rat aortic ring sprouting assay. The assay was performed according to the standard protocol [2], with minor modifications. Seven-week-old male Sprague-Dawley rats were humanely sacrificed via cervical dislocation under anesthesia with diethyl ether. The thoracic aorta was excised, rinsed with HBSS, cleaned of fibroadipose tissue and cross sectioned into thin rings of 1-mm thickness. Afterwards, 120 μl of Matrigel was loaded in each 48-well plate and one aortic ring was seeded in each well and covered with another layer of Matrigel matrix (50 μl). After polymerization for 30 min, serum-free M199 media was added to each well. VEGF with or without PAHWE was then added to the wells to a final volume of 600 μl of human endothelial serum-free medium M199. On day 7, aortic rings were photographed at ×40 magnification using a Nikon eclipse TS100 inverted microscope. The angiogenic response was quantified by measuring the number of neovessels that sprouted out of the rings during the incubation period. 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 data were analyzed for statistically significant differences between groups by using the Student's t-test and one-way analysis of variance (ANOVA). A p-value lower than 0.05 was considered to be significant.

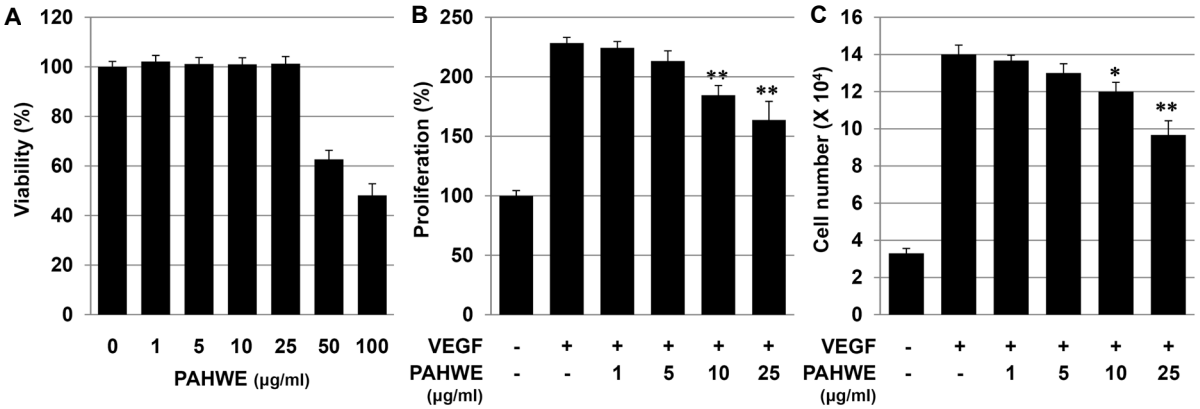

Fig. 1. Effect of PAHWE on cytotoxicity and proliferation of endothelial cells. (A) HUVECs were exposed to PAHWE at various concentrations (1, 5, 10, 25, 50, and 100 μg/ml). After 24 hr, cytotoxicity was quantified by an MTT assay. The vehicle-treated cells were used as the control (100%). (B, C) HUVECs were treated with various concentrations (1, 5, 10, and 25 μg/ml) of PAHWE for 40 min before exposure to VEGF (20 ng/ml). The number of proliferating cells was quantified by an MTT assay (B) and counted with an automated cell counter (C) 48 hr later. Each bar represents the average ± SE of three independent experiments. *p<0.05 and **p<0.01 versus VEGF alone.

Results

Effect of PAHWE on cytotoxicity and proliferation of endothelial cells

Because VEGF-stimulated endothelial cell proliferation is a key component of the angiogenic response, we first examined the effect of PAHWE on the mitogenesis of HUVECs. In the cell proliferation assay, VEGF-induced proliferation of HUVECs was significantly suppressed by PAHWE treatment in a clear dose-dependent manner (Fig. 1B and 1C). These inhibitory effects were not due to cytotoxicity because up to 25 μg/ml PAHWE had no effect on the normal growth of HUVECs in the absence of VEGF (Fig. 1A). A significant inhibitory effect on cell viability was observed in response to PAHWE at concentrations ≥50 μg/ml in the cytotoxicity assay.

Effect of PAHWE on migration of endothelial cells

To determine whether PAHWE was capable of influencing endothelial cell migration, we further examined the an-

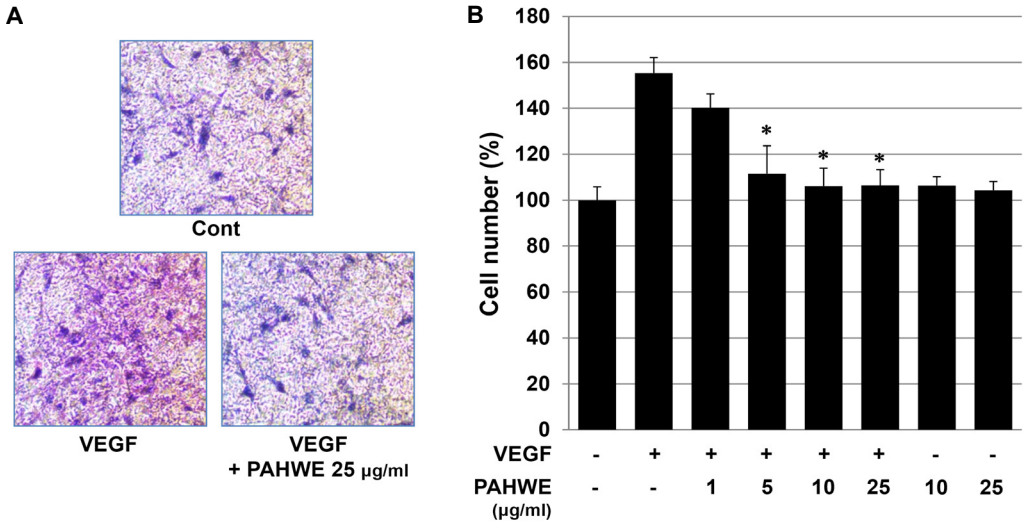

Fig. 2. Effect of PAHWE on VEGF-induced migration of endothelial cells. HUVEC migration as determined by using the Transwell culture plate. HUVECs were pretreated for 40 min at various concentrations (1, 5, 10, and 25 μg/ml) of PAHWE before exposure to VEGF (20 ng/ml). After incubation for 4 hr, chemotactic migration was evaluated. Cells that migrated to the bottom of the filter were photographed (A) and counted (B) using inverted optical microscopy. Data are expressed as mean ± SE (n=3). *p<0.01 versus VEGF alone.

ti-migration activity of PAHWE on HUVECs. We observed that PAHWE treatment remarkably inhibited VEGF-induced endothelial cell migration in comparison with corresponding untreated HUVECs in a non-cytotoxic range of concentrations (Fig. 2). However, PAHWE alone had no significant effect on the basal migration of endothelial cells.

Effect of PAHWE on invasive activity of endothelial cells

Next, we evaluated the ability of PAHWE to inhibit the invasion of human endothelial cells using a Matrigel chemoinvasion assay. Results showed that the invasive activity of HUVECs in response to VEGF was significantly inhibited in a dose-dependent manner by PAHWE, whereas no suppressive activity was observed in cells treated with PAHWE alone (Fig. 3A).

Effect of PAHWE on expression of MMP-2 and MMP-9 in endothelial cells

Migration and invasion, including degradation of the basement membrane (BM) and extracellular matrix (ECM), is an essential pattern in angiogenesis. To verify whether PAHWE could also down-regulate the expression of the pro-angiogenic MMPs, we quantified the extracellular levels of MMP-2 and -9 that were released by HUVECs using a zymogram assay. 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 staining. As shown in Fig. 3B, VEGF-stimulated expression of MMP-2 and -9 in HUVECs was decreased by PAHWE treatment in a dose-dependent manner, and 25 μg/ml of PAHWE completely inhibited their expression as compared to vehicle treatment. In addition, we used conditioned medium from PMA-stimulated HUVECs to distinguish between different MMPs.

Effect of PAHWE on tube formation of endothelial cells

Tube formation is another key step in angiogenesis. We evaluated the effects of PAHWE on two-dimensional vessel formation by HUVECs on Matrigel matrix-coated surfaces after addition of non-toxic doses of extract. PAHWE exposure suppressed the VEGF-responsive formation of tubular structures in a concentration-dependent manner (Fig. 4). In the absence of PAHWE, the HUVECs stimulated with VEGF constructed strong and thick vessel tubes by connecting to neighboring cells, but in the presence of PAHWE, intercellular connections were limited and the HUVEC cells failed to form tubes. To quantify the degree of tube formation, photographs were taken under an inverted microscope and the tube area was calculated using image analysis software. This result suggests that PAHWE may have

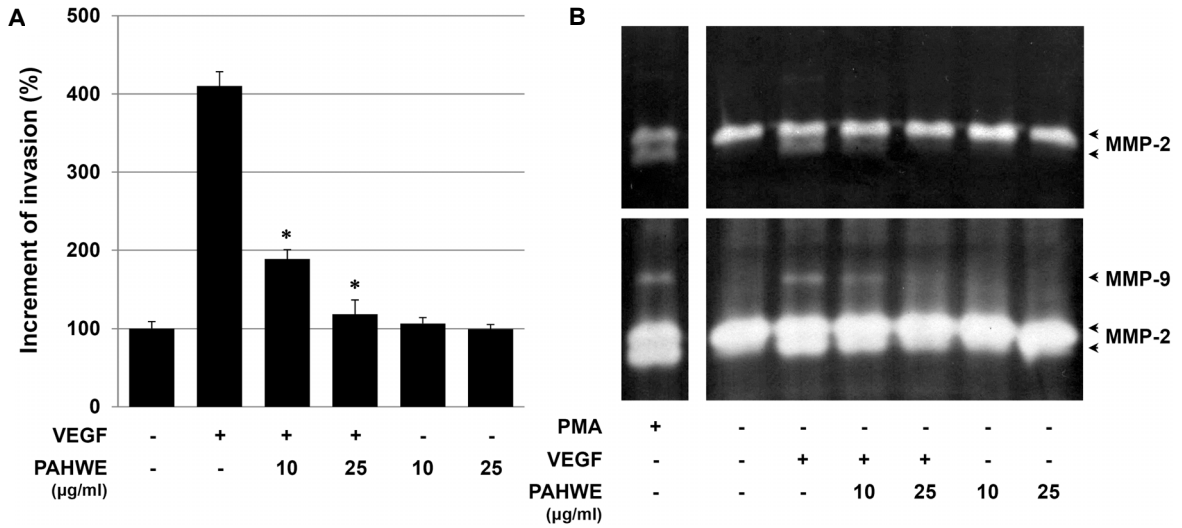

Fig. 3. Effect of PAHWE on VEGF-induced invasion of endothelial cells and activity of MMP. HUVECs were pretreated for 40 min with PAHWE (10 and 25 μg/ml) before exposure to VEGF (20 ng/ml). HUVECs were then exposed to VEGF (20 ng/ml) for 24 hr and chemotactic invasion was evaluated. Invading cells were counted using inverted optical microscopy (A). Changes in MMP-2 and MMP-9 activity were measured by gelatinolytic zymogram analysis (B). 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. Data are expressed as the mean ± SE from triplicate experiments. *p<0.01 versus VEGF alone.

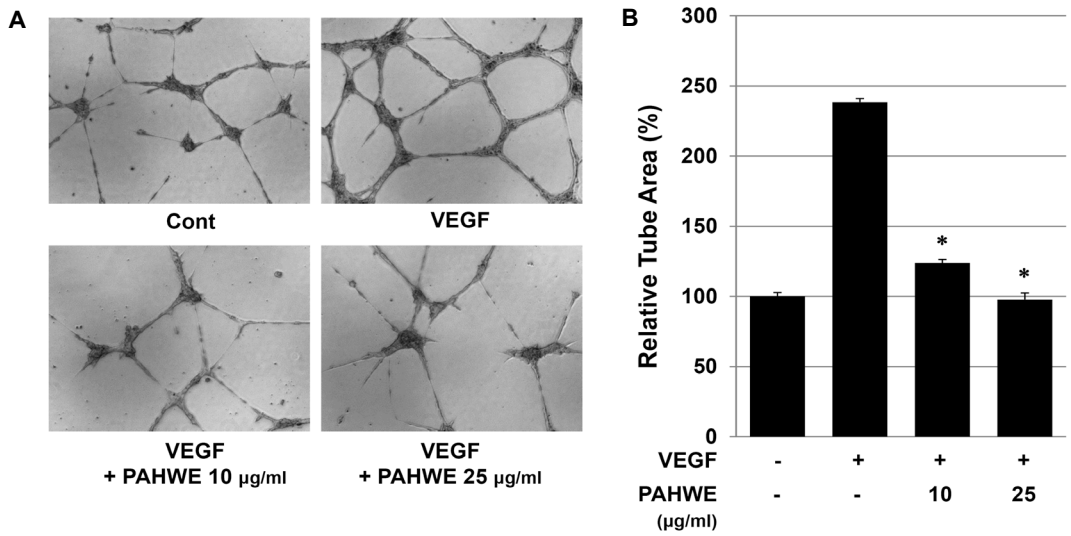

Fig. 4. Effect of PAHWE on VEGF-induced tube formation of endothelial cells. HUVECs were pre-incubated for 40 min with PAHWE (10 and 25 μg/ml), plated on Matrigel matrix-coated plates at a density of 2×10^5 cells per well, and then incubated in the presence or absence of 20 ng/ml VEGF for 20 hr. VEGF treatment alone served as a positive control. (A) Photographs were obtained after 20 hr using an inverted phase contrast microscope (×40). (B) The area covered by the capillary-like tubes was measured using Image-Pro Plus software. Data shown are the mean ± SE of triplicate determinations. *p<0.01 versus VEGF alone.

an inhibitory effect on angiogenesis

Effect of PAHWE on microvessel outgrowth from the rat aorta

In order to evaluate the antiangiogenic properties of PAHWE, we performed the rat aortic ring assay with 10 and 25 μg/ml PAHWE. The rat aortic ring sprouting assay is a widely used *ex vivo* anti-angiogenic model that mimics several stages of angiogenesis, including vascular endothelial cell proliferation, migration and tube formation. Treat-

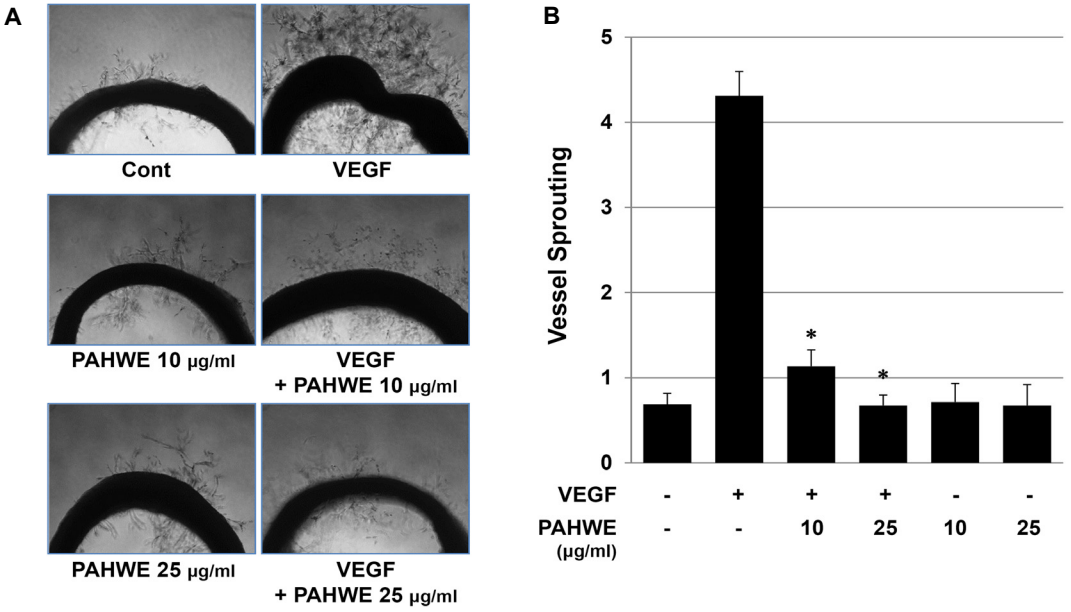

Fig. 5. Effect of PAHWE on microvessel outgrowth from the rat aorta *ex vivo*. Aortas in Matrigel were exposed to VEGF (20 ng/ml) in the absence or presence of PAHWE (10 and 25 μg/ml) and photographed via microscopy on day 7. (A) Representative aortic rings were photographed. (B) PAHWE blocks VEGF-induced neovessel formation from the aorta. The assay was scored from 0 (least positive) to 5 (most positive), and the data are mean ± SE values (n=6). *p<0.01 versus VEGF alone.

ment with VEGF stimulated vessel outgrowth when compared to the results with medium alone. However, the microvessel outgrowth from VEGF-treated aortic rings was significantly reduced by treatment with PAHWE in a dose-dependent manner (Fig. 5). This result indicated that PAHWE may be a useful anti-angiogenic drug candidate that blocks the VEGF signaling pathway.

Discussion

Anti-angiogenic cancer therapy that targets tumor-induced angiogenesis by affecting endothelial cells has yielded promising outcomes in clinical trials [43]. Angiogenesis is comprised of multiple tightly controlled, complex processes and is triggered by an interaction with pro-angiogenic factors, especially VEGF, and its receptors [37]. This angiogenic process requires a series of sequential events; after activation, BM and ECM are degraded by proteases such as matrix metalloproteinases (MMPs). Then, endothelial cells migrate and proliferate into new areas, differentiating to form new vascular tube structures [35]. VEGF and their receptors have been implicated in tumor angiogenesis that occurs in many solid tumors, such as breast cancer [27], colon cancer [44], hepatoma [51], ovarian cancer [5], gastric cancer [22] and prostate cancer [1]. Thus, anti-angiogenic agents, especially those targeting the VEGF-signaling pathway in vascular endothelial cell, are highly promising anticancer agents [10, 13, 21, 24, 41].

P. amurense has a long history of ethnobotanical use among native peoples in East Asia and is known to be a rich source of berberine and aphorphine alkaloids, flavonoids, various coumarins, lignans and limonoids [30]. Earlier reports showed that *P. amurense* extract has an anxiolytic effect in rodent stress studies [40]. It also has chemotherapeutic potential, and arrests cell cycle progression at the G0/G1 phase in prostate cancer cells [15]. The extract exhibits various biological effects, such as an inhibitory effect on prostatic contractility [49], an anti-osteoarthritic effect [23], an anti-inflammatory property [48], and anticancer activity [16, 18, 26], but antiangiogenic effects have not been reported yet. This present study aimed to elucidate the effect of the hot water extract of *P. amurense* on angiogenesis. We tested cell biologic functions such as extracellular matrix degradation, cell motility, cell proliferation, cell invasion and tube formation *in vitro,* as well as *ex vivo* neovessel formation associated with angiogenesis. We are the first group to demonstrate that PAHWE inhibits VEGF-induced angiogenesis in HUVECs and the rat aorta. Our experimental results demonstrate that PAHWE inhibited VEGF-responsive HUVEC proliferation in a concentration-dependent manner without cytotoxicity (Fig. 1). The interaction of VEGF with its receptors stimulates the intrinsic receptor tyrosine kinase activity and subsequently mediates the activation of MAP kinases including ERK and p38, which are important for angiogenesis [4, 38, 42, 52]. However, in these experiments, we did not observe that VEGF-stimulated phosphorylation of receptor tyrosine kinase (VEGFR2), ERK, and p38 was downregulated by PAHWE treatment. MMPs are implicated in the tissue remodeling that occurs during angiogenesis via the degradation of ECM and basement membrane components, and are also key enzymes involved in the migration and invasion of endothelial cells [6, 14, 47]. PAHWE exposure attenuated the VEGF-stimulated increase the number of migrating and invading cells in a dose-dependent manner (Fig. 2 and 3A). In addition, VEGF-induced activation of MMP-2 and MMP-9 was markedly reduced by treatment with PAHWE (Fig. 3B). Taken together, these inhibitory effects of PAHWE on the activity of MMPs and cellular proliferation, migration, and invasion resulted in global inhibition of HUVEC cell tube formation *in vitro* on a Matrigel matrix (Fig. 4). The final step in this study was to test if the PAHWE can exhibit its antiangiogenic properties *ex vivo,* as it did in *in vitro* assays. The VEGF-induced neovessel formation from rat aorta was significantly inhibited by treatment with PAHWE (Fig. 5). The results show that PAHWE inhibited many crucial steps of the angiogenesis process; therefore, this plant may provide a new source of antiangiogenic agents as potential candidates for treatment of angiogenesis related diseases, such as cancer, psoriasis, rheumatoid arthritis and diabetic retinopathy.

Acknowledgments

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초록 : *In vitro*와 *ex vivo*에서 황백 온수추출물의 신생혈관 억제효과

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신생혈관형성을 억제하는 것은 최근들어 많은 고형암의 치료에 있어서 매우 유용한 접근 방법이다. 현재까지 가장 잘 알려진 접근 방법은 신생혈관형성의 핵심인자인 혈관내피세포성장인자(VEGF)를 표적으로 하는 방법이다. 다양한 화학예방 물질들을 포함하는 많은 자연의 생산물들이나 추출물들은 그들의 항신생혈관형성 성질을 통하여 악성종양의 성장을 억제하고 있다. 황백은 항종양, 항균, 항염증 및 그외 다른 생물학적 작용을 가지고 있는, 오래전부터 널리 사용되어온 한국 전통 약재이다. 우리는 종양의 성장, 침투, 전이에 있어서 매우 중요한 과정인 신생혈관형성에 미치는 황백 온수추출물의 효과를 연구하였다. 황백 온수추출물의 항신생혈관형성 효과를 확인하기 위해서 혈관 내피세포의 성장, 이동, 침투, 관형성 그리고, 자이모그램 분석을 수행하였으며, 흰쥐 대동맥 주변 미세혈관 발아실험을 진행하였다. 그 결과, 황백 온수추출물은 혈관내피세포성장인자(VEGF)에 의해 유도되는 혈관내피세포의 성장, 이동, 침투, 관형성 그리고, 대동맥의 혈관발아를 억제하는 효과를 *in vitro*와 *ex vivo* 실험을 통해서 확인하였다. 또한, 황백 추출물은 VEGF에 의해 유도되는 기질금속단백질분해효소 (MMP)-2와 -9의 활성화를 저해하였다. 본 연구 결과들은 황백 온수추출물의 신생혈관형성 억제작용이 암과 같은 혈관신생과 관련된 질병을 치료하는데 좋은 소재가 될 수 있음을 시사한다.