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Journal of Cellular Biochemistry 113:1478–1487 (2012)

Journal of Cellular Biochemistry

Decursin Inhibits Vasculogenesis in Early Tumor Progression by Suppression of Endothelial Progenitor Cell Differentiation and Function

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ABSTRACT

Endothelial progenitor cells (EPCs) contribute to the tumor vasculature during tumor progression. Decursin isolated from the herb *Angelica gigas* is known to possess potent anti-inflammatory activities. Recently, we reported that decursin is a novel candidate for an angiogenesis inhibitor [Jung et al., 2009]. In this study, we investigated whether decursin regulates EPC differentiation and function to inhibit tumor vasculogenesis. We isolated AC133+ cells from human cord blood and decursin significantly decreased the number of EPC colony forming units of human cord blood-derived AC133+ cells that produce functional EPC progenies. Decursin dose-dependently decreased the cell number of EPC committing cells as demonstrated by EPC expansion studies. Decursin inhibited EPC differentiation from progenitor cells into spindle-shaped EPC colonies. Additionally, decursin inhibited proliferation and migration of early EPCs isolated from mouse bone marrow. Furthermore, decursin suppressed expression of angiopoietin-2, angiopoietin receptor Tie-2, Flk-1 (vascular endothelial growth factor receptor-2), and endothelial nitric oxide synthase in mouse BM derived EPCs in a dose-dependent manner. Decursin suppressed tube formation ability of EPCs in collaboration with HUVEC. Decursin (4 mg/kg) inhibited tumor-induced mobilization of circulating EPCs (CD34+/VEGFR-2+ cells) from bone marrow and early incorporation of Dil-Ac-LDL-labeled or green fluorescent protein (GFP)+ EPCs into neovessels of xenograft Lewis lung carcinoma tumors in wild-type- or bone-marrow-transplanted mice. Accordingly, decursin attenuated EPC-derived endothelial cells in neovessels of Lewis lung carcinoma tumor masses grown in mice. Together, decursin likely affects EPC differentiation and function, thereby inhibiting tumor vasculogenesis in early tumorigenesis. J. Cell. Biochem. 113: 1478–1487, 2012.

KEY WORDS: DECURSIN; ENDOTHELIAL PROGENITOR CELL; SDF-1α; TUMOR MICROVESSELS

Endothelial progenitor cells (EPCs) comprise a heterogeneous subpopulation of bone marrow (BM) mononuclear cells (MNCs) that have been selected for their enhanced potential to differentiate into endothelial cells. Marking the first identification of adult EPCs in peripheral blood (PB), EPCs play a pivotal role in vascular formation and are crucially involved in wound healing, cardiac regeneration, and many other biological processes, as well as pathogenic conditions such as tumor growth and metastatic spread [Folkman et al., 1989; Losordo et al., 1998; Asai et al., 2006]. In adult humans, blood vessel formation in response to normal and pathological conditions occurs through neovascularization, which comprises two processes: Angiogenesis (i.e., sprouting and growth from the pre-existing microvasculature) and vasculogenesis (i.e., de novo assembly of blood vessels), which is the primary mechanism of blood vessel development during embryogenesis [Lyden et al., 2001].

Abbreviations: EPC, endothelial progenitor cell; eNOS, endothelial nitric oxide synthase; SDF-1α, stromal cell-derived factor-1 α; GFP, green fluorescent protein; BM, bone marrow; MNC, mononuclear cell; PB, peripheral blood.

Grant sponsor: The Korean Ministry of Education, Science and Technology; Grant number: 2009-0066170.

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Received 19 January 2012; Accepted 23 January 2012 • DOI 10.1002/jcb.24085 • © 2012 Wiley Periodicals, Inc.
Published online 1 February 2012 in Wiley Online Library (wileyonlinelibrary.com).

In tumor growth, development of the neovasculature is the most crucial process to provide cells with oxygen, nutrients, and growth factors [Folkman, 1990; Fidler and Ellis, 1994]. Angiogenic sprouting from pre-existing vessels is initiated by tumor-derived growth factors such as vascular endothelial growth factor (VEGF) secreted following exposure to a hypoxic stimulus or cytokines [Ferrara, 2002]. Over the past 30 years, inhibition of angiogenesis has been accepted to be an effective strategy to treat human cancer and active searches for angiogenic inhibitors have been conducted. Extensive research has led to identification and isolation of regulators of angiogenesis, some of which represent therapeutic targets. However, a smaller spotlight exists regarding the vasculogenic process for the treatment of cancer than angiogenesis. Concerning vasculogenesis, in cancer patients, the number of circulating EPCs is increased [Bertolini et al., 2003; Ho et al., 2006; Dome et al., 2006] and, in various animal tumor models, transplanted EPCs are incorporated functionally into tumor-associated vessels. Most of the newly formed tumor vessels are derived from the genetically marked donor, mainly during the early stages of tumor growth. Other studies have also highlighted the important contribution of EPCs during the early phase of tumor neovascularization [Spring et al., 2005; Nolan et al., 2007].

The root of the Korean medicinal herb *Angelica gigas* Nakai (Umbelliferae) has been used to treat female afflictions and anemia [Guo et al., 2007; Lee et al., 2003] and harbors pyranocoumarin compounds as major active principles, including decursin [Sarker and Nahar, 2004], its isomer decursinol angelate [Ahn et al., 1996], and decursinol [Konoshima et al., 1968]. Recently, anticancer activities of decursin have been reported against the human prostate carcinoma cells K562 (erythroleukemia cells) and U937 (myeloleukemia cells) [Kim et al., 2005] We also reported that decursin and decursinol angelate inhibited VEGF-induced angiogenesis by blocking the VEGFR-2 signaling pathway [Jung et al., 2009] and activation of extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase [Son et al., 2009] Therefore, decursin is a novel candidate for an angiogenesis inhibitor. However, its inhibitory activity on EPC populations and differentiation in the early steps of tumor vasculature formation is not yet well defined.

In the present study, we examined the anti-vasculogenic activity of decursin in the early phase of tumor progression. We isolated human AC133+ cells and mouse BM-derived EPCs, and determined the effect of decursin on proliferation, differentiation, and migration of EPCs. Additionally, we further investigated the effect of decursin on tumor growth, mobilization of EPCs from mouse BM to peripheral circulation, and incorporation of EPCs into tumor neovessels in a mouse tumor model.

MATERIALS AND METHODS

ANIMAL

Animal experiments (C57BL/6J mice, SLC, Japan) were performed according to the guidelines for care and use of laboratory animals approved by the institutional ethical animal care committee of the Kyungpook National University (Daegu, Korea). Transgenic mice universally expressing the green fluorescent protein (GFP) under the chick β-actin promoter (C57BL/6J) were kindly provided by Dr. Goo-Taeg Oh in Ewha University.

PLANT EXTRACTS AND PURIFICATION

Decursin from the roots of *Angelica gigas* Nakai (Umbelliferae family) was kindly provided by Dr. Eun Mi Ahn (Department of Herbal Foodceutical Science Daegu Haany University, Korea) as described previously [Jung et al., 2009].

ISOLATION OF HUMAN CORD BLOOD-DERIVED AC133+ CELLS USING THE AUTOMACS SYSTEM

The protocol used was approved by the Tokai University Research Ethics Committee, Japan, and informed consent was obtained from all participants. Human umbilical cord blood was mixed with Dulbecco's phosphate-buffered saline (DPBS) at a ratio of 1:1, and then the mixture was added very carefully on top of a Ficoll-PaqueTM PLUS gradient (GE Healthcare Life Science, Uppsala, Sweden) to isolate MNCs. After centrifugation, the plasma layer was aspirated. The upper MNC layer was transferred to a tube, which was filled with PBS supplemented with 2 mM ethylenediaminetetraacetic acid (EDTA). After mixing gently, the sample was washed and the pellet was resuspended in 2 mM EDTA/PBS. To deplete red blood cells, ammonium chloride solution (Stem Cell Technologies, Vancouver, Canada) was added, mixed gently, and incubated at 37°C in a water bath for 5 min. To isolate human cord blood-derived AC133+ cells with high purity (>99%), a MACS Separator (Miltenyi Biotec) was used. Freshly isolated MNCs were incubated with Fc receptor blocking reagents and CD133 microbeads. Cells were washed with 2% fetal bovine serum (FBS; HyClone Laboratories, Inc., Logan, UT) containing MACS buffer.

ISOLATION OF MOUSE MNCs FROM BM OR PB, AND CELL CULTURE

Mice were treated with 2% avertin (2,2,2-tribromethanol) intraperitoneally (0.2 ml/10 body weight) once for anesthesia and isolated all long bones and blood from the mice. After isolation of bones and blood, mice were sacrificed automatically. MNCs were isolated from BM or PB of C57BL/6J mice using a Histopaque-1083 density gradient medium (Sigma). Light-density MNCs were harvested and washed twice with PBS supplemented with 5 mM EDTA. Cells were incubated with 0.8% ammonium chloride (Stem Cell Technologies) for 5 min at 4°C to lyse contaminating erythrocytes. Next, freshly isolated MNCs were suspended in EGM-2 MV medium (Lonza, Basel, Switzerland). Cell suspension was then plated onto 1% gelatin-coated culture dishes. After 4 days, adherent cells were cultured for another 3 days. These cells were considered to be early EPCs and were used for further investigation in real-time polymerase chain reaction (PCR) analysis, migration and tube formation assays, or western blot analysis. MNCs from PB were used for analysis of circulating EPCs.

EPC COLONY FORMING ASSAY (EPC-CFA)

EPC vasculogenic CFAs were performed and each EPC colony was characterized as previously described [Kamei et al., 2010; Kwon et al., 2009; Kwon et al., 2011]. Briefly, the frequency of round- or spindle-shaped EPC colony formation was assessed in triplicate by culturing 500 AC133+ cells for 18 days in methyl cellulose-

containing medium H4236 (Stem Cell Technologies) with 20 ng/ml stem cell factor (SCF; Kirin, Tokyo, Japan), 50 ng/ml VEGF, 20 ng/ml interleukin-3 (IL-3; Kirin), 50 ng/ml basic fibroblast growth factor (Wako, Osaka, Japan), 50 ng/ml epidermal growth factor receptor (EGFR) (Wako), 2 U/ml heparin (Ajinomoto, Tokyo, Japan), 30% FBS, and antibiotics. The colony forming units (CFUs) were identified as spindle-shaped or round-shaped CFUs by visual inspection with an inverted microscope under 40× magnification.

EPC EX VIVO EXPANSION ASSAY

Ex vivo expansion of EPC-enriched cells was performed according to procedures established in our laboratory (Masuda et al., unpublished data). Briefly, AC133+ cells were seeded onto 24-well plates and cultured in Stem Span media (StemCell Technologies), supplemented with 50 ng/ml VEGF (R&D Systems), 20 ng/ml IL-6 (R&D Systems), 100 ng/ml SCF (Kirin), 20 ng/ml thrombopoietin (TPO; Wako), 100 ng/ml Flt-3 ligand (Wako), and antibiotics. After 7 days in culture, cells were harvested and counted.

QUANTITATIVE REAL-TIME REVERSE TRANSCRIPTION-PCR

Total RNA was extracted from mouse cultured EPCs using the Qiagen RNeasy Micro Kit according to the manufacturer's instructions. cDNA synthesis from diluted RNA and amplification of cDNA were performed using the ExScript RT reagent Kit (TaKaRa). Real-time RCR was performed using a SYBR Green PCR Master Mix (Applied Biosystems). The conditions for real-time PCR comprised 40 cycles of denaturation (15 s at 95°C) and annealing (1 min at 60°C), followed by a cycle of dissociation (15 s at 95°C, 30 s at 60°C, and 15 s at 95°C). To evaluate target gene expression, the mean cycle threshold (Ct) values were calculated from quadruplicate measurements with normalization to β-actin as an internal control. Table I summarizes the sequences of the primers used in this study.

WESTERN BLOT ANALYSIS

Western blot analysis was performed as previously described [Jung et al., 2009]. Proteins were transferred to nitrocellulose membranes and blots were probed with Akt, phosphorylated Akt, phosphorylated ERK, ERK, phosphorylated endothelial nitric oxide synthase (eNOS), eNOS, Flk-1, Ang-1, Tie-2, CXCR-4 and α-tubulin antibodies, followed by incubation with horseradish peroxidase-conjugated mouse or rabbit immunoglobulin. Blots were then developed using the West Pico Chemiluminescent Substrate (Pierce, Woburn, MA).

FLOW CYTOMETRY

AC133+ cells cultured for 7 days were harvested with 2 mM EDTA/PBS (PBSE), and then suspended in PBSE with 3% FBS. Cells were stained for 30 min on 4°C with fluorescein isothiocyanate (FITC)-conjugated anti-human CD34 monoclonal, allophycocyanin (APC)-conjugated anti-human CD133 monoclonal, and phycoerythrin (PE)-conjugated anti-human KDR, or VEGFR-2 monoclonal antibodies (all from BD Biosciences, San Diego, CA). MNCs from the PB of mice were stained with an FITC-conjugated anti-CD34 monoclonal antibody (GeneTex, Irvine, CA) and a PE-conjugated anti-VEGFR2 monoclonal antibody (BD Biosciences). After staining, cells were analyzed by FACS Calibur (BD Biosciences).

LEWIS LUNG CARCINOMA CELL CULTURE AND TRANSPLANTATION

Lewis lung carcinoma (LLC) cells were maintained in Dulbecco's modified Eagle's Medium (HyClone) supplemented with 10% FBS (HyClone) and 1% antibiotics. LLCs (5×10^4–1×10^6) were inoculated subcutaneously into C57BL/6J mice and the tumor size was measured with a caliper every other day [volume (cm^3) = height (cm) × length (cm) × depth (cm)].

BONE MARROW TRANSPLANTATION (BMT) ASSAY

C57BL/6J mice were exposed to a lethal dose of total body irradiation (10 Gy), and then injected intravenously with 1×10^7 BM-derived MNCs, which had been collected from 8- to 12-week-old GFP transgenic mice. After 4 weeks, we analyzed the blood of BMT mice using flow cytometry to determine the presence of a GFP signal. GFP+ BMT mice were inoculated with 1×10^6 LLCs subcutaneously and injected with 4 mg/kg decursin or PBS every day intraperitoneally for 2 weeks. Tumors were fixed overnight with 4% paraformaldehyde, followed by 15, 30, and 50% sucrose/PBS solution, and then were frozen for cryosection assays. Immunohistochemistry was performed using the anti-mouse CD31 antibody (BD Biosciences). Fluorescent images of endothelium that contained donor-derived ECs were obtained using a fluorescence microscope (Zeiss).

EPC MIGRATION ASSAY

The migration assay was performed as previously described1 using a 24-well micro chemotaxis chamber. Briefly, cells (5×10^4 cells per well) were placed in the upper chamber, and the lower chamber was filled with medium containing 50 ng/ml of murine recombinant stromal-derived factor-1α (SDF-1α; PeproTech, Rocky Hill, NJ). The chamber was incubated for 6 h at 37°C and 5% CO2. The migrated cells on the lower side of the filter were stained with 4′,6-diamidino-2-phenylindole (DAPI) and counted under a light microscope at 200× magnification. Each sample was assayed in triplicate and repeated twice.

TABLE I. Primer Sequences for Real-Time PCR

Gene name	Forward	Reverse
Ang1	5′–AACCTCACCCTGCAAAGATG-3′	5′-CACAGATGGCCTTGATGTTG-3′
Ang2	5′–CAAGGCACTGAGAGACAC-3′	5′–TGCGCTTCAGTCTGGTACAC-3′
TIE2	5′–GCTTGCTCCTTTCTGGAACTGT-3′	5′–CGCCACCCAGAGGCAAT-3′
VEGF	5′–GGGCAGAGCTGAGTGTTAGC-3′	5′–TCTCCCAGATCGGTGACAGT-3′
FLK1	5′–CACCACTCAAACGCTGACATGTA-3′	5′–GCTCGTTGGCGCACTCTT-3′
eNOS	5′–CGGCATCACCAGGAAGAAGA-3′	5′–CATGAGCGAGGCGGAGAT-3′
β-actin	5′–AAGTCCCTCACCCTCCCAAAAG-3′	5′–AAGCAATGCTGTCACCTTCCC-3′

TUBE FORMATION ASSAY

Mouse BM derived EPCs were mixed with human umbilical vein endothelial cells (HUVECs; Cambrex, UK) (HUVECs:mouse BM derived EPCs $= 1 \times 10^4$:2×10^3 cells in 100 μl of a solution containing 0.5% FBS in endothelial basal medium-2 [EBM-2]). The cell mixture was seeded onto a 96-well culture plate coated with growth factor-reduced Matrigel (10 mg/ml; BD Biosciences) and further incubated for 8 h. After incubation, the tube length was measured using one picture per well at 40× magnification by light microscopy.

IN VIVO EPC INCORPORATION ASSAY

To determine putative EPCs to incorporate into tumor vessels, DiI-Ac-LDL-labeled EPCs (1×10^6 cells) were injected into the tail vein of tumor-bearing C57BL/6J mice. Tumor tissues were fixed with paraformaldehyde and embedded in paraffin. Paraffin sections (5 μm) were stained with goat anti-mouse CD31 antibody (Santa Cruz Biotech., Santa Cruz, CA) followed by staining with FITC-conjugated anti-goat antibody to identify capillaries in tumors. Double-stained cells positive for FITC and DiI were observed using fluorescence microscopy (Zeiss, Gemany) at 400× magnification and considered to be EPC-derived endothelial cells.

STATISTICAL ANALYSIS

ANOVA tests were performed to assess significant differences between control and experimental groups. The level of significance was set at $P < 0.01$ or $P < 0.05$. Results are presented as the mean ± standard deviation (SD).

RESULTS

DECURSIN INHIBITS EPC DIFFERENTIATION OF AC133+ CELLS FROM HUMAN CORD BLOOD

To determine whether decursin inhibits differentiation of AC133+ stem cells derived from human cord blood, we first tried to perform a colony formation assay. As previously described, we can discriminate early and late EPC-differentiated cells from stem cells—i.e., round- and spindle-shaped EPC-CFUs, respectively—using our novel assay [Kwon et al., 2009; Kamei et al., 2010; Kwon et al., 2011]. Round- and spindle-shaped EPC-CFUs expressed EC markers [Kwon et al., 2011] and showed specific morphology (Fig. 1). After isolation of AC133+ cells from human cord blood, cells were cultured in a specific medium as described in Materials and Methods section for 18 days. Decursin significantly inhibited spindle-shaped EPC-CFUs in a dose-dependent manner, but not round-shaped EPC-CFUs (Fig. 1). This result suggests that decursin might inhibit a late step of EPC differentiation rather than an early step.

Because during EPC differentiation, stem cells should proliferate, commit, and differentiate into EPCs, we tried to determine where this inhibition of EPC differentiation by decursin occurs. Thus, we next examined the proliferation ability of AC133+ cells during the entire differentiation process. As shown in Figure 2A, when AC133+ cells were treated with decursin during proliferating and committing stages for 7 days using the ex vivo expansion assay, decursin significantly inhibited proliferation of AC133+ cells and commitment into EPCs (Fig. 2A), indicating that decursin might inhibit a step for proliferation and commitment into EPCs. However, the characteristics of EPCs during differentiation were not changed by the treatment of decursin, because the expression level of EPC surface markers, such as CD34, KDR, or AC133 was the same in control and decursin-treated groups (data not shown). When we allowed decursin-treated committed EPCs to be differentiated into EPC-CFUs after washing out decursin, the number of round- and spindle-shaped EPC-CFUs was not altered compared to control, suggesting that the inhibitory effect of decursin on EPC differentiation from AC133+ cells might be reversible (Fig. 2B). When EPC-committed cells were treated with decursin, late EPC-CFUs were significantly inhibited (Fig. 2C). Together with the data in Figure 1, these results suggest that inhibition of EPC differentiation by decursin might be caused by a decreased number of committed EPCs and decreased ability of differentiation.

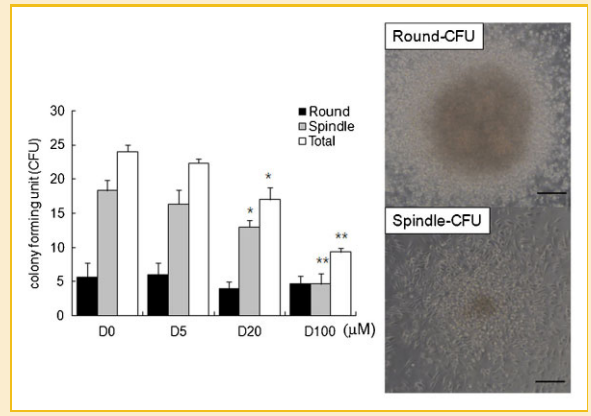

Fig. 1. Decursin inhibits differentiation of AC133+ cells isolated from human cord blood. MNCs were isolated from human cord blood and CD133 microbeads were used for isolation of AC133+ MNCs using autoMACS and FACSAria systems. AC133+ cells were treated with decursin (5–100 μM) and EPC-CFAs were performed in methylcellulose media for 18 days. Representative round- or spindle-shaped CFUs are shown in the right panel (40×) Scale bar = 100 μm. Round-shaped, spindle-shaped, and total CFUs are expressed as the colony number generated from 500 cells. $^{*}P < 0.05$, $^{**}P < 0.01$.

DECURSIN INHIBITS EXPRESSION OF CYTOKINES, GROWTH FACTORS, AND THEIR RECEPTORS RESPONSIBLE FOR EPC DIFFERENTIATION

To identify which genes are responsible for inhibition of EPC differentiation by decursin, we analyzed the mRNA levels of some factors and their receptors involved in EPC differentiation with mouse BM-derived EPCs. Decursin increased the mRNA expression of *angiopoietin-1* (*Ang-1*) and *VEGF*, but suppressed that of *Ang-2*, angiopoietin receptor *Tie-2*, *Flk-1* (KDR, or VEGFR-2), and *eNOS* in a dose-dependent manner (Fig. 3A). Although decursin has been reported to inhibit the VEGFR-2 signaling pathway [Jung et al., 2009], we found that the *VEGF* mRNA level in mouse BM-derived EPCs was significantly increased by decursin, but the level of its receptor, *VEGFR-2*, was clearly diminished (Fig. 3A). Increased *Ang-1*, decreased *Ang-2*, and decreased *Tie-2* mRNA expression would attenuate EPC differentiation, because binding of Ang-2 to the Tie-2 receptor increases angiogenic sprouting, which competes

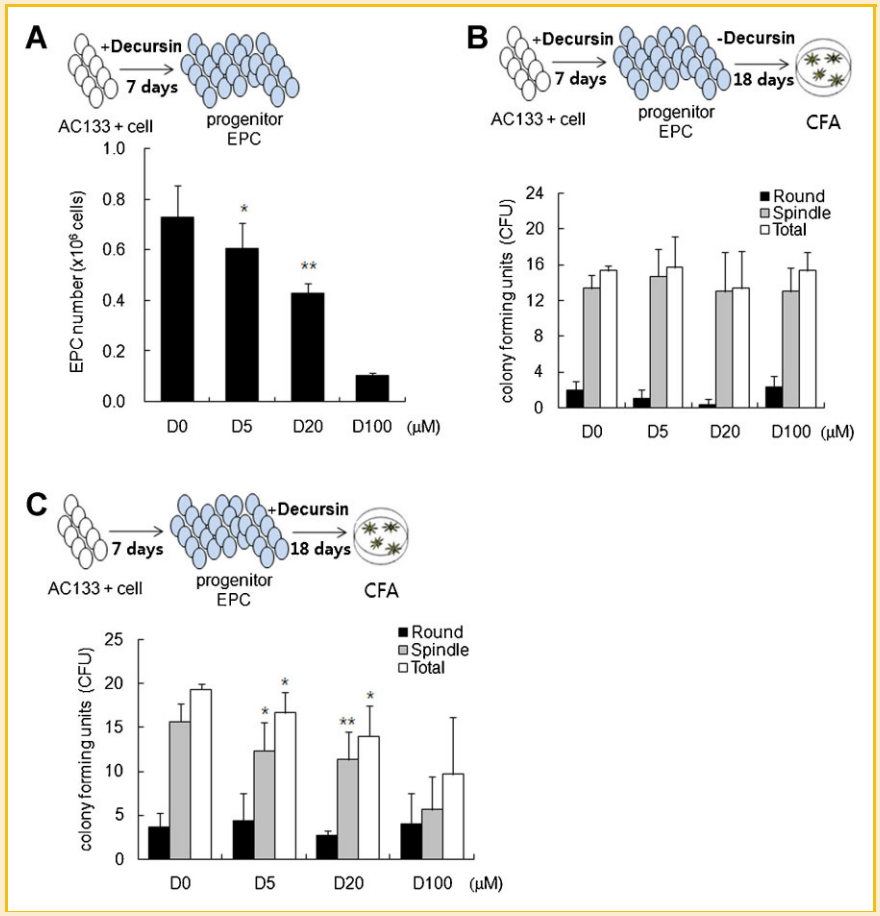

Fig. 2. Decursin inhibits the proliferation and differentiation ability of AC133+ cells. A: After isolation of AC133+ cells from human cord blood, cells were treated with indicated doses of decursin in the early EPC committing stage for 7 days in Stem Span media and the EPC number was counted. B: Decursin-treated cells the same as described in (A) were further cultured after washing out decursin for 18 days and assayed for EPC-CFUs. Round-, spindle-shaped, and total CFUs are expressed as the colony number generated from 500 cells. C, AC133+ cells were cultured in Stem Span media for 7 days and treated with decursin for a further 18 days. EPC-CFAs were performed and round-shaped, spindle-shaped, and total-CFUs are expressed as the colony number generated from 500 cells. Compared with control, $^{*}P < 0.05$; $^{**}P < 0.01$.

with Ang-1, having a controversial effect against Ang-2 [Yancopoulos et al., 2000]. Although the mRNA level of *SDF-1* was unaltered, that of *CXCR4* was significantly decreased by decursin dose-dependently (Fig. 3), indicating that decursin inhibits the SDF-1/CXCR4 signaling pathway in EPC differentiation. We confirmed that protein level of VEGF, Flk-1, Tie-2, and CXCR4 was inhibited by the treatment of decursin (Fig. 3B). The expression of *eNOS* was significantly suppressed by decursin (Fig. 3), which might also contribute to attenuate EPC differentiation.

DECURSIN INHIBITS SDF-1α- INDUCED MIGRATION AND ITS DOWNSTREAM SIGNALING PATHWAY

VEGF and SDF-1α are key regulators of EPC differentiation and function in vasculogenesis [Spring et al., 2005; Zheng et al., 2007; Kwon et al., 2008] and decursin has already been reported to suppress the VEGFR-2 signaling pathway [Jung et al., 2009; Kim et al., 2009a, b]. As expected, expression of CXCR4 mRNA was clearly diminished (Fig. 3), and thus we investigated whether decursin affects SDF-1α-induced migration and signaling pathway of EPCs. When we treated mouse BM-derived EPCs with decursin in the transwell migration assay, decursin significantly inhibited SDF-1α-induced EPC migration dose-dependently (Fig. 4A). SDF-1α increased migration of EPCs by twofold; however, treatment of decursin attenuated SDF-1α-induced EPC migration to control levels (Fig. 4A). Moreover, SDF-1α activated Akt (phospho-Akt), ERK1/2 (phospho-ERK1/2), and eNOS (phospho-eNOS), which was consistent with the results obtained by Zheng et al. (2007) whereas decursin inhibited SDF-1α-induced activation of Akt, ERK1/2, and eNOS in a dose-dependent manner (Fig. 4B). These results indicate that decursin suppresses EPC differentiation and function through inhibition of SDF-1α and its signaling pathway.

DECURSIN INHIBITS THE TUBE FORMATION ABILITY OF EPC

To confirm whether decursin blocks the ability of EPC to promote formation of EC-derived tubes, we co-cultured EPCs with HUVECs. As shown in Figure 5, EPCs only did not form tube structure whereas HUVECs only did successfully. But co-culture of EPCs with HUVECs dramatically promoted tube formation by HUVECs (D0). It seems that structural basis for the observed tube structure came from HUVECs, with EPCs playing a supporting role. However, decursin

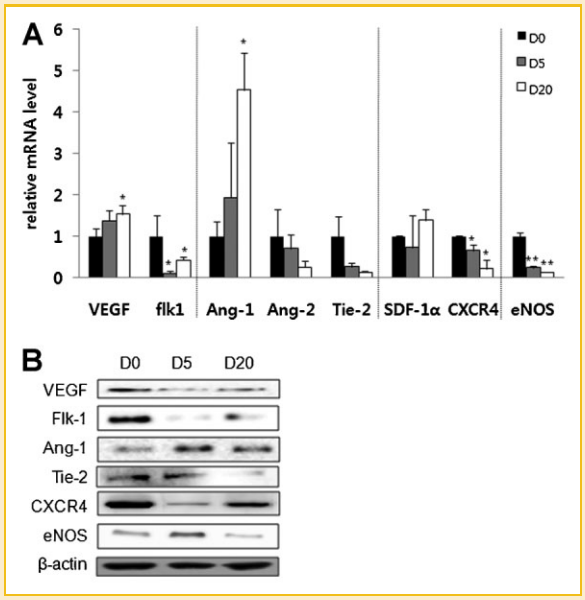

Fig. 3. Decursin inhibits expression of cytokines, growth factors, and their receptors for EPC function. A: MNCs isolated from mouse BM were cultured in EBM-2 medium supplemented with EGM-2MV SingleQuots for 7 days. After decursin (5 and 20 μM) treatment for 3 days in EGM-2MV media, total RNA was collected and real-time PCR analysis to determine the mRNA expression level of VEGF, Flk-1, Ang-1, Ang-2, Tie-2, SDF-1, CXCR4, and eNOS was performed. Quantitation of each gene expression level relative to β-actin from three different experiments was graphed. Compared with control, $^{*}P < 0.05$, $^{**}P < 0.01$. B: Western blot analysis for VEGF, Flk-1, Ang-1, Tie-2, CXCR-4, and eNOS from same samples as (A) was performed with respective antibodies. β-actin was examined as an internal control.

significantly disrupted the ability of EPCs to form EC-derived tubes dose-dependently (Fig. 5A,D5,D20,D100). When we counted the length of tubes formed using the Matrigel assay, decursin significantly blocked a tubular structure (Fig. 5B).

DECURSIN ATTENUATED VASCULOGENESIS IN XENOGRAFT LUNG CANCER PROGRESSION

To clarify whether decursin inhibits EPC-derived microvessels in tumor progression, we performed LLC tumor xenograft experiments in mice. When we injected 5×10^4 LLC cells into the mouse flank followed by treatment with decursin (4 mg/kg), the control tumor mass was palpated approximately 12 days after inoculation. However, the decursin-treated tumor mass was palpated approximately 18 days after inoculation (Fig. 6A). Because circulating EPCs are increasing when the tumor mass is growing [Chakroborty et al., 2008], we measured circulating MNCs in LLC tumor-inoculated mice at 7 days after inoculation. As shown in Figure 6B left graph, the circulating MNC number in LLC tumor-bearing mice was significantly enhanced compared with those in normal control mice. When we checked for EPCs using the CD34/VEGFR2 double-labeling assay with circulating MNCs, no difference was found in the number of CD34 + /VEGFR2+ cells among the groups (Fig. 6B right panel); thus, the number of circulating EPCs might be also decreased following treatment with decursin. Previously, we found that the microvessel density as determined by immunohistochemistry against platelet endothelial cell adhesion molecule-1 (CD31) is attenuated in LLC tumor masses obtained at 3 weeks after decursin treatment [Jung et al., 2009].

The day after 1×10^6 LLC cells inoculation in the mouse flank, we injected DiI-Ac-LDL-labeled mouse BM-derived EPCs into the mice through the tail vein and examined EPC-derived microvessels in the tumor mass. At 6 days after tumor inoculation, the tumor mass was

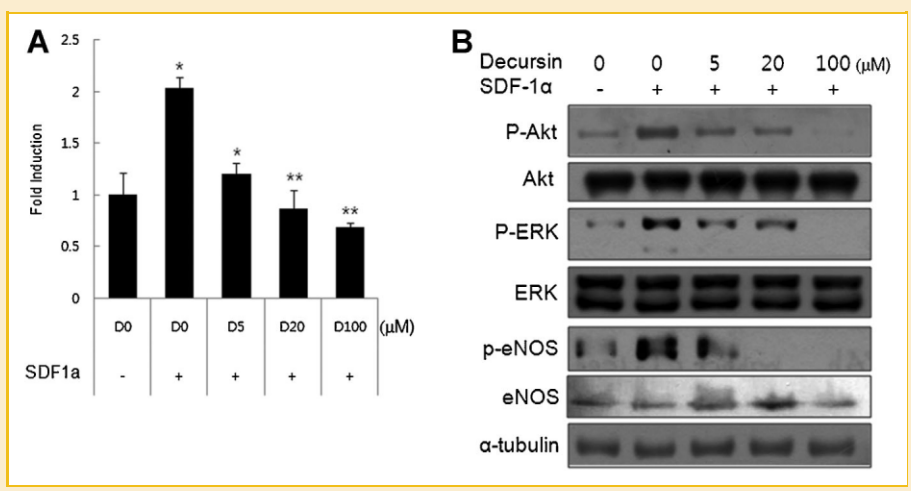

Fig. 4. Decursin inhibits SDF-1α-induced migration and its signaling pathway. A: Mouse BM-derived EPCs (5×10^4 cells per well) were plated onto a 24-well transwell membrane coated with collagen and treated with or without decursin (5–100 μM) in the presence of SDF-1α (50 ng/ml) for 6 h. Cells that migrated into the bottom part of the membrane were stained with DAPI and counted. The relative migration ability was calculated compared with control (without decursin and SDF-1α). Compared with control, $^{*}P < 0.05$, $^{**}P < 0.01$. B: Mouse BM-derived EPCs were treated with SDF-1α (50 ng/ml) for 30 min in the presence or absence of decursin (5–100 μM) and proteins were collected for western blot analysis. Phosphorylated and native forms of Akt, ERK, and eNOS were detected using antibodies. The total protein and α-tubulin were examined as an internal control.

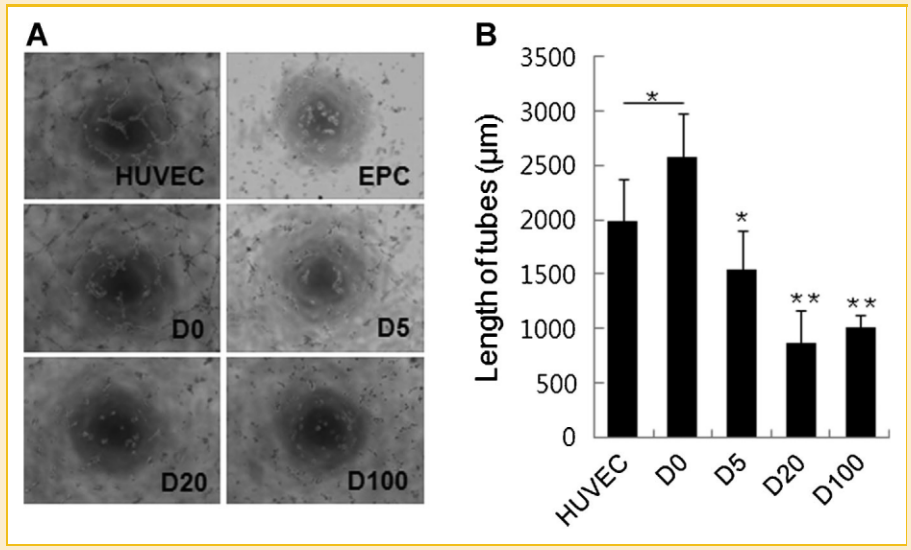

Fig. 5. Decursin inhibits the tube formation ability of EPCs. A: Mouse BM-derived EPCs mixed with HUVECs were plated onto a 96-well plate coated with growth factor-reduced Matrigel (10 mg/ml) and further incubated for 8 h. HUVEC: HUVEC only, EPC:EPC only, D0–D100: Decrusin (0–100 μM) treatment in HUVEC plus EPC (1:1). The length of tubes was measured after each well was pictured at 40× magnification by light microscopy. B: Quantitation of loop length was graphed (compared with control, $^{*}P < 0.05$, $^{**}P < 0.01$).

fixed and stained with anti-mouse CD31 antibody. Double-positive cells for both DiI-Ac-LDL and CD31-FITC were counted as EPC-derived microvessel endothelial cells. Compared with the LLC tumor mass, in the decursin-treated tumor, double-positive cells were significantly decreased as well as CD31 single-positive cells, indicating that decursin inhibited incorporation of labeled EPCs into tumor microvessels (Fig. 6C).

To confirm this result, we performed a BMT experiment. After irradiation with 10 Gy of γ-rays, we injected BM MNCs obtained from GFP transgenic mice (C57BL/6-Tg) into lethally irradiated age-matched, syngeneic, non-GFP recipient mice intravenously. Analysis of BM and PB (4 weeks post-transplantation) showed >95% reconstitution of recipient hematopoiesis by the donor BM-derived GFP+ transplanted progenitor cells (data not shown), indicating stable replacement of original host stem cell population by the donor cells. The reconstituted mice were then challenged with LLC cells subcutaneously. We assessed the contribution of BM-derived EPCs to early stages of LLC tumor growth. Using immunohistochemistry, tumors were stained with CD31, an endothelial cell marker. As expected, BM-derived GFP+ cells were observed infiltrating the tumor bed (Fig. 6D). During the early stage of tumor growth (days 4–6), we observed a marked recruitment of BM-derived GFP+ cells expressing CD31 (Fig. 6D). By contrast, in decursin-treated mice tumors, these GFP + CD31+ cells were clearly diminished (Fig. 6D). However, proliferating cells (PCNA+ cells) among GFP + CD31+ cells were not different between the two groups. At the later stage of tumor formation (days 14–16), we also assessed the recruitment of BM-derived cells. Interestingly, the late tumor mass had less BM-derived GFP + CD31+ cells whereas many CD31+ cells were found. Decursin treatment also decreased CD31+ cells without GFP, implying an angiogenic inhibitory effect as reported previously [Jung et al., 2009]. Together, these results suggest that decursin clearly blocks the early-phase vasculogenic process through inhibition of EPC differentiation and function as well as through angiogenesis in tumor progression.

DISCUSSION

Decursin treatment (4 mg/kg body weight) in mice delayed tumor formation and dramatically decreased tumor growth by inhibition of angiogenesis through VEGFR-2 signaling pathway (Fig. 6A). When we observed tumor microvessels at 21 days after inoculation of LLC cells, the microvessel density of tumor tissue was significantly diminished compared with that of control LLC tumor tissue [Jung et al., 2009]. This suggests that a low dose of decursin clearly inhibits angiogenic process in tumor growth. However, in that experiment, we ignored the early event of tumor growth treated with decursin. Delayed palpation of the tumor mass in decursin-treated mice was experienced: i.e., the mass was touched around 6 days later than in control mice (Fig. 6A). This delayed onset of tumor mass may be due to inhibition of EPC recruitment from BM because EPCs contribute from the early stages of tumorigenesis rather than the angiogenic process from neighboring host tissues [Nolan et al., 2007]. Here, we clearly demonstrated that decursin blocked recruitment of BM-EPCs into an early tumor mass when we excised the tumor and performed immunostaining with tissues at 14 days after inoculation (Fig. 6A,C). Vasculogenic neovessel formation in the tumor mass was clearly suppressed by decursin.

In this study, we showed that decursin significantly attenuated EPC differentiation from human cord blood-derived stem cells (Figs. 1 and 2). Particularly, it blocked the late EPC-CFUs, suggesting that early steps of EPC differentiation are not affected by decursin compared with late steps of differentiation. Late EPC-CFUs represent

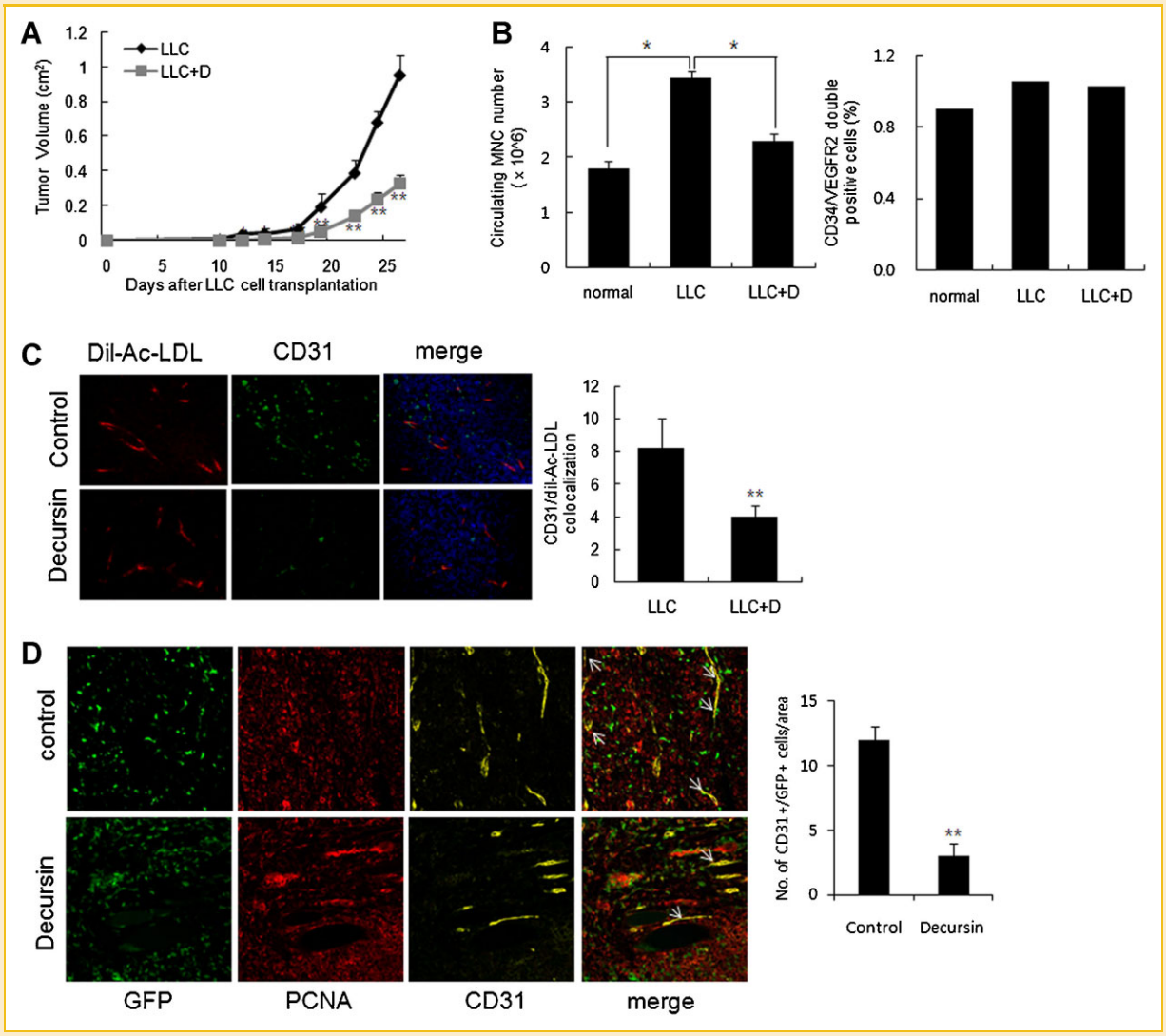

Fig. 6. Decursin inhibits vasculogenesis in xenograft lung cancer progression. A: LLCs (5×10^4 cells) were inoculated subcutaneously into C57BL/6J mice and tumor size was measured with a caliper every other day. Mice were treated with decursin (4 mg/kg body weight) or vehicle (PBS) every day for 4 weeks. Compared with LLC (vehicle) control, $^{*}P < 0.05$, $^{**}P < 0.01$. B: Left graph shows the number of MNCs from BM measured at 14 days after tumor cell (one inoculation in the same mice described in (A) treated with vehicle (LLC) or decursin (LLC + D). $^{*}P < 0.05$. Right graph shows the number of circulating EPCs determined by CD34 + /Flk-1+ cells from PB measured at 14 days after tumor cell inoculation in the same mice described in the left graph treated with vehicle (LLC) or decursin (LLC + D). $^{*}P < 0.05$. C: Dil-Ac-LDL-labeled EPCs (1×10^6 cells) were injected into the tail vein of tumor-bearing mice 4 days after tumor cell inoculation. Five days after injection, tumor tissues were fixed with paraformaldehyde and embedded in paraffin. Paraffin sections (5 μm) were stained with goat anti-mouse CD31 antibody overnight at 4°C, followed by staining with FITC-conjugated anti-goat antibody. Double-stained cells with FITC and Dil were observed under a fluorescence microscope at 400× magnification (left panel). Double-labeled cells were counted and graphed. $^{*}P < 0.05$ versus control. D: After mice were irradiated with γ-rays (10 Gy), MNCs isolated from the BM of GFP mice were injected into mice via the tail vein. After 4 weeks, successfully transplanted (GFP +) mice were inoculated with 1×10^6 LLC cells subcutaneously and treated with decursin (4 mg/kg body weight) or vehicle (PBS) every day (intraperitoneally) for 2 weeks. Tumors were fixed and immunostained with rat anti-mouse CD31 and mouse anti-PCNA antibodies, followed by an Alexa Fluor 647-conjugated anti-rat IgG and a Texas red-conjugated anti-mouse IgG, respectively. Fluorescent cells were observed under a confocal microscope at 200× magnification (left panel). Yellow fluorescent positive cells (CD31 +) among GFP cells were counted and graphed. $^{**}P < 0.01$ versus control (vehicle).

an increased ability to form functional vessels in an ischemic model [Masuda et al., 2011]. Thus, our data suggest that decursin might control the maturation steps into functional EPCs rather than the commitment steps into the EPC lineage from BM progenitor cells.

Decursin inhibits VEGFR-2 activation and its signaling pathways in HUVECs [Jung et al., 2009]. Moreover, decursin inhibits migration and proliferation of retinal endothelial cells, and resultant retinal angiogenesis [Kim et al., 2009a] as well as blood retinal barrier breakdown in an oxygen-induced retinopathy mouse model via blocking VEGFR-2 signaling pathways [Kim et al., 2009b]. Decursin might inhibit transcription of VEGFR-2 or its activation through regulation of VEGFR-2 phosphorylation directly or blocking the binding of VEGF to its receptor VEGFR-2. When we performed real-time PCR for growth factors or cytokines involved in the recruitment, mobilization, and differentiation of EPCs, expression of VEGF protein and VEGFR-2 (Flk1) mRNA was significantly

inhibited in mouse BM-derived EPCs (Fig. 3). An increased VEGF mRNA level might be a compensatory mechanism for inhibition of the VEGFR-2 mRNA level and its signaling pathway.

As shown in Figure 4A, SDF-1α-induced migration was significantly decreased by decursin treatment dose-dependently. The SDF-1α-induced activation of Akt, ERK, and eNOS was dramatically attenuated. These results indicate that the effect of decursin on inhibition of EPC differentiation is due to a mechanism that inhibits VEGF/VEGFR-2 and SDF-1α/CXCR4 signaling pathways. Inhibition of these signaling pathways might originate from transcriptional inhibition of the receptors VEGFR-2 and CXCR4 (Fig. 3). A reduced receptor pool in the cell membrane might decrease signaling activation that is necessary for making functional EPCs. However, the precise mechanism to inhibit these signaling pathways remains to be investigated, such as inhibition of phosphorylation of the receptors or stereotypical blocking of binding to the receptors.

Endothelial NOS is essential for the BM microenvironment and increases in BM NO levels result in mobilization of EPCs from BM niches to the circulation, ultimately allowing their participation in tissue-level vasculogenesis and wound healing [Murohara et al., 1998; Aicher et al., 2003; Li et al., 2008]. SDF-1α induced-activation of phosphoinositide 3-kinase (PI3K)-Akt increases phosphorylation of eNOS Ser 1177, increasing NO production in EPCs [Li et al., 2008]. Because eNOS-derived NO can promote differentiation of many cell types, including EPCs [Aicher et al., 2003] neovascularization or endothelization from EPCs is inhibited by treatment with decursin through inhibition of the PI3K-Akt-eNOS signaling axis. EPC recruitment substantially depends on ischemia-induced upregulation of SDF-1α in tissues [Ceradini et al., 2004]. Therefore, our findings strongly indicate that decursin treatment may decrease SDF-1α-induced EPC mobilization from the BM under hypoxic conditions in tumors and the incorporation of EPCs into the tumor neovasculature might be inhibited, resulting in inhibition of tumor growth and neovessel formation (Fig. 6).

Because several reports indicate that mobilization of EPCs from the BM to the peripheral circulation and its subsequent incorporation into neovessels are important for pathogenesis of many diseases, including cancer [Asahara et al., 1999; Lyden et al., 2001; Duda et al., 2006], targeting EPC mobilization may be an important therapeutic intervention for treatment of various pathological conditions. EPC functions in vasculogenesis in tumor vasculature as well as in ischemic tissues, such as cardiac ischemia owing to the obstruction of coronary artery [Asahara et al., 1999; Ceradini and Gurtner, 2005]. In the prevention or treatment of cancer, benefits of EPC contribution to increase healing process or ischemic diseases would be disregarded. For example, side effect of VEGF blockade therapy in cancer patient is a hypertension and thrombosis. However, the side effect of anti-angiogenic therapy should be overcome by alternative regimens, such as prevention or optimal treatment of ischemic injury or hypertention. This is to be accounted by the choice of risk or benefit.

In conclusion, here, we identified the effect of decursin purely isolated from the root of *Angelica gigas* on inhibition of BM-derived EPCs in vasculogenesis in the early stage of tumor formation. Additionally, this inhibitory effect was caused by inhibition of the differentiation ability from BM-derived or cord blood-derived stem cells to EPCs, mobilization from BM to tumor tissues, and EPC function. Thus, decursin may have therapeutic value in cancer and other diseases in which EPC mobilization is stimulated. Furthermore, decursin might be used for therapeutics in combination with anti-cancer agents and for health foodceuticals to prevent and block early progression of tumor development.

ACKNOWLEDGMENTS

This work was supported by the Korean Research Foundation Grant by the Korean Ministry of Education, Science and Technology (2009-0066170). We would like to thank Dr. Eun-Mi Ahn in Daegu Hanni University for generously providing purified decursin and Dr. Goo-Taeg Oh in Ewha University for GFP-transgenic mice.

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