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Anti-metastatic and anti-angiogenic activities of sulfated polysaccharide of *Sepiella maindroni* ink

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ABSTRACT

A previous study demonstrated that SIP-SII, a sulfated *Sepiella maindroni* ink polysaccharide, suppressed the invasion and migration of cancer cells *via* the inhibition of the proteolytic activity of matrix metalloproteinase-2 (MMP-2). Therefore, this study investigated the anti-metastatic effect of SIP-SII *in vivo*. SIP-SII (15 and 30 mg/kg d) markedly decreased B16F10 pulmonary metastasis in mice models by 85.9% and 88.0%, respectively. Immunohistochemistry showed that SIP-SII decreased the expression of the intercellular adhesion molecule 1 (ICAM-1) and basic fibroblast growth factor (bFGF) in lung metastasis nodules. In addition, SIP-SII inhibited neovascularization in chick chorioallantoic membrane assay at 0.08–2 mg/mL. In the *in vitro* experiments, SIP-SII (0.8–500 μg/mL) significantly decreased the protein and mRNA expression of ICAM-1 and bFGF in SKOV3 and EA.hy926 cells, respectively. These results suggested that SIP-SII might suppress melanoma metastasis *via* the inhibition of the tumor adhesion mediated by ICAM-1 and the angiogenesis mediated by bFGF, as well as resulting in depression of the invasion and migration of carcinoma cells.

1. Introduction

Cancer metastasis occurs through a complex multistep process consisting of entrance into the circulation from the primary tumor, migration to distant organs, adhesion to endothelial cells lining the blood vessels, and infiltration into the underlying tissue to form a new focus (Geiger & Peeper, 2009). The metastasizing ability of malignant tumors is accountable for the poor prognosis and high mortality rate in cancer patients (Balch et al., 2004). Hence, the development of therapeutic agents that can inhibit metastasis is crucial for improving the management of cancer.

Matrix metalloproteinases (MMPs) are the most important proteinases in ECM degradation and angiogenesis (Rundhaug, 2005) during metastatic process and are found to be overexpressed in a variety of malignant tumor types (Deryugina & Quigley, 2006; Lukaszewicz-Zajac, Mroczko, & Szmitkowski, 2011; Roy, Yang, & Moses, 2009; Szarvas, vom Dorp, Ergün, & Rübben, 2011). Among them, MMP-2 and MMP-9 are thought to be mostly associated with tumor metastasis (Kessenbrock, Plaks, & Werb, 2010). The adhesion of tumor cells to the ECM or other cells in the metastatic cascade is mediated by cell adhesion molecules on cell surface (Hood & Cheresh, 2002). Cell adhesion molecules such as ICAM-1 and E-cadherin have been proposed as pharmacological targets for decreasing the invasiveness of cancer cells (Hehlgans, Haase, & Cordes, 2007; Jeanes, Gottardi, & Yap, 2008; Rambaruth & Dwek, 2011). Angiogenesis plays a critical role in tumor growth and metastasis (Ichihara, Kiura, & Tanimoto, 2011). Among the known angiogenic factors, basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) are potent and representative factors involved in tumor development (Schmitt & Matei, 2012). Therapeutic strategies targeting various aspects of the inhibition of invasion, adhesion and angiogenesis could be potential methods to prevent or inhibit cancer metastasis.

Recently, a new heteropolysaccharide composed of a hexasaccharide repeating unit with a molecular weight of 1.13×10^4 Da was isolated from the ink of the cuttlefish *Sepiella maindroni* de Rochebruns, which was designated as *S. maindroni* ink polysaccharide (SIP) (Liu et al., 2008). The sulfated SIP (SIP-SII), with a sulfate

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content of 34.7%, was prepared using chlorosulfonic acid (Geresh, Mamontov, & Weinstein, 2002). In previous studies, SIP-SII was found to significantly inhibit the expression of MMP-2 in SKOV3 and ECV304 cells and the invasion and migration of these cell lines (Wang et al., 2008). In another test, SIP-SII was shown to inhibit the expression of VEGF and tube formation *in vitro* (data not shown). In this study, it was determined whether SIP-SII inhibits the metastasis in a melanoma mouse model and its possible mechanisms were further explained.

2. Materials and methods

2.1. Reagents and antibodies

SIP and SIP-SII were prepared as described previously (Liu et al., 2008; Wang et al., 2008), and were dissolved in serum-free medium for the *in vitro* assay and in normal saline (NS) for the *in vivo* assay. Mouse anti-ICAM-1 monoclonal antibodies (against amino acids 258–365 of ICAM-1 of human origin, SC-8439), goat anti-ICAM-1 monoclonal antibodies (against the C-terminal domain of ICAM-1 of mouse origin, SC-1511), and goat anti-FGF-2 polyclonal antibodies (against the C-terminal amino acids of FGF-2 of human origin, SC-1360) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Mouse anti-E-cadherin antibodies (against the human E-cadherin C-terminal recombinant protein) were purchased from BD Biosciences (BD Transduction Laboratories, San Diego, CA, USA). Rabbit anti-FGF basic polyclonal antibodies (against amino acids 1–23 of rat FGF basic, ab8880) were purchased from Abcam (Cambridge, MA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse, goat anti-rabbit or rabbit anti-goat IgG secondary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

2.2. Animals and cell lines

The use of animals was approved by the Institutional Animal Care Committee of Shandong University, with firm adherence to the Ethical Guidelines for the Care and Use of Animals. Wild-type female C57BL/6 mice (6–8-week-old, 16–18 g) were obtained from the Laboratory Animal Center, Shanghai Institute of Materia Medica, Chinese Academy of Sciences (Shanghai, China).

Mouse melanoma cell line B16F10, human ovarian carcinoma cell line SKOV3 and human endothelial cell hybridoma line EA.hy926 cells were obtained from the Shanghai Cell Bank, the Institute of Cell Biology, China Academy of Sciences (Shanghai, China). B16F10 and SKOV3 cells were maintained in RPMI-1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin–streptomycin (100 IU/mL–100 μg/mL), 2 mM glutamine, and 10 mM HEPES. EA.hy926 cells were cultured in DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin–streptomycin (100 IU/mL–100 μg/mL), 2 mM glutamine, and 10 mM HEPES. All cells were cultured in a humid atmosphere (5% CO_2, 95% air) at 37 °C, fed every 2–3 d and harvested by brief incubation in 0.02% EDTA–0.25% trypsin.

2.3. Western blot analysis

SKOV3 cells (1×10^5 per well) seeded in 6-well plates were treated with different doses of SIP-SII (500, 100, 20, 4, 0.8 μg/mL) or SIP (100, 20 μg/mL) for 48 h. The medium was removed and the cells were washed with PBS. Cells were then lysed in 50 μL of lysis buffer at 0 °C (ice-bath) for 30 min with intense shock for 30 s every 10 min. Total protein was determined using the Bradford method. Equal amounts of protein in the cell extracts were fractionated by 8% SDS-PAGE and then electrotransferred onto polyvinylidene fluoride (PVDF) membranes. After blocking with TBST buffer (20 mM Tris-buffered saline and 0.1% Tween) containing 5% non-fat dry milk for 1–2 h at room temperature, the membranes were incubated with monoclonal anti-ICAM-1 (Santa Cruz Biotechnology), monoclonal anti-E-cadherin (BD Biosciences) or polyclonal anti-bFGF-2 (Santa Cruz Biotechnology) antibodies for 2 h, followed by washing 3 times and reacting with HRP-conjugated secondary antibodies (Santa Cruz Biotechnology). The proteins were then detected using chemiluminescence agents (ECL, Amersham).

2.4. Real-time reverse-transcription polymerase chain reaction assay

EA.hy926 cells (1×10^5 per well) seeded in 6-well plates were treated with different doses of SIP-SII (500, 100, 20, 4 and 0.8 μg/mL) or SIP (100 and 20 μg/mL) for 48 h. The medium was removed and the cells were washed with PBS. Cells were then harvested and total mRNA was isolated using the TRIzol reagent (Bio Basic Inc., Markham, Ontario, Canada) according to the manufacturer's instructions. RNA purity was assessed by spectrophotometric measurements. Total RNA (1 μg) from each well was subjected to reverse transcription with Oligo (dT), deoxynucleotide triphosphates (dNTPs), Maloney murine leukemia virus (M-MLV) reverse transcriptase (ReverTra Ace®, Toyobo Co., Ltd., Osaka, Japan) and RNase inhibitor in a total reaction volume of 20 μL. The synthesized cDNA was used immediately for real-time PCR amplification using primers specific for ICAM-1 (forward primer: 5′-CAG TCA CCT ATG GCA ACG AC-3′, reverse primer: 5′-ATT CAG CGT CAC CTT GGC TC-3′), bFGF (forward primer: 5′-CAT ACA GCA GCC TAG CAA C-3′, reverse primer: 5′-TTC GGC AAC AGC ACA CAA ATC C-3′) or the control glyceraldehyde-3-phosphate dehydrogenase (GAPDH; forward primer: 5′-GAA GGT GAA GGT CGG AGT-3′, reverse primer: 5′-CAT GGG TGG AAT CAT ATT GGA A-3′). The real-time PCRs (20 μL) consisted of 10 μL of SYBR® Green Real-time PCR Master Mix (Toyobo Co., Ltd., Osaka, Japan), 2 μL of mixed primers (10 μM), 2 μL of cDNA and 6 μL of double-distilled water. The real-time PCR analysis was performed with the iQ5 Real-Time PCR Detection System (Bio-Rad, Bio-Rad Laboratories, Hercules, CA, USA). The results of relative expression of mRNA were semi-quantitated using the comparative Ct method (Livak & Schmittgen, 2001).

2.5. Chick chorioallantoic membrane (CAM) assay

Fertilized eggs were incubated in a constant-temperature incubator (Heraeus, Germany) maintained at 37 °C and 40–60% humidity for 4 d. A hole was then drilled into the air sac of the egg, a small piece of egg shell from the middle was removed using a pinhead without destroying the chicken chorioallantoic membrane under it, and 20 μL of normal saline was dropped onto the exposed membrane. The incubation of eggs was continued using the same conditions for 3 d to create a false air sac directly over the middle membrane, and a 1–2 cm^2 window was created after carefully removing the egg shell separated from the membrane around the false air sac. The windows were sealed with adhesive tape and the eggs were incubated for a further 48 h.

Round gelatin sponge (Ø5 mm) saturated with SIP-SII (0.08, 0.4 and 2 mg/mL) or normal saline was placed on the areas between pre-existing vessels, and the embryos were further incubated for 48 h. The zones of neovascularization under and around the gelatin sponge were photographed using an anatomic microscope (Leica, MS5, Switzerland). Quantitative analyses were performed with the Image-pro plus 6.0 image analysis software.

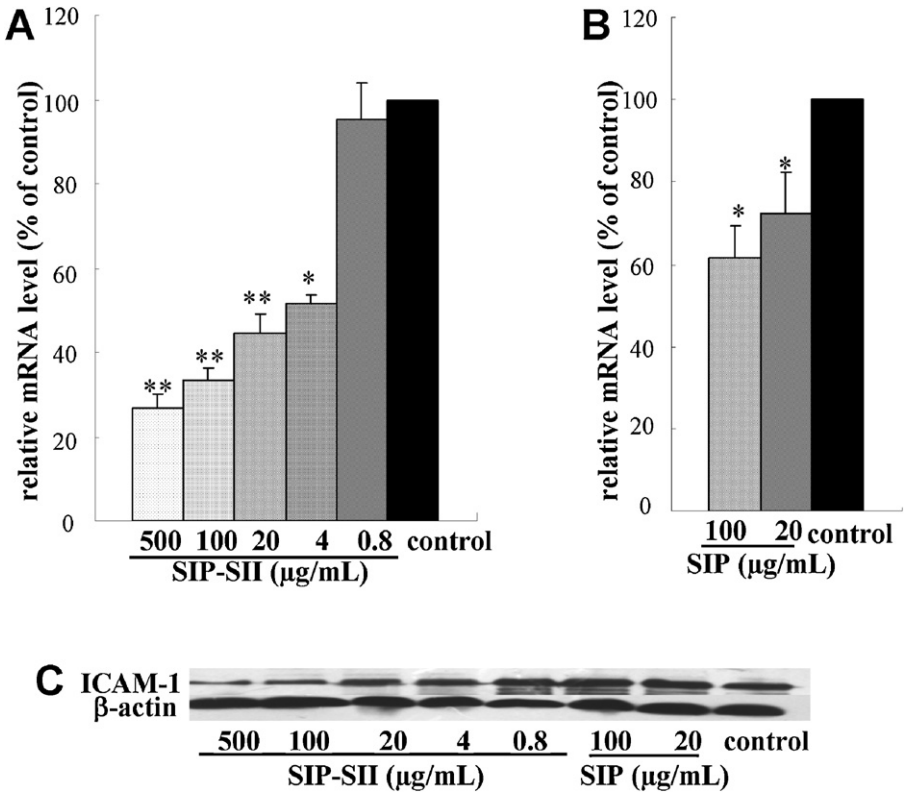

Fig. 1. Effects of SIP and SIP-SII treatment on the expression of ICAM-1 in SKOV3 cells. SKOV3 cells were treated with varying doses of SIP or SIP-SII for 48 h, and then the protein expression and mRNA levels of ICAM-1 were detected by Western blot analysis and real-time PCR, respectively. (A) The relative mRNA level of ICAM-1 in SKOV3 cells treated with SIP-SII and (B) the relative mRNA level of ICAM-1 in SKOV3 cells treated with SIP. Values were obtained by computerized image analysis of the specific RT-PCR products compared to that in the controls and normalized to GAPDH. (C) The protein expression of ICAM-1 in SKOV3 cells treated with SIP-SII or SIP. Each of the blots shown was demonstrated to have equal protein loading by re-probing with the monoclonal antibody for β-actin. $^{*}P < 0.05$, $^{**}P < 0.01$, significant difference compared with control group.

2.6. Animal metastasis experiments

Single-cell suspensions were prepared from 0.25% trypsin-treated monolayer of B16F10 melanoma cultures, washed, and diluted in normal saline. A volume of 200 μL cell suspensions (approximately 2×10^6 tumor cells per animal) was injected into the tail vein of female C57BL/6 mice between 6 and 8 weeks old. A volume of 0.2 mL of SIP-SII (15, 30 mg/kg) was administered intraperitoneally to the mice once a day for 10 d consecutively 1 d after the tumor cell inoculation. The same volume of normal saline was given as a control at each administration time-point for SIP-SII. The mice were killed on the 12th day and the number of lung metastatic foci was counted.

2.7. Immunohistochemistry assay

Mouse lungs containing metastatic nodules were fixed in 10% formaldehyde and embedded in paraffin wax. The paraffin blocks were serially sectioned into 4 μm pieces. After deparaffinization with dimethyl benzene and rehydration in an ethanol gradient, the sections were incubated in H_2O_2 (3%) for 10 min at room temperature to block the endogenous peroxidase activity and were then heated in 0.01 M citrate buffer (pH 6.0) for antigen retrieval. Subsequently, the slides were incubated with goat anti-ICAM-1 monoclonal antibodies (Santa Cruz Biotechnology) or rabbit anti-FGF basic polyclonal antibodies (Abcam) overnight at 4 °C and washed with PBS. The slides were then incubated with HRP-tagged rabbit anti-goat or goat anti-rabbit antibodies (Santa Cruz Biotechnology, USA) for 30 min at room temperature and washed with PBS. Finally, the sections were treated with stable 3,3′-diaminobenzidine (DAB) as a peroxidase substrate and restained with hematoxylin. The sections were then dehydrated, cleared, and mounted with neutralgum.

2.8. Statistical analysis

Data was described as mean ± S.D., and analyzed by Student's two-tailed *t*-test. The limit of statistical significance was $P < 0.05$. Statistical analysis was done using SPSS/Win11.0 software (SPSS, Inc., Chicago, IL, USA).

3. Results and discussion

3.1. SIP-SII inhibits protein and mRNA expression of ICAM-1

During metastasis, tumor cells adopt enhanced plasticity in the interactions with neighboring tumor cells, the ECM, and other cell types within the microenvironment. Cell adhesion molecules are important regulators of such homotypic and heterotypic interactions and are essential for transducing the intracellular signals responsible for adhesion, migration, invasion, angiogenesis, and

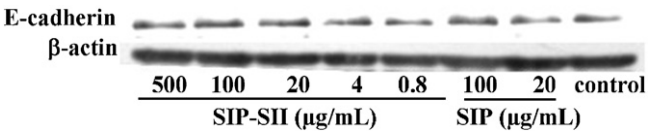

Fig. 2. Effects of SIP and SIP-SII treatment on the expression of E-cadherin in SKOV3 cells. Each of the blots shown was demonstrated to have equal protein loading by re-probing with the monoclonal antibody for β-actin.

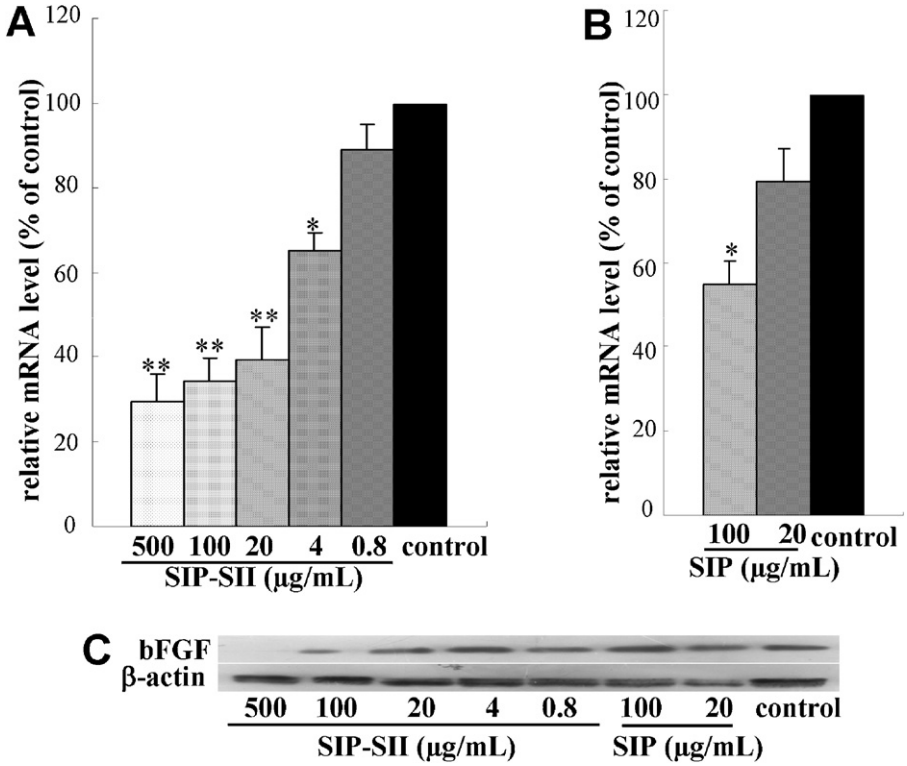

Fig. 3. Effects of SIP and SIP-SII treatment on the expression of bFGF in EA.hy926 cells. EA.hy926 cells were treated with varying doses of SIP or SIP-SII for 48 h, and then the protein expression and mRNA levels of bFGF were detected by Western blot analysis and real-time PCR, respectively. (A) The relative mRNA level of bFGF in EA.hy926 cells treated with SIP-SII and (B) the relative mRNA level of bFGF in EA.hy926 cells treated with SIP. Values were obtained by computerized image analysis of the specific RT-PCR products compared to that in controls and normalized to GAPDH. (C) The protein expression of bFGF in EA.hy926 cells treated with SIP-SII or SIP. Each of the blots shown was demonstrated to have equal protein loading by re-probing with the monoclonal antibody for β-actin. $^{*}P<0.05$, $^{**}P<0.01$, significant difference compared with control group.

organ-specific metastasis (Li & Feng, 2011). Cadherins are a large family of adhesion molecules that mediate cell–cell adhesion within the primary tumor mass. The abrogation of E-cadherin has been shown to be associated with the metastatic phenotype and taken as a novel target for anti-cancer therapies (Blaschuk & Devemy, 2009). ICAM-1, a member of the immunoglobulin superfamily, has been found to be up-regulated in many human cancers (Buitrago et al., 2012; Hayes & Seigel, 2009). Several studies have suggested that ICAM-1 facilitates the spread of metastatic cancer cells to secondary sites by recruiting inflammatory cells (Lin, Shun, Wu, & Chen, 2006; Skelding, Barry, & Shafren, 2009; Yamada et al., 2006). Interaction of ICAM-1 on tumor cells and

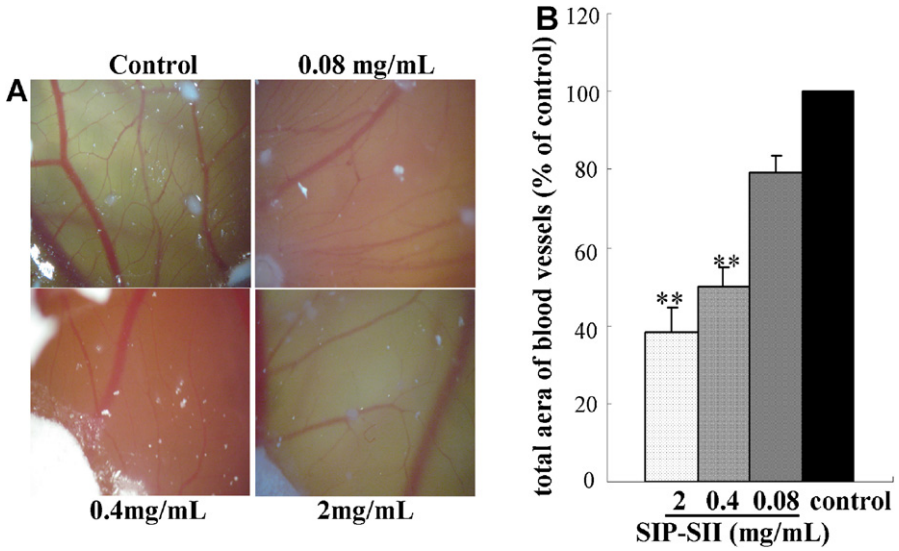

Fig. 4. Suppression of the blood vessel formation within CAM by SIP-SII. The CAMs were treated with increasing concentrations of SIP-SII (0.08, 0.4 and 2 mg/mL) or normal saline for 48 h. (A) Effect of SIP-SII on angiogenesis in CAMs. (B) Total area of blood vessels analyzed with angiogenesis-measuring software. Statistical significance of differences between control and experimental groups was determined by using two group two-tailed Student's *t*-test; $^{**}P<0.01$ was taken as the level of statistical significance.

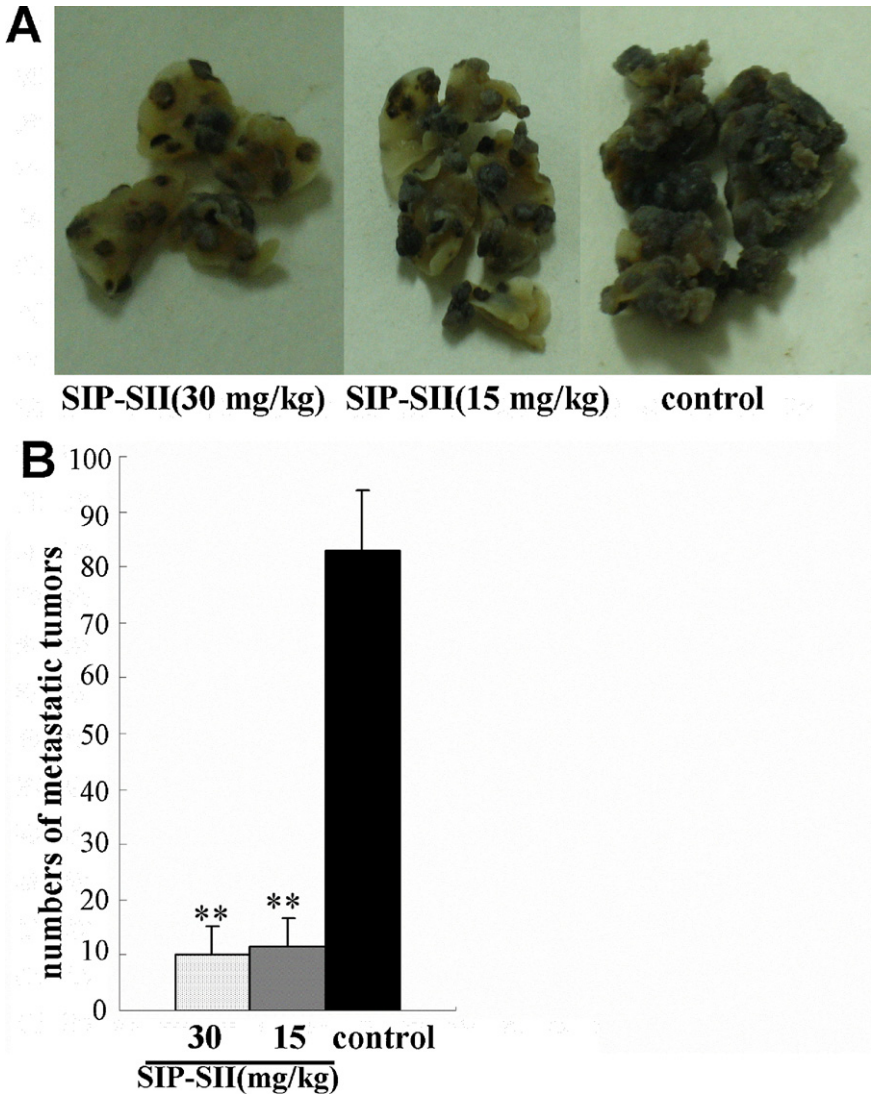

Fig. 5. Effect of SIP-SII on the formation of experimental metastases. 2×10^6 melanoma cells B16F10 were injected into the tail vein of C57BL/6 mice. SIP-SII (15 and 30 mg/kg d) or normal saline was infused consecutively by an intraperitoneal injection on the next day after the B16F10 cell injection. After administration for 10 d, lungs were collected and the number of pulmonary metastatic foci was counted. (A) Representative photos of the lung with the metastatic colonies obtained from 15 and 30 mg/kg SIP-SII-treated and control groups (the tissues were fixed with 10% formaldehyde). (B) Lung metastatic nodules of each group. Each bar represents the mean ± S.D. ($n = 8$). Statistical analysis was performed by *t*-test. $^{**}P < 0.01$, significant difference compared with control animals.

β2 integrins on infiltrating lymphocytes leads to detachment from the tumor mass and migration into the blood circulation (Strell, Lang, Niggemann, Zaenker, & Entschladen, 2010). Circulating cancer cells expressed high levels of ICAM-1 that can bind to β2 integrins expressed by circulating leukocytes, which serve as linking cells for the adhesion of carcinoma cells to endothelium (Liang, Slattery, Wagner, Simon, & Dong, 2008; Strell, Lang, Niggemann, Zaenker, & Entschladen, 2007). Inhibition of ICAM-1 on tumor cells lines leads to a strong suppression of metastasis (Brooks, Coleman, & Vitetta, 2008; Rosette et al., 2005). TNF-α can induce ICAM-1 expression through activating the NF-κB pathway, and as a result enhance tumor metastasis *in vivo* (Miele, Bennett, Miller, & Welch, 1994). This TNF-α inducible increase in metastasis can be reversed by knocking down ICAM-1 on tumor cells (Miele et al., 1994) or through the suppression of NF-κB (Moon, Choi, Moon, Kim, & Kim, 2011).

The effects of SIP-SII on the expression of ICAM-1 and E-cadherin in SKOV3 cells were evaluated using a real-time PCR assay and Western blot analysis. As shown in Fig. 1A, the level of ICAM-1 mRNA was decreased in a dose-dependent manner after 48 h incubation with SIP-SII. The inhibition rates of SIP-SII at doses of 0.8, 4, 20, 100 and 500 μg/mL were 4.6%, 48.3%, 55.6%, 66.5% and 73%, respectively. SIP also demonstrated a down-regulating effect on ICAM-1 mRNA levels with inhibition rates of 27.7% and 38.6% at 20 and 100 μg/mL, respectively, but the inhibitory efficacy of SIP was lower than that of SIP-SII (Fig. 1B). Consistent results were observed for the protein expression of ICAM-1 (Fig. 1C). At a concentration range of 0.8–500 μg/mL of SIP-SII, the rates of decrease were from 1% to a maximum of 58.1%. However, the inhibition of the expression of the ICAM-1 protein by SIP was not detected (Fig. 1C). No noticeable change in E-cadherin expression was observed in SKOV3 cell lines after treatment with SIP-SII or SIP (Fig. 2).

3.2. SIP-SII inhibits protein and mRNA expression of bFGF and reduces neovascularization

Tumor angiogenesis plays a very important role in cancer growth and metastasis (Makrilia, Lappa, Xyla, Nikolaidis, & Syrigos, 2009). Targeting angiogenesis has become an established therapeutic approach in cancer patients (Dong, Han, & Yang,

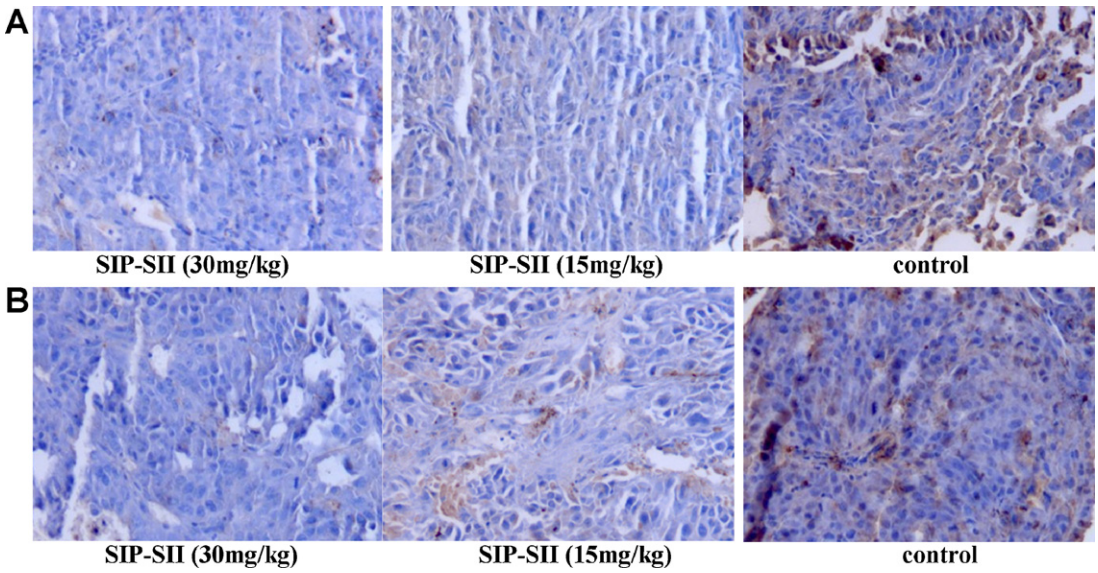

Fig. 6. SIP-SII inhibits ICAM-1 and bFGF expression in lung metastasis nodules. (A) Representative images of ICAM-1 immunohistochemistry staining in sections of lung metastasis nodules obtained from 15 and 30 mg/kg SIP-SII-treated and control groups. (B) Representative images of bFGF immunohistochemistry staining in sections of lung metastasis nodules. Sections were counterstained with hematoxylin (original magnification, 200×).

2007; Mahabeleshwar & Byzova, 2007; Szala, Mitrus, & Sochanik, 2010). The suppression of bFGF expression can cause the inhibition of angiogenesis and tumor progression (Fujimoto, 2008; Mamou et al., 2006). bFGF triggers a complex "proangiogenic phenotype" in endothelium and induce the neovascularization process (Presta et al., 2005). Besides, bFGF induces the expression of a variety of angiogenic growth factors in endothelial cells (Andrés et al., 2009). It also works synergistically with VEGF in inducing angiogenesis (Cao et al., 2004). bFGF regulates the expression of cadherins, integrins, proteases, and various ECM components that contribute to the maturation of the new blood vessels by regulating lateral cell–cell and substrate adhesions of ECs (Presta et al., 2005).

Elevated levels of bFGF have been found in the serum and urine of patients with active metastatic cancers including ovarian cancer and have been shown to correlate significantly with extent of disease, clinical status and risk of future mortality (Nguyen et al., 1994). In addition, gene expression profiling of advanced ovarian tumors indicates that bFGF signaling plays a central role throughout the carcinogenesis process (De Cecco et al., 2004). Inhibition of the FGF/FGFR system in tumor cells by transfection (Auguste et al., 2001), gene knockout (Polnaszek et al., 2003), or monoclonal antibodies (Hori et al., 1991) results in inhibition of tumor growth by both angiogenesis-dependent and -independent mechanisms.

Real-time PCR assays and Western blot analysis demonstrated that the relative levels of bFGF mRNA and protein expression in EA.hy926 cells were markedly decreased by SIP-SII after 48 h incubation (Fig. 3A and C). At a concentration range of 0.8–500 μg/mL, the inhibition rates of SIP-SII on bFGF mRNA levels and protein expression were increased from 17.9% to a maximum of 88.9%, and from 10.9% to 70.7%, respectively. SIP presented a weaker inhibitory effect on the expression of bFGF compared with SIP-SII (Fig. 3B and C).

The anti-angiogenic activity of SIP-SII was investigated *in vivo* using the CAM assay. The results in control eggs indicated that blood vessels formed dense branching vascular networks. In contrast, CAM neovascularization, which presented as increased blood vessel numbers and branching patterns, was dose-dependently suppressed by SIP-SII (Fig. 4A). The inhibition rates of 0.08, 0.4 and 2 mg/mL of SIP-SII were 21.0%, 49.9% and 61.5%, respectively (Fig. 4B).

3.3. SIP-SII inhibits lung metastasis of melanoma

Cancer invasion and metastasis are the leading causes of mortality in cancer patients. Inhibition of ICAM-1 (Brooks et al., 2008) or bFGF (Giavazzi et al., 2001) results in inhibition of tumor metastasis *in vivo*.

The anti-metastasis effect of SIP-SII was detected in mice implanted with metastatic B16F10 melanoma cells. After administration for 10 d, lung metastatic colonies in SIP-SII-treated mice were much fewer than those in the control, showing that lung metastasis formation was inhibited significantly by SIP-SII (Fig. 5A). The inhibition rates of 15 and 30 mg/kg of SIP-SII were 85.9% and 88% respectively (Fig. 5B). The expression levels of ICAM-1 and bFGF in lung metastasis nodules were analyzed by immunohistochemistry (Fig. 6). In comparison with the control group, the expression of ICAM-1 (Fig. 6A) and bFGF (Fig. 6B) in lung metastasis nodules in the SIP-SII-treated group showed a significant decrease.

4. Conclusion

In this study, SIP-SII was demonstrated to have significant anti-metastatic activity against B16F10 melanoma *in vivo*. The inhibition rate of low doses (15 mg/kg) of SIP-SII was higher than 85%. The anti-metastatic activity was attributed mainly to its suppression of ICAM-1 expression and anti-angiogenic activity mediated through the down-regulation of bFGF expression. These results suggest that SIP-SII may be a candidate anti-metastatic drug that deserves further research.

Acknowledgments

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