Bee venom inhibits tumor angiogenesis and metastasis by inhibiting tyrosine phosphorylation of VEGFR-2 in LLC-tumor-bearing mice

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ABSTRACT

Bee venom (BV) treatment is the therapeutic application of honeybee venom (HBV) for treating various diseases in Oriental medicine. In the present work, the authors investigated the functional specificity of BV as an angiogenesis inhibitor using *in vitro* models and *in vivo* mouse angiogenesis and lung metastasis models. BV significantly inhibited the viability of Lewis lung carcinoma (LLC) cells but did not affect peripheral blood mononuclear lymphocytes (PBML) cells. BV also inhibited vascular endothelial growth factor (VEGF)-induced proliferation, migration and capillary-like tube formation of human umbilical vein endothelial cells (HUVECs). Western blotting analysis showed that BV inhibited AKT and MAPK phosphorylation in LLC cells and HUVECs and down regulated expression of VEGF and VEGFR-2 of LLC cells and HUVECs. Also, BV effectively disrupted VEGF-induced neovascularization in Matrigel plugs in our *in vivo* angiogenesis assay. When given subcutaneously, BV also significantly suppressed tumor angiogenesis through inhibition of VEGF and VEGFR-2 in LLC model. Mice bearing subcutaneous LLC tumors were treated with 1 μg/ml or 10 μg/ml of BV. They showed reductions ranging between 49% and 62% in primary tumor volume and reduction of spontaneous pulmonary metastasis occurrences. Furthermore, BV treatment in the spontaneous lung metastases model after primary tumor excision prolonged their median survival time from 27 to 58 days. These results suggest that the tumor-specific anti-angiogenic activity of BV takes effect during different stages of tumor progression by blocking the tyrosine phosphorylation of VEGFR-2, and validate the application of BV in lung cancer treatment.

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1. Introduction

Angiogenesis is a major feature of tumor growth and metastasis. As such, targeting tumor neovascularization is an attractive strategy for cancer therapy. A number of angiogenesis inhibitors including endostatin and angiostatin have been identified and tested in preclinical models, and several inhibitors have also been tested in clinical trials [1,2]. Furthermore, angiogenesis plays an important role in lung cancer and increasing numbers of anti-angiogenesis agents are being investigated in all types of pulmonary malignancies. Targeting angiogenesis is an exciting and attractive area in the treatment of lung cancer, and the results of ongoing trials are eagerly awaited [3–5]. Although existing anti-angiogenesis therapies show lower toxicity than conventional treatments such as radiotherapy, they are often associated with limited tumor regression and other clinical side effects [6]. Therefore, much effort has been focused on identifying novel angiogenesis inhibitors and evaluating new approaches to maximize the effects of anti-angiogenesis therapies [7].

Bee venom treatment (BVT) is the therapeutic application of honeybee venom (HBV) to the treatment of various diseases. BVT has been used in Oriental medicine to treat a variety of conditions, such as arthritis, rheumatism, back pain, cancerous tumors, and skin diseases [8,9]. BV contains at least 18 active components, including melittin, apamin, adolapin, the mast-cell-degranulating (MCD) peptide, enzymes (i.e., phospholipase [PL] A_2), biologically active amines (i.e., histamine and epinephrine), and non-peptide components which have a variety of pharmaceutical properties [10]. In Oriental medicine, the use of BV combined with forms of acupuncture is increasing in practice and is used primarily for pain relief in inflammatory diseases [11,12]. Direct injection of BV into acupoint ST36 (known as Zusanli) in an animal model of chronic arthritis produced a potent anti-nociceptive effect compared with injection at a non-acupoint, which suggests that this alternative form of acupoint stimulation for pain relief using BV has validity [13,14]. Furthermore, subcutaneous injections of BV and its fractions did not have any significant side effects on the general physiological functions of the central nerves, cardiovascular respiratory and gastrointestinal functions at the highest dose tested (200 times and 100 times higher doses than that used clinically, respectively) in rodent models [15]. BV has also been reported to induce apoptosis in cancer cells both in vitro and in vivo. The induction of apoptotic cell death through several cancer cell death mechanisms, including the activation of caspase and matrix metalloproteinases (MMP), is important in the BV anti-cancer mechanism [16–19]. In preclinical models, agents that target the tumor vasculature have been shown to prevent or delay tumor growth and even to promote tumor regression or dormancy. Therefore, in the present work, the authors evaluated the anti-angiogenic and anti-tumor activities of BV in both in vitro and in vivo, and investigated the effectiveness of BV in prevention and treatment of cancer.

2. Materials and methods

2.1. Reagents and animals

BV (Apis mellifera L.) purchased from You-Miel company (Hwasoon, South Korea). The composition of the BV was as follows: 50–55% melittin, 2.5–3% apamin, 2–3% MCD peptide, 12% PLA_2, 1% lyso-PLA, 0.5–1% histidine, 4–5% 6 pp lipids, 0.5% secarpin, 0.1% tertiapin, 0.1% procamine, 1.5–2% hyaluronidase, 2% amine and 10–17% other, including protease inhibitor, glucosidase, invertase, acid phosphomonoesterase, dopamine, nonepinephrine, with >99.5% purity. Anti-VEGF polyclonal rabbit antibody was purchased from Santa Cruz Biotechnology (Santa Cruze, CA, USA); anti-VEGFR-1 monoclonal mouse monoclonal antibody, anti-VEGFR-2 monoclonal mouse antibody were purchased from R&D system (Minneapolis, MN, USA); anti-p44/42 MAP kinase monoclonal rabbit antibody, anti-phospho-p44/42 MAP kinase monoclonal rabbit antibody, anti-AKT monoclonal rabbit antibody and anti-phospho-AKT monoclonal rabbit antibody were purchased from Cell Signaling Laboratories (Beverly, MA, USA); anti-PCNA

monoclonal mouse antibody and Factor VIII related antigen monoclonal mouse antibody were obtained from BD Pharmingen (La Jolla, CA, USA); C57BL/6JmsSlc mice were obtained from Jackson Laboratory (SLC, Shizoka, Japan). This study was conducted according to the "Guiding Principles for the Care and Use of Laboratory Animals" and all procedures were approved by the ethics Committee of KyungHee University Medical Center.

2.2. Cells

Lewis lung carcinoma (LLC) cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Human peripheral blood mononuclear lymphocytic (PBML) cells were obtained by Ficoll-hypaque technique from the blood of donors. Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical vein using type II collagenase [20].

2.3. Cell viability assay

Cells were plated in 96-well plates, and subconfluent cells were subsequently treated with BV (0.2–20 μg/ml) or vehicle (saline) for 48 h. After treatment, cell viability was measured by the Cell Counting Kit-8 (CCK-8) system according to the manufacturer's instructions. Briefly, the CCK-8 solution (10 μl per 100 μl of medium in each well) was added, the plates were incubated at 37 °C for 1 h, and the absorbance of each well was read at 450 nm using a microplate reader.

2.4. Human umbilical vein endothelial cell (HUVECs) culture

The cells were cultured in M199 supplemented with 20% fetal bovine serum (FBS), 3 ng/ml bFGF, 5 units/ml heparin and 100 units/ml antibiotic–antimycotic (complete culture medium) in 0.1% gelatin coated flasks. Cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2.

2.5. Cell proliferation assays

HUVECs were grown through passage 7 in M199 complete culture medium. For proliferation assays, cells were trypsinized, washed, suspended in culture medium (DMEM containing 10% heat inactivated fetal bovine serum and 5 μg/ml gentamycin), plated in triplicate onto 96-well plates, and incubated in a humidified incubator for 24 h. After being starved for 6 h in M199 containing 5% heat-inactivated FBS, the cells were exposed to various concentrations of BV (0.2–20 μg/ml) in the presence or absence of VEGF (20 ng/ml) incubated for 48 h at 37 °C. Then, 10 μl of BrdU was added to each well, and the cells were further incubated for 6 h at 37 °C. The cells were fixed and incubated with anti-BrdU, and then the substrate reaction was measured. The reaction was stopped by addition of 25 μl of 1 M H_2SO_4 and the absorbance was measured using a microplate reader (Molecular Devices Co., Sunnyvale, USA) at 450 nm with 690 nm correction.

2.6. Chemotactic migration assay

Chemotaxis assay was carried out in 48 well Boyden chambers. Briefly, the chamber was divided into two compartments by a polyvinyl-pyrrolidone-free filter, 25×80 mm, and 12 μm pore size. The polycarbonate filter was coated with 0.2% gelatin. The lower compartment of the chamber was filled with 30 μl of basal medium (0.2% BSA containing M199) in the presence of BV (1 μg/ml or 10 μg/ml) or VEGF (20 ng/ml). Then 3×10^5 cells suspended with control medium were loaded on the upper compartment of the chamber. After 4 h at 37 °C in 5% CO_2, the filter was fixed and stained with Diff Quick solution (hematoxylin and eosin). Non-migrating cells on the upper surface of the filter were removed by wiping with a cotton swab, and chemotaxis was quantified by counting the number of cells that migrated to the lower side of the filter using optical microscopes (100×, Nikon, Tokyo, Japan). Ten fields were counted for each assay. Each assay was conducted with three subject wells and similar results were reproduced atleast four times.

2.7. Capillary-like tube formation assay

Growth factor-reduced Matrigel was added to 24-well plates with a total volume 250 μl in each well and allowed to polymerize for 30 min at 37 °C. HUVECs, 1×10^5 cells/well, in a final volume of 500 μl M199 basal medium treated with different concentrations of BV (1 μg/ml or 10 μg/ml) or VEGF (20 ng/ml) were plated on the Matrigel. After 18 h of incubation in 5% CO_2 at 37 °C, the area covered by the tube network was determined using an optical imaging technique in which pictures of the tubes were quantified using the I-solution program (IMT I-solution Inc., USA).

2.8. Western blot analysis

Cells were lysed with protein lysis buffer. Protein concentration was determined by the Bradford protein assay. The proteins (15 μg/lane) were size-fractionated by 10% SDS–polyacrylamide gel electrophoresis under reducing conditions, and transferred onto Hybond-C nitrocellulose membranes. The membranes were blocked for 2 h in 5% dried milk in TBST at room temperature and proofed overnight at 4 °C with rotation in primary antibody. Blots were washed for 15 min with TBST and incubated with a horseradish peroxidase-conjugated species-specific antibody diluted in blocking buffer for 1 h at room temperature with rotation. After additional washes, blots were developed with enhanced chemiluminescent substrate and exposure to Kodak X-OMAT auto radiographic film.

2.9. Mouse Matrigel plug angiogenesis assay

The Matrigel assay was performed as described in a previous study [21]. Briefly, 6-week-old C57BL/6JmsSlc mice were subcutaneously injected with 0.5 ml of growth factor-reduced Matrigel containing BV (1 μg or 10 μg) or VEGF (100 ng) and heparin (5 U). After 7 days, mice were sacrificed, and the Matrigel plugs were removed, fixed in 10% neutral buffered formalin solution and embedded in paraffin. Tissues were sectioned (5 μ thickness) and slides were stained by hematoxylin–eosin (H&E) staining. To quantify the formation of functional blood vessels, the amount of hemoglobin (Hb) was measured using the Drabkin reagent Kit 525 (Sigma, St. Louis, MO, USA).

2.10. LLC tumor-induced angiogenesis model

A highly metastatic subline of LLC (hm-LLC) was generated by three rounds of serial in vivo passage and recovered from spontaneous lung metastasis after subcutaneous inoculation of LLC cells. After in vivo expansion to sufficient numbers, cells were frozen until use for in vivo implantation. hm-LLC cells (5×10^5/50 μl) were intradermally inoculated on the backs of 7-week-old male C57BL/6JmsSlc mice. Three days later, BV (0.01, 0.1 or 1 mg/kg) was subcutaneously (s.c.) administered for four consecutive days. Seven days after tumor inoculation, the mice were sacrificed and the tumor-inoculated skin was separated from the underlying tissues. Tumor-induced angiogenesis was quantified by counting the newly formed blood vessels around LLC cells under a dissecting microscope. The tumors were snap frozen for RNA extraction or fixed in 4% paraformaldehyde for histologic and immunohistochemical analysis.

2.11. LLC spontaneous pulmonary tumor growth model

Seven-week-old male mice were anesthetized and received subcutaneous injection with hm-LLC cells (1×10^6 cells/100 μl). Starting 3 days after tumor inoculation, the mice were given s.c. injections of BV (0.01, 0.1, or 1 mg/kg) above the thoracic vertebra every other day. Tumor volume was estimated (in mm^3) twice weekly as the product of two-dimensional caliper measurements and calculated using this formula: ($0.52 \times L$ (longest perpendicular length) $\times W^2$ (width)) [22,23]. All mice were sacrificed and samples were harvested 21 days after tumor implantation. Lung samples were collected and fixed in Bouin's solution, and tumor nodules on the lung were counted under a dissecting microscope.

2.12. LLC spontaneous pulmonary tumor metastasis model

hm-LLC cells (5×10^6/100 μl) were injected s.c. into 7-week-old male C57BL/6JmsSlc mice. Primary ectopic tumors reached a diameter of 5 mm at 1 week. Animals were anesthetized, and the subcutaneous tumors were surgery excised along with surrounding skin tissue. After wound closure, animals were randomized into three groups, and s.c. injection of BV (0.01 or 0.1 mg/kg) was applied above the thoracic vertebra every other day. Animals were humanely euthanized when they showed inability to reach food or water, symptoms of emaciation, or a 15% decrease from normal body weight. Six mice from each group were sacrificed 21 days after surgery, and lung tissue were harvested for histologic examination. The rest of the mice were sacri-

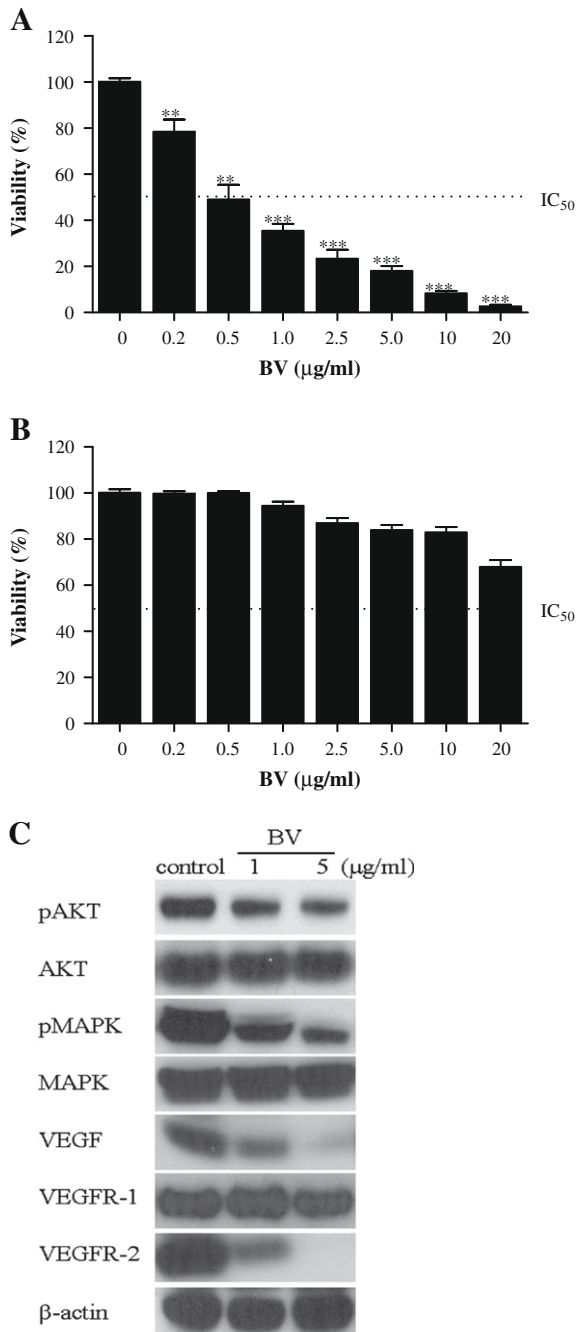ficed at the end of the experiment (10-week post-treatment), and their lungs were examined.

2.13. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA extraction was performed according to the manufacturer's recommendations for use of TRIzol® (invitrogen). Final RNA concentrations were determined by OD at 260 nm, and integrity was verified by ethidium bromide staining of ribosomal 18 S and 28 S bands on an agarose gel. Total RNA (1 μg) was reverse transcribed into single-strand cDNA using superscript II reverse transcriptase according to the manufacturer's instructions. Amplification of VEGF, VEGFR-2(Flk-1/KDR), VEGFR-1(Flt-1) and GAPDH in each reaction was carried out by PCR with the following primers and protocol: to avoid amplification of contaminating genomic DNA, one of the two primers for Flk-1/KDR was chosen at the junction between two exons (10, accession number AF063658AF063658). The primer se-

Fig. 1. Bee venom inhibits cell viability of LLC cells and angiogenesis signaling pathway. (A and B) Cells were treated with BV at concentrations from 0 to 20 μg/ml for 24 h. Cell viability of BV on LLC cells (A) and peripheral blood mononuclear lymphocytes (B) was determined by the CCK-8 assay. (C) Western blot analysis using β-actin to normalize samples showed reduced phosphorylation of AKT and p42/44 MAPK and protein expression of VEGF and VEGFR-2 in BV-treated LLC cells compared to control-treated cells. Bars, SEM; $n = 8$; $**p < 0.01$ and $***p < 0.001$ versus control.
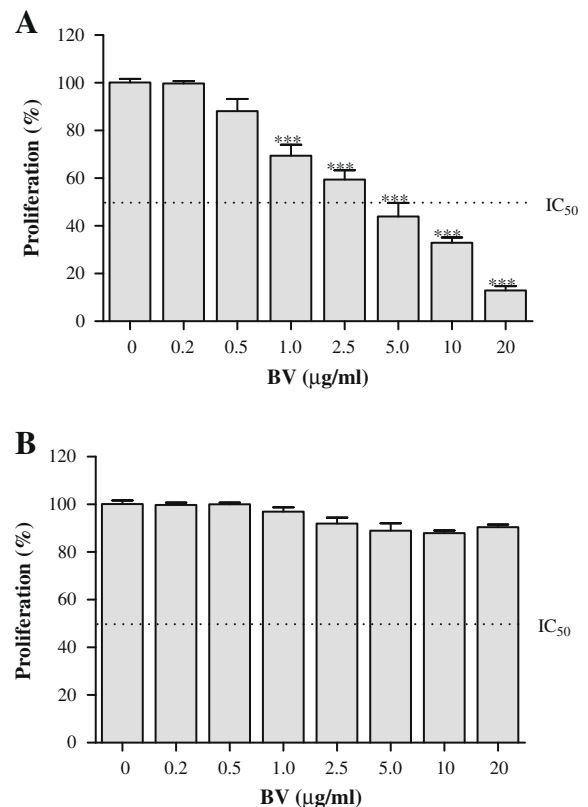

Fig. 2. Bee venom inhibits VEGF-induced proliferation, migration and tube formation of HUVECs via modulating the growth, survival, and angiogenesis signaling pathways. (A and B) HUVECs were treated with various concentrations of BV in the presence (A) or absence (B) of VEGF (20 ng/ml) for 48 h. Proliferation assay was performed using BrdU incorporation method. (C) The migrated cells were stained and counted as described in Section 2. (D) The tube formation assay showed interference of network assembly of HUVECs on Matrigel treated with BV in the presence of VEGF. (E) Western blot analysis using β-actin to normalize samples showed reduced phosphorylation of AKT and p42/44 MAPK and expression of VEGF and VEGFR-2 of BV-treated HUVECs compared to control cells. Values represent means ±SEM, $n = 8$; $###p < 0.001$ versus VEGF untreated control; $*p < 0.05$ and $***p < 0.001$ versus VEGF treated control.

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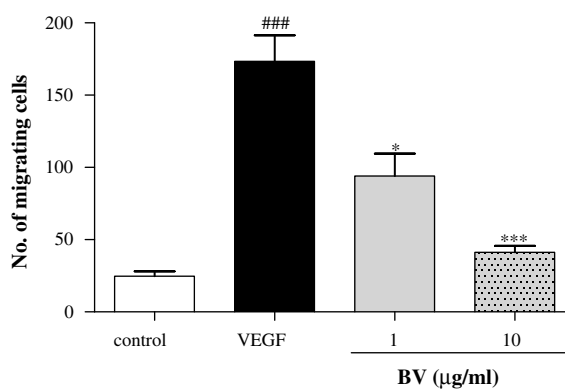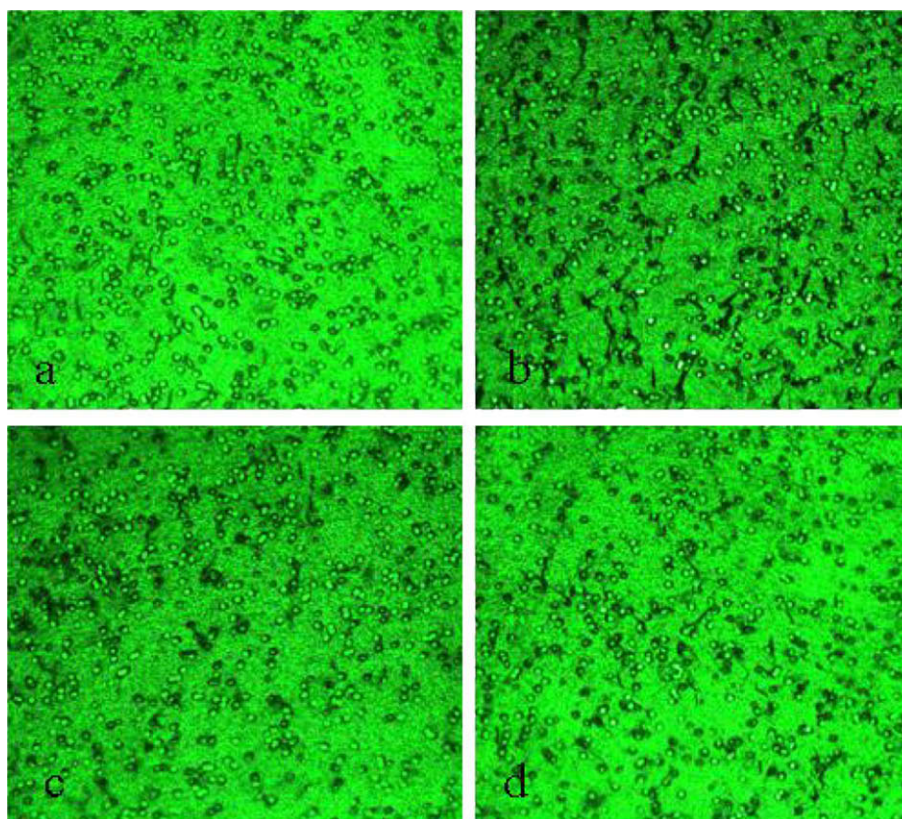
Fig. 2 (*continued*)

quences for Flk-1/KDR were as follows: forward 5′-TCTCAATGTGGTCAACCTTCTAGG-3′; reverse 5′-TTTAAACG-TCTTAAGGGTGTTAGTGG-3′. The primer sequences for Flt-1 were as follows: forward 5′-CGACGTGTGGTCTTACG-GAGTA-3′; reverse 5′-CTTCCCTCAGGCGACTGC-3′. The primer sequences for VEGF were as follows: forward, 5′-CTGTGCAGGCTGCTGTAACG-3' reverse, 5′-GTTCCCGAAA- CCTGAG GAG-3′. An equal volume from each PCR was analyzed by 1.8% agarose gel electrophoresis, and ethidium bromide-stained PCR products were evaluated. Signal

intensity was quantified with the Gel Doc EQ (BIO-RAD Laboratories, Milan, Italy).

2.14. Immunohistochemistry

Immunohistochemical staining of tumor was performed on paraffin-embedded samples that had been deparaffinized and rehydrated. After treatment with trypsin for 10 min or heat for 25 min, tissue sections were incubated with primary antibody and then blocked with 3%
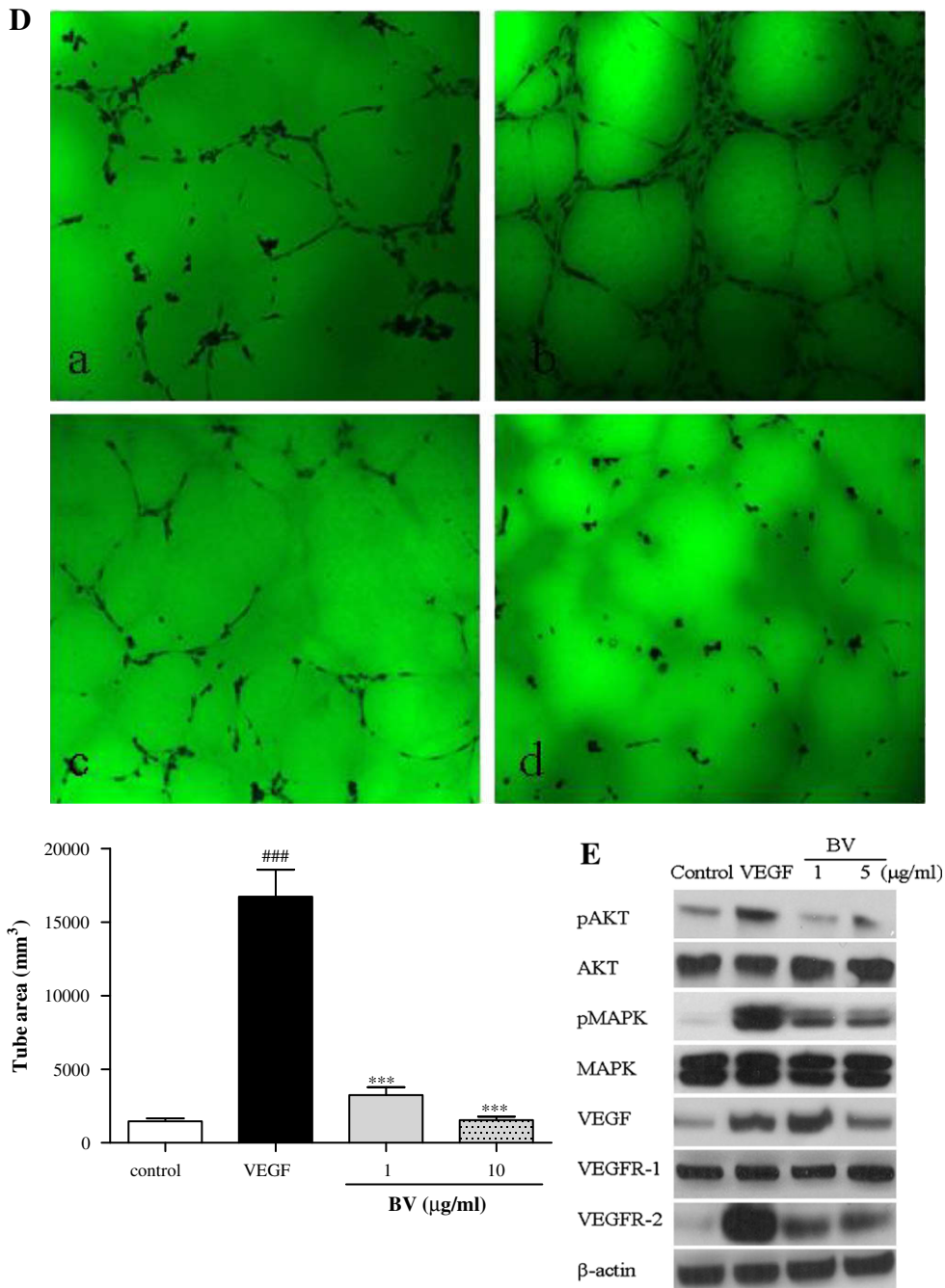
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goat serum in Tris-buffered saline. Purified monoclonal mouse anti-PCNA antibody, mouse anti-Factor VIII related antigen antibody, and mouse anti-VEGFR-2 antibody were used for staining. Sections were subcutaneously incubated with horseradish peroxidase-conjugated secondary antibody for 30 min. Stain was developed using peroxidase 3,3′-diaminobenzidine (DAB) substrate and counterstained with hematoxylin.

2.15. Analysis of proliferation index and angiogenic index

Quantitative analysis of images was conducted using Image-Pro Plus analysis software. PCNA and endothelial cells were counted at a minimum of 10 randomly selected high power fields ($200\times$) from representative tumor sections. VEGF expression was assessed by scanning under an Axiovert S 100 light microscope (Carl Zeiss, Inc., USA) at low

power fields (100×), and three fields with the highest intensity were selected. Indices were calculated as below:

Proliferative index (%)

$$= (\text{no. of PCNA positive cells/total cells}) \times 100$$

Endothelial cell index (%)

$$= (\text{no. of Factor VIII positive cells/total cells}) \times 100$$

VEGFR-2 index (%)

$$= (\text{no. of VEGFR-2 positive vessels/total cells}) \times 100$$

2.16. Statistical analysis

All values represent means (SD statistically significant differences between control and sample groups were calculated by the Student's t-test. The Wilcoxon rank sums test was used to evaluate difference in tumor growth rates. Kaplan–Meier survival curves were compared using the log-rank test in GraphPad Prism.

3. Results

3.1. BV inhibits viability of LLC cells and angiogenesis related signaling pathway

To evaluate the cytotoxic effect of BV on cancer cells and normal cells, the authors analyzed cell viability using the CCK-8 assay. BV significantly inhibited LLC cell growth in a dose-dependent manner (Fig. 1A). The IC_{50} value for inhibition of LLC cell growth was 0.5 µg/ml. To discriminate the difference in BV susceptibility between cancer cells and normal cells, the authors explored the effects of BV in PBML. BV did not reduce cell viability in PBML cells at doses below 10 µg/ml (Fig. 1B). The authors sought to determine whether if BV could affect viability or proliferation of tumor cells by interacting with proliferation and angiogenesis signaling pathways through examination of phosphorylated AKT and p44/42MAPK, VEGF, VEGFR-1 and VEGFR-2 using western blotting. As shown in Figs. 1C, BV at a dose of 5 µg/ml markedly inhibited AKT and P44/42 MAPK phosphorylation of tumor cells. Also, there was significant downregulation of VEGFR-2 expression in LLC cells, which were concordant with downregulated phospho-AKT and phospho-MAPK (Fig. 1C).
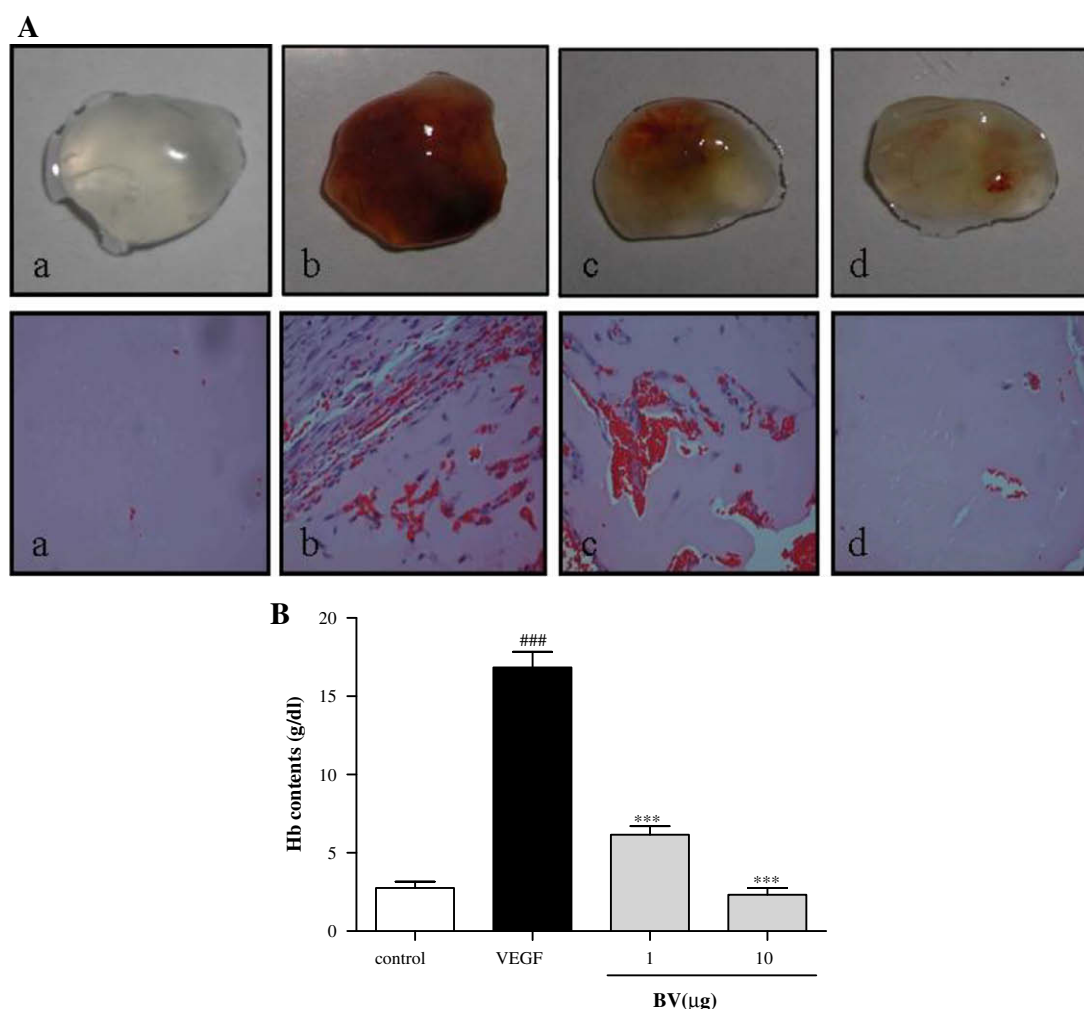

Fig. 3. Bee venom suppresses the angiogenesis in VEGF-induced Matrigel plugs assay. (A) Growth factor-reduced Matrigel was mixed with VEGF (100 ng) alone or in combination with BV (1 µg or 10 µg) and subcutaneously injected into C57BL/6JmsSlc mice. After 7 days, the animals were sacrificed and the Matrigel plugs were removed, the histological sections were performed as described in the Section 2. (B) To quantify functional blood vessels, the amount of hemoglobin (Hb) was measured. Values represent means ±SEM, n = 7; ###p < 0.001 versus VEGF untreated control; ***p < 0.001 versus VEGF treated control.

3.2. BV inhibits proliferation, migration and tube formation of endothelial cells via interacts with angiogenesis related signaling pathways

The inhibitory effect of BV on proliferation of VEGF-treated HU-VECs was examined. BV significantly inhibited the proliferation of VEGF-treated HUVECs in a dose-dependent manner, with an IC_{50} value of 4 μg/ml (Fig. 2A). This inhibitory effect was not attributable to the cytotoxicity of BV in HUVECs, since BV did not have any significant cytotoxic effect on HUVECs in doses under 20 μg/ml (Fig. 2B). BV significantly inhibited VEGF-induced migration of HUVECs (4.6-fold and 7.4-fold reduction at 1 μg/ml and 10 μg/ml, respectively) (Fig. 2C). BV was also tested for its inhibitory potency on the differentiation of endothelial cells into tube-like structures. BV significantly reduced the capillary-like tube formation of HUVECs (4050 ± 120.5 tube area/field, 1620 ± 92.2 tube area/field, respectively; 74% and 89% inhibition at 1 μg/ml and 10 μg/ml, respectively) (Fig. 2D). As shown in Figs. 2E, BV at a dose of 5 μg/ml markedly inhibited AKT and p44/42 MAPK phosphorylation of endothelial cells. Also, there were significant downregulations of VEGFR-2 expression in HUVECs, which were concordant with downregulated phospho-AKT and phospho-MAPK (Fig. 2E).

3.3. BV inhibits VEGF-induced Matrigel plug in vivo angiogenesis

The anti-angiogenic activity of BV was also confirmed by performing an established in vivo angiogenesis model test, the Matrigel plug assay. In the Matrigel plug assay, VEGF loaded plugs from mice exhibited reddish color indicating abundant red blood cells and therefore hemoglobin in the newly formed vasculature, whereas light yellowish color was ob-served in the BV/VEGF loaded plugs (Fig. 3A). Control Matrigel plugs containing VEGF exhibited formation of capillary-like tube network under histologic examination. However, BV strongly inhibited VEGF-induced angiogenesis (Fig. 3A). The Hb contents of the Matrigel plugs were also measured in order to quantify functional vasculature. Control plugs showed a mean Hb content of 2.4 ± 0.74 g/dl and VEGF containing plugs showed a mean content of 16.7 ± 1.4 g/dl. However, BV significantly lowered the content level of Hb in both control and VEGF plugs to 6.4 ±1.0 g/dl and 2.2 ± 0.44 g/dl, respectively (Fig. 3B).

3.4. BV inhibits tumor-induced neovascularization

To assess the effect of BV on tumor cell induced angiogenesis, LLC cells were intradermally inoculated on the region of skin above the thoracic vertebra for tumor angiogenesis assay. As shown in Fig. 4A and B, BV significantly reduced the number of vessels around LLC cells compared with untreated control. To confirm the anti-angiogenic activity of BV, RT-PCR and immunohistochemical examinations were carried out. As shown in Fig. 4C and D, BV inhibited the expression level of VEGF and VEGFR-2 mRNA, and reduced the expression of PCNA, Factor VIII and VEGFR-2 protein in the angiogenesis tumor model. The proliferation index of tumors treated with BV was significantly decreased compared with control (mean index of 72.2 ± 10.4% versus 57.4 ± 6.7%, 37.5 ± 4.4%, 18.9 ± 2.3%, Fig. 4E). A significant decrease in angiogenic index in mice treated with BV was observed compared with that of control mice (Factor VIII; mean index of 18.8 ± 1.3% versus 11.4 ± 0.7%, 6.4 ± 0.4%, 2.9 ± 0.3%, VEGFR-2; mean index of 64.4 ± 5.7% versus 48.4 ± 4.6%, 18.2 ± 3.9%, 14.1 ± 1.2%, Fig. 4E).
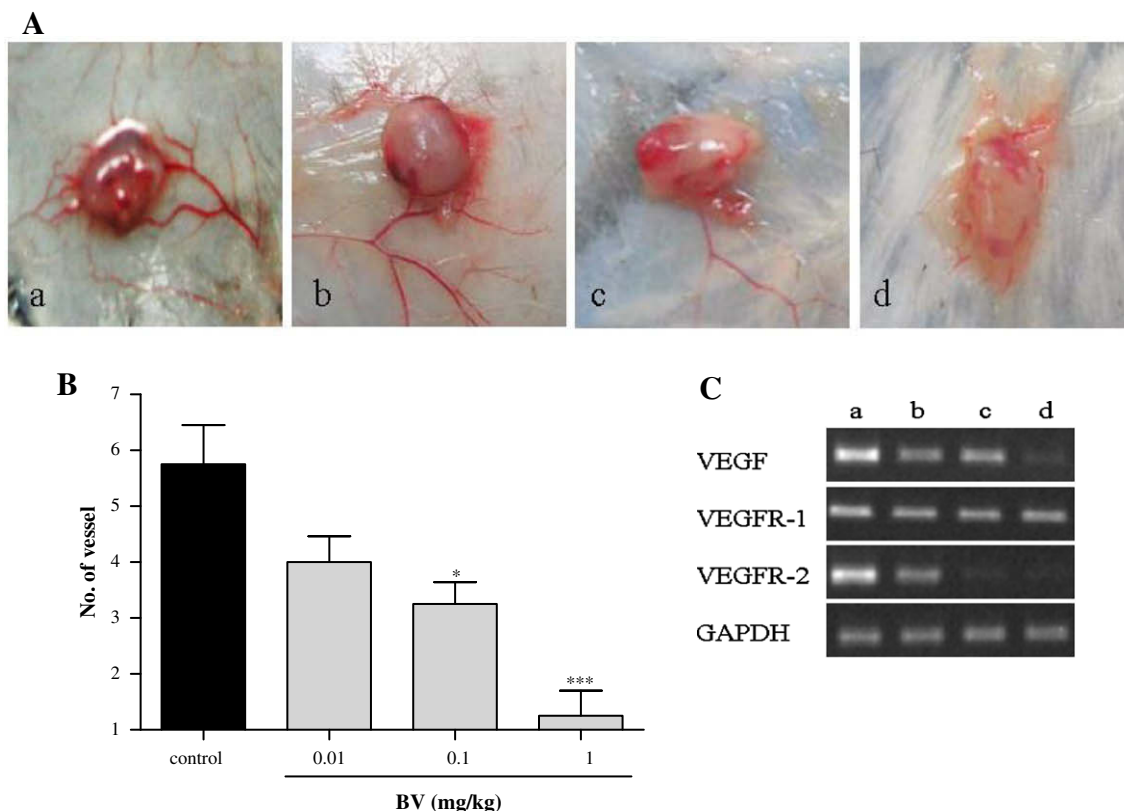

Fig. 4. Bee venom inhibits the angiogenesis in tumor-induced angiogenesis in vivo. (A) Tumors displayed varying degree of neo-vessel formation along the undersurface of the surrounding dermis at 7 days post inoculation control (a), 0.01 mg/kg of BV (b), 0.1 mg/kg of BV (c), 1 mg/kg of BV (d). (B) Number of tumor-supplying vessels feeding tumors pretreated with saline or BV. (C) Relative expression of VEGF, VEGFR-1 and VEGFR-2 in tumor tissues by semiquantitative reverse transcription-PCR. (D) Representive immunohistochemical staining for anti-PCNA, anti-Factor VIII related antigen, and anti-VEGFR-2. Control (a, c, e), 0.1 mg/kg of BV (b, d, f). (E) The proliferative and angiogenic (Factor VIII related antigen and VEGFR-2) indexes were calculated by the percentage of positive cells to total stained cells in tumor sections. Values represent means ±SEM, n = 7; $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ versus saline treated control.
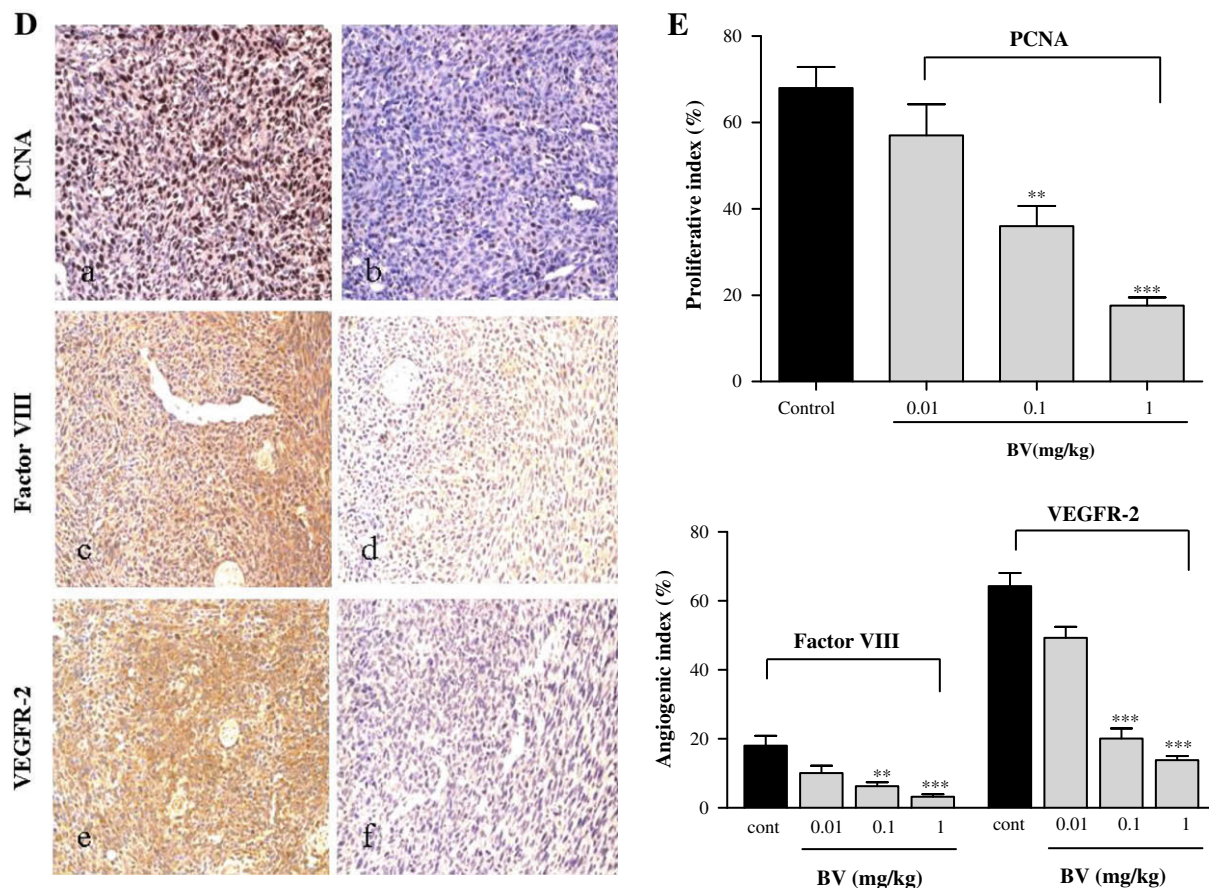
Fig. 4 (*continued*)

3.5. BV suppresses subcutaneous tumor growth and metastasis

To test if BV can serve as an anti-angiogenesis factor and suppress tumor growth in *in vivo*, BV (0.01, 0.1 or 1 mg/kg) or saline was s.c. administered to the back of mice every other day for 18 days from 3 days after subcutaneous inoculation with LLC at the flank region. Growth of tumor mass in the control group was rapid, whereas tumor growth from BV-treated LLC cells was delayed. As a result, BV showed significant inhibition of tumor volume over time (Fig. 5A). As LLC cells can readily metastasize from the subcutaneous primary tumor site to the lung by the 21st day, the authors evaluated the effect of BV on modulating metastatic progression. Mice in the control group displayed numerous distinguishable pulmonary metastatic nodules, while BV treated group showed fewer visible tumor nodules. The total surface area of tumor nodules was reduced by 47.5% and 76.1% with application of 0.01 mg/kg and 0.1 mg/kg of BV, respectively (Fig. 5B).

3.6. BV suppressed expansion of lung metastasis and improved survival rate

To evaluate the anti-metastasis effect and survival enhancement of BV in *in vivo*, the authors used a spontaneous pulmonary metastasis model wherein hm-LLC cells were inoculated s.c. to initiate primary tumor and subsequent pulmonary metastases. When the primary tumor reached approximately 0.5 cm in maximum diameter at 1 week postinoculation, the primary tumor was surgically removed, a procedure that resulted in accelerated metastasis development in the lung. When mice were sacrificed 30 days after BV treatment or were humanely euthanized during this period, the lung were harvested for examination of metastatic incidence and severity. All the mice in control groups exhibited massive hemothorax and developed metastatic nodules in their lungs. In control

animals, large, vascularized tumors with hemorrhage were found in the majority of lungs (Fig. 6A, a). In contrast, the lung surface of one in four mice receiving 10 μg/ml of BV was tumor free (Fig. 6A, c), whereas the others had several small metastasis nodules (Fig. 6A, b). Histologic analysis showed that large tumors from the control group were composed of more heterogenous cell populations and hemorrhage at the lung (Fig. 6B, a). Contrarily, minimal vascularity was observed in lungs of mice treated with BV (Fig. 6B, b and c). Upon termination of the experiment at 9 weeks after treatment, significantly prolonged survival was observed in BV treated tumor-bearing mice (Fig. 6D). In an independent set of experiments, the authors investigated animals 30 days after receiving BV. The authors had confirmed, by RT-PCR, that the expression levels of VEGF and VEGFR-2 were reduced in lungs (Fig. 6C). As shown in Fig. 6D, the median survival time was 27 days in the control group and was significantly improved in BV-treated mice, with a median survival of 51.5 days at 0.01 mg/kg of BV and 58 days at 0.1 mg/kg of BV.

4. Discussion

Angiogenesis plays a vital role in growth, intravasation, and metastatic spread of cancer [24,25]. Inhibition of angiogenesis provides a good chance of preventing cancer from becoming malignant [24–27]. It is widely accepted that tumor-induced angiogenesis is initiated by angiogenic cytokines such as bFGF and VEGF that are expressed in the tumor itself [26,27]. This process depends on vascular cell proliferation, migration and tube formation of endothelial
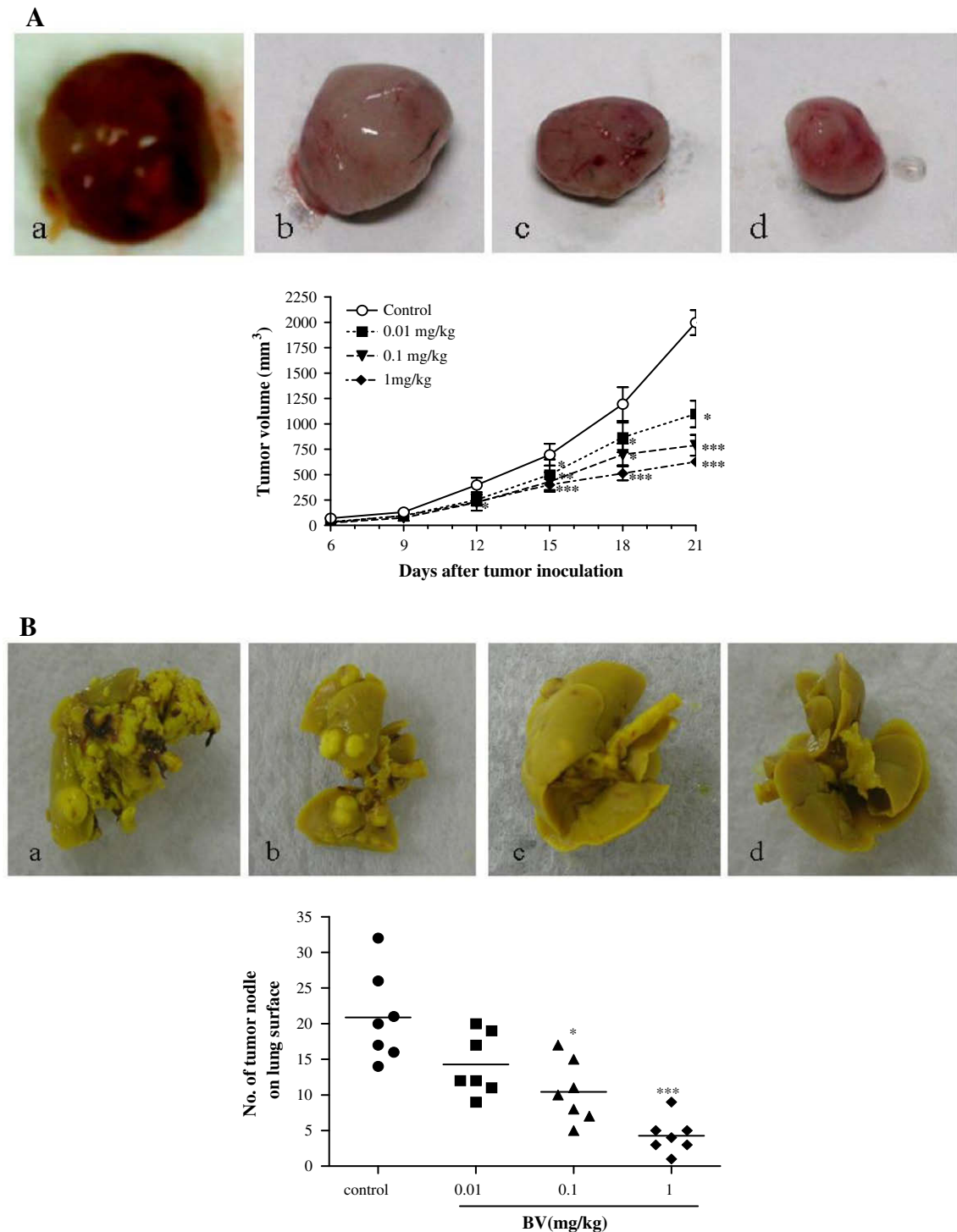
A

B

Fig. 5. Bee venom inhibits subcutaneous tumor growth and pulmonary metastasis in LLC-bearing mice. LLC cells were subcutaneously injected into the right flank of C57BL/6JmsSlc mice. Three days after tumor inoculation, mice were given subcutaneously injection of BV (0.01 mg/kg, 0.1 mg/kg and 1 mg/kg) or control (saline). (A) Tumor volumes were measured every 3 days for 21 days after tumor implantation with a caliper; control (a), 0.01 mg/kg of BV (b), 0.1 mg/kg of BV (c), 1 mg/kg of BV (d). (B) Pulmonary metastatic burden was assessed by counting the number of tumor nodules on the lung surface at 21 days postinoculation. Values represent means ±SEM, $n = 7$; $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ *versus* saline treated control.

cells. This provides a motive to determine whether if anti-angiogenic activities of BV work by inhibiting HUVECs growth, migration and tube formation.

In this study, the authors demonstrated, for the first time, that BV played a remarkable role in inhibiting angio-genesis and metastasis via down regulation of VEGF and
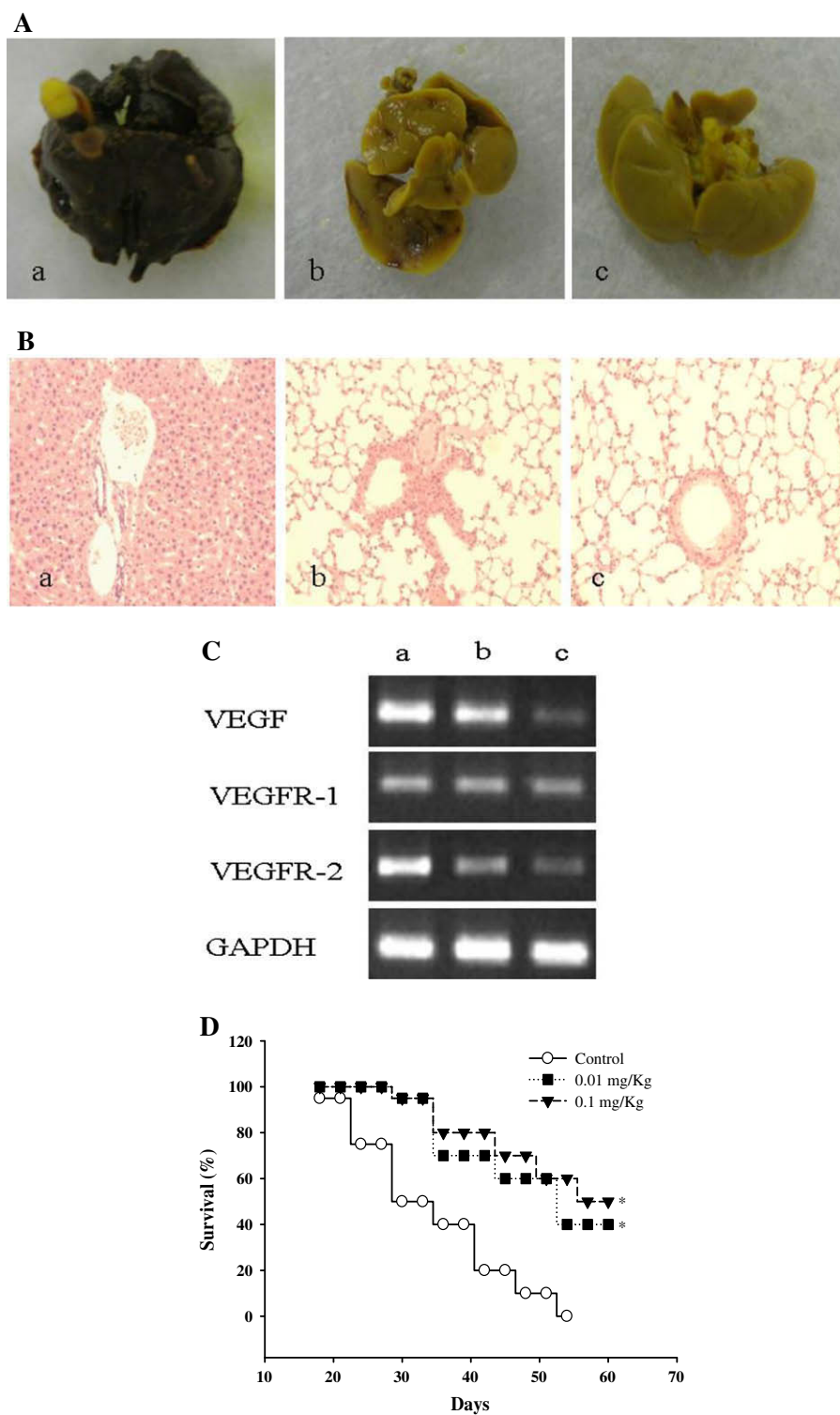
Fig. 6. Bee venom suppresses pulmonary metastasis and prolonged survival after removal of primary tumor in mice. (A) Representative lung samples bearing metastatic tumor nodules from mice sacrificed 30 days after BV treatment initiation; control (a), 0.01 mg/kg of BV (b), 0.1 mg/kg of BV (c). (B) H&E staining of lung sections from mice sacrificed 30 days after BV treatment initiation. (C) The mRNA levels of VEGF, VEGFR-1 and VEGFR-2 were lowered by BV treatment. The mRNA was extracted from the lung tissue at 30 days after BV treatment initiation and quantified by semiquantitative reverse transcription-PCR analysis. (D) BV treatment prolonged the survival of hm-LLC tumor-bearing mice after primary tumor removal. Values represent means ±SEM, n = 6; [*]p < 0.05 versus saline treated control.

VEGFR-2 in lung cancer treatment: (i) BV inhibited the viability of cancer cells without exhibiting cytotoxicity; (ii) BV downregulated the expression of VEGF-R2 in LLC cells and HUVECs and blockaded the VEGFR-2 signaling pathways by interfering with the activation of AKT and p42/44 MAPK; (iii) BV inhibited VEGF-induced proliferation, migration and capillary-like tube formation of endothelial cells. These results are further supported by the tumor-associated angiogenesis and metastasis in the Lewis lung carcinoma model induced by VEGFR-2 in vivo; (iv) BV suppressed neovascularization in the Matrigel plug angiogenesis model and tumor-induced angiogenesis model; (v) BV suppressed tumor growth of LLC cells in our subcutaneous tumor model; (vi) BV delayed metastatic spread in mice which underwent primary tumor resection in the LLC tumor model; (vii) BV could potentially offer long-term survival benefit when combined with surgery in the LLC tumor model.

In vitro, in vivo, and clinical trials have demonstrated that BV has a variety of pharmacological effects and may be an important traditional medicine in cancer treatment. BV has anti-cancer activities on several types of cancer cells including lung, liver, breast, prostate, and mammary cancer cells as well as leukemia cells [19,28–31]. Previous studies reported that the cytotoxic effects on cancer cells work through the activation of PLA_2, caspase and MMP, which destroy cancer cells and is suggested as an important basic mechanism for the anti-cancer activity of BV [18,19,29–31]. However, the mechanisms of BV which interfere with cancer induced angiogenesis are complex and not completely understood.

In in vitro angiogenesis models, the authors have revealed that the inhibition of HUVECs with BV leads to reductions in cell proliferation as well as chemotactic motility and strongly inhibits the formation of capillary tube networks. Furthermore, the authors observed that BV has no effect on the normal physiological angiogenesis that is essential for embryonic development. This suggests that the doses of BV in the therapeutic range or higher are safe in clinical studies, implying that conventional BVT is also safe, even though more in-depth clinical studies will be needed to determine the effectiveness of BV for practical indications. In our western blotting experiments, the authors found that BV can interfere with events in VEGF and VEGFR-2 signaling in cancer cell and endothelial cells. The inhibition of VEGF and VEGFR-2 expression is closely associated with angiogenesis and metastasis in several models [32–35]. The binding of VEGFR-2 promotes endothelial cell proliferation and migration via the phosphatidylinositol(PI)-3 kinase/AKT and p42/44 MAPK pathways. Therefore, blockade of the VEGF and VEGFR-2 signaling pathways could effectively inhibit tumor angiogenesis and metastasis.

The authors demonstrated that BV interferes with angiogenesis in the VEGF-induced Matrigel model in which VEGF-containing Matrigel implants showed decreases in capillary tube network formation and Hb contents. These in vivo results are supported by in vitro studies showing that BV inhibited in vitro HUVECs cell proliferation, migration, and formation of capillary-like structures that play essential roles in the angiogenesis process. BV also pre-

vented tumor-induced angiogenesis. In this study, immunohistological staining of LLC tumors revealed that BV inhibited the proliferating cells, endothelial cells and vascular-associated cells as well as some cancer cells. The authors suggest that the proliferation index has correlation with the angiogenic index, and has no significant cytotoxicity. Previous preclinical studies of anti-angiogenesis inhibitors suggest that treatment at early stages of tumor progression is more effective than intervention at later stages due to the complexity of cellular interactions in malignant tumors [36]. The authors also observed a delay in the initiation of tumor growth through angiogenesis inhibition by BV treatment. The results of this study suggest that BV acts during the onset of metastasis to inhibit the angiogenic switch, resulting in the delay of tumor progression and metastasis. Angiogenesis provides a principle route for tumor cells to exit the primary site to a distant site [37]. When cancer has spread into the bloodstream, surgical removal of visible tumors is often not beneficial. Surgical excision of lung tumor raises the risk of relapse from micrometastasis due to insufficient activity of endogenous angiogenesis inhibitors [38]. For instance, an increase in systemic VEGF levels after pulmonary surgery or local radiation therapy results in the acceleration of the metastasis from LLC primary tumors [39,40].

In summary, the authors have provided positive data for the safety and efficacy of BV as a potential anti-angiogenesis strategy against lung cancer through downregulation of VEGF and VEGFR-2. The effectiveness of BV in disrupting lung tumor growth and metastasis provides a promising basis toward further studies and clinical application of BV for lung cancer treatment.

Conflicts of Interest

The authors declare that they have no competing interests.

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