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Abietic acid isolated from pine resin (Resina Pini) enhances angiogenesis in HUVECs and accelerates cutaneous wound healing in mice☆

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ABSTRACT

Ethnopharmacological relevance: Resin known as Resina Pini is listed in the Korean and Japanese pharmacopoeias and has been used for treating skin wounds and inflammation. Resin is composed of more than 50% abietic acid and 10% neutral substances.

Objective: In the present study, the wound-healing effects of abietic acid and the possible underlying mechanism of action were investigated in various in vitro and in vivo models.

Materials and methods: The effects of abietic acid on tube formation and migration were measured in human umbilical vein vascular endothelial cells (HUVECs). Protein expression of mitogen-activated protein kinase (MAPK) activation was evaluated via Western blotting analysis. The wound-healing effects of abietic acid were assessed using a mouse model of cutaneous wounds.

Results: The results showed that abietic acid enhanced cell migration and tube formation in HUVECs. Abietic acid induced significant angiogenic potential, which is associated with upregulation of extracellular signal-regulated kinase (ERK) and p38 expression. Additionally, 0.8 μM abietic acid-treated groups showed accelerated wound closure compared to the controls in a mouse model of cutaneous wounds.

Conclusion: The current data indicate that abietic acid treatment elevated cell migration and tube formation in HUVECs by the activation of ERK and p38 MAPKs. We suggest that abietic acid can be developed as a wound-healing agent.

1. Introduction

Wound healing is an important process that repairs and regenerates tissue structure and function that has been disrupted or wounded by physical, bacterial, chemical, or viral insults (Muralidhar et al., 2011; Hwang et al., 2016). In general, there are four major stages of wound healing: clot formation, inflammation, proliferation, and remodeling (Hosemann et al., 1991; Watelet et al., 2002; Kim et al., 2015a).

Wound healing processes include the stimulation of new blood vessel formation simultaneously, proliferation of cells such as fibroblasts and keratinocytes, and production of basement membrane zones and connective tissues (Bates and Jones, 2003; Hwang et al., 2004). The production of a number of growth factors in the wound area affects its healing processes by influencing the activation, recruitment, pro-

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liferation, differentiation, and migration of various cells (Tsuboi and Rifkin, 1990; Choi et al., 2015).

Angiogenesis plays an essential role in physiological and pathological processes such as wound healing, embryonic development, tumor growth, and chronic inflammation (Pandya et al., 2006; Ha et al., 2016). Angiogenesis is regulated by several growth factors, including basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF); these factors promote proliferation, migration, and other angiogenic activities of endothelial cells (Scharpfenecker et al., 2007). However, the side effects or toxic effects of the growth factors are of concern owing to their multidistribution and multiple functions (Fu et al., 2005; Choi et al., 2016). Consequently, the identification of non-toxic natural products with pro-angiogenic activity would be very useful for the development of a potential alternative agent for wound healing (Kim et al., 2011).

Resin, also known as Resina Pini, is a natural resin obtained from plants belonging to the Pinaceae family and it is commercially produced from 7 *Pinus* species including *P. palustris* Mill., *P. pinaster* Ait., *P. sylvestris* L., *P. laricio* Poiret, *P. longifolia* Roxb., *P. densiflora* Siebold et Zucc., and *P. thunbergii* Parlatore. Resin is listed in the Korean and Japanese pharmacopoeias and has been used for treating wounds in traditional Korean medicine (Simbirtsev et al., 2002; Sipponen and Lohi, 2003; Sipponen et al., 2007). Resin is composed of approximately 90% resin acids and 10% neutral substances. In particular, resin is composed of more than 50% abietic acid (Fig. 1(A)). Abietic acid has been reported to show anti-inflammatory, anti-allergic, phytoalexin-like, and anti-convulsant activities and inhibitory effects on melanoma cancer metastasis (González et al., 2010; Hsieh et al., 2015; Gao et al., 2016). During a search for angiogenic substances produced by natural products, we observed that pine resin and abietic acid stimulated the migration of human endothelial cells. In this study, we demonstrated that abietic acid stimulated angiogenic properties in vitro and enhanced cutaneous wound healing in mice.

2. Materials and methods

2.1. Chemicals, reagents, and plant sample

Endothelial cell basal medium-2 (EGM-2) was purchased from Lonza Inc. (Walkersville, MD, USA). Pine resin (500 g) was purchased from KANTO CHEMICAL CO., INC. (Tokyo, Japan) in March 2016. The material was confirmed by one of the authors (K. H. Kim). A voucher specimen (SKKU-RS-2016-03) was deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Korea. The standard abietic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). Primary antibodies for p38, phosphorylated-p38, ERK and phosphorylated-ERK, as well as all secondary antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). U0126 and p38 kinase inhibitor SB203580 were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

2.2. Quantitative analysis of abietic acid by LC-MS

The detection of abietic acid was analyzed by LC-MS, Agilent 1200 Series analytical system equipped with a photodiode array (PDA) detector combined with a 6130 Series ESI mass spectrometer. Briefly, rosin was purchased from KANTO CHEMICAL CO., INC. (Tokyo, Japan). The rosin (5.0 mg) was dissolved in MeOH (500 μL) and filtered through a 0.50 μm syringe filter. The filtered sample was analyzed using a Kinetex C18 column (2.1×100 mm, 5 μm; Phenomenex, Torrance, CA, USA) set at 25 °C. The mobile phase was a gradient program from mixtures of 0.1% formic acid in water (A) and MeOH (B), which was as follows: 0–30 min from 10% to 100% B; 30–40 min at 100% B; followed by a rapid drop to 10% B at 41 min, and then isocratic condition with 10% B to 52 min. The flow rate was set at 0.3 mL/min, and the injection volume was 10 μL. Abietic acid was detected at 30.2 min of retention time. Calibration curves and linear regression equations were generated for the external standard,

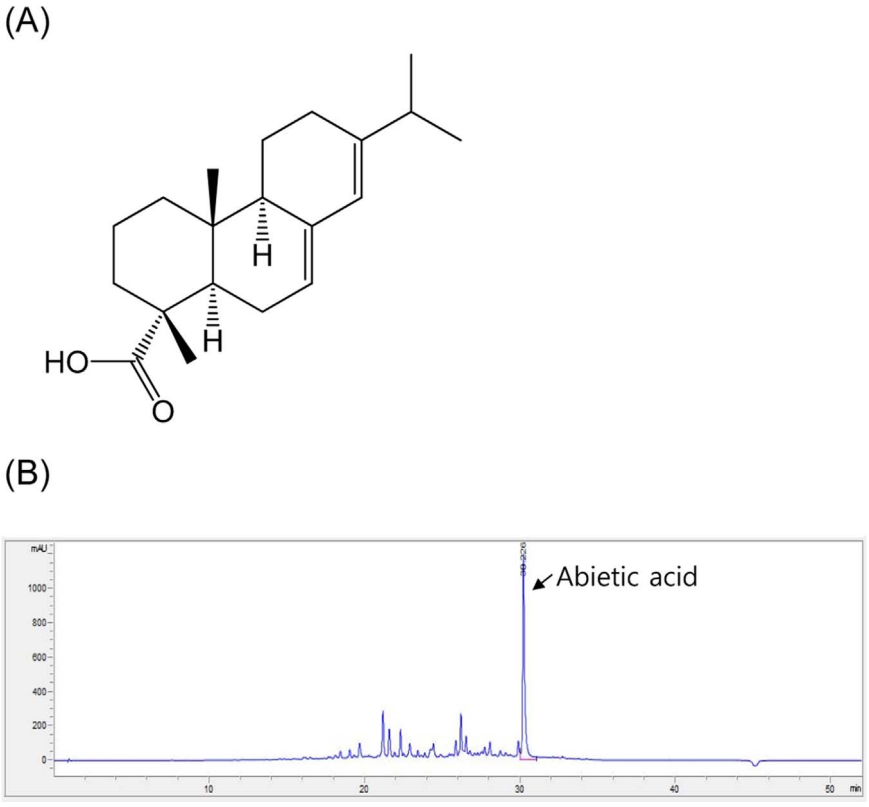

Fig. 1. Quantitative analysis of abietic acid by LC-MS. (A) Chemical structure of abietic acid. (B) UV chromatogram of LC/MS (detection wavelength was set as 254 nm) of resin extract.

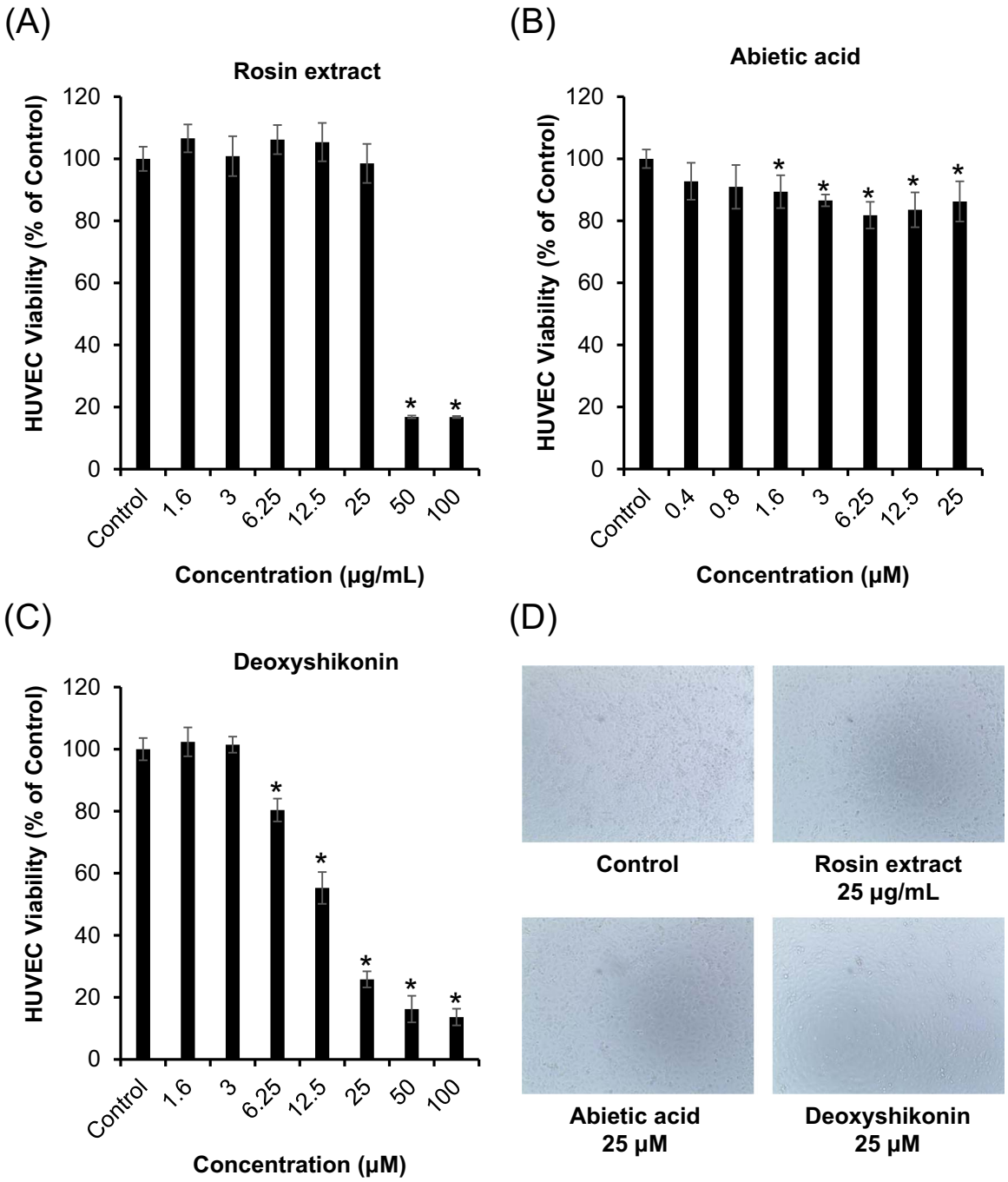

Fig. 2. Comparison in the effects of resin, abietic acid, and deoxyshikonin on HUVECs proliferation. (A) HUVECs were treated with resin at a series of concentrations (1.6–100 μg/mL) or with the DMSO vehicle (control) for 24 h, followed by the evaluation of cell proliferation through the MTT assay. (B) HUVECs were treated with abietic acid at a series of concentrations (0.4–25 μM) or with the DMSO vehicle (control) for 24 h, followed by the evaluation of cell proliferation through the MTT assay. (C) HUVECs were treated with deoxyshikonin at a series of concentrations (1.6–100 μM) or with the DMSO vehicle (control) for 24 h, followed by the evaluation of cell proliferation through the MTT assay. (D) Photographs of HUVECs after incubation with or without resin (25 μg/mL), abietic acid (25 μM), and deoxyshikonin 25 μM) after 24 h.

abietic aicd. Quantification of abietic acid was based on the peak area obtained from the MS detection (negative-ion mode) and calculated as equivalents of the standard. The content of abietic aicd is expressed as gram per 10 g of the extract weight. The result is the mean of three replicated measurements.

2.3. Cell culture, proliferation, migration and tube formation assays

Human umbilical vein vascular endothelial cells (HUVECs) (ATCC, Manassas, VA, USA) were maintained using the Clonetics EGM-2 in a humidified atmosphere (5% CO_2, 95% air). The cells were fed every 2–3 days and sub-cultured once they reached 70–80% confluence. The effect of abietic acid on the proliferation of HUVECs was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT, Sigma Aldrich, Saint Louis, MO, USA) as reported previously (Han et al., 2016).

The migration of endothelial cells was determined using Falcon cell culture PET inserts with a pore size of 8 μm and a 24-well format (No 353097; Falcon, Franklin Lakes, NJ, USA) following reported methods (Jeon et al., 2016).

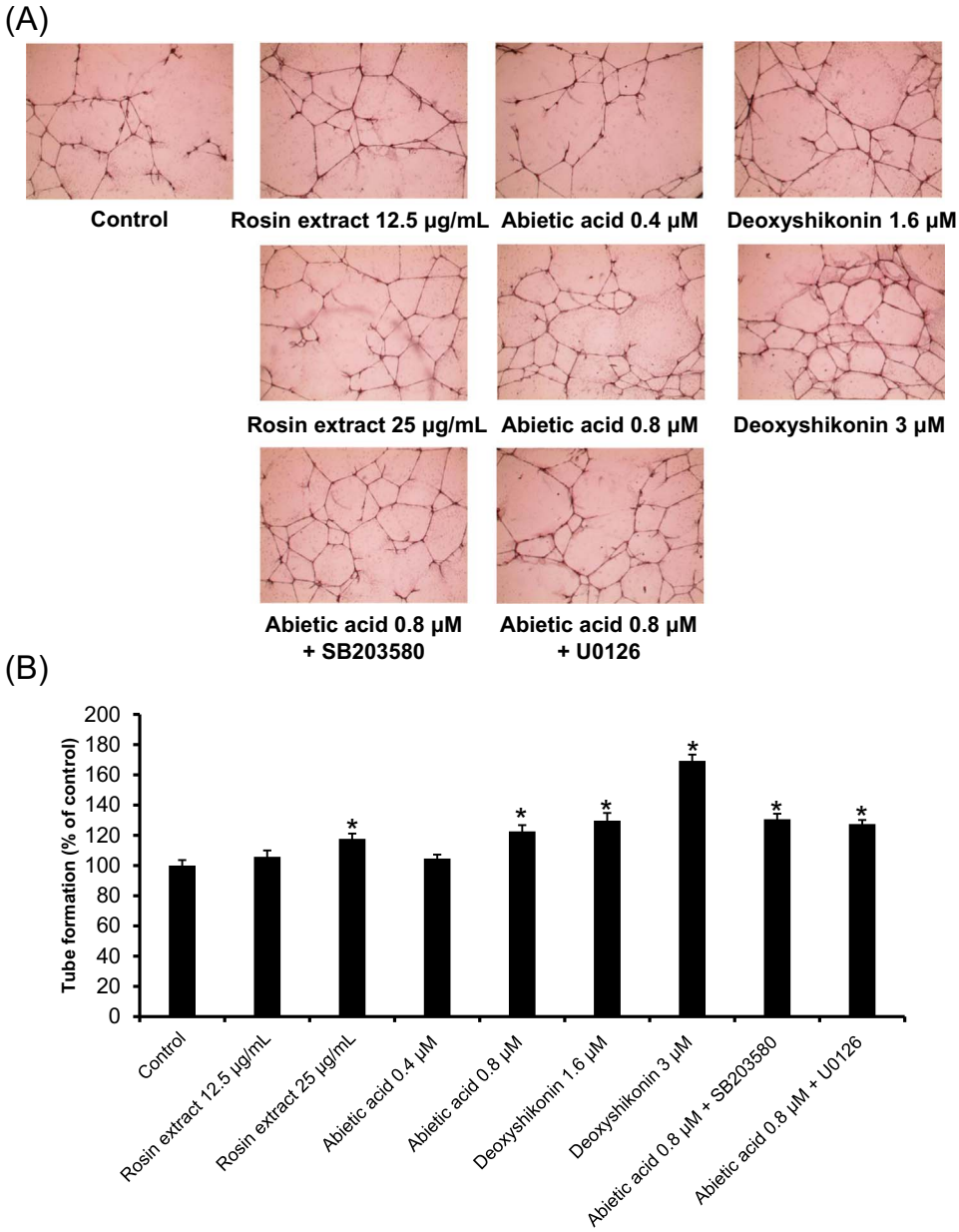

Fig. 3. Comparison in the effects of resin, abietic acid, and deoxyshikonin on HUVEC tube formation. (A) Photographs of tube formation in HUVECs on Matrigel after incubation with or without resin (12.5 and 25 μg/mL), abietic acid (0.4 and 0.8 μM), and deoxyshikonin (1.6 and 3 μM) after 24 h. (B) The relative lengths of tubes were measured using the ImageJ software.

Tube-formation assay in HUVECs was performed as reported previously (Lee et al., 2016). Briefly, HUVECs were seeded onto the Matrigel-coated plate and treated either 0, 0.4, or 0.8 μM of the abietic acid. The degree of tube formation was quantified by measuring the lengths of the tubes in the images captured using an ImageJ program (National Institutes of Health, Bethesda, USA).

2.4. Cell scratch wound healing assay

The scratch wounds were created in the cell monolayer by using a sterile pipette tip (Kim et al., 2015b). After treatment of abietic acid (0.4 and 0.8 μM) for 24 h, the wound width was determined using a light microscope equipped with a digital camera, and four measurements of wound width were made at randomly chosen points. The extent of wound closure was presented as the percentage by which the original scratch width had decreased at each measured time point.

2.5. Western blotting analysis

Western blotting was performed as reported previously (Park et al., 2016). In brief, proteins were separated by electrophoresis in a precast 4–15% Mini-PROTEAN TGX gel (Bio-Rad, CA, USA), blotted onto polyvinylidene fluoride (PVDF) transfer membranes, and analyzed with epitope-specific primary and secondary antibodies.

2.6. Wound healing effect in a mouse model of cutaneous wounds

The Guidelines for Animal Experimentation approved by Gachon University were followed in these experiments. Male ICR mice (4–5 weeks of age) were obtained from Orient Bio Co., Ltd. (Seongnam, Korea). Wound healing effect in a mouse model of cutaneous wounds was performed as reported previously (Park et al., 2017). In brief, a 5-mm full-thickness excisional skin wound was made on the back of each

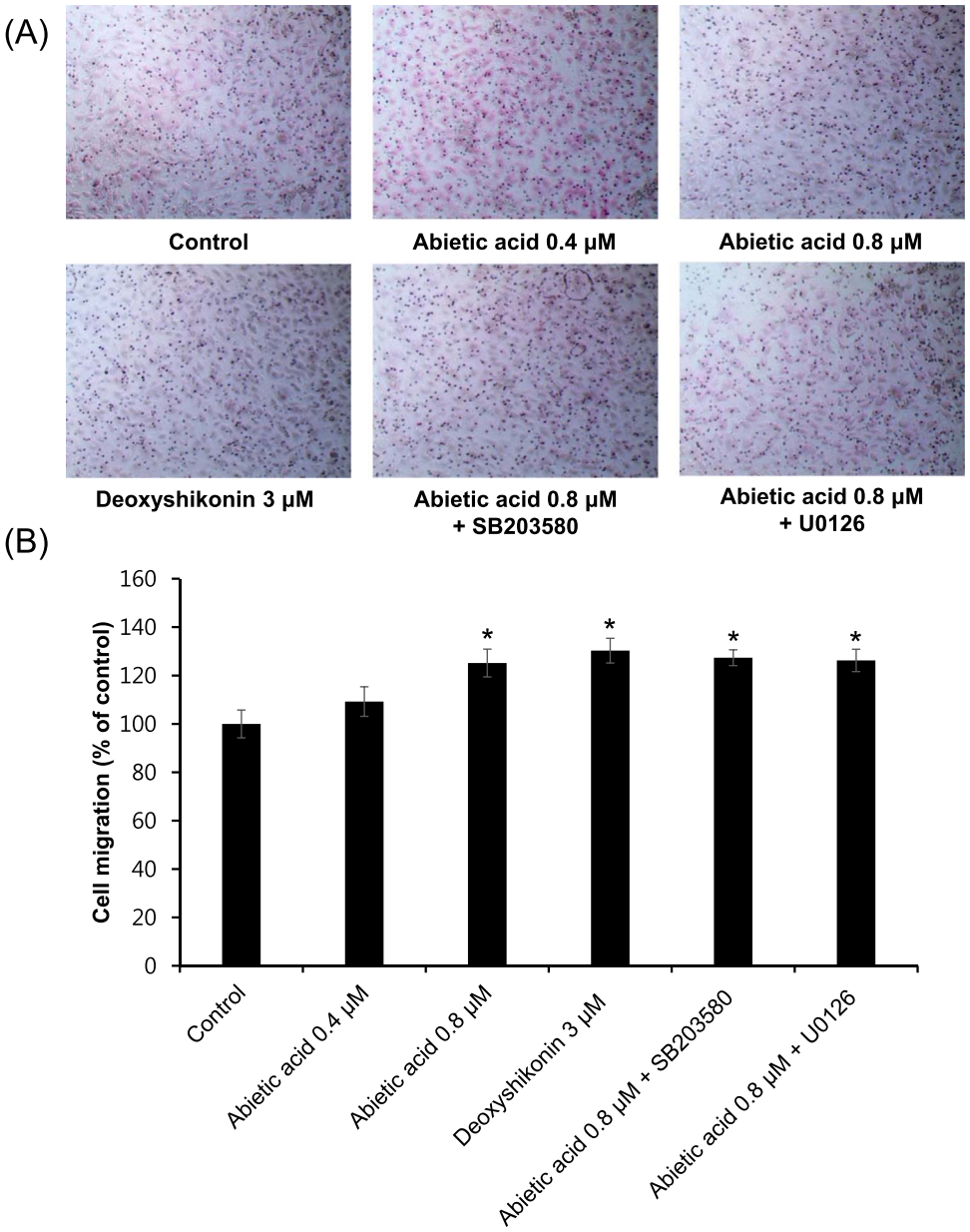

Fig. 4. Effects of abietic acid on HUVECs migration. (A) Photographs of HUVEC migration. (B) HUVECs (2×10^3 cells μL^{-1}·per well) were seeded into the insert of each well, and treated with abietic acid or the DMSO vehicle (control) for 24 h. Migrated cells underwent a migration assay and were then counted.

mouse. Each wound was treated with phosphate buffered saline (PBS, control) or abietic acid (0.8 μM) daily. Digital images of the wounds were captured on day 0, 2, 4, 6, 8, and 10 by using a 10-megapixel digital camera. The ImageJ software was used to calculate measurements of the wound. The percentage of wound closure was calculated as follows: [(area of original wound−area of actual wound)/area of original wound]×100.

2.7. Statistical analysis

Statistical significance was determined through analysis of variance (ANOVA) followed by a multiple comparison test with a Bonferroni adjustment. P values of less than 0.05 were considered statistically significant.

3. Results and discussion

The process of wound healing involves several phases, including the formation of granulation tissue, re-epithelialization, neovascularization, and tissue reorganization (Broughton et al., 2006). Among these phases, neovascularization, also known as angiogenesis, plays a critical role in early phases of wound healing (Kim et al., 2015; Martin and Nunan, 2015). Agents that control angiogenesis are beneficial in the development of wound-healing and tissue-regenerating agents (Pandya et al., 2006; Baek et al., 2016). In this study, we have demonstrated that abietic acid enhances angiogenesis in vitro and wound healing in vivo.

3.1. Resin, abietic acid, and the positive control deoxyshikonin inhibited HUVEC cell viability

Proliferation of cells such as fibroblasts and endothelial cells is essential for effective wound healing (Nissen et al., 1998; Chung et al., 2015). Therefore, we investigated the effects of various concentrations of resin, abietic acid, and deoxyshikonin on the proliferation of HUVECs using MTT assay. The content of abietic aicd in the rosin extract was 4.9 g

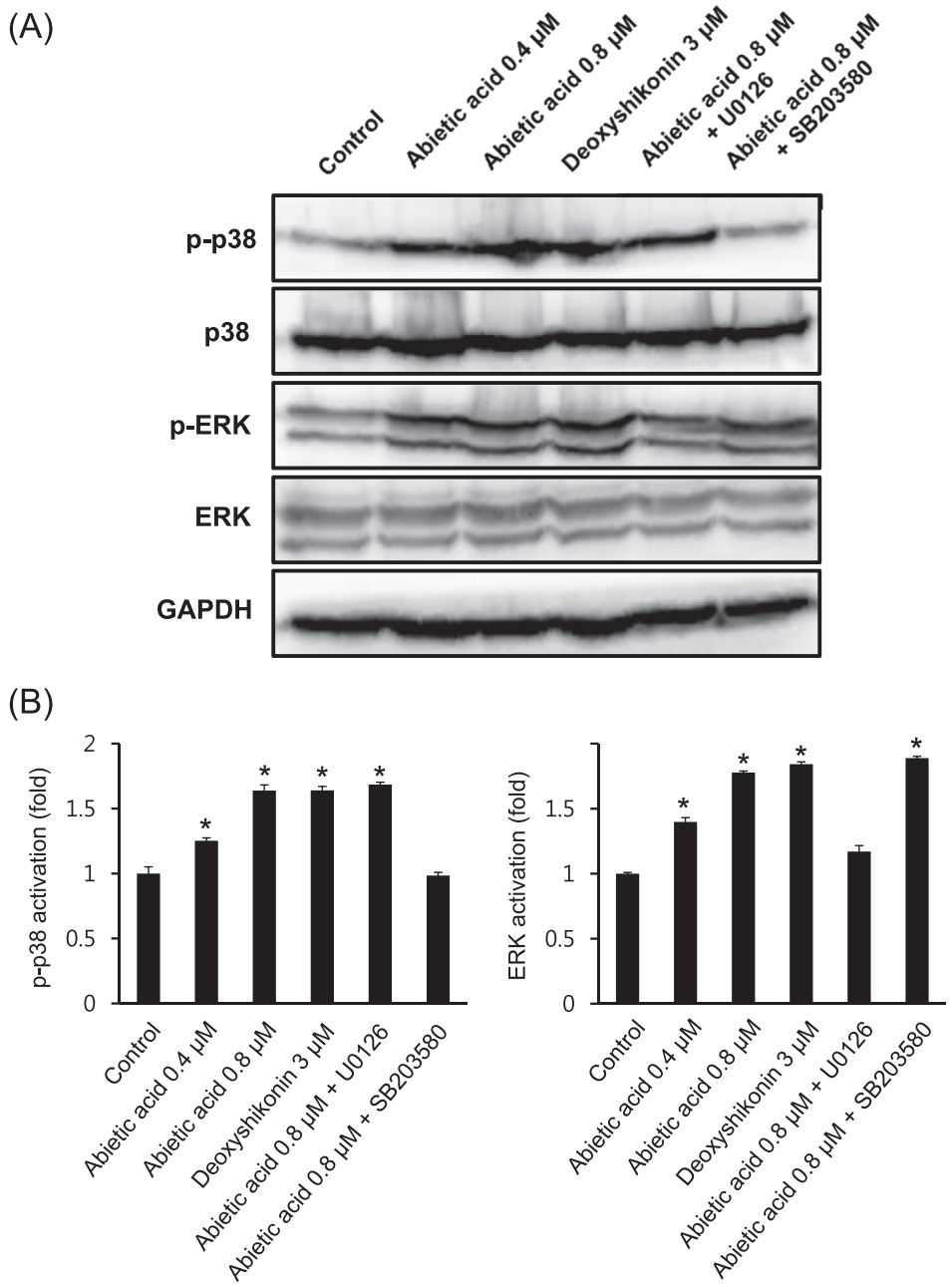

Fig. 5. Effect of abietic acid on MAPK activation in HUVECs. Results of the Western blot show the levels of phosphorylated-p38, p38, phosphorylated-extracellular-signal-regulated kinases (ERK), and ERK in HaCaT cells treated with abietic acid at different concentrations for 24 h. Whole cell lysates (20 μg) were separated via SDS-PAGE, transferred onto PVDF transfer membranes, and probed with the indicated antibodies. Proteins were visualized using an ECL detection system.

per 10 g, thus 49% of the total extract (Fig. 1(B)). As shown in Fig. 2(A), resin inhibited the proliferation of HUVECs in a dose-dependent manner. The cell viability (% of control) ranged from 106.59 ± 3.26% (1.6 μg/mL) to 16.79 ± 0.52% (100 μg/mL). Resin inhibited the viability of HUVECs by 83.21% at a concentration of 100 μg/mL. Treatment with up to 25 μg/mL resin had no effect on HUVEC proliferation, whereas treatment with 50–100 μg/mL decreased cell viability and IC50 value was evaluated to be 39.8 μg/mL. As shown in Fig. 2(B), abietic acid inhibited the proliferation of HUVECs in a dose-dependent manner. The cell viability (% of control) ranged from 92.75 ± 4.69% (0.4 μM) to 86.25 ± 4.31% (25 μM). Abietic acid inhibited the viability of HUVECs by 13.75% at a concentration of 25 μM. Treatment with up to 0.8 μM abietic acid had no effect on HUVEC proliferation, whereas treatment with 1.6–25 μM abietic acid decreased cell viability. Previous studies have shown that deoxyshikonin treatment elevated tube formation in HUVECs, and that deoxyshikonin-induced proliferation and migration in HaCaT cells were mediated by the activation of ERK and p38, respectively (Park et al., 2017). Thus, we used deoxyshikonin as a positive control. As shown in Fig. 2(C), deoxyshikonin inhibited the proliferation of HUVECs in a dose-dependent manner. The cell viability (% of control) ranged from 102.35 ± 2.19% (1.6 μM) to 13.63 ± 1.89% (100 μM). Deoxyshikonin inhibited the viability of HUVECs by 86.37% at a concentration of 100 μM. Treatment with up to 3 μM deoxyshikonin had no effect on HUVEC proliferation, whereas treatment with 6.25–100 μM deoxyshikonin decreased cell viability and its IC50 value was evaluated to be 14.7 μM. The representative images shown in Fig. 2(D) demonstrate that the morphology of HUVECs are normal after treatment with non-toxic doses of resin and abietic acids, but characteristic of the apoptotic morphology (cell rounding and shrinkage) with toxic dose of deoxyshikonin.

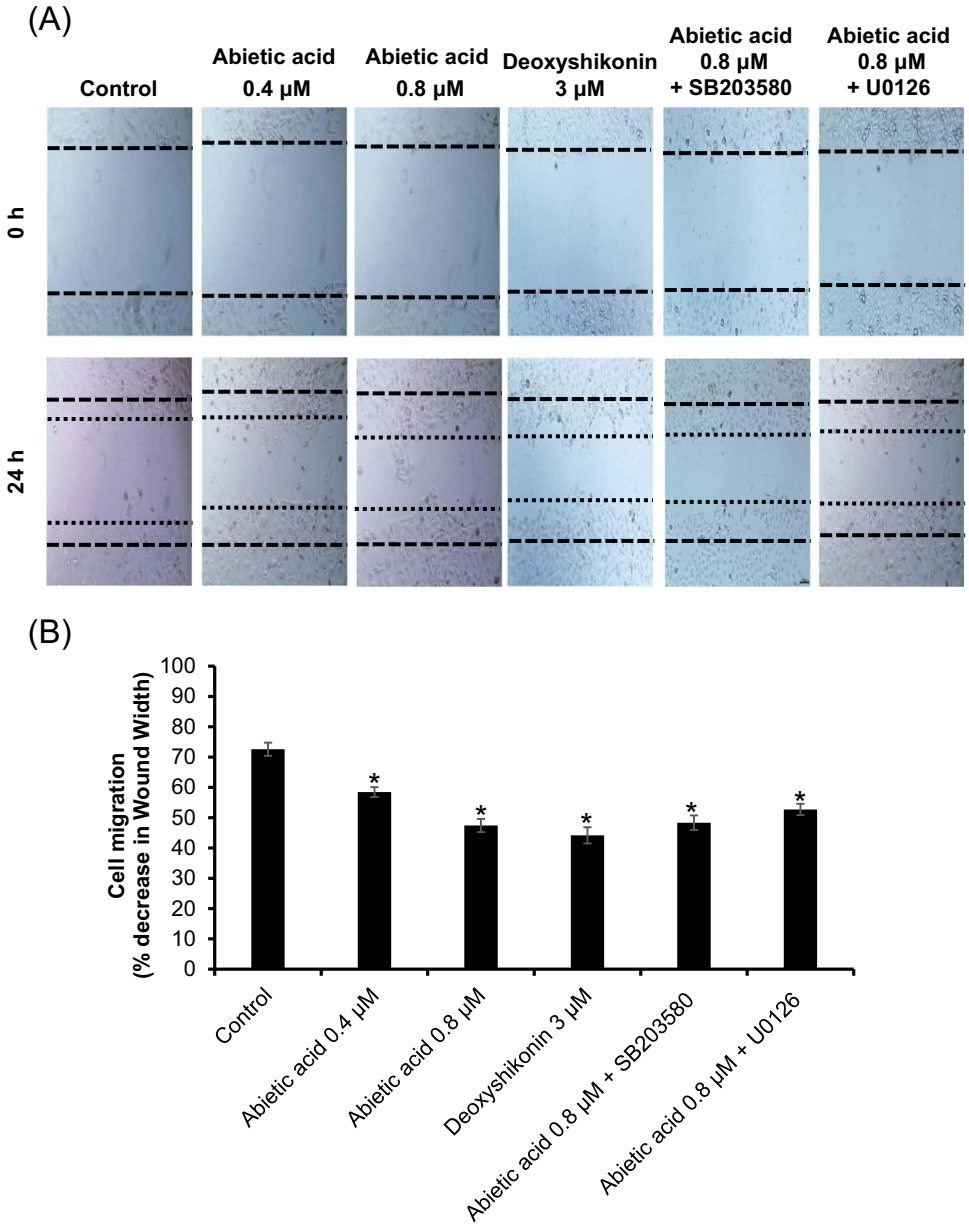

Fig. 6. Effects of abietic acid on HUVEC scratch wound healing. (A) Scratch wound healing of HUVECs was evaluated using cell scratch wound healing assay. Images from the same area were captured at 0 and 24 h after wound infliction. (B) Scratch wound healing of abietic acid-treated HUVECs was assessed using the scratch wound healing assay. Quantification of migration is expressed as a percentage of the wound closure.

3.2. Resin, abietic acid, and deoxyshikonin inhibited tube formation in HUVECs

The effects of resin (doses of 12.5 and 25 μg/mL), abietic acid (doses of 0.4 and 0.8 μM), and deoxyshikonin (doses of 1.6 and 3 μM) on the ability of HUVECs to form tubes (Fig. 3(A)) were tested. As shown in Fig. 2, treatment with resin, abietic acid, and deoxyshikonin increased tube formation in HUVECs, quantified by the number of branching points (Fig. 3(B)). Compared with the control, 17.63% enhancement of tube formation was achieved with 25 μg/mL resin, 24.24% enhancement with 0.8 μM abietic acid, and 69.24% enhancement with 3 μM deoxyshikonin. To study whether the p38 and ERK are involved in abietic acid-induced tube formation, we used the p38 and ERK inhibitors, SB203580 and U0126, respectively. However, the inhibition of ERK or p38 had no obvious effect on tube formation (Fig. 3B).

3.3. Abietic acid inhibited HUVEC cell migration

Cell migration is a step in the angiogenic response; thus, we assessed the effect of abietic acid on HUVEC migration. As shown in Fig. 4, abietic acid treatment increased cell migration. Compared with the control, 0.4 and 0.8 μM abietic acid resulted in cell migration increases of 9.23% and 25.17%, respectively. In particular, 0.8 μM abietic acid significantly increased cell migration ($p < 0.05$). However, SB203580 and U0126 exerted no obvious effect on abietic acid-induced migration of HUVECs (Fig. 4(B)).

3.4. Abietic acid increased angiogenic protein expression in HUVECs

The phosphorylation in Akt and MAP kinase signaling pathways is known to be involved in the signaling pathways for proliferation and migration of endothelial cells (Barrientos et al., 2008; Lee et al., 1994).

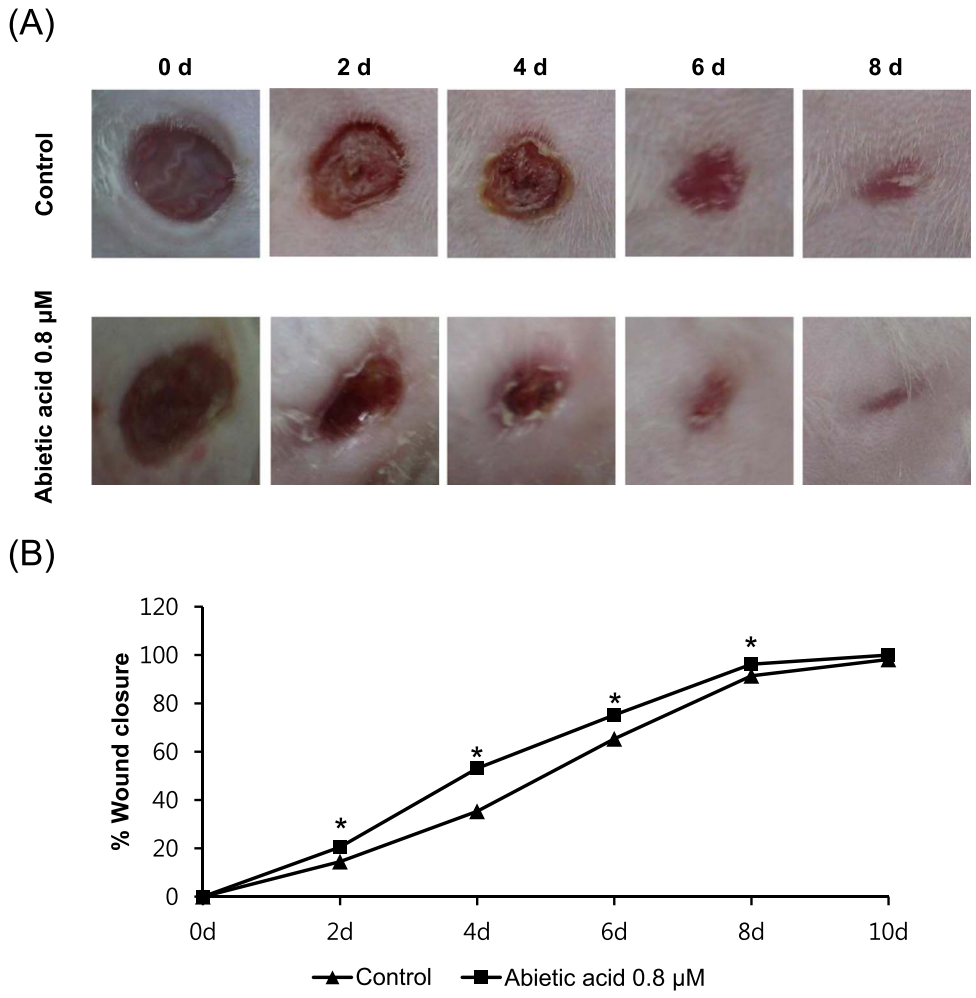

Fig. 7. Effects of abietic acid on wound healing in a mouse model of cutaneous wounds. (A) Comparison of wound closure among the three groups (mice treated with 0.8 μM of abietic acid and the control). Representative photographs of the wounds at day 0, 2, 4, 6, and 8 after grafting. (B) Quantitative analysis showed that wound size reduced in the abietic acid-treated groups compared to the control after 2 days.

Thus, we investigated the effects of abietic acid on the phosphorylation of Akt and MAP kinases. Western blotting analysis was performed to further explore the effect of abietic acid on the MAPK signaling pathway in HUVECs (Fig. 5(A)). As shown in Fig. 5(B), the Western blot analysis showed that the expression of p-p38 (1.14 ± 0.01 and 1.16 ± 0.01-fold at 0.4 and 0.8 μM, respectively) and p-ERK (1.50 ± 0.02 and 2.01 ± 0.01-fold at 0.4 and 0.8 μM, respectively) were increased in the cells treated with abietic acid and deoxyshikonin, compared to the control. In addition, the treatments of specific inhibitors for p38 (SB203580) and ERK (U0126) deceased the protein expressions of p-p38 and p-ERK (Fig. 5).

3.5. Abietic acid ameliorated cell scratch wound healing in HUVECs

The effect of abietic acid on the scratch wound healing of HUVECs was tested in an in vitro wound healing model, in which scratch wounds were generated in confluent cell cultures (Fig. 6(A)). The repair rate of scarification in the presence of 0.4 and 0.8 μM of abietic acid reached 58% and 47%, respectively, at 24 h (Fig. 6(A) and (B)). Incubation of HUVECs with SB203580 and U0126 slightly increased the cell migration induced by 0.8 μM of abietic (Fig. 6B)). However, these effects were statistically not significant. Therefore, p38 and ERK are not the direct factors that regulate abietic acid-induced decrease in migration of HUVECs.

3.6. Abietic acid improved wound healing in mouse model

As shown in Fig. 7(A), the 0.8 μM abietic acid-treated group exhibited accelerated wound healing compared to the controls. The wound closure was observed 2 days post-treatment, and became more evident after 4 days. Quantitative analysis demonstrated that 0.8 μM abietic acid had an effect on wound closure compared to control groups at 2, 4, 6, and 8 days after treatment (Fig. 7(B)).

In conclusion, this study demonstrated that abietic acid enhanced wound healing of skin in an animal model. Abietic acid also promoted proliferation of human fibroblast cells and angiogenesis in vitro; these processes may contribute to the enhancement of wound healing. Our results demonstrated for the first time the cutaneous wound healing properties of abietic acid, which may be associated with angiogenic activity and the proliferation of fibroblast cells. Our findings warrant further investigation of abietic acid to identify the phytoconstituents responsible for wound healing, as well as investigation of agents for the topical treatment of wounds.

Conflict of interest

The author(s) declare no competing interest.

Authors' contributions

Ki Sung Kang was the primary investigator in this study. Yun Kyung

Lee, Jeong-Eun Yoo, Seok Sun Roh, Jun Yeon Park, Myoung-Sook Shin, Hae-Jeung Lee, Seulah Lee, Ki Hyun Kim and Ki Sung Kang designed and performed experimental work, analyzed the statistical data, and interpreted results. Jun Yeon Park, Yun Kyung Lee, Su-Nam Kim, Dong-Soo Lee, Noriko Yamabe, Ki Hyun Kim and Ki Sung Kang drafted and critically evaluated the manuscript. All the authors read and approved the final manuscript.

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