

Parthenolide exerts inhibitory effects on angiogenesis through the downregulation of VEGF/VEGFRs in colorectal cancer

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Abstract. Parthenolide (PT) is responsible for the bioactivities of feverfew (*Tanacetum parthenium*). Apart from its potent anti-inflammatory effects, this compound has been reported to induce apoptosis in various cancer cells. However, little is known about its role in the process of tumor angiogenesis. In the present study, we investigated the effects and potential mechanisms of action of PT on angiogenesis in human colorectal cancer (CRC). The anti-angiogenic effects of PT were evaluated in cultured human umbilical vein endothelial cells (HUVECs) and in the human CRC cell lines, HT-29, SW620 and HCT116. PT markedly inhibited vascular cell migration and capillary-like structure formation even at a dose which had not effects on cell viability. PT also suppressed the expression of angiogenic biomarker proteins [vascular endothelial growth factor (VEGF), VEGF receptor (VEGFR)1 and VEGFR2] in both the HUVECs and CRC cells. Additionally, PT effectively inhibited tumor neovascularization in a HT-29 xenograft model. These results indicate that PT suppresses angiogenesis by reducing the expression of VEGF and its receptors and may be a viable drug candidate in anti-angiogenesis therapies for human CRC.

Introduction

The process of new blood vessel formation or angiogenesis has become widely recognized as an important target in the treatment of various types of cancer, including metastatic colorectal cancer (mCRC). Folkman's hypothesis of tumori-

genesis states that tumor growth, invasion and metastasis depend on angiogenesis, which is regulated by pro-angiogenic factors and inhibitors (1-3). Therefore, the blocking of angiogenesis in cancer therapy has become a focus of cancer research. Tumor angiogenesis is closely related with vascular endothelial cells and angiogenesis-related factors. The induction of angiogenesis is mediated by a variety of molecules secreted from cells within tumors. It is well known that vascular endothelial growth factor (VEGF) plays a crucial role in regulating angiogenesis and has become an essential step in anti-angiogenic therapy for the growth and metastasis of cancer (4,5). The agent directly targeting VEGF, bevacizumab, has become part of the standard treatment regimen for mCRC. A number of studies have demonstrated an advantage in favor of the addition of bevacizumab to chemotherapeutic regimens in terms of overall survival, progression-free survival and response rate (6). However, some side-effects attributable to bevacizumab therapy and bevacizumab-resistant tumors have been reported (7,8). Therefore, it is necessary to find a new anti-angiogenic treatment strategy for CRC.

Parthenolide (PT), a natural product, has been used for the treatment of fevers and inflammatory diseases (9). It is responsible for the bioactivities of feverfew (*Tanacetum parthenium*). Studies have indicated that PT has strong antitumor activities, such as the inhibition of proliferation and the promotion of the apoptosis of tumor cells in various types of cancer (10-14). In a previous study, using xenograft models, we demonstrated that PT may be a potential chemopreventive and therapeutic agent for CRC (15). Moreover, we have demonstrated that combination treatment with PT enhances sensitivity to 5-fluorouracil and promotes apoptosis in CRC (16). However, little has been reported on the effects of PT on vascular endothelial cells from the point of view of tumor angiogenesis. In particular, PT as an anti-angiogenic agent has not been evaluated using an animal model of CRC. Therefore, in this study, we examined whether PT exerts anti-angiogenic effects on human endothelial cells, human CRC cells and in an *in vivo* mouse model.

Materials and methods

Chemicals and reagents. PT was obtained from Calbiochem (San Diego, CA, USA). Propidium iodide (PI) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Growth factor-

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reduced Matrigel was purchased from BD Biosciences (San Diego, CA, USA). Mouse recombinant VEGF was purchased from R&D Systems Inc., (Minneapolis, MN, USA). Anti-VEGF, anti-VEGF receptor (VEGFR)1, anti-VEGFR2 and anti-actin antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). PT was dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich) to a concentration of 100 μ M and stored in the dark at -20°C. An aliquot of DMSO was stored under the same conditions and used as a control treatment.

Cell culture and treatment. Human umbilical vein endothelial cells (HUVECs) were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA) and cultured in endothelial cell growth medium (EGM[®]-2 MV Bullet kit; Lonza Inc., Walkersville, MD, USA) containing 2% fetal bovine serum (FBS). The HUVECs were used at passages 3–4.

The human CRC cell lines, HT-29, SW620 and HCT116, were obtained from ATCC and cultured in RPMI-1640 medium supplemented with 10% FBS, 100 units penicillin, and 100 units streptomycin. For the treatment of the cells with PT, the cells were subcultured in RPMI-1640 medium without FBS for 12 h. PT dissolved in DMSO was diluted with FBS-free medium to achieve the indicated concentrations. The same concentrations of DMSO were applied to the cells as a control.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric survival assay. The HUVECs and CRC cells were plated at a density of 1×10^4 cells/well in 96-well plates. The cells were treated with PT for 24 h, the medium was removed, and 200 μ l of fresh medium plus 20 μ l of MTT, 2.5 mg dissolved in 50 μ l of DMSO were added to each well. Following incubation for 4 h at 37°C, the culture medium containing MTT was withdrawn and 200 μ l of DMSO were added, followed by shaking until the crystals were dissolved. Viable cells were determined by measuring the absorbance at 570 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Cell cycle and sub-G1 analysis. Cell cycle and sub-G1 distribution were determined by the staining of DNA with PI (Sigma-Aldrich) [excitation (Ex)/emission (Em), 488/617 nm]. In brief, 1×10^6 cells were incubated with PT for 24 h. The cells were then washed with phosphate-buffered saline (PBS) and fixed in 70% ethanol overnight. The cells were washed again with PBS and then incubated with PI (10 μ g/ml) and simultaneously treated with RNase at 37°C for 1 h. The percentages of cells in the different phases of the cell cycle or the sub-G1 DNA content were measured using a BD LSR flow cytometer and analyzed using CellQuest software (Becton Dickinson, New York, NY, USA).

Endothelial cell migration assay: wound healing. Cell migration was evaluated by a wound healing assay, according to the method described in the study by Reischer *et al.* (17). HUVECs (1×10^6 /well) were seeded in 6-cm culture plates and allowed to form a confluent monolayer. The monolayer was then scraped with a P200 pipette tip to generate a wound of approximately 1 mm in diameter. Subsequently, the medium was changed to

fresh endothelial basal medium (EBM-2; supplemented with 20 ng/ml of VEGF) in the absence or presence of PT. Images of the wounds were captured after 24 h of incubation at x40 magnification, and the wound area was determined using an inverted microscope (IX71; Olympus, Center Valley, PA, USA). The ability of the cells to close the wound, that is their motility, was evaluated by determining the healed area. The percentage of the healed area was calculated as follows:

$$\text{Migration percentage (\%)} = \frac{\text{number of cells in wound area (group treated with PT)}}{\text{number of cells in wound area (untreated group)}} \times 100$$

Tube formation assay with HUVECs on Matrigel. Matrigel (200 μ l/well) was added to a 24-well plate and polymerized for 30 min at 37°C. The HUVECs (1×10^4 cells/well) were seeded onto each well of the Matrigel-coated 24-well plate and then incubated in EGM-2 containing 2% FBS. Following overnight incubation, the medium was changed to fresh EBM-2 (supplemented with 20 ng/ml of VEGF) in the absence or presence of PT. The formation of endothelial cell tubular structure was visualized under an inverted microscope and photographed at x40 magnification. Furthermore, the tube formation was quantified by calculating the number of polygons and was expressed as a percentage by normalization with PT-untreated control cells.

Cell extraction and western blot analysis. The cells were collected, washed twice with PBS, and then lysed for 30 min on ice in a lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM EDTA, 1% Triton X-100, 0.5% SDS and protease inhibitor cocktail). The protein concentration in the cell lysates was measured using a Protein Quantification kit (Bio-Rad, Hercules, CA, USA). Samples (50 μ g protein/well) were loaded onto a SDS-PAGE gel. After transferring and blocking, the membrane was probed with anti-VEGF, anti-VEGFR1, anti-VEGFR2 and anti-actin antibodies. The signal was detected using WEST-one[™] enhanced chemiluminescence solution (Intron Biotechnology Inc., Daejeon, Korea), captured and analyzed by a Luminescent Image Analyzer (LAS-3000; Fujifilm, Tokyo, Japan).

Animal experiments. All animal experiments were approved by the Chonbuk National University Animal Care and Use Committee (Approval no: CBU 2012-0035). The HT-29 (6×10^6) cells were injected into nude mice. The mice were randomly assigned to the control and treatment group and intraperitoneally injected 3 times a week with the vehicle (DMSO) or 4 mg/kg PT. Treatment with PT or the vehicle commenced 7 days after tumor cell implantation (25 mm³ tumor volume). The experiment was terminated at 28 days, and the tumors were harvested for immunohistochemistry. Immunohistochemistry was carried out in paraffin-embedded 5- μ m-thick tissue sections. The sections were immunostained with an antibody against mouse CD31 (sc-507; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), visualized by appropriate biotin-conjugated secondary antibodies followed by immunoperoxidase detection with the Vectastain ABC Elite kit (Linaris, Dossenheim, Germany) and diaminobenzidine

(DAB) substrate (Vector Laboratories, Peterborough, UK). Five equal-sized fields were randomly selected. Four fields at x40 magnification were selected and the CD31-positive cells were counted.

Statistical analysis. The data are presented as the means \pm standard error (SE) of at least 3 independent experiments performed in duplicate. Representative blots are shown. All the data were entered into Microsoft Excel 5.0 and SPSS software was used to perform the two-tailed t-tests or the analysis of the variance, where appropriate. P-values <0.05 were considered to indicate statistically significant differences.

Results

Effect of PT on the viability of HUVECs. To determine the anti-angiogenic effects of PT *in vitro*, we used MTT assay to examine HUVEC viability and survival. Treatment of the HUVECs with PT at a dose of up to 2.5 μM did not decrease cell viability. However, at a dose of $>5 \mu\text{M}$ PT, the viability of the HUVECs was significantly reduced in a dose-dependent manner ($P<0.05$), showing $2.94\pm1.31\%$ viability at 20 μM (Fig. 1A). We also evaluated the effects of PT on cell cycle modifications. After 24 h of incubation of the HUVECs with or without PT, the cells were analyzed by PI staining and flow cytometric analysis. Treatment with PT resulted in the presence of a sub-G1 cell population, suggestive of cell death. The sub-G1 cell population was also increased in a dose-dependent manner (Fig 1B).

Effects of PT on endothelial cell migration and capillary structure formation. Cell migration is a key step in angiogenesis (18). We therefore investigated the effects of PT on the motility of the endothelial cells by wound healing assay. The results revealed that PT significantly reduced the migration ability of the HUVECs at 2.5 μM ($P<0.05$; Fig. 2A).

To further investigate the effects of PT on endothelial cells, we examined the VEGF-stimulated tube formation of HUVECs using Matrigel. It is well known that endothelial cells are able to spontaneously form a capillary-like network on Matrigel *in vitro*, an important feature in the process of angiogenesis (19). HUVECs spontaneously formed a capillary-like tube structure following 24 h of incubation on Matrigel (Fig. 2B). However, treatment with PT (2.5 μM) markedly inhibited the VEGF-stimulated tube formation of the HUVECs, resulting in less elongated, broken and foreshortened tubes.

Effects of PT on the expression of angiogenic biomarkers in HUVECs. VEGF induces endothelial cell proliferation, migration and survival through its cell surface receptors, VEGFR1/VEGFR2 (20). To elucidate the underlying mechanisms responsible for the anti-angiogenic activity of PT, the effects of PT on the expression of VEGF and its receptors in the HUVECs were investigated. Western blot analyses revealed that the treatment of the HUVECs with PT markedly reduced the protein levels of VEGF, VEGFR1 and VEGFR2 compared to the levels in control cells (Fig. 3).

Effects of PT on cell viability and the expression of angiogenic biomarkers in CRC cells. To assess the anti-angiogenic effects

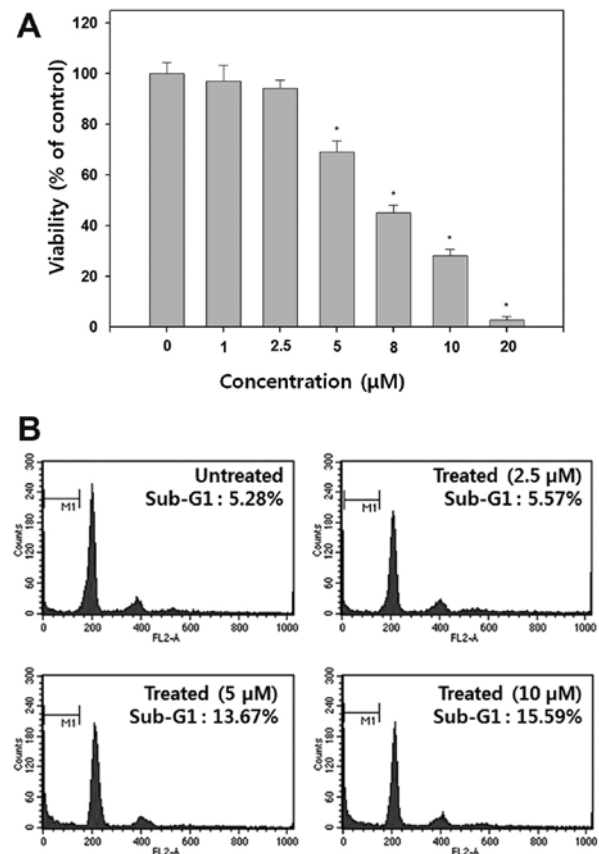


Figure 1. Effect of parthenolide (PT) on the growth of human umbilical vein endothelial cells (HUVECs). (A) Twenty-four hours of exposure to PT resulted in the inhibition of proliferation. Cell viability is expressed as the percentage compared to the vehicle-treated control (100%). Data are presented as the means \pm standard error (SE) of 3 independent experiments. * $P<0.05$ vs. control. (B) PT-induced cell death of HUVECs. Flow cytometric detection of apoptosis of HUVECs after 24 h of exposure to 0, 2.5, 5 and 10 μM PT. The percentage of the sub-G1 population is shown in each histogram. The experiments were performed at least 3 times and the result of one representative experiment is shown.

of PT in human CRC cells, we investigated its effects on cell viability by MTT assay. The HT-29, SW620 and HCT116 cells were treated with various concentrations (0, 5, 10 and 20 μM) of PT for 24 h. The viability of these 3 cell lines was decreased in a dose-dependent manner, indicating that the IC_{50} of PT is approximately 20 μM (Fig. 4A).

We also determined the expression levels of VEGF and its receptors in human CRC cells. In addition, western blot analyses revealed that the levels of VEGF and VEGFR2 were significantly decreased in a dose-dependent manner following treatment with PT (Fig. 4B); the results obtained for the CRC cells are in accord with the results obtained for the HUVECs.

Effect of PT on angiogenesis *in vivo*. To examine the effects of PT on angiogenesis *in vivo*, we used a xenograft nude mouse tumor model in which mice were subcutaneously implanted with HT-29 cells. Immunohistochemical analysis of CD31 revealed that the blood vessel network was well developed in the tumors from the control (vehicle-treated) mice, whereas the development of the blood vessel network was substantially

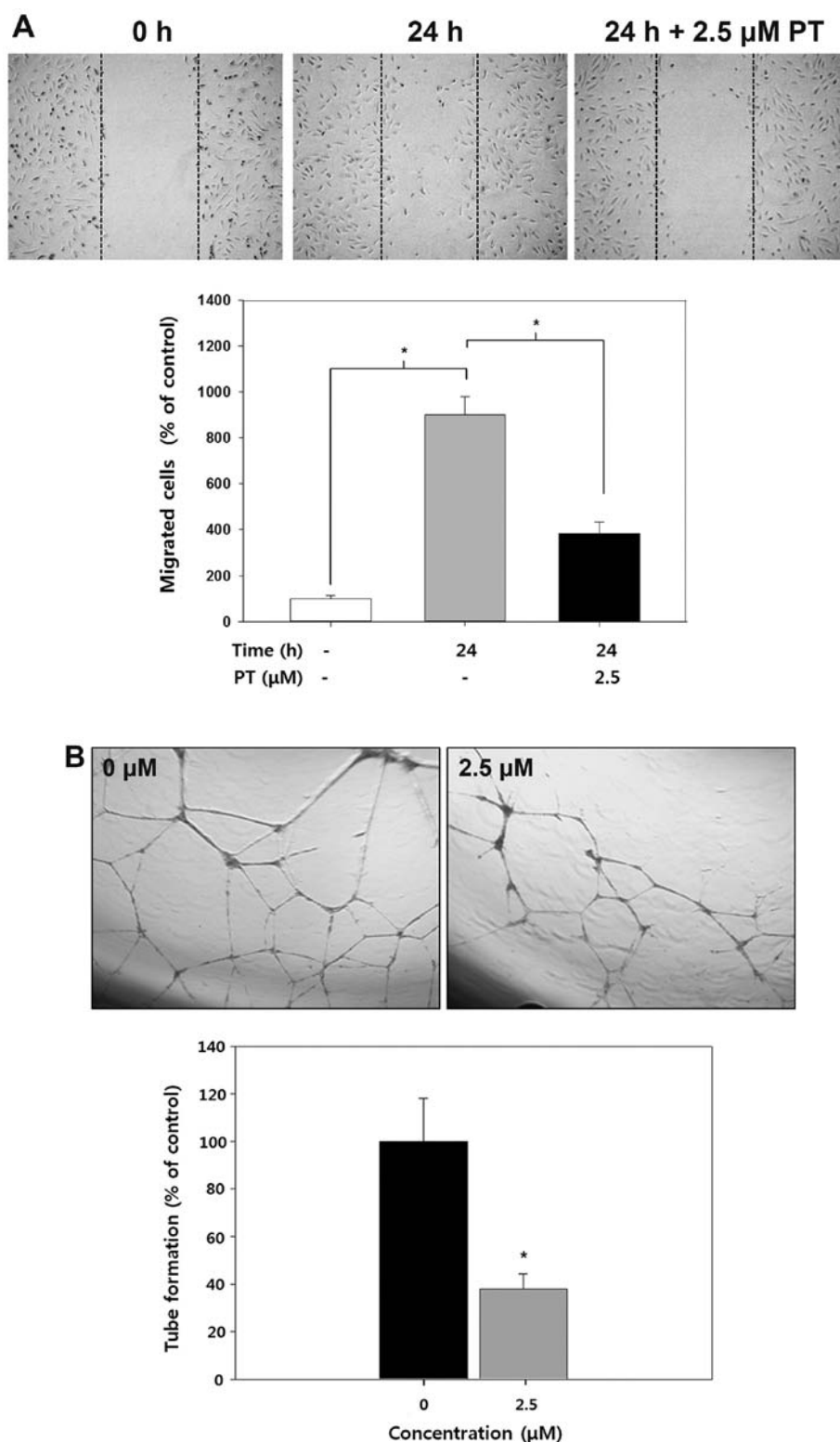


Figure 2. Inhibitory effects of parthenolide (PT) on capillary structure formation and migration of human umbilical vein endothelial cells (HUVECs). (A) PT inhibited HUVEC migration. Cells were grown to confluency in 6-well plates, wounded, and treated with the indicated concentrations of PT for 24 h. The migrated cells were counted. (B) PT inhibited the tube formation of HUVECs. Cells were placed in 24-well plates coated with Matrigel. Following incubation for 4-8 h in the absence or presence of PT, the tubular structures were photographed. The number of polygons was quantified by manual counting. Data are represented as the means \pm standard error (SE). * $P < 0.05$.

inhibited by the administration of PT (Fig. 5). Moreover, the tumor tissues from the mice treated with PT displayed a significantly lower number of CD31-positive stained cells, as compared to those from control mice ($P < 0.01$).

Discussion

In this study, we investigated the biological functions of PT as an inhibitor of angiogenesis. Our results revealed that PT

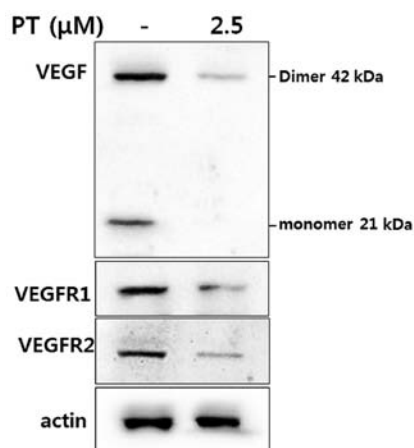


Figure 3. Effects of parthenolide (PT) on the expression of angiogenic biomarkers in human umbilical vein endothelial cells (HUVECs). Cell lysates were prepared following treatment with PT for 24 h and then analyzed by western blot analysis with vascular endothelial growth factor (VEGF), VEGF receptor (VEGFR)1 and VEGFR2 antibodies. The expression levels of the angiogenic biomarker proteins, VEGF, VEGFR1 and VEGFR2, were significantly decreased following treatment with PT. Actin was used as a loading control.

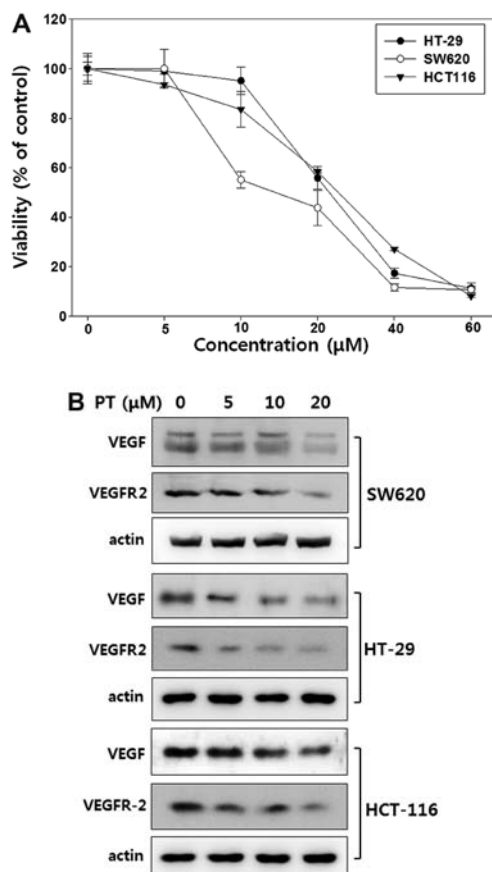


Figure 4. Inhibitory effects of parthenolide (PT) on angiogenesis in human colorectal cancer (CRC) cells. (A) Effect of PT on growth of human CRC cells. Twenty-four hours of exposure to PT resulted in the inhibition of proliferation in a concentration-dependent manner. SW620, HT-29 and HCT116 cell viability is expressed as a percentage compared to the vehicle-treated control and is expressed as the mean \pm standard error (SE). (B) Effects of PT on the expression angiogenic biomarkers. Cell lysates were prepared following treatment with PT for 24 h and then analyzed by western blot analysis with vascular endothelial growth factor (VEGF) and VEGF receptor (VEGFR)2 antibodies. The expression levels of the angiogenic biomarker proteins, VEGF and VEGFR2, were significantly decreased following treatment with PT in a dose-dependent manner. Actin was used as a loading control.

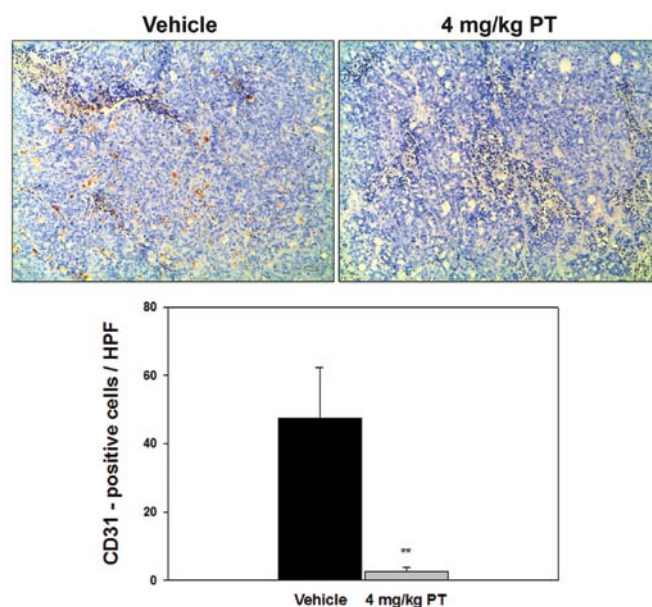


Figure 5. Effects of parthenolide (PT) on angiogenesis in a subcutaneous xenograft tumor model. HT-29 cells were subcutaneously implanted in nude mice. Mice bearing HT-29 tumor xenografts were randomly divided into the vehicle and PT treatment groups. Each treatment group consisted of 6-8 mice treated with the vehicle or 4 mg/kg PT for 28 days. Tumor sections from animals were fixed and subjected to immunohistochemistry for CD31. Brown spots in the image representing the control (vehicle-treated) mice show positive staining for CD31. The number of CD31-positive cells in tumors was counted at $\times 40$ magnification. Columns represent the means \pm standard error (SE). ** $P < 0.01$ vs. vehicle-treated group.

inhibited various aspects of angiogenesis, including endothelial cell proliferation, migration, capillary structure formation and the expression of angiogenic biomarkers. Moreover, PT markedly reduced the viability of human CRC cells and the protein levels of angiogenic biomarkers in CRC cells. PT also inhibited the tumor volume and angiogenesis in a mouse xenograft tumor model of human CRC (21,22).

CRC is one of the most common causes of mortality due to malignant tumors worldwide. However, the common therapeutic regimens for CRC, including surgery, chemotherapy and radiation, are insufficient to eliminate the tumor burden and CRC continues to inflict individuals worldwide. Therefore, there is an urgent need to develop novel therapeutic approaches to combat this devastating disease.

A number *in vitro* studies using PT as a single treatment demonstrated that treatment with 10-100 μ M PT for 24 h resulted in the downregulation of anti-apoptotic genes and in the upregulation of pro-apoptotic genes (13,15,23-25). Of note, in this study, we demonstrated that incubation with PT (2.5 μ M) for 24 h markedly inhibited tube formation and migration, as well as the expression of VEGF/VEGFRs in HUVECs. Moreover, the viability of the HUVECs was not affected at this concentration. Our results suggest that the angiogenesis-related genes or pathway respond to low concentrations of PT.

Previous studies, including our own, have suggested the involvement of multiple pathways in PT-induced apoptotic cell death in human cancer cells, and the mechanisms involved include oxidative stress, the inhibition of DNA synthesis, the activation of STAT and nuclear factor- κ B and mitochondrial dysfunction (10,23,24,26-29). However, to our knowledge,

there is no study available on its ability to inhibit tumor growth *in vivo* through the inhibition of angiogenesis. In our previous study, we demonstrated an anticancer property of PT using CRC cells and a xenograft tumor model (15). Moreover, we also showed that PT participates in suppressing VEGF expression, using immunohistochemistry for CD31 (15). A study on renal cell carcinoma demonstrated that PT reduces VEGF production *in vitro* and *in vivo* (30). PT has also been shown to inhibit proliferation and induce cell cycle arrest at the G0/G1 phase in vascular smooth muscle cells (31). Moreover, as previously demonstrated, PT inhibits the proliferation, motility and capillary formation ability of HUVECs and has anti-angiogenic effects on multiple myeloma and breast cancer *in vitro* (32,33). In this study, we therefore focused on the regulatory effects on angiogenesis and mechanisms of action of PT in CRC. To the best of our knowledge, the present study is the first to demonstrate that PT inhibits angiogenesis through the downregulation of the VEGF/VEGFR pathway.

VEGF, a major regulator of angiogenesis, binds and activates two tyrosine kinase receptors, VEGFR1 (Flt-1) and VEGFR2 (KDR/Flk-1) (34-36). These receptors regulate physiological, as well as pathological angiogenesis. Although VEGFR1 and VEGFR2 are expressed in endothelial cells, it is generally believed that VEGFR2 is the functional receptor that transduces active signals to trigger angiogenic and vascular permeability activity (37,38). VEGFR2 has stronger tyrosine kinase activity than VEGFR1, and transduces the major signals for angiogenesis (39-41). VEGFR2 expression is 3-5-fold higher in the tumor vasculature than in the normal vasculature (42), VEGFR1 is selectively expressed in endothelial cells and macrophage-lineage cells (43-46). Thus, VEGFR2 itself and VEGFR2 signaling appear to be critical targets for the suppression of cancer. In our study, treatment with PT inhibited the expression of VEGF and VEGFR1/VEGFR2 in HUVECs. Treatment with PT also reduced the expression of VEGF and VEGFR2 in CRC cells; however, VEGFR1 was not detected in CRC cells. These observations indicate that PT significantly inhibits angiogenesis in CRC through the downregulation of VEGF/VEGFRs mainly in the VEGFR2 signaling pathway. The VEGFR2 signaling pathway involves a number of effectors, including extracellular signal-regulated kinases (ERKs), sarcoma oncogene/tyrosine kinase (Src), phosphoinositide 3 kinase (PI3K)/Akt, focal adhesion kinase (FAK), protein kinase C (PKC), phospholipase C γ (PLC γ) and p38 mitogen-activated protein kinase (MAPK) (47-49). However, this requires further investigation, in order to fully elucidate whether PT affects the activation of VEGFR2-mediated downstream signaling.

In conclusion, we found that PT inhibits angiogenesis through the downregulation of the VEGF/VEGFR signaling pathway which plays multiple roles in regulating neovascularization. Therefore, our results strongly suggest that PT may be an effective antagonist of VEGF/VEGFR-mediated angiogenesis and therefore may be a potential candidate for the development of multifunctional anticancer agents for the treatment of CRC.

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