Decursin and decursinol angelate inhibit VEGF-induced angiogenesis via suppression of the VEGFR-2-signaling pathway

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Inhibition of angiogenesis is an attractive approach for the treatment of angiogenic diseases, such as cancer. Vascular endothelial growth factor (VEGF) is one of the most important activators of angiogenesis and interacts with the high-affinity tyrosine kinase receptors, VEGFR-1 and VEGFR-2. The pyranocoumarin compounds decursin and decursinol angelate isolated from the herb, *Angelica gigas*, are known to possess potent anti-inflammatory activities. However, little is known about their antiangiogenic activity or their underlying mechanisms. Here, we show the antiangiogenic effects of decursin and decursinol angelate using *in vitro* assays and *in vivo* animal experiments. Decursin and decursinol angelate inhibited VEGF-induced angiogenic processes *in vitro*, including proliferation, migration and tube formation of human umbilical vein endothelial cells. Decursin and decursinol angelate significantly suppressed neovessel formation in chick chorioallantoic membrane and tumor growth in a mouse model. The microvessel density in tumors treated with decursin for 14 days was significantly decreased compared with a vehicle control group. Decursin and decursinol angelate inhibited VEGF-induced phosphorylation of VEGFR-2, extracellular signal-regulated kinases and c-Jun N-terminal kinase mitogen-activated protein kinases. Taken together, these results demonstrate that decursin and decursinol angelate are novel candidates for inhibition of VEGF-induced angiogenesis.

Introduction

The dependence of tumor growth on the development of neovasculature is now a well-established aspect of cancer biology. Angiogenesis, the process by which capillaries sprout from pre-existing vasculature, is important for the supply of oxygen, nutrients, growth factors, hormones and proteolytic enzymes (1,2). The angiogenic process is highly complex, dynamic and regulated by a large number of molecules. The 'switch' to an angiogenic phenotype is considered to be a hallmark of the malignant process whereby proangiogenic mechanisms overwhelm or circumvent negative regulators of angiogenesis (3). Tumors lacking adequate vasculature become necrotic (4) or apoptotic (5), while tumors that have undergone neovascularization may not only enter a phase of rapid growth but may also have increased metastatic potential. Thus, in 1971, Folkman (6) proposed that antiangiogenesis might be an effective therapeutic strategy in the treatment of cancer.

One of the major molecules involved in the angiogenic process is the vascular endothelial growth factor (VEGF) family of proteins and their receptors. The VEGF family currently includes six known members: VEGF-A, placenta growth factor, VEGF-B, VEGF-C, VEGF-D and VEGF-E. These are secreted as dimeric glycoproteins and all contain the characteristic regularly spaced eight cysteine residues referred to as the 'cysteine knot' motif. VEGF family members regulate vascular development, angiogenesis and lymphangiogenesis by binding to their receptors, such as VEGFR-1, VEGFR-2 and VEGFR-3. VEGFR-1 is required for the recruitment of hematopoietic stem cells and the migration of monocytes and macrophages while VEGFR-2 regulates vascular endothelial proliferation, migration and invasion and VEGFR-3 mediates lymphangiogenesis. Over the last decade, considerable progress has been made in delineating the VEGFR-2-specific intracellular signaling cascades that lead to proliferation, migration, cell survival, increased permeability and the angiogenic response. Furthermore, therapeutic inhibition of the VEGFR-2-signaling pathway is now having a clinical impact on the treatment and prevention of a number of angiogenic diseases (7).

VEGF and VEGF receptors have been implicated in the angiogenesis that occurs in many solid tumors including breast cancer (8), colon cancer (7), hepatoma (9), bladder cancer (10), gastric cancer (11) and prostate cancer (12). Several strategies have been developed for targeting the VEGF-signaling pathway as a part of anticancer therapy (13). Potential approaches for blocking VEGF-signaling pathways include the inhibition of endogenous VEGF secretion, neutralization of VEGF in the microcirculation and prevention of VEGF from binding to receptors and subsequent signal transduction.

Angelica gigas Nakai (Umbelliferae) root has been traditionally used in Korean folk medicine as a tonic and for treatment of anemia and other common diseases (14). There are some reports about the pharmacological properties of this plant showing antibacterial and antiamnestic effects, an inhibitory effect on acetylcholinesterase, depression of cardiac contraction, activation of protein kinase C (PKC) and antitumor activity against sarcoma cancer cells (15). Based on its therapeutic potential, efforts have been made to isolate the active principles from this plant, leading to the isolation of many coumarin compounds (16). It is also known that this herbal medicine contributes to healthy pregnancies and easy deliveries and that coumarins such as decursin and decursinol angelate are the major active constituents of this plant (17,18). Recently, it has been reported that decursinol and decursin exhibit significant neuroprotecitve activity against glutamate-induced neurotoxicity in primary cultures of rat cortical cells and improved scopolamine-induced amnesia *in vivo* with another coumarin constituent, nodakenin (19). Anticancer activities of decursin have been also reported against human prostate carcinoma, human K562 erythroleukemia and U937 myeloleukemia cells (20).

In the present study, we isolated decursin and decursinol angleate from the roots of *A.gigas* and examined their antiangiogenic effects *in vitro* and *in vivo*. We further investigated the underlying mechanisms of their inhibitory effects toward VEGF-induced angiogenesis. These two compounds significantly inhibited VEGF-signaling pathways through VEGFR-2 leading to inhibition of tumor growth in a mouse animal model. Therefore, we suggest that decursin and decursinol angelate may be utilized to target active angiogenesis in tumors as well as in angiogenic diseases.

Materials and methods

Chemicals and antibodies

Recombinant human VEGF and basic fibroblast growth factor were purchased from R&D systems (Wiesbaden, Germany). Mouse monoclonal antibodies against VEGFR-2 and α-tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Zymed (San Francisco, CA), respectively. Rabbit polyclonal antibodies against phosphorylated (p)-VEGFR-2, p-extracellular signal-regulated kinase (ERK), ERK, p-c-Jun N-terminal kinase (JNK), JNK, p-p38, p38, p-Akt and Akt were purchased from Cell Signaling (Danver, MA). Goat polyclonal antibody against CD31 was purchased from Santa Cruz. Other chemicals were obtained from Sigma (St Louis, MO).

Abbreviations: CAM, chorioallantoic membrane; ERK, extracellular signal-regulated kinase; GFP, green fluorescent protein; HUVEC, human umbilical vein endothelial cell; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; PMA, phorbol 12-myristate 13-acetate; VEGF, vascular endothelial growth factor.

Plant extracts and purification

The roots of *A.gigas* Nakai (Umbelliferae family) were extracted serially with methanol, ethylacetate and *n*-butanol and fractionated. From the ethylacetate fraction, decursin and decursinol angelate were isolated using silica gel column chromatography. After column chromatography, the structure of purified coumarin compounds, decursin ($C_{19}H_{20}O_5$) and decursinol angelate with a molecular weight of 328 g were characterized by gas chromatography (Shimadzu, Kyoto, Japan), nuclear magnetic resonance (JEOL JNM-LA 400, Japan) and mass spectroscopy (JEOL-AX 505WA, Japan) at Daegu Hanny University, Daegu, Korea. The structures and nuclear magnetic resonance spectra of decursin and decursinol angelate are shown in Figure 1A and B, respectively.

Cell culture

Human umbilical vein endothelial cells (HUVECs) (passage 2–5) were grown on 1% gelatin-coated culture plates in M199 (Gibco, Rockville, MD) supplemented with 20% fetal bovine serum (Hyclone, Logan, UT), $1 \times$ antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, Invitrogen, Calsbad, CA), basic fibroblast growth factor (2 ng/ml) and heparin (2 U/ml, Sigma). Lewis lung carcinoma cells (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium (Hyclone) supplemented with 10% fetal bovine serum and $1 \times$ antibiotics.

Western blot analysis

Western blot analysis was performed as described previously (21). Proteins were transferred to nitrocellulose membrane and blots were probed with anti-VEGFR-2 p-VEGFR-2, p-ERK, ERK, p-JNK, JNK, p-p38, p38, p-Akt and Akt, followed by incubation with horseradish peroxidase-conjugated mouse or rabbit immunoglobulin. Blots were then developed using the West Pico Chemiluminescent Substrate (Pierce, Woburn, MA).

Zymography

Supernatant from cell cultures was analyzed for gelatin degradation activity by sodium dodecyl sulfate–polyacrylamide gel electrophoresis under non-reducing conditions. One milligram per milliliter gelatin was prepolymerized on a 10% polyacrylamide gel as a substrate. Electrophoresis was carried out at 4°C. The gel was washed with washing buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl and 2.5% Triton X-100), followed by incubation with a buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$, 0.02% NaN_3 and 1 μM $ZnCl_2$) at 37°C for 16 h and visualized with Coomassie Blue R-250.

Proliferation assay

HUVECs were seeded in gelatin-coated 48-well plates, 1×10^4 cells per well. The medium was replaced by low-serum medium (1% fetal bovine serum in M199) containing VEGF and/or decursin or decursinol angelate. After 24 h, 0.5 μCi per well of [^{3}H]-thymidine was added and incubated for 16 h. Cells were fixed with methanol, washed with distilled water, treated with 5% trichloroacetic acid and solubilized with sodium hydroxide (0.3 N). The cell-associated radioactivity was determined by liquid scintillation counter (Perkin Elmer, Waltham, MA).

In vitro migration assay

Migration was measured with 8 μm porosity polycarbonate filters (Costar, Lowel, MA). The lower side of filters was coated with type I collagen (0.5 mg/ml). The lower part of filter contained low-serum media with decursin or decursinol angelate in the presence or absence of VEGF. In the upper part of transwell plate, 5×10^4 cells were resuspended in 100 μl Dulbecco's modified Eagle's medium and plated. Cells were fixed with methanol and stained with hematoxylin and eosin (Sigma). Cells on the upper surface of the filter were removed and the cells that had migrated to the lower part were counted under light microscopy with a $\times 200$ magnification. Each sample was assayed in triplicate and repeated twice.

Tube formation assay

HUVECs were seeded on 96-well culture plates that were coated by Matrigel (10 mg/ml, BD Biosciences, San Diego, CA) for 30 min at 37°C. HUVECs were stimulated with decursin or decursinol angelate in the presence or absence of VEGF incubated for 24 h. Morphologic changes of cells were observed under a microscope and photographed at $\times 100$ magnification.

Zebrafish strains and growth conditions

Standard AB strain zebrafish (*Danio rerio*) and hemizygous transgenic Tg(*flk-1:EGFP*)^{s843} embryos were maintained in an oxygenated fish tank on a 14/10 h (light/dark) cycle at 28°C as described previously (22). Ages were given in hours post-fertilization based on the standard developmental stage.

Chorioallantoic membrane assay

On day 3.5 after fertilization, chick eggshell membrane and egg albumin was removed. At 4.5 day, decursin or decursinol angelate in the presence of phorbol 12-myristate 13-acetate (PMA) on Thermanox (Nunc, Rochester, NY) was air-dried and loaded onto chorioallantoic membrane (CAM) surface. Two days later, a 10% fat emulsion was injected into the chorioallantois and the CAM was observed under a microscope (Olympus BX 40). PMA was used as a positive control. The response was scored as positive and the percentage of positive eggs to total numbers of eggs tested was calculated. Fifteen eggs per group were used in each experiment and three independent experiments were performed.

In vivo tumor xenograft experiment in mouse

Lewis lung carcinoma cells were mixed with Matrigel and immediately inoculated subcutaneously at the right flank of C57BL/6J mice. Treatment with vehicle or decursin (4 mg/kg) was administered intraperitoneally everyday for 21 days. Tumor growth was measured with a caliper every three days using the formula, volume (V, cm³) = height $\times$ length $\times$ depth (cm).

Immunohistochemistry

The tumor tissue was fixed with 4% paraformaldehyde (pH 7.4) and embedded in paraffin or OCT compound. Serial sections (5 μm) were mounted on poly-L-lysine-coated slides and processed for either histology or for immunohistochemistry. Each section was immunostained with antibodies against mouse CD31 (BD, Biosciences), p-ERK, p-JNK, p-Akt and p-VEGFR-2 (Cell Signaling) and visualized by appropriate biotin-conjugated secondary antibodies followed by immmunoperoxidase detection with the Vectastain ABC Elite kit (Vector, Linaris, Germany) and diaminobenzidine substrate (Vector). Counterstaining was performed with hematoxylin.

Fig. 1. Structure and nuclear magnetic resonance spectra of decursin and decursinol angelate. (**A**) The chemical structure of decursin and decursinol angelate is depicted. (**B**) Decursin and decursinol angelate were isolated from the roots of *Angelica gigas* Nakai as described in Materials and Methods. Purified decursin and decursinol angelate were characterized by nuclear magnetic resonance and mass spectroscopy. ¹H-nuclear magnetic resonance spectra of decursin and decursinol angelate in CDCl₃ is shown.

Results

Decursin and decursinol angelate inhibits proliferation of endothelial cells induced by VEGF

After isolation of decursin and decursinol angelate from *A.gigas* Nakai, the purity of the chemicals was identified by gas chromatography and nuclear magnetic resonance (Figure 1B). Because 25–50 μM doses of decursin have a cytotoxic effect on DU145 and PC-3 prostate
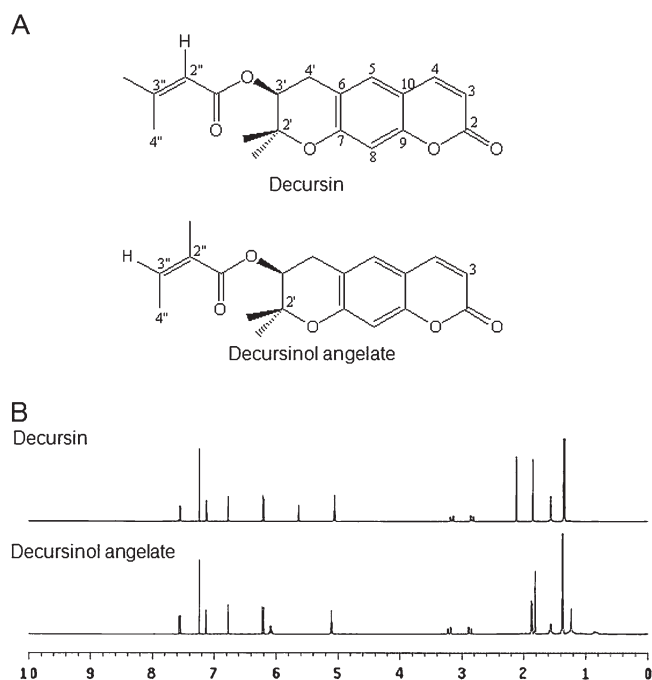
cancer cells with 15–25% cell death in 24 h (23), we examined the cytotoxicity of decursin and decursinol angelate on endothelial cells by 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide assay. Cytotoxicity of decursin or decursinol angelate was not observed in HUVECs at doses ranging from 10 to 100 µM (data not shown). To determine whether decursin and decursinol angelate inhibit the activation of endothelial cells induced by VEGF, the proliferation rate was analyzed after stimulation with VEGF (10 ng/ml). Both decursin and decursinol angelate significantly inhibited proliferation induced by VEGF in a dose-dependent manner, from 2 to 20 µM (Figure 2, $P < 0.001$). The inhibition of proliferation by 20 µM decursin or decursinol angelate was far less than in the quiescent control cells. Because these concentrations did not show any cytotoxicity, this result suggests that decursin or decursinol angelate may inhibit the proliferation rate of HUVECs through inhibition of cell cycle progression.

Decursin and decursinol angelate inhibits in vitro angiogenesis induced by VEGF

Migration of endothelial cells toward a chemoattractant is an important step in angiogenesis. When VEGF alone was present in the bottom chamber of transwell plate, the endothelial cell efficiently migrated through the micropores to the bottom of the membrane ($P < 0.01$). However, the addition of decursin ($P < 0.002$) or decursinol angelate ($P < 0.005$) to the top chamber significantly inhibited VEGF-induced migration of endothelial cells similar to control cells (Figure 3A).

Maturation of migrated endothelial cells into a tube-like structure is a critical step for formation of functional vessels. HUVECs were seeded onto Matrigel and stimulated to form capillary networks with VEGF (20 ng/ml). As shown in Figure 3B, robust and complete tube network formation was observed in VEGF-stimulated HUVECs. However, this effect of VEGF was significantly inhibited by decursin or decursinol angelate. There was incomplete sprouting or branching or broken network between tubes of HUVECs. These results indicate

Fig. 2. Decursin and decursinol angelate inhibit VEGF-induced proliferation of HUVECs. HUVECs were seeded into gelatin-coated 48-well plates at a density of 1×10^4 cells per well. A cell proliferation assay with [^{3}H]-thymidine was performed 24 h after treatment with decursin (**A**) or decursinol angelate (**B**) at the indicated doses, in the presence or absence of VEGF (10 ng/ml), after 1 day starvation with 1% fetal bovine serum-containing media. $^\#P < 0.001$, significance between control and VEGF; $^*P < 0.001$, significance between VEGF and VEGF plus decursin or decursinol angelate.

that decursin and decursinol angelate have some roles in blocking migration and maturation of endothelial cells induced by VEGF.

Decursin and decursinol angelate inhibits blood vessel formation in vivo

To confirm the effect of decursin or decursinol angelate on the formation of blood vessels *in vivo*, we used *flk-1-GFP* transgenic zebrafish embryos. Decursin and decursinol angelate significantly inhibited the blood vessel development in the trunk region (Figure 4A). Trunk intersomite vessels and dorsal longitudinal anastomotic vessels were incomplete (arrows). The dorsal aorta and pericardial vein were more closed and showed weak green fluorescent protein (GFP) fluorescence by decursin or decursinol angelate (Figure 4A).

To verify this result in a different model system, we performed a chick CAM assay. Decursin and decursinol angelate significantly inhibited the PMA-induced angiogenic formation of new capillaries from the pre-existing vascular network, as compared with PMA-treated positive control eggs. PMA alone demonstrated 2.3-fold activation of angiogenesis compared with the vehicle control, whereas treatment with decursin and decursinol angelate showed 80–85% inhibition of angiogenesis of CAM induced by PMA (Figure 4B). These results demonstrate that decursin and decursinol angelate efficiently block *in vivo* neovascularization.

Decursin and decursinol angelate inhibit the VEGFR-2-signaling pathway in endothelial cells

Because decursin and decursinol angelate inhibit VEGF-induced proliferation, migration and tube formation, we next determined whether they can inhibit VEGF-induced signaling cascade pathways. When VEGF binds to VEGFR-2, endothelial cells are activated for proliferation, migration and vessel maturation. Thus, autophosphorylation of VEGFR-2 was monitored just after the treatment of VEGF. VEGF-induced VEGFR-2 phosphorylation was significantly increased 5 min after treatment, but pretreatment with decursin or decursinol angelate for 2 h significantly inhibited VEGFR-2 phosphorylation (Figure 5A). VEGFR-2 activation leads to activation of several mitogen-activated protein kinase (MAPK) pathways, such as p42/44 ERK, JNK, p38 MAPK and phosphoinositide-3 kinase–Akt (24). VEGF treatment activated p42/44 ERK and JNK–MAPK. Decursin and decursinol angelate significantly suppressed VEGF-induced phosphorylation of p42/44 ERK and JNK–MAPK in endothelial cells (Figure 5B). MAPK activation by VEGFR-2 phosphorylation further leads to matrix metalloproteinase (MMP) activation and potentiation of endothelial cell migration (25). Thus, activation of MMPs was determined after VEGF treatment. Decursin and decursinol angelate significantly suppressed VEGF-induced MMP-2 activation, as observed by gelatin zymography (Figure 5C).

Decursin suppresses tumor growth and angiogenesis in nude mice

Lewis lung carcinoma lung cancer cells were subcutaneously inoculated into the flank of mice and decursin was injected intraperitoneally everyday for 3 weeks. Vehicle-injected control mice showed rapid increase of tumor growth as shown in Figure 6A. However, decursin suppressed tumor growth at a dose of 4 mg/kg/day (Figure 6A). The tumor volume in decursin-treated mice was significantly decreased from 16 days after treatment (∼65–70%) compared with the vehicle-treated group. The *in vivo* dosage of decursin was determined from the *in vitro* effective dose of 5–20 µM for the inhibition of endothelial cell proliferation, migration and tube formation (Figures 2–4). To determine whether decursinol, a metabolite of decursin, is the main compound affecting the inhibition of angiogenesis, we determined the ratio of decrusin to decursinol in mouse plasma by high-performance liquid chromatography as well as *in vitro* proliferation and migration assays with decursinol. In plasma, the decursin concentration was 10–40 times higher than decursinol at Tmax after oral administration of decursin (data not shown). In addition, decursinol had similar anti-angiogenic activity in proliferation and migration induced by VEGF in HUVEC (supplementary Figure 1 is available at *Carcinogenesis* Online). The significant decrease in tumor microvessel density
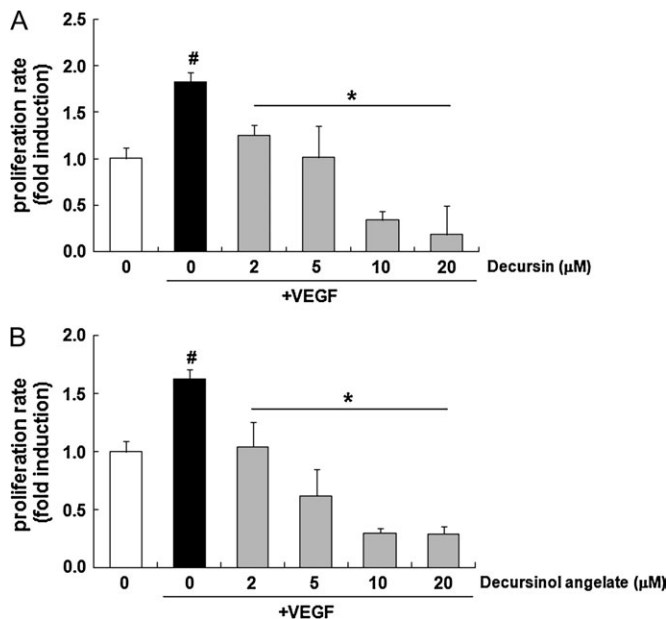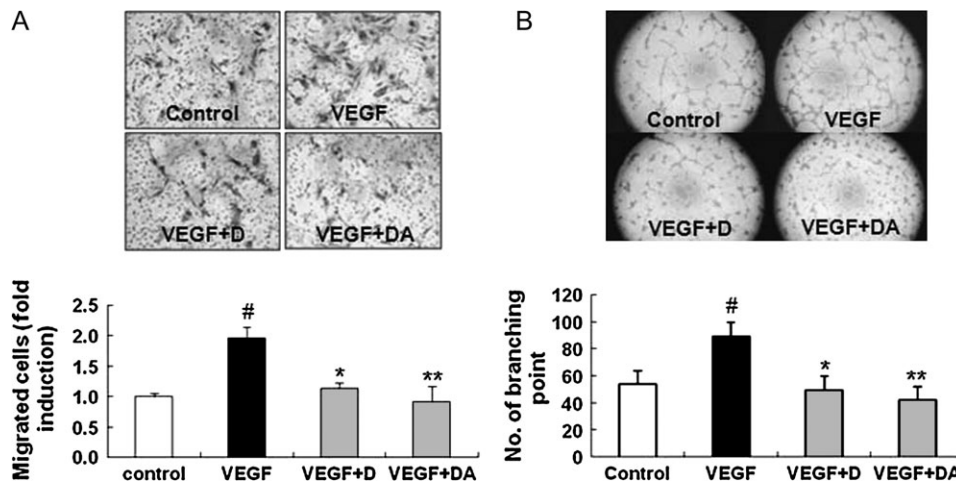
Fig. 3. Decursin and decursinol angelate inhibit VEGF-induced migration and tube formation in HUVEC. (**A**) HUVECs were plated onto 24-well transwell coated with type I collagen at opposite side of membrane. After 24 h, cells migrated into the bottom of the membrane were counted. Graph represents fold induction of the number of migrated cells compared with control. [#]$P < 0.010$, between control and VEGF; [*]$P < 0.002$, between VEGF and VEGF plus decursin; [**]$P < 0.005$, between VEGF and VEGF plus decursinol angelate. (**B**) HUVECs were seeded at a density of 1.5×10^4 cells per 96-well plate. Plates were previously coated with Matrigel and stimulated with VEGF in the presence or absence of decursin or decursinol angelate for 16 h. Graph represents fold induction of the number of branching sites in capillary networks. [#]$P < 0.005$, between control and VEGF; [*]$P < 0.005$, between VEGF and VEGF plus decursin; [**]$P < 0.002$, between VEGF and VEGF plus decursinol angelate.
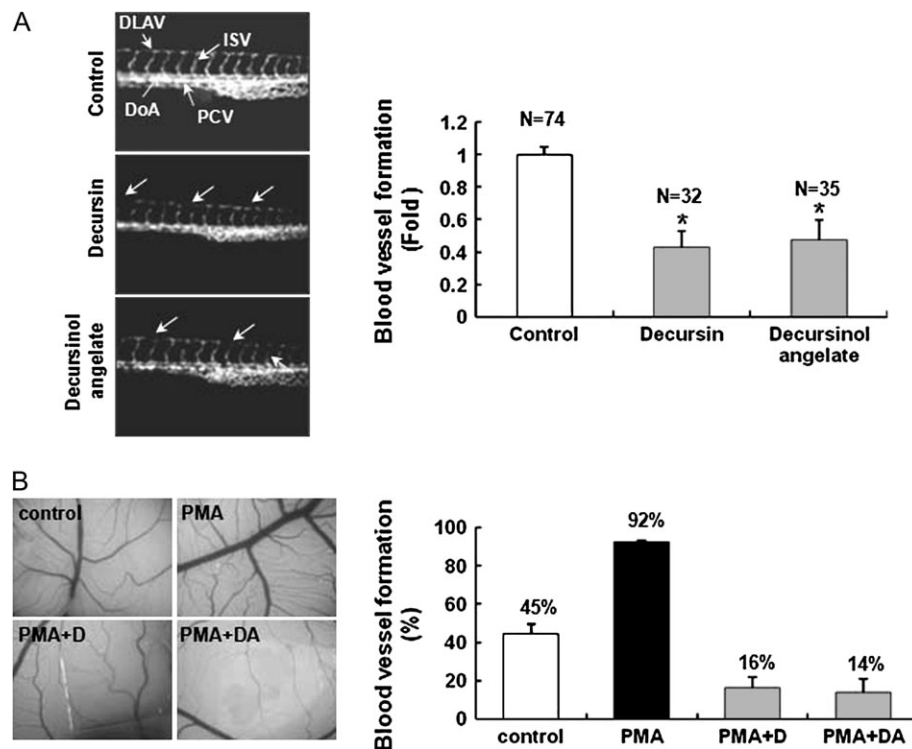

Fig. 4. Decursin and decursinol angelate inhibit angiogenesis in *flk-1*-GFP transgenic zebrafish. (**A**) *Flk-1*-GFP transgenic zebrafish was treated with decursin (20 µM) or decursinol angelate (20 µM) at bud stage (10 h post-fertilization). Images acquired with dark-field illumination of embryos show GFP fluorescence of the trunk microvessels in *flk-1*-GFP transgenic zebrafish embryos at 36 h post-fertilization. Graph represents fold induction of the number of embryos with normal vessel formation. [*]$P < 0.001$, significance between control and decursin or decursinol angelate (**B**) PMA (100 ng per egg) and decursin or decursinol angelate (6 µg per egg) were treated on the CAM surface of 4-day-old chick embryos. Decursin and decursinol angelate inhibited PMA-induced new blood vessel formation in CAM. Graph represents the percentage of positive eggs relative to the total number of eggs tested. [#]$P < 0.005$, between control and PMA; [*]$P < 0.001$, significance between control and decursin or decursinol angelate.

determined by immunohistochemistry against PECAM-1 (CD31) was observed compared with the vehicle control group (Figure 6B and C), suggesting that the reduced tumor growth was due to the vessel regression caused by decursin. Moreover, decursin dramatically decreased the expression level of phospho-VEGFR-2, p-ERK, p-JNK, as well as MMP-2 compared with vehicle-treated tumors (Figure 6B). VEGFR-2 was strongly expressed in the endothelial cell lining of tumor microvessels, but treatment of decursin decreased
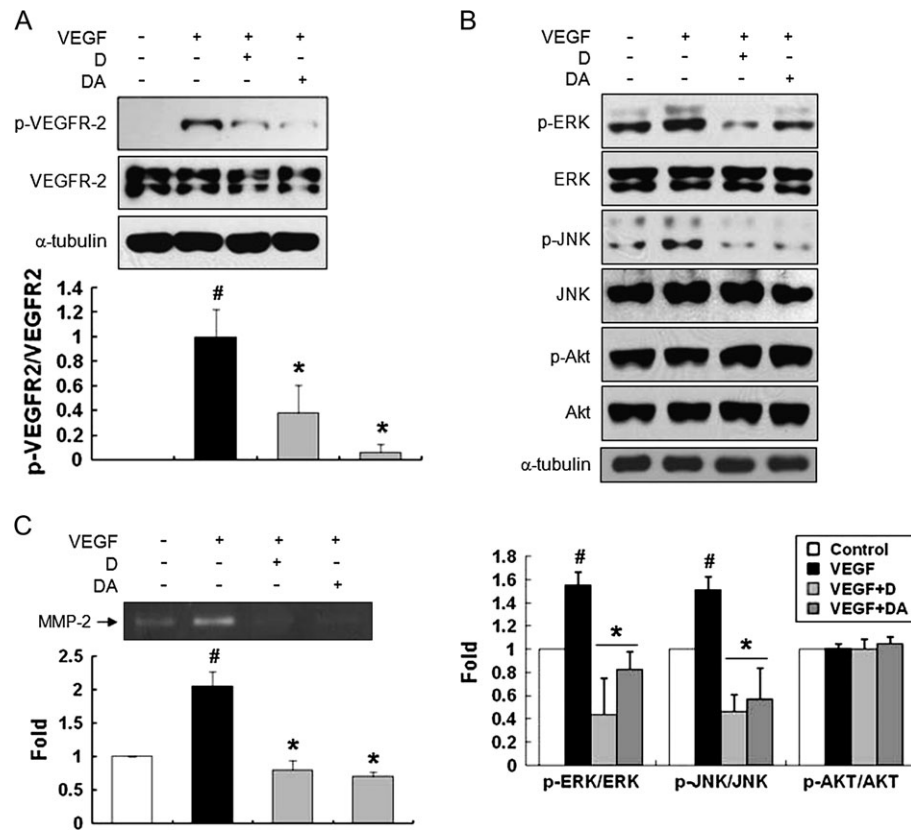
Fig. 5. Decursin and decursinol angelate suppress VEGF-induced VEGFR-2-signaling pathways in HUVECs. HUVECs were pretreated with decursin or decursinol angelate for 2 h and stimulated with VEGF (10 ng/ml) for 5 min. Western blot analysis was performed using anti-p-VEGFR-2, VEGFR-2 (**A**), p-ERK, ERK, p-JNK, JNK, MAPK, p-Akt and Akt antibodies (**B**). α-Tubulin was probed as a protein loading control. HUVECs were seeded at a density of 2×10^5 cells per 60 mm dish. Dishes were previously coated with 1% gelatin and stimulated with VEGF (10 ng/ml) in the presence of decursin or decursinol angelate for 16 h. Conditioned medium was concentrated with Centricon® (Millipore, Billerica, MA) at a ratio of 1:5. Supernatants were analyzed for gelatin degradation activity by sodium dodecyl sulfate–polyacrylamide gel electrophoresis under non-reducing conditions. A clear zone of gelatin digestion indicates the presence of MMP-2. Graphs represent the fold induction of western blot or zymography bands analyzed by ImageJ from three independent experiments (National Institutes of Health, Bethesda, MD) compared with non-treated control. $^{\#}P < 0.005$, significance between control and VEGF; $^{*}P < 0.001$, significance between control and decursin or decursinol angelate.

microvessel density and the VEGFR-2 level. In conclusion, these results suggest that decursin and decursinol angelate may inhibit microvessel development and suppress tumor progression through inhibition of the VEGFR-2-signaling pathway.

Discussion

The activity of VEGF is mediated by receptor tyrosine kinases, VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR) with a high affinity (26–28). Among these two receptors, VEGFR-2 appears to be a major transducer of VEGF signals in endothelial cells, leading to cell proliferation, migration, differentiation, capillary like formation and vascular permeability. Inhibition of the VEGFR-2-mediated signaling pathway, therefore, represents an excellent approach to an antiangiogenic intervention (29–31). Here, we have shown that decursin and decursinol angelate successfully inhibited VEGFR-2 activation and the downstream signaling pathways (Figure 5). In particular, the p42/44 ERK and JNK–MAPK pathways were blocked by decursin and decursinol angelate but not p38 MAPK or Akt pathways. It has been reported that VEGF activate Ras-GTPase following p42/44 ERK phosphorylation, but not p38-MAPK or Akt activation. Ras-ERK activation by VEGF causes active angiogenic endothelial cell migration, proliferation and tube formation (32). In particular, activation of p42/44 ERK is reported to be an absolute requirement for VEGF-stimulated proliferation. Therefore, it is suggested that blocking ERK activation by decursin or decursinol angelate might contribute to the inhibition of endothelial cell proliferation induced by VEGF (Figure 2). Actu-

ally, JNK–MAPK activation results in nuclear activation of c-Jun as a component of activator protein 1 (AP-1) which activates AP-1-mediated gene transcription involved in cell proliferation (33). Our results suggest that ERK–MAPK and JNK–MAPK inactivation by decursin and decursinol angelate might cooperatively contribute the inhibition of proliferation in HUVECs.

Disruption of the integrity of the basement membrane is a key histological marker of tumor transition to an invasive carcinoma. The basement membrane is made up of a complex mixture of extracellular matrix proteins, such as laminin, collagens and gelatins. MMPs are key molecules that degrade extracellular matrix, and therefore have been implicated in migration and invasion of cancer in addition to their involvement in remodeling and angiogenesis in normal tissue. Among them, MMP-2 overexpression and activity have been implicated in the invasive potential of human tumors. Active MMP-2 is detected more frequently in malignant than benign carcinomas (34). MMP-2 has also been associated with angiogenesis during the development of brain metastasis (35). MAPK pathways have been related to MMP activation and expression. The correlation between MMP-2 expression and activity and the ERK pathway was identified by treatment with PD184352 (36, 37, 38). Because the promoters of MMPs including MMP-1, MMP-2, MMP-3, MMP-7 and MMP-9 all have AP-1 consensus sequence, it is speculated that the JNK–MAPK pathway might also participate in increased MMP activity. Thus, inhibition of the p42/44 ERK–MAPK and JNK–MAPK pathways by decursin and decursinol angelate might be involved in the attenuation both of proliferation and migration in HUVECs.
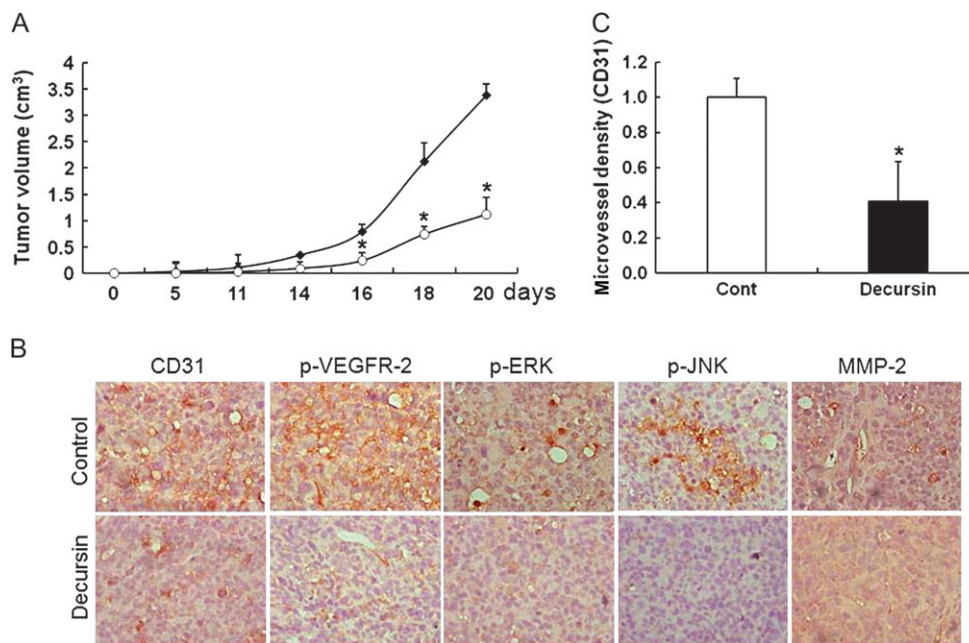
Fig. 6. Decursin attenuated tumor growth and VEGFR-2 phosphorylation in mice models. (**A**) C57BL/6J mice were treated with vehicle or decursin (4 mg/kg) intraperitoneally everyday for 3 weeks. Tumor growth was measured with calipers every 3 days using the formula V = height × length × depth (cm³). (**B**) The tumor tissue was removed from mice at 20 days after treatment and fixed with 4% paraformaldehyde at 4°C and embedded in paraffin. Serial sections (5 μm) were processed for immunohistochemistry with antibodies against p-VEGFR-2, p-ERK, p-JNK and MMP-2. Counterstaining was performed with hematoxylin. $^*P < 0.002$, between control and decursin; $^{**}P < 0.007$, between control and decursin.

A growing list of inhibitors of angiogenesis, including small molecule kinase inhibitors or monoclonal antibodies, is currently being evaluated as potential therapeutic agents for a number of human diseases, including cancer and proliferative retinopathies. For example, Avastin®, a monoclonal antibody against VEGF, has shown clinical benefits in many cancer patients including those with carcinomas of the colorectum, lung and breast (39). Here, we showed novel candidates for the anti-VEGF-signaling inhibitor, decursin and decursinol angelate, leading to complete inhibition of a variety of biological events in endothelial cells stimulated by VEGF. Decursin and decursinol angelate inhibited *in vitro* angiogenesis as well as *in vivo* angiogenesis in animal models (Figures 3 and 4). Furthermore, decursin significantly attenuated tumor growth in mice inoculated with lung cancer cells. The antitumorigenic activity is shown at 50–100 mg/kg (15), but in our experiment, we tried to determine only the antiangiogenic activity in reference to tumor growth without any cytotoxic activity on the cancer cells. The attenuation of tumor growth by decursin was due to the inhibition of microvessel density and the expression of the VEGFR-2-signaling molecule, as well as MMP-2 identified by immunohistochemistry (Figure 6). These results clearly demonstrate that decursin and decursinol angelate can be utilized as anticancer drugs through the blocking of VEGF-signaling pathways in endothelial cells leading to inhibition of neovessel growth.

Because decursin is metabolized into decursinol in animal model (40), a question may arise that an antiangiogenic effect of decursin is owing to an effect of its metabolite decursinol. When we analyzed mouse plasma with high-performance liquid chromatography after oral administration of decursin, we could detect both decursinol and decursin peaks at 9 and 16 min retention time, respectively. However, a paper reported that decursin is not detected in rat plasma samples after oral administration of decursin (40). A shorter retention time for decursinol than for decursin might lead to miss a decursin peak at later time in high-performance liquid chromatography chromatogram. And we performed *in vitro* angiogenic activity with decursinol and found that decursinol was effective to inhibit the *in vitro* angiogenesis in HUVEC (supplementary Figure 1 is available at *Carcinogenesis*

Online). Thus, the *in vivo* antiangiogenic effect of decursin might be from the effects both of decursin and decursinol.

Decursin and decursinol angelate have antiestrogenic and antiandrogenic activity owing to their chemical structures (41,42). As such, they have an anticancer effect on breast (41) and prostate cancer cells (43). Moreover, these compounds affect cytotoxic activity in leukemia cells via activation of PKC (20).

In summary, our results demonstrate a novel function of decursin and decursinol angelate as antiangiogenic agents by targeting the VEGF-signaling pathways, including p42/44 ERK–MAPK and JNK–MAPK, through the inhibition of VEGFR-2 phosphorylation in endothelial cells. Therefore, our results strongly suggest that decursin and decursinol angelate may be effective antagonists of VEGF/VEGFR-2-stimulated angiogenesis.

Supplementary materials

Supplementary Figure 1 can be found at http://carcin.oxfordjournals.org/

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