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Antiangiogenic effect of licochalcone A

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ABSTRACT

To date, no antiangiogenic activity has been demonstrated for licochalcone A (LicA), a major phenolic constituent of *Glycyrrhiza inflata*, although it shows significant antitumor activity in human malignant cell lines. Our previous work demonstrated that LicA down-regulates inflammatory responses to lipopolysaccharide in murine macrophages. The purpose of the present study was to evaluate whether LicA inhibits angiogenesis, which is crucial for cancer development and progression. LicA significantly inhibited proliferation (20 μM), migration (5–20 μM), and tube formation (10–20 μM) of human umbilical vascular endothelial cells (HUVECs) as well as microvessel growth from rat aortic rings (10–20 μM). Furthermore, LicA significantly inhibited the growth of CT-26 colon cancer implants in BALB/c mice, with fewer CD31- and Ki-67-positive cells but more apoptotic cells. The underlying antiangiogenic mechanism of LicA correlated with down-regulation of vascular endothelial growth factor receptor (VEGFR)-2 activation. Our findings provide the first evidence that LicA inhibits angiogenesis *in vitro* and *in vivo*, perhaps by blocking VEGF/VEGFR-2 signaling. Inhibition of tumor growth may be attributed, at least in part, to decreased angiogenesis in LicA-treated mice. These findings emphasize the potential use of LicA against tumor development and progression in which angiogenesis is stimulated.

1. Introduction

Angiogenesis is the development of new blood vessels from pre-existing ones through a complex multistep progression that includes proliferation, migration, and tube formation of endothelial cells [1]. It plays an important role in physiologic and pathologic processes, such as embryonic development, wound healing, tumor growth, metastasis, and various inflammatory disorders [2]. Angiogenesis is crucial for tumor progression and sustains malignant cells with nutrients and oxygen. Inhibition of new blood vessel networks reduces tumor size and metastases [3]. Since angiogenesis is required for tumor development and tumor vasculature is a supreme target for anticancer strategies, anti-angiogenic compounds may act as cancer treatment agents or adjuncts to standard chemotherapeutic regimens [4–6].

One of the most important factors regulating angiogenesis is vascular endothelial growth factor (VEGF) and its endothelial tyrosine kinase receptors, VEGF receptors (VEGFR). VEGF transduces their signals to the nucleus principally through VEGFR-1, -2, and -3 [1,7,8]. Of these receptors, VEGFR-2 appears to play a critical role in the regulation of angiogenesis through signal transduction pathways that control proliferation, migration and tube formation of endothelial cells [8–10]. Phosphorylation of VEGFR-2 is necessary for the activation of AKT, which is vital for following activation of endothelial cell survival, migration as well as proliferation [9] and is also essential for the activation of cSrc, which regulates cell migration [10]. In contrast, VEGFR-1 is poorly autophosphorylated in response to VEGF in endothelial cells and also seems to participate in pathological angiogenesis [8]. VEGFR-3 is responsible for lymphangiogenesis [8]. Several angiogenesis inhibitors, including VEGFR-2 inhibitors, are currently being evaluated in phase I or phase II clinical trials for cancer therapy [11].

Bioactive compounds in traditional spices and herbs can protect and/or prevent cancer development by reducing angiogenesis. These bioactive compounds are generally safe and efficacious, given that they have been consumed by humans for centuries [12]. However, understanding their mechanisms of action as therapeutic modalities is a major challenge for contemporary science.

Licochalcone A (LicA; $C_{21}H_{22}O_4$, MW 338.4, Fig. 1) is the main active compound of the licorice species *Glycyrrhiza inflate*, and is an estrogenic flavonoid with antitumor and antiparasitic properties [13–16]. LicA reduces Bcl-2 protein expression and induces apoptosis in several human cancer cell lines [14]. It also interferes with the parasite mitochondrial electron transport chain and

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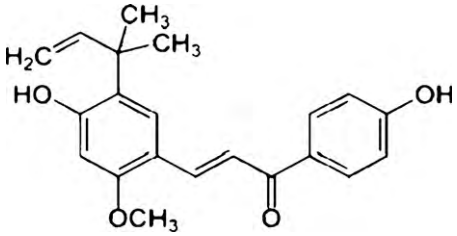

Fig. 1. Structure of LicA.

energy metabolism [15,16]. We previously demonstrated that LicA exerts anti-inflammatory effects by suppressing nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) signaling [17]. As inflammation is closely linked to tumor angiogenesis [18], we asked whether LicA inhibits angiogenesis, a central step in tumor growth and metastasis. Here we show that LicA inhibits the migration and tube formation of endothelial cells *in vitro* and angiogenesis *in vivo*.

2. Materials and methods

2.1. Reagents and cells

LicA was purchased from Calbiochem (San Diego, CA). 40 mM LicA was prepared in DMSO (Sigma–Aldrich, St. Louis, MO), stored at −20 °C, and then diluted as needed with cell culture medium for *in vitro* experiments or with PBS for animal experiments. Human umbilical vascular endothelial cells (HUVECs) were obtained from Lonza (Walkersville, MD) and cultured in EGM (Lonza). CT-26 colon cancer cells were obtained from the Korean Cell Bank (Seoul, Korea) and cultured in DMEM (Hyclone, Logan, UT) containing 10% FBS (Hyclone), 100 U/ml penicillin (Hyclone), and 100 μg/ml streptomycin (Hyclone) at 37 °C in a 5% CO_2 atmosphere. Recombinant human VEGF was obtained from eBioscience (San Diego, CA). Matrigel was ordered from BD Biosciences (San Jose, CA).

2.2. MTS-tetrazolium salt assay

The Promega CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI) was used to measure the cytotoxicity of LicA by determining the number of viable cells in HUVECs culture.

2.3. Migration assay

Migration of HUVECs was analyzed using a previously described method [19]. Briefly, HUVECs grown up to 90–95% confluence in gelatin (0.1%)-coated 6-well plates were incubated with 10 μg/ml mitomycin C (Sigma–Aldrich) at 37 °C for 2 h. The HUVECs were then wounded by scratching with a 0.1-ml pipette tip and fresh medium containing different concentrations of LicA was added. Migrated images were taken by AxioImager M1 microscope (Carl Zeiss, Gottingen, Germany) after 8 h of incubation at 37 °C.

2.4. Tube formation assay

Tube formation of HUVECs was measured as described [19]. Briefly, Matrigel was added to a 24-well plate and left to polymerize for 45 min at 37 °C. HUVECs (4×10^4 cells) were added in 1 ml EGM with LicA at 37 °C in a humidified 5% CO_2 atmosphere for 16 h. After incubation, the medium was removed and rhodamine-labeled phalloidin (Thermo SCIENTIFIC, Rockford, IL) was added to stain F-actin (Thermo SCIENTIFIC). Images of fluorescently labeled cells were collected with a ThermoScientific Cellomics ArrayScan High Content Screening Reader (Cellomics, Pittsburgh, PA) and analyzed by an automated algorithm that identified the tubes formed by the association and clustering of the endothelial cells [20].

2.5. Aortic ring assay

Rat aortic ring explant cultures were prepared by modification of protocols previously described [19]. 48-well plates were covered by 100 μl of Matrigel and left to polymerize for 45 min at 37 °C. Aortic rings were prepared from Sprague Dawley rats (KOATECH, Pyeongtek, Korea). Aortas were sectioned into 1–1.5 mm-long cross-sections, rinsed several times with PBS, placed on Matrigel in wells and covered with an additional 100 μl of Matrigel. The rings were cultured in 1.5 ml of EGM without serum for 24 h, and then the medium was replaced with 1.5 ml of EGM with vehicle or LicA (5, 10 and 20 μM). The medium was changed every 2 days. After 7 days of incubation, the microvessel growth was measured in photographs taken with an AxioImager ZI inverted microscope (Carl Zeiss) with a 4× objective lens.

2.6. Measurement of angiogenic growth factors

The amount of IL-6, IL-8, TGF-β and VEGF in cell-culture supernatant was measured using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN). HUVECs were plated in a 24-well cell culture plate at a density of 5×10^4 cells/well and incubated with vehicle or LicA for 24 h. The culture supernatant was collected and assayed according to the manufacturer's instructions. Each sample was assessed in triplicate.

2.7. In vivo murine tumorigenesis assay

All animal studies were approved by the Institutional Animal Care and Use Committee of Hallym University. Five-week-old BALB/c male mice (KOATECH) weighing 20 g were divided into groups (10 mice/group). CT-26 cells were subcutaneously injected (1×10^5 cells/mouse) into the mice. Starting the next day, vehicle or LicA at 5, 15, or 30 mg/kg were orally administered daily. Body weight and tumor volume were determined every 2 days by direct measurement with calipers (Mitutoyo Corporation, Japan). Tumor volume was determined by measuring two perpendicular diameters and using the formula [(smallest diameter^2 × largest) × 0.52]. Fifteen days after CT-26 cell injection, tumor tissues were harvested for further analysis.

2.8. Histology and immunohistochemistry

Tumors were removed 15 days after CT-26 cell injection and fixed with 4% paraformaldehyde for at least 24 h. Fixed tumors were embedded in paraffin, sectioned into 6-μm-thick sections, deparaffinized, and stained with hematoxylin and eosin. Apoptotic cells in the tumors were labeled by the terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick end-labeling (TUNEL) method using an In Situ Apoptosis Detection Kit (R&D Systems) according to the manufacturer's instructions. Proliferating cells and platelet endothelial cell adhesion molecule-positive cells were assessed by the immunoperoxidase technique using an anti-Ki-67 antibody (Lab Vision, Fremont, CA) or anti-CD31 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Images were taken with an AxioImager M1 microscope (Carl Zeiss) and quantified by counting the number of positively stained cells in 15 randomly chosen fields at 200× or 400× magnifications.

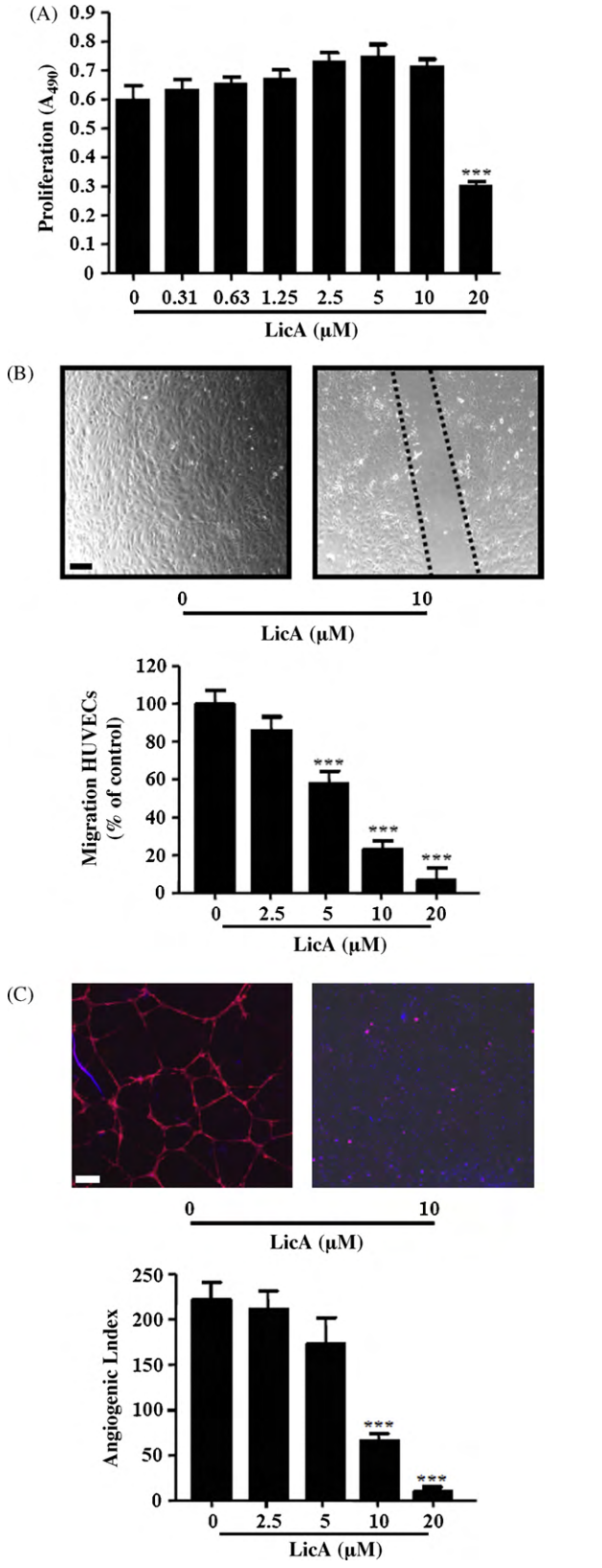

Fig. 2. Inhibition of endothelial cell proliferation, migration, and capillary-like tubule formation by LicA. (A) HUVECs were plated in 96-well plates, allowed to attach overnight, and then cultured for 24 h with vehicle or the indicated concentration of LicA. The proliferation was determined as described in the Materials and Methods section. (B) For cell migration, a monolayer of inactivated HUVECs was wounded by scratching with a 0.1-ml pipette tip, and fresh medium containing vehicle or LicA was added. After 24 h, migration of HUVECs was quantitated. Original magnification, 40×. Scale bar 200 μm. (C) For capillary-like tubule formation, HUVECs (4×10^4/well) were seeded onto Matrigel-coated 24-well plates and incubated with vehicle or LicA at 37 °C for 16 h. Endothelial tubules were photographed and quantitated. Original magnification, 40×. Scale bar 200 μm. The results are means ± S.E.M. of four independent experiments in triplicate. $^{**}P < 0.01$, $^{***}P < 0.001$ versus 0 μM LicA-treated cells.

2.9. Western blot analysis

HUVECs pretreated with 0–20 μM LicA for 60 min were treated with 10 ng/ml VEGF for 5 min. A 10 μg sample of each total cellular protein preparation was subjected to Western blotting with anti-VEGFR-2 (Cell Signaling Technology, Danvers, MA), anti-phospho-VEGFR-2 (Cell Signaling Technology), anti-cSrc (Cell Signaling Technology), anti-phospho-cSrc (Cell Signaling Technology), and anti-β-actin monoclonal antibody (Sigma). Immunoreactive proteins were detected using a chemiluminescence Western blotting detection system (ECL Plus™ Western Blotting Reagents, Amersham Biosciences, Boston, MA).

2.10. Measurement of VEGFR-2 tyrosine kinase

VEGFR-2 tyrosine kinase activity was investigated using the HTScan® VEGF Receptor 2 kinase assay kit (Cell Signaling Technology) according to the manufacturer's instructions.

2.11. Transfection of small interfering RNA into HUVECs

HUVECs were transfected with indicated concentrations of VEGFR-2 small interfering RNA (siRNA, ON-TARGET plus SMART-pool; Dharmacon, Inc., Chicago, IL) or nontargeted siRNA (ON-TARGET plus Non-targeting Pool siRNA, Dharmacon) using DharmaFECT-4 (Dharmacon) as described by the vendor. Inhibition of VEGFR-2 protein expression was verified by Western blot analysis.

2.12. Statistical analysis

The data are means ± S.E.M. of at least three independent experiments. The values were evaluated by one-way analysis of variance (ANOVA) with Bonferroni multiple comparison post-tests using GraphPad Prism 4.0 software (GraphPad Software Inc., San Diego, CA). P values < 0.05 were considered statistically significant.

3. Results

3.1. LicA inhibits migration and tube formation of endothelial cells

Sprouting angiogenesis includes successive phases of microvessel formation, neovessel growth, and neovessel stabilization [21]. These steps require the migration of endothelial cells from the parent vessel toward angiogenic growth factors, proliferation of endothelial cells behind the migration front, and the organization of endothelial cells into capillary-like structures. These multistep processes can be recapitulated with *in vitro* assays.

We first tested whether LicA treatment affected HUVECs proliferation. Lower doses of LicA (up to 10 μM) had no effect, but 20 μM LicA reduced cell proliferation (Fig. 2A). LicA also dose-dependently inhibited the migration of HUVECs in a wound healing migration assay (Fig. 2B), a model of the migration required for angiogenesis [22]. When HUVECs are plated on a basement membrane matrix (Matrigel) in a short-term culture, they align into networks of tubules (Fig. 2C), a process that is dependent upon proteolytic degradation of the matrix, cell realignment, and apoptosis; directed cell migration and proliferation, however, are not involved in this process [21]. LicA dose-dependently reduced HUVECs tubule formation (10 and 20 μM, Fig. 2C).

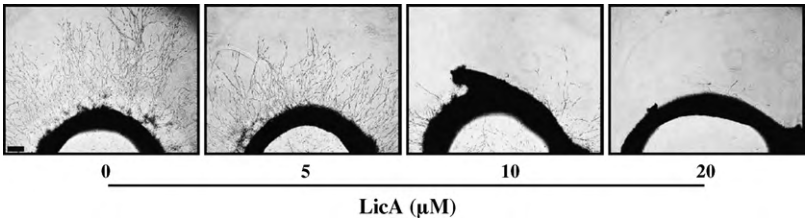

Fig. 3. Effect of LicA on microvessel outgrowth arising from rat aortic rings. Aortic rings isolated from SD rats were embedded in Matrigel in 48-well plates, and then fed with medium containing LicA for 7 days. Photographs are representative of three independent experiments. Original magnification, 40×. Scale bar 200 μm.

3.2. LicA inhibits angiogenesis in vitro

To examine the inhibitory effect of LicA on angiogenesis, we performed aortic ring assays using isolated rat aortas. The 1–1.5 mm-long aortic rings were put on Matrigel and covered with another Matrigel layer and endothelial cell growth medium (EGM) with or without LicA. After 7 days of incubation, the numbers of microvessels growing from the aortic rings were compared. 20 μM of LicA completely inhibited neovascular outgrowth (Fig. 3).

3.3. LicA suppresses the release of angiogenic growth factors

The angiogenic and metastatic potential of cancer cells is regulated by multiple angiogenic growth factors [23–25]. We therefore determined the levels of these molecules in medium from HUVECs cultured for 24 h in the presence of vehicle or LicA (Fig. 4). LicA decreased the levels of interleukin (IL)-6 (2.5–20 μM LicA) and IL-8 (5–20 μM LicA) in the medium, but did not affect TGF-β. Additionally, the spontaneous release of VEGF was not detected in the conditioned medium of HUVECs culture (Fig. 4).

3.4. LicA inhibits tumor angiogenesis and tumor growth in vivo

Tumor angiogenesis provides oxygen, nutrients, and routes for tumor growth and metastasis and acts as a rate-limiting step in tumor development and progression [26]. The *in vivo* antiangiogenic and antitumor activity of LicA was assessed with murine colon carcinoma CT-26 cells inoculated subcutaneously into syngeneic BALB/c mice. Since our previous data showed 15–30 mg/kg LicA exerted antitumor effect in colitis-associated colon cancer model [26], we examined the antiangiogenic effect with similar dosage of LicA. The CT-26-bearing mice were treated orally with LicA (vehicle, 5, 15 and 30 mg/kg) every day for 2 weeks and tumor growth was measured. LicA treatment did not change body weights (Fig. 5A), indicating minimal toxicity. At day 15 after tumor cell injection, the average tumor size of the control group was $899 \pm 59\ mm^3$, whereas that of the 30 mg/kg LicA group was $333.9 \pm 53.8\ mm^3$ (Fig. 5B), indicating that LicA significantly inhibited tumor growth.

To examine the effect of LicA on tumor angiogenesis, we stained the 6-μm tumor sections with the specific endothelial cell marker,

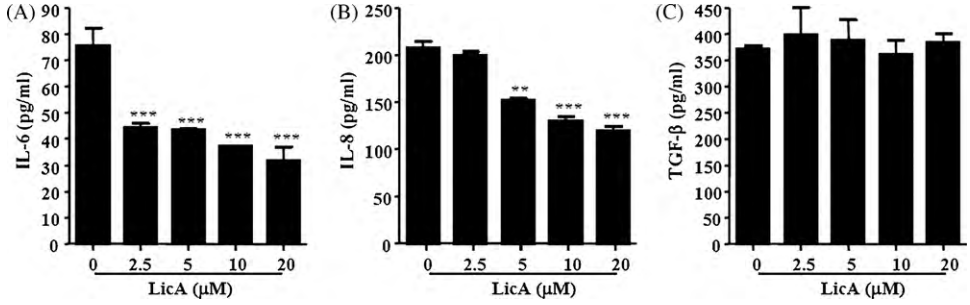

Fig. 4. Effect of LicA on the production of angiogenic growth factors in HUVECs. HUVECs were plated in a 24-well cell culture plate at a density of 5×10^4 cells and incubated with vehicle or LicA for 24 h. The levels of IL-6 (A), IL-8 (B) and TGF-β (C) were determined as described in Section 2. The results are means ± S.E.M. of four independent experiments in triplicate. $^{**}P < 0.01$, $^{***}P < 0.001$ versus 0 μM LicA-treated cells.

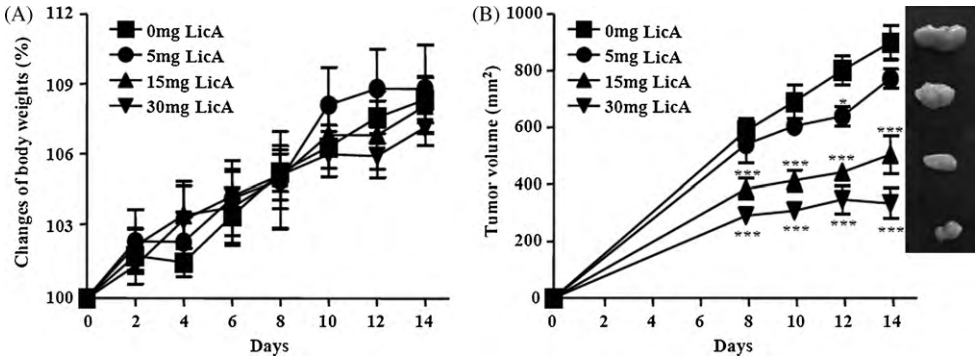

Fig. 5. LicA inhibits the growth of mouse tumors. CT-26 cells (1×10^5 cells/100 μL) were injected subcutaneously into the right flank, followed by oral treatment with vehicle or LicA. (A) Changes in body weight of mice treated with vehicle or LicA. (B) Tumor growth after LicA treatment. Tumor sizes were measured every 2 days with a caliper. Results are mean ± S.E.M. (10 mice/group). $^{*}P < 0.05$, $^{***}P < 0.001$ versus 0 μM LicA-treated animals.

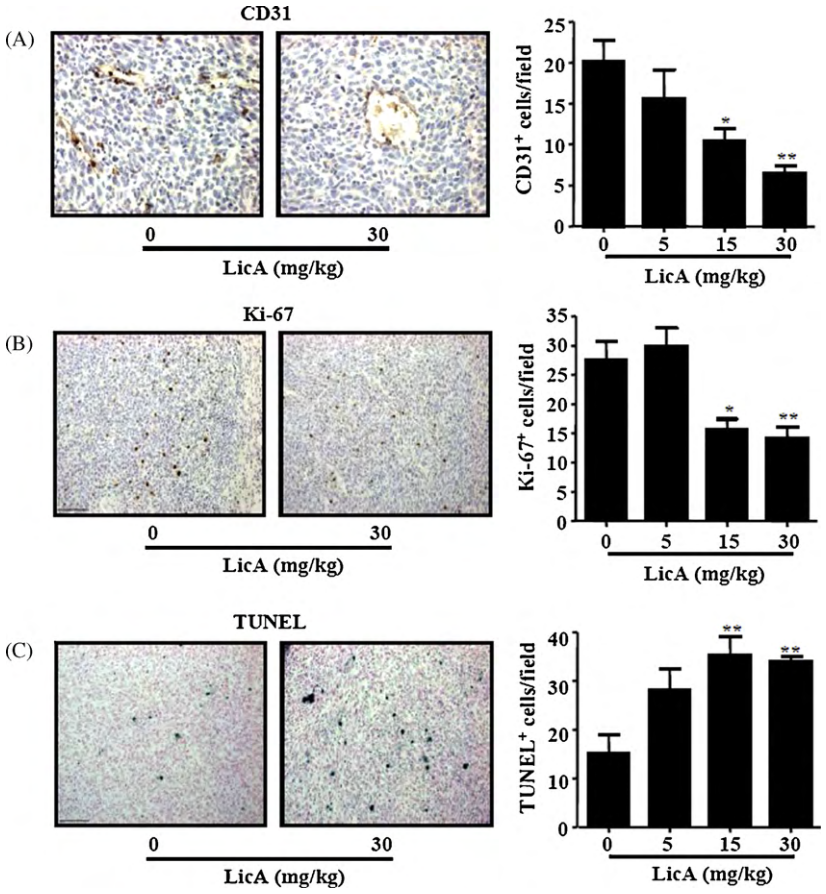

Fig. 6. *In vivo* mechanism of LicA in the mouse tumor model. Mice injected with CT-26 cells were randomly assigned and treated with vehicle or LicA. Tumors were collected 15 days after tumor implantation, fixed in formalin, and then analyzed by immunohistochemistry. Histologic examination of CT-26 tumor grafts stained with (A) anti-CD31 vessel staining, original magnification, 400×. (B) anti-Ki-67 nuclear antigen, original magnification, 200×. (C) Apoptosis by TUNEL assay, original magnification, 200×. Scale bar 100 μm. Quantitation of CD31 vessel staining, Ki-67 staining, and TUNEL staining from immunohistochemical analysis are shown on right panels. Results are mean ± S.E.M. of 15 sections per group. $^{*}P < 0.05$, $^{**}P < 0.01$ versus 0 μM LicA-treated animals.

anti-CD31. Vehicle-treated mice showed 20.1 ± 2.6 CD31-immunoreactive cells per field, whereas LicA-treated mice showed 7.3 ± 1.3 per field (30 mg/kg; Fig. 6A), indicating that LicA significantly inhibited tumor angiogenesis and thereby prevented tumor growth.

We next tested whether LicA causes histological changes in tumor tissue by measuring proliferation and apoptosis using Ki-67 and TUNEL staining, respectively. In similar fields of view, LicA mice showed fewer proliferative cells and more apoptotic cells than control mice (Fig. 6B and C). Collectively, these data suggest that LicA (15 and 30 mg/kg) inhibits tumor angiogenesis, and subsequently promotes apoptosis and reduces tumor progression.

3.5. LicA inhibits VEGFR-2 signaling

VEGFR-2 is the primary receptor for VEGF that mediates angiogenic activity, endothelial cell proliferation, migration, differentiation, and tube formation [7–10]. We therefore tested whether LicA interacted with the VEGF/VEGFR-2 signaling pathway. VEGFR-2 was phosphorylated by exogenous VEGF in HUVECs (Fig. 7A), and LicA blocked this phosphorylation. LicA also dose-dependently decreased the VEGF-stimulated phosphorylation of cSrc, a downstream signaling molecule, reaching almost complete inhibition at 10 μM. The total steady state levels of VEGFR-2 and cSrc proteins remained unchanged, indicating that LicA specifically interferes with receptor activation.

To verify the inhibitory effect of LicA on VEGFR-2 tyrosine kinase activity, we examined the effects of various concentrations of LicA on the specific activation of VEGFR-2 using the HTScan® VEGFR-2 kinase assay kit (Cell Signaling Technology). LicA inhibited VEGFR-2 kinase activity with an IC_{50} of 13.3 μM (Fig. 7B), indicating that LicA is a potent VEGFR-2 inhibitor.

3.6. VEGFR-2 signaling is necessary for the inhibition of angiogenesis by LicA

To directly assess the functional role of VEGFR-2 in LicA-induced inhibition of angiogenesis, VEGFR-2 expression was inhibited by introducing short interfering RNA (siRNA) into HUVECs. As a control, a nontargeted siRNA that targets a nonhuman mRNA sequence was introduced into HUVECs as well. As shown in Fig. 8A, almost complete inhibition of VEGFR-2 protein expression was shown at 48 h after transfection (Fig. 8A). A concentration of 25 nM siRNA was chosen for subsequent experiments because this concentration could significantly inhibit VEGFR-2 protein expression, whereas the nontargeted siRNA had no effect. LicA inhibited endothelial cell proliferation, migration and tube formation in cells transfected with nontargeted siRNA (Fig. 8B–D). In contrast, LicA treatment did not suppress the proliferation, migration and tube formation of HUVECs in which VEGFR-2 had been depleted by VEGFR-2 siRNA (Fig. 8B–D). These data indicate that VEGFR-2 is necessary for LicA-induced inhibition of HUVEC proliferation, migration as well as tube formation.

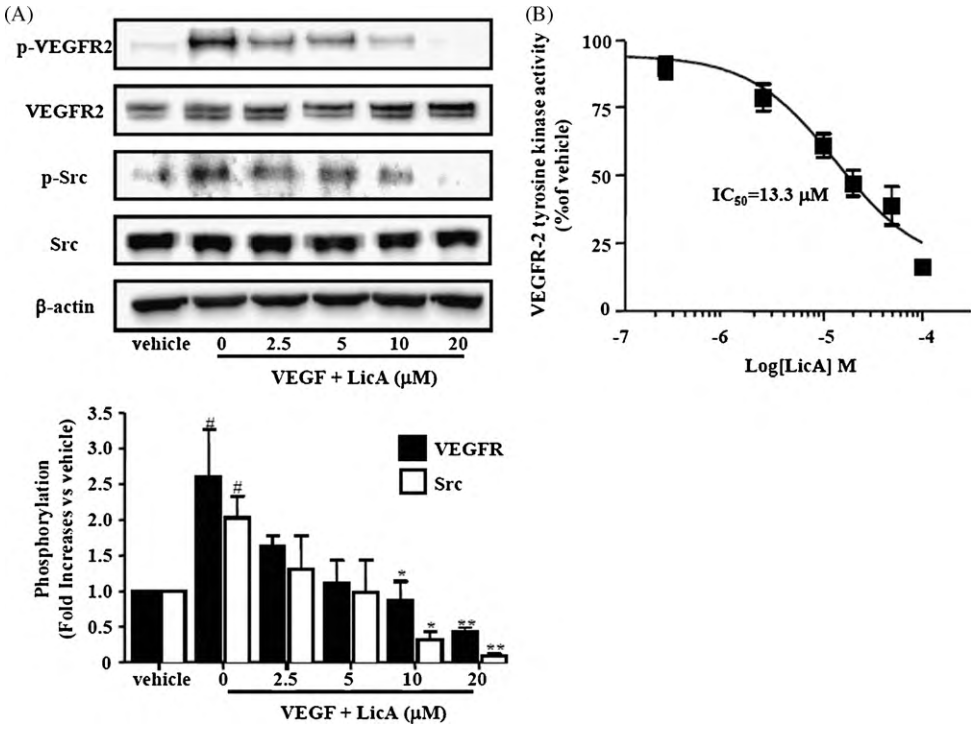

Fig. 7. Inhibition of VEGFR-2 signaling by LicA. (A) HUVECs were incubated with VEGF, for 5 min, with LicA. Total cell lysates were prepared, proteins were separated by SDS–PAGE, and Western blot analysis was performed. Photographs of chemiluminescent detection of the blots, representative of 4 independent experiments, are shown. Band intensity was quantitated by densitometry and each bar represents the mean ± S.E.M. ($n = 4$). $^{*}P < 0.05$, $^{**}P < 0.01$ versus VEGF-treated cells. $^{\#}P < 0.01$ versus vehicle alone. (B) Inhibition of VEGFR-2 activation by LicA in a specific VEGFR-2 inhibition assay. The results are reported as mean ± S.E.M. of triplicate assays.

4. Discussion

Angiogenesis is one of the hallmarks of cancer, playing a fundamental role in tumor growth, invasion, and metastasis [1–3]. Persistent upregulated angiogenesis is a common feature in many pathological conditions, including chronic inflammation, diabetic retinopathy, rheumatoid arthritis, and atherosclerosis [22,27]. Thus, understanding the central importance of angiogenesis and how new blood vessels are formed has led to novel therapies designed to interrupt this process [3–6,28,29]. As part of our search

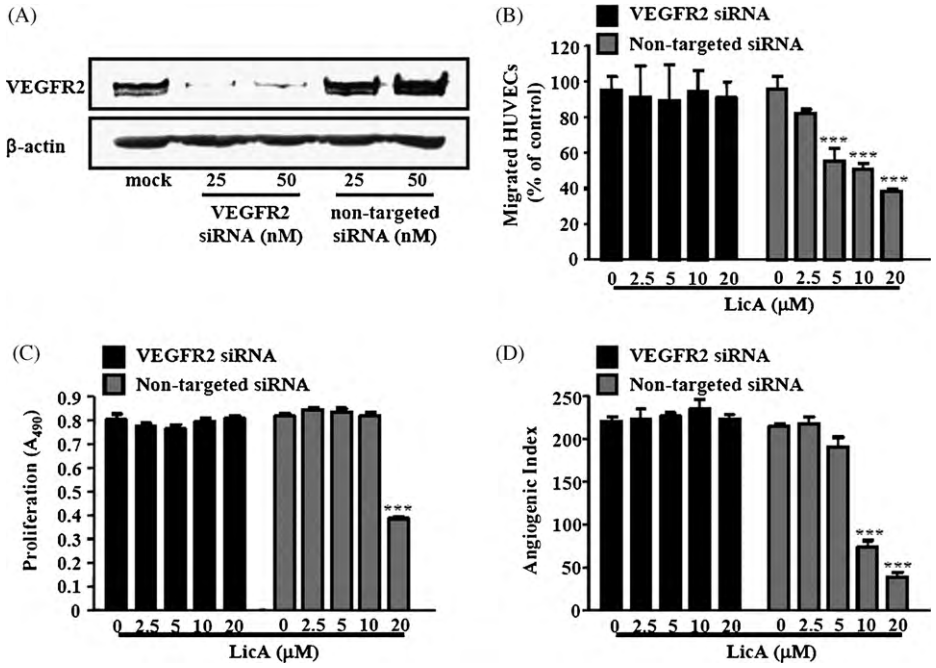

Fig. 8. Requirement of VEGFR-2 for LicA-induced inhibition of angiogenesis. HUVECs were transfected with indicated concentrations of VEGFR-2 siRNA or nontargeted siRNA. (A) Inhibition of VEGFR-2 protein expression was verified by Western blot analysis, (B) endothelial cell proliferation, (C) migration and (D) capillary-like tubule formation were measured as described in Fig. 2. The results are means ± S.E.M. of three independent experiments in triplicate. $^{***}P < 0.001$ versus 0 μM LicA-treated cells.

for natural product-based antiangiogenic agents, we first studied the effects of LicA obtained from licorice on the angiogenic course, namely cell proliferation, migration, and capillary-like structure formation of HUVECs. Our *in vitro* studies using HUVECs demonstrated that LicA inhibited the angiogenesis (Fig. 2). In addition, a similar phenomenon was observed in the rat aortic ring assay (Fig. 3), suggesting that LicA has potent antiangiogenic effects on endothelial cells. To the best of our knowledge, this is the first study demonstrating that LicA inhibits angiogenesis.

IL-6 and IL-8 exert essential roles in angiogenic processes, including proliferation, migration, invasion, and tube formation [30–32]. Huang et al. demonstrated that IL-6 effectively promoted HUVEC proliferation and tube formation *in vitro* and Matrigel plug vascularization *in vivo* primarily by inducing VEGF in gastric carcinoma [30]. IL-8 functions as an important autocrine growth and angiogenic factor in regulating biological activities in endothelial cells [31]. Recombinant human IL-8 induces endothelial cell proliferation and capillary tube organization, while neutralization of IL-8 blocks IL-8-mediated capillary tube organization [32]. Here, spontaneous secretion of IL-6 and IL-8 in HUVECs was blocked by LicA treatment (Fig. 4), leading to suppressed angiogenesis.

Based on acute toxicity test (Supplementary Tables 1 and 2), oral administration of LicA (2000 mg/kg body weight) had no toxic effect and no death occurred, thus lethal dose of LicA for BALB/c mice is higher than 2000 mg/kg. In addition, pharmacokinetic study by using a high-performance liquid chromatographic method demonstrated that following oral administration of 30 mg LicA and i.p. administration of 10 mg LicA, the peak concentration was 200 μg/ml after 15 min (Supplementary Fig. 1) and 13 μg/ml after 5 min [33], respectively. Based on this information, we postulate that LicA might influence various biological functions including angiogenesis *in vivo* upon oral administration.

Using a nontoxic dose of LicA (15–30 mg/kg), we showed that LicA inhibited tumor growth as well as angiogenesis in a mouse model (Figs. 5 and 6). Interestingly, LicA also suppressed proliferation and induced apoptosis of inoculated CT-26 cancer cells in mice (Fig. 6B and C). Several *in vitro* studies in cancer cell lines demonstrated that LicA inhibited cell cycle progression and induced apoptosis [14,34,35]. Thus, LicA may induce cell cycle arrest and apoptosis to decrease tumor growth. It remains to be determined whether cell cycle arrest and apoptosis are direct effects of LicA or the results of decreased angiogenesis.

VEGF and its receptors regulate vessel formation in both physiological and pathological processes such as tumor angiogenesis [7–10]. VEGF primarily uses VEGFR-2 to induce angiogenic responses by activating a variety of signaling cascades [7,8]. Given the critical role of VEGFR-2 signaling in angiogenesis, regulation of VEGFR-2 activity/activation may represent an important mechanism for the control of angiogenesis. Inhibition of VEGFR-2 activity/activation by LicA might be a potential strategy to regulate angiogenesis for the prevention and/or treatment of cancer. Therefore, we observed the effects of LicA on VEGFR-2 expression and VEGFR-2 phosphorylation in HUVECs. We found that LicA suppressed VEGF-induced VEGFR-2 phosphorylation and a downstream VEGFR-2 signaling pathway (cSrc) in HUVECs. In addition, LicA suppressed the VEGFR-2 tyrosine kinase activity; therefore, the inhibition of VEGFR-2 phosphorylation by LicA could be explained by the inhibition of VEGR-2 tyrosine kinase. Additionally, targeted knockdown of VEGFR-2 by siRNA demonstrated that VEGFR-2 is essential for LicA-mediated inhibition of proliferation, migration as well as network formation on Matrigel of HUVECs (Fig. 8).

We previously showed that LicA strongly inhibits the production of inflammation-associated mediators by regulating NF-κB and AP-1 signaling in murine macrophages [17]. Also, in a recent study by Funakoshi et al. [36], LicA strongly inhibited tumor necrosis factor (TNF)-α-induced nuclear localization, DNA binding activity, and the transcriptional activity of NF-κB. Moreover, the NF-κB-regulated mediators, IL-6 and IL-8, were reduced by LicA treatment in HUVECs in the present study (Fig. 4). NF-κB is a transcription factor that controls the expression of a variety of angiogenesis-related molecules [37] and mediates endothelial cell tube formation [38]. According to Mohan et al. [39], NF-κB-specific inhibitors inhibit endothelial cell proliferation as well as NF-κB DNA binding, exerting anti-endothelial cell sprouting activity. Based on these observations, LicA probably inactivates NF-κB to reduce angiogenesis in HUVECs.

Although the relationship between anti-inflammatory and antiangiogenic activities is not fully understood, several anti-inflammatory agents such as epigallocatechin-3-gallate in green tea exert antiangiogenic effects [18,38,40] via obstructing VEGF signaling pathway [41–43]. Here, we showed that LicA, an anti-inflammatory agent [17], inhibited angiogenesis *in vitro* and *in vivo* by suppressing VEGFR-2 signaling, suggesting that it may have chemopreventive and/or chemotherapeutic potential.

In conclusion, the present study shows that LicA inhibits VEGFR-2 signaling, leading to the inhibition of angiogenesis and tumorigenesis. This is the first study to show that LicA inhibits HUVEC migration and tube formation, as well as its antiangiogenic activity *in vitro* and *in vivo*. Our data suggest a new mechanism of action for LicA and its potential as an antiangiogenic and anticancer agent.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2010.07.006.

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