Anti-angiogenic effects of *Siegesbeckia glabrescens* are mediated by suppression of the Akt and $p70^{S6K}$-dependent signaling pathways

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Received August 11, 2014; Accepted November 12, 2014

DOI: 10.3892/or.2014.3630

Abstract. *Siegesbeckia glabrescens* (SG) Makino (Compositae) has been used as a traditional medicine for the treatment of allergic and inflammatory diseases. In the present study, we report the effects and molecular mechanism of an ethanolic extract of SG on cell proliferation, migration and tube formation in vascular endothelial growth factor-A (VEGF-A)-treated human umbilical vein endothelial cells. SG treatment inhibited VEGF-A-stimulated endothelial cell proliferation through downregulation of cyclin D and upregulation of cyclin-dependent kinase inhibitors such as $p27^{Kip1}$ and $p21^{WAF1/Cip1}$. In addition, SG inhibited VEGF-A-stimulated endothelial cell migration and tube formation. These anti-angiogenic activities of SG were mediated by inactivation of the Akt- and $p70^{S6K}$-dependent signaling pathways. Collectively, our findings demonstrate the pharmacological roles and molecular mechanism of SG in regulating angiogenic responses and support further evaluation and development of SG as a potential therapeutic agent for the treatment and prevention of angiogenesis-related diseases including cancer.

Introduction

Angiogenesis, the formation of new blood vessels which includes activation of endothelial cells and recruitment of pericytes, plays important roles in cancer growth and metastasis. This response is controlled by changes in a delicate balance between angiogenic and anti-angiogenic factors (1,2). Vascular endothelial growth factor (VEGF) and the VEGF receptor (VEGFR) have been well characterized as key factors that trigger signaling events in angiogenesis. VEGF-A, one of the most common members of the VEGF family, binds to its cognate receptor VEGFR-2 and activates signaling pathways, resulting in endothelial cell proliferation, migration, survival and vascular permeability associated with cancer progression. Thus, selective inhibition of VEGF-A/VEGFR-2 activation or the downstream signaling pathways is appreciated as a potent strategy, compared to conventional chemotherapy. Many drugs that target VEGF, VEGFR and the downstream signaling pathways are currently in clinical trials or use (3-6).

Siegesbeckia glabrescens (SG) Makino (Compositae) has been used as a traditional medicine for the treatment of acute hepatitis, paralysis, hypertension, asthma and rheumatoid arthritis. The extracts and bioactive components of SG have anti-inflammatory, anti-allergic and anticancer activities (7-10). We previously reported that the anticancer activity of SG against ovarian cancer and non-small cell lung cancer cells is mediated through downregulation of receptor tyrosine kinases and their signaling pathways (11,12). In addition, a recent study demonstrated that SG has anti-angiogenic and anti-adipogenic activities (13). However, no detailed mechanisms of SG responsible for the regulation of angiogenesis have been clearly elucidated to date. In the present study, we evaluated the regulatory effects and signaling pathways of SG on proliferation, adhesion, migration and tube formation in human umbilical vein endothelial cells (HUVECs). We showed for the first time that the anti-angiogenic activity of SG in VEGF-A-treated HUVECs is mainly mediated through inactivation of the VEGF-A/VEGFR-2 downstream signaling pathways such as Akt and $p70^{S6K}$.

Materials and methods

Cell culture conditions. Primary cultures of HUVECs were purchased from Lonza Walkersville Inc. (Walkersville, MD, USA) and used between passages 4 and 6 for all experiments. Cells were cultured in EGM-2® BulletKit media, according to the manufacturer's instructions (Lonza).

Reagents. The following pharmacological agents and antibodies were purchased from commercial sources: VEGF-A and LY294002 [an inhibitor of the phosphatidylinositol3-kinase

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Key words: *Siegesbeckia glabrescens*, angiogenesis, vascular endothelial growth factor-A, human umbilical vein endothelial cells

(PI3-K)/Akt pathway; Merck Millipore, Billerica, MA, USA]; rapamycin [an inhibitor of the mammalian target of rapamycin (mTOR)/$p70^{S6K}$ pathway; Sigma-Aldrich, St. Louis, MO, USA]; anti-phospho-extracellular signal-regulated kinase (ERK) (T202/Y204), anti-phospho-Akt (S473), anti-phospho-$p70^{S6K}$ (T421/S424) and anti-phospho-pRb (S780) (Cell Signaling Technology Inc., Beverly, MA, USA); anti-$p27^{Kip1}$ (BD Biosciences, Bedford, MA, USA); anti-ERK, anti-Akt, anti-$p70^{S6K}$, anti-Cdk4, anti-Cdk2, anti-cyclin D, anti-cyclin E, anti-$p27^{WAF1/Cip1}$, anti-actin antibodies, and mouse and rabbit IgG-horseradish peroxidase conjugates (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Preparation of the SG extract. SG was purchased from Dae Kwang Herb Medicine Co. (Chuncheon, Gangwon-do, Korea) and deposited at the herbarium of the Radiant Research Institute (Radiant Inc., Chuncheon, Gangwon-do, Korea). One hundred grams of SG was extracted with 1 liter of ethanol and stirred for 90 min. The extract of SG was obtained as previously reported (11,12).

Cell viability and proliferation assay. Subconfluent cells, plated on 6-well plates ($1x10^5$ cells/well; BD Biosciences), were serum-starved for 14 h to synchronize cells in the G_1/G_0 phase of the cell cycle, pretreated with SG (0.01-1 μg/ml) for 30 min in the presence or absence of LY294002 (10 μM) or rapamycin (50 nM) as indicated and further incubated with VEGF-A (10 ng/ml) for 24 h. Following culture for 24 h, cell viability was determined by a Muse™ cell analyzer using a cell count and viability assay kit (Merck Millipore), and the cell proliferation was quantified as previously described (14). The results from triplicate determinations (mean ± standard deviation) are presented as the fold-increase of the untreated controls or the percentage of viable cells of the total cell count.

Cell cycle analysis. Serum-starved cells were pretreated with SG (1 μg/ml) for 30 min, followed by VEGF-A (10 ng/ml) for 24 h. Cells were harvested with trypsin-EDTA, rinsed with phosphate-buffered saline (PBS, pH 7.4) and then fixed with ice-cold 70% ethanol for 3 h. After washing with PBS, cells were stained with Muse™ cell cycle reagent. The profile of cells in the G_1/G_0, S and G_2/M phases of the cell cycle was analyzed with a Muse™ cell analyzer (Merck Millipore) (15).

Western blot analysis. Subconfluent cells in 100-mm dishes ($1x10^6$ cells/dish; BD Biosciences) were serum-starved for 14 h, pretreated with SG for 30 min followed by VEGF-A (10 ng/ml) for 15 min or 24 h, as indicated. Cells were rinsed twice with ice-cold PBS and lysed by incubation in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 100 μg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml pepstatin A, 0.5 μg/ml leupeptin, 80 mM β-glycerophosphate, 25 mM sodium fluoride and 1 mM sodium orthovanadate for 30 min at 4˚C. Cell lysates were clarified at 12,500 x g for 20 min at 4˚C, and the supernatants were subjected to western blot analysis as described previously (16,17). All western blot analyses were performed at least in triplicate experiments and representative gels are shown. Bands of interest were integrated and quantified by the use of National Institutes of Health (NIH) ImageJ Version 1.34s software.

Adhesion assay. Subconfluent cells were detached with trypsin-EDTA and allowed to recover in EGM-2® BulletKit media for 1 h at 37˚C with gentle rocking. After recovery, the cells were collected by low-speed centrifugation and resuspended in serum-free EBM-2 media (Lonza). The cell suspension was pretreated with or without SG (1 μg/ml) for 30 min followed by VEGF-A (10 ng/ml) treatment. The cells were plated on 96-well plates ($1.5x10^4$ cells/well) and further incubated for 2 h at 37˚C. Following the incubation, the unattached cells were removed by washing the wells 3 times with ice-cold PBS. Attached cells were fixed with methanol and then stained with 0.04% Giemsa staining solution (Sigma-Aldrich). The cells were photographed and counted. The results (mean ± standard deviation) are presented as the numbers of adherent cells (18).

Migration assay. Cell migration was quantified in the *in vitro* wound-healing assay as described previously (14,19). After cells were plated on 48-well plates and grown to confluence, a single wound was created in the center of the cell monolayer by the gentle removal of the attached cells with a sterile plastic pipette tip. Following serum starvation with EBM-2 for 2 h, cells were pretreated with SG (0.01-1 μg/ml) for 30 min in the presence or absence of LY294002 (10 μM) or rapamycin (50 nM) as indicated, followed by VEGF-A (10 ng/ml) stimulation for 15 h. Cells were fixed with methanol and then stained with 0.04% Giemsa solution. The migration of the cells into the wound was observed with still images captured at the indicated time-point.

Tube formation assays. Matrigel® basement membrane matrix (10.4 mg/ml, BD Biosciences) was thawed overnight at 4˚C, and each well of pre-chilled 24-well plates was coated with 200 μl Matrigel and then incubated at 37˚C for 30 min. Following serum starvation with EBM-2 for 2 h, cells ($4x10^4$ cells/ml) were added to the Matrigel-coated plates and pretreated with SG (1 μg/ml) for 30 min, followed by VEGF-A (10 ng/ml) for 6 h. Tube formation was observed with an Olympus CKX41 inverted microscope (CAchN 10/0.25php objective) and ToupTek Toupview software (version x86, 3.5.563, Hangzhou ToupTek Photonics Co., Zhejiang, China).

Statistical analysis. Statistical analysis was performed using the Student's t-test and was based on at least 3 different experiments. The results were considered to be statistically significant at $P<0.05$.

Results

SG suppresses VEGF-A-stimulated endothelial cell proliferation through regulating the expression of cyclin D and cyclin-dependent kinase inhibitors. We first examined the effect of SG on cell proliferation of HUVECs. SG treatment suppressed VEGF-A-stimulated endothelial cell proliferation in a dose-dependent manner (Fig. 1A). In addition, treatment of non-stimulated HUVECs with SG at the highest concentration used in this study did not alter the viability or the proliferative response of HUVECs (Fig. 1B), indicating that SG inhibition of cell proliferation was not mediated by induction of apoptosis or cytotoxicity. This finding is similar to the patterns of

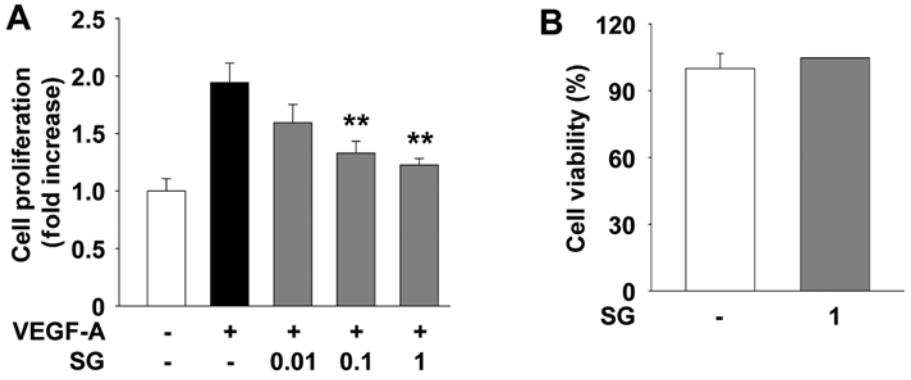

Figure 1. SG inhibits VEGF-A-stimulated cell proliferation in HUVECs. (A) Quiescent cells were pretreated with SG at different concentrations (0.01-1 μg/ml) for 30 min, followed by VEGF-A (10 ng/ml) stimulation for 24 h. Values are the mean ± SD of 3 independent experiments. Statistical significance is indicated (**P<0.01, compared with VEGF-A-treated cells). (B) Cells were treated with SG (1 μg/ml) for 24 h. Cell viability was assessed as described in Materials and methods. The results from triplicate determinations (mean ± SD) are presented as the percentage of viable cells of the total cell count.

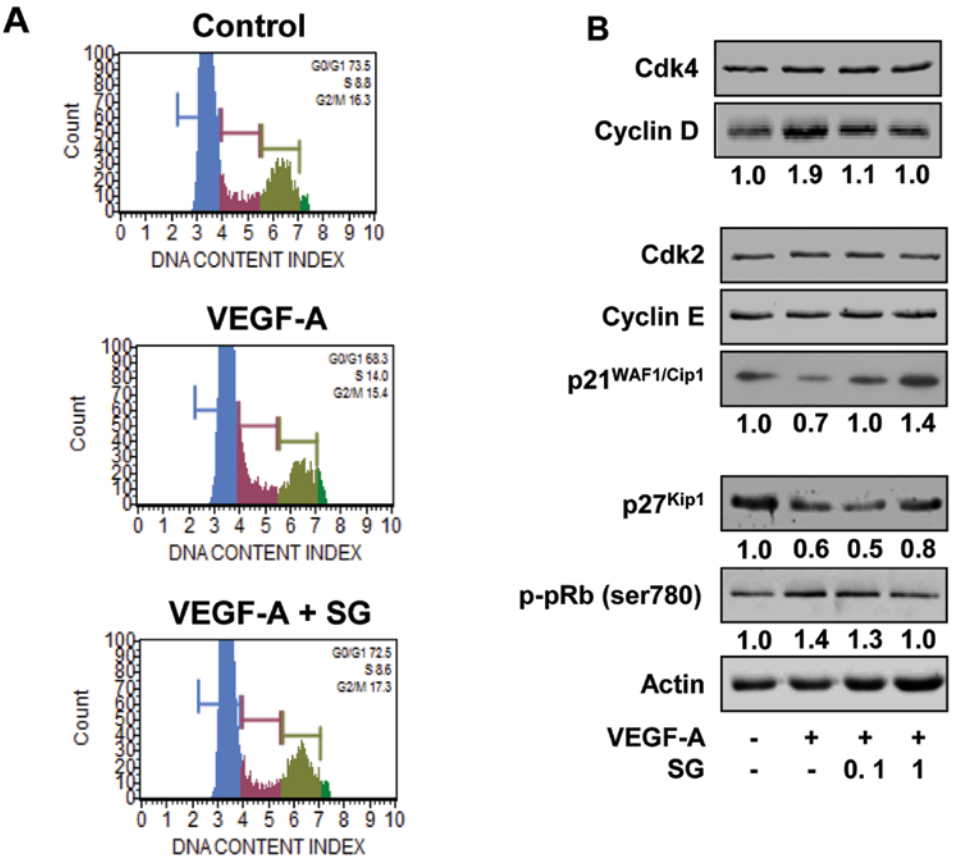

Figure 2. Anti-proliferative effect of SG on VEGF-A-stimulated HUVECs is mediated by downregulation of cyclin D and upregulation of Cdk inhibitors. (A) Quiescent cells were pretreated with SG (1 μg/ml) for 30 min, followed by VEGF-A (10 ng/ml) stimulation for 24 h. Cell cycle analysis was performed as described in Materials and methods. (B) Cells were treated with SG (0.1 and 1 μg/ml) as described in A, and western blotting with anti-Cdk4, anti-Cdk2, anti-cyclin D, anti-cyclin E, anti-p21$^{WAF/Cip1}$, anti-p27^{Kip1}, anti-phospho-pRb (S780) or anti-actin antibodies was carried out. Integrated density values were normalized to the untreated controls. Results shown are representative of at least 3 independent experiments.

SG in other cell types as previously reported (11,12). We next investigated the effect of SG on the cell cycle by DNA content analysis (Fig. 2A). VEGF-A stimulation for 24 h increased the percentage of cells in the S phase, compared with the untreated controls (8.8 vs. 14.0%) and resulted in the concomitant decrease of cells in the G_1 phase (73.5 vs. 68.3%). SG treatment prevented the increase in the percentage of cells in the S phase (14.0 vs. 8.6%) and the decrease in the percentage of cells in the G_1 phase (68.3 vs. 72.5%) associated with VEGF-A stimulation, similar to those of the untreated controls. These findings indicate that SG inhibits the transition from G_1 to S phase, leading to G_1 arrest, which is well correlated with inhibition of cell proliferation (Fig. 1A). SG has previously been reported to inhibit proliferation by downregulation of

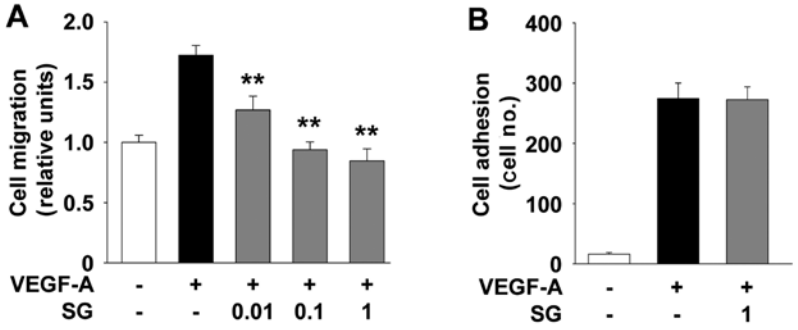

Figure 3. SG inhibits VEGF-A-stimulated endothelial cell migration, but not adhesion. Cells were pretreated with SG (0.01-1 μg/ml) for 30 min, followed by VEGF-A (10 ng/ml) stimulation for 15 h (A) and 2 h (B). (A) Cell migration results from six independent experiments (mean ± SD) are presented as the fold-increase of the untreated controls. Statistical significance is indicated (**P<0.01, compared with VEGF-A-treated cells). (B) Cell adhesion results from six independent experiments (mean ± SD) are presented as the numbers of attached cells.

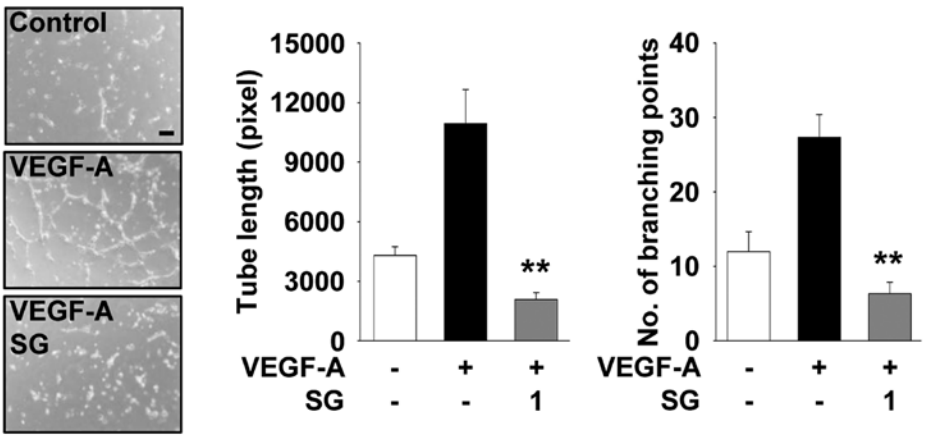

Figure 4. SG inhibits VEGF-A-stimulated formation of capillary-like structures in HUVECs. Tube formation was performed as described in Materials and methods and quantified by measuring the lengths of the tubes and the numbers of branching points per unit area. Cells were pretreated with SG (1 μg/ml) for 30 min, followed by VEGF-A (10 ng/ml) stimulation for 6 h. Scale bar, 100 μm. Statistical significance is indicated (**P<0.01, compared with VEGF-A-treated cells).

cyclin-dependent kinase 4 (Cdk4), cyclin D and cyclin E and upregulation of $p27^{Kip1}$ in SKOV-3 ovarian cancer cells (11). In addition, SG-mediated inhibition of proliferation in A549 and H1299 non-small cell lung cancer cells was mediated by suppression of Cdk4 and Cdk2 (12). Based on these observations, we analyzed the changes in cell cycle-related proteins such as Cdks, cyclins and Cdk inhibitors in the SG-treated HUVECs. As shown in Fig. 2B, SG treatment markedly suppressed the expression of cyclin D and induced the levels of Cdk inhibitors such as $p21^{WAF1/Cip1}$ and $p27^{Kip1}$, resulting in inhibition of pRb phosphorylation in response to VEGF-A stimulation. Although the molecular mechanism of SG in regulating cell cycle progression appears slightly different in various cell types, these findings clearly demonstrate the anti-proliferative activity of SG against various types of cells.

SG abrogates VEGF-A-stimulated endothelial cell migration and tube formation. Interaction of endothelial cells with extracellular matrix molecules plays important roles in cell migration, adhesion and capillary-like structure formation which are associated with cancer growth and progression (1,2). SG treatment inhibited VEGF-A-stimulated cell migration in a dose-dependent manner (Fig. 3A). In contrast to cell migration, SG did not alter the VEGF-A-induced endothelial cell adhesion (Fig. 3B), indicating that stable adhesiveness may contribute, at least in part, to reduced cell migration as previously reported (20,21). However, in SKOV-3 ovarian cancer cells SG was found to markedly simultaneously block mitogen-induced cell adhesion as well as migration (11). Collectively, these findings indicate that SG may differentially regulate cell adhesion and migration, depending on the type of cells or growth factor. We next investigated the ability of SG to regulate the formation of capillary-like structures in HUVECs. As shown in Fig. 4, SG completely inhibited VEGF-A-stimulated tube formation to the levels observed in the untreated controls, similar to inhibition of cell migration (Fig. 3A). These observations suggest that the ethanolic extract of SG possesses a variety of biologically active components which act on multiple targets and mechanisms involved in the regulation of cell proliferation, migration and tube formation in HUVECs.

Anti-proliferative and anti-migratory effects of SG are mediated through inhibition of the Akt- and $p70^{S6K}$-dependent signaling pathways. To investigate the molecular mechanisms by which SG regulates VEGF-A-induced endothelial cell

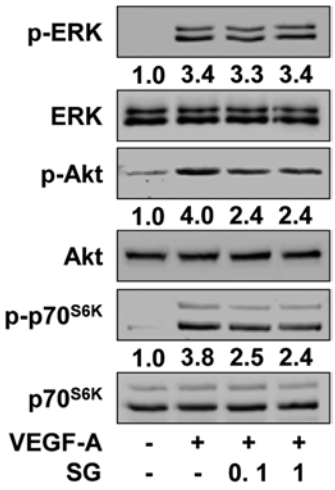

Figure 5. SG inhibits VEGF-A-stimulated activation of Akt and $p70^{S6K}$. (A) Quiescent cells were pretreated with SG (0.1, 1 μg/ml) for 30 min, followed by VEGF-A (10 ng/ml) stimulation for 15 min. Cell lysates were subjected to western blotting with anti-phospho-ERK, anti-ERK, anti-phospho-Akt, anti-Akt, anti-phsopho-$p70^{S6K}$ or anti-$p70^{S6K}$ antibodies. Integrated density values were normalized to the untreated controls. Results shown are representative of at least 3 independent experiments.

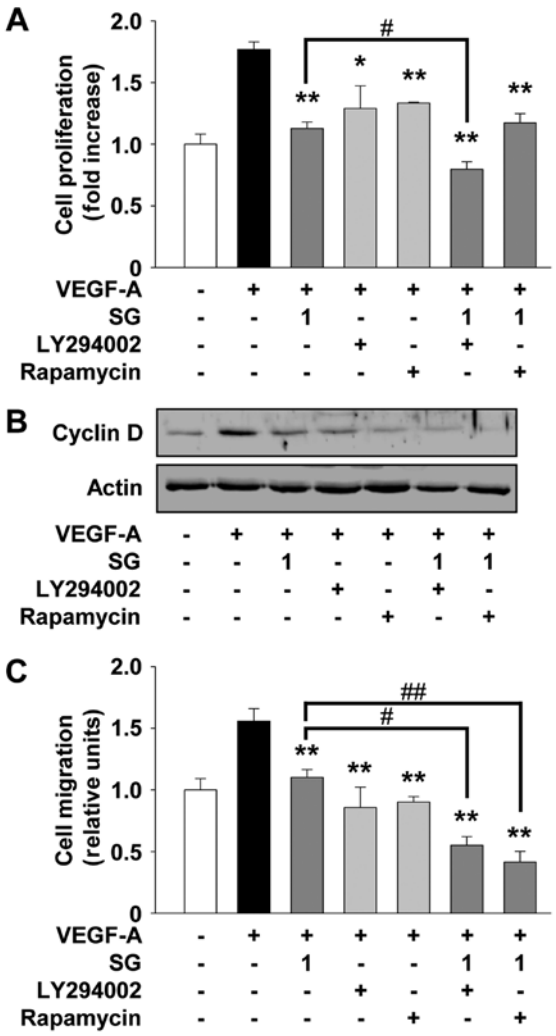

Figure 6. SG inhibits VEGF-A-stimulated cell proliferation and migration through the inactivation of Akt and $p70^{S6K}$. Quiescent cells were pretreated with SG (1 μg/ml) for 30 min in the presence or absence of LY294002 (10 μM) or rapamycin (50 nM), followed by VEGF-A (10 ng/ml) stimulation for 24 h (A and B) or 15 h (C). Results shown are representative of at least 3 independent experiments. Statistical significance is indicated (*P<0.05, **P<0.01, compared with VEGF-A-treated cells; $^{\#}$P<0.05, $^{\#\#}$P<0.01, compared with VEGF-A plus SG-treated cells).

responses, we examined the changes in the activation of VEGF-A/VEGFR-2 downstream signaling pathways including ERK, PI3-K/Akt and mTOR/$p70^{S6K}$, which play important roles in cellular fate (22). Compared with the unstimulated controls, VEGF-A treatment markedly increased the phosphorylation/activation of ERK, Akt and $p70^{S6K}$ in HUVECs (Fig. 5). However, SG treatment significantly inhibited VEGF-A-stimulated phosphorylation of Akt and $p70^{S6K}$, but not that of ERK. To directly examine the contribution of inactivation of Akt and $p70^{S6K}$ activity to the anti-angiogenic activity of SG, we studied the changes in cell proliferation and migration in the presence of LY294002 and rapamycin (Fig. 6). Pretreatment of cells with LY294002 or rapamycin mimicked the suppressive effect of SG on VEGF-A-stimulated cell proliferation and migration (Fig. 6A and C). As shown in Fig. 6B, both LY294002 and rapamycin also mimicked the SG-mediated suppression of cyclin D expression, with a high correlation with previous cell proliferation and cell cycle-related protein expression experiments (Fig. 1A and 2B). The addition of LY294002 significantly enhanced the ability of SG to inhibit cell proliferation and cyclin D expression as well as migration (Fig. 6). Rapamycin treatment enhanced the inhibitory effect of SG on cell migration, but not cell proliferation (Fig. 6A and C). Collectively, these observations suggest that SG may contain pharmacologically effective components similar to these inhibitors and share the roles and mechanisms of action in regulating angiogenic responses *in vitro*.

Discussion

Siegesbeckia glabrescens has been used as a traditional medicine for the treatment of several diseases including acute hepatitis, paralysis, hypertension, asthma and rheumatoid arthritis. These applications are well supported by previous investigations that SG possesses biologically active components to reduce allergic and inflammatory responses (8,9,23). In addition, SG has been reported to have anticancer activity against several different cell lines including breast cancer, ovarian cancer and non-small cell lung cancer (10-12). A recent study demonstrated that SG exerts anti-angiogenic and anti-adipogenic activities (13). However, the effects and molecular mechanisms of SG in angiogenesis have not yet been clearly identified. In the present study, we demonstrated for the first time that the ethanol extract of SG inhibited VEGF-A-stimulated endothelial cell proliferation, migration and capillary-like structure formation. These anti-angiogenic activities of SG in the VEGF-A-treated HUVECs were found to be mediated through the inactivation of VEGF-A/VEGFR-2 downstream signaling pathways such as Akt and $p70^{S6K}$.

Angiogenic stimulation by the VEGF-A/VEGFR-2 signaling pathways includes the secretion and activation of matrix metalloproteinases (MMPs), resulting in the degradation of the extracellular matrix and remodeling of the tissue microenvironment associated with cell growth, migration

and invasion (20,24-28). Based on the regulatory effects of SG on angiogenic responses *in vitro*, we examined the ability of SG to alter the levels of MMP-2 and tissue inhibitors of metalloproteinases-2 (TIMP-2), an endogenous inhibitor of MMPs, in VEGF-A-treated HUVECs (29-31). SG treatment showed little or no change of MMP-2 and TIMP-2 expression (data not shown), similar to previous studies in other types of cells (11,12). These findings indicate that the anti-angiogenic activity of SG may not require the regulation of MMP-2 and TIMP-2, however, it does not rule out the possibility that SG might modulate the expression and activity of other MMP and TIMP family members.

In conclusion, this study describes the pharmacological roles and mechanisms of SG in the regulation of angiogenesis. Further investigation of SG is warranted in regards to the prevention and treatment of a variety of diseases associated with angiogenesis.

Acknowledgements

This study was supported by the research fund of Dankook University in 2013.

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