Note

Inhibition of Migration and Invasion in Melanoma Cells by β -Escin via the ERK/NF- κ B Signaling Pathway

HyeongSeob Kwak,^{a,#} Hongyan An,^{a,#} Md Badrul Alam,^b Won-Sik Choi,^{c,d} Sang Yong Lee,^e and Sang-Han Lee^{*,a,b,d}

^aDepartment of Food Science & Biotechnology, Kyungpook National University; Daegu 41566, Korea; ^bFood & Bio-Industry Research Institute, Kyungpook National University; Daegu 41566, Korea; ^cResearch Institute, JeonjinBio Co., Ltd.; Daegu 42713, Korea; ^dDepartment of Nano Science and Technology, Graduate School, Kyungpook National University; Daegu 41566, Korea; and ^eDepartment of Neuro Surgery, Pennsylvania State University; Hershey, PA 17033, U.S.A.

Received March 28, 2018; accepted July 30, 2018

β -Escin, a natural triterpene saponin was extracted from *Aesculus hippocastanum* seeds, which have been widely used to treat inflammation in traditional medicine. In an effort to study the possible anti-tumor effects of β -escin, we performed wound healing, invasion, and adhesion assays to examine the effects of β -escin on cell migration, invasion, and angiogenesis. Our results revealed that β -escin inhibits cell migration as well as motility in B16F10 and SK-MEL5 cells in a dose-dependent manner. RT-PCR and Western blot analysis showed that β -escin increased *TIMP-1*, *-2* while significantly downregulated *phosphorylated extracellular signal-regulated kinase (p-ERK)* expression, and suppressing nuclear factor-kappa B (NF- κ B) and inhibitor of nuclear factor-kappa B (I κ B) expression. Overall, the data from the current study suggest that β -escin has the potential for inhibiting both metastatic and angiogenic activities, and are the earliest evidence for the involvement of the NF- κ B/I κ B signaling in β -escin-induced anti-tumor effects.

Key words β -escin; extracellular signal-regulated kinase; nuclear factor-kappaB (NF- κ B); cell migration; invasion

β -Escin is a type of triterpene saponin isolated from *Aesculus hippocastanum* seeds. It has been widely used to treat inflammation for a long time in traditional medicine in China, Korea, and Japan.¹⁾ Recent studies have investigated and demonstrated the protective effects of β -escin in vascular, lung, and liver injuries.^{2–4)} Moreover, β -escin has been found to possess anti-cancer activity because of its ability to inhibit cell growth in several cancer cell lines.^{5–7)} However, till date, the effects of β -escin on wound healing, migration, and angiogenesis in both mouse and human melanoma have not been studied and are yet to be investigated. Hence, the purpose of this study is to examine the anti-tumor activities of β -escin using the mouse (B16F10) and human (SK-MEL5) melanoma cell lines, with respect to its regulation of the migration, invasion, and adhesion properties.

Here, we report that β -escin decreases cell migration, invasion, and adhesion in melanoma cell lines and that these effects may include the nuclear factor-kappa B/inhibitor of nuclear factor-kappa B (NF- κ B/I κ B) signaling event. Our findings provide the first insight into the signaling-mediated anti-tumor potential of β -escin, and hence, we strongly suggest that β -escin should be considered as a potential candidate for development as a preventive/therapeutic agent.

MATERIALS AND METHODS

Chemicals and Reagents All chemicals, solvents, reagents, and β -escin ($\geq 95\%$ pure, E1378) used in the experiments were purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). β -Escin was dissolved in dimethyl sulfoxide

(DMSO) prior to use.

Cell Culture B16F10 and SK-MEL5 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, U.S.A.; no. CRL-6475; no. HTB-70). Cell culture media and fetal bovine serum (FBS) were purchased from Hyclone (GE Healthcare Life Science, Logan, UT, U.S.A.). The B16-F10 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin, while the SK-MEL5 were cultured in Roswell Park Memorial Institute Medium (RPMI 1640) supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in a humidified atmosphere with 5% CO₂.

Cytotoxicity and Anti-tumor Assays To assess cytotoxicity, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was carried out as described earlier elsewhere.⁸⁾ For the wound healing assay, cells seeded on a 6-well plate (3×10^5 cells/well) were incubated for 24 h, and wounding was performed with a yellow pipette tip. They were then washed twice with PBS. New media, with or without β -escin, was filled up in the wells and the cells were further incubated for 24 h at 37°C. As a positive control, 50 μ M of dykellic acid was used. Following this, images of the assayed cells were captured at 0, 12, and 24 h using a microscope (ECLIPSE TE2000-U, Nikon, Tokyo, Japan). For the invasion assay, Transwell inserts (pore size 8 μ m, BB Biosciences, NY, U.S.A.) were coated with 100 μ L Matrigel (1 mg/mL) and incubated for 6 h at 37°C. Cells treated with various concentrations of β -escin were seeded on the Transwell inserts with serum-free medium, and 750 μ L of complete medium with 1% bovine serum albumin (BSA) as chemoattractant was added to the lowest chamber of the plate. After 24 h of incubation, the invaded cells were fixed with 3.5% formaldehyde and

[#]These authors contributed equally to this work.

*To whom correspondence should be addressed. e-mail: sang@knu.ac.kr

methanol, followed by staining with 0.1% crystal violet. The images of the assayed cells were captured another five parts of membrane by a microscope (ECLIPSE TE2000-U), and the invasive cells were counted. Adhesion assay was carried out as described previously elsewhere.^{9,10)}

RT-PCR Total cellular RNA was isolated from β -escin-treated melanoma cells using the TRIzol reagent (Life Technology, CA, U.S.A.) according to the commercially available manufacturing protocol as described previously. cDNA was synthesized from 2 μ g of total RNA using RT-&GO Mastermix kit (MP Biomedicals, CA, U.S.A.), and PCR amplification was performed with the following specific primers: mouse tissue inhibitor of metalloproteinase-1 (*timp-1*) forward-ACC AGA ACA CCA TCG AG ACC and reverse-AAAGCA TCA TCC ACG GTT TC; mouse *timp-2* forward-GAG TGCCAG ATG TTG CAG AA and reverse-CCA TCA AAG GGG AAG CTGT; mouse matrix metalloproteinase-7 (*mmp-7*) forward-GGG TTT CTG TCC AGA CCA AG and reverse-GGA TGCCGT CTA TGT CGT CT; mouse *mmp-13* forward-TTG ATG GCA AAG GTG GTA CA and reverse-CGA AAT GTG CTG GGG TTA AG; human *timp-1* forward-CCG CCA TGCA AAA AGT TCT AT and reverse-GCC TTG ATCTCA GTCCAAA; human *timp-2* forward-GAC ATT CGC CTC TCT TTCCA and reverse-AGG TCCCCT CAG TCC AGA GT; human *mmp-2* forward-GAC ATT CGC CTC TCT TTCCA and reverse-AGG TCCCCT CAG TCC AGA GT; human *vegf* forward-GAC ATT CGC CTC TCT TTCCA and reverse-AGG TCCCCT CAG TCC AGA GT; mouse glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) forward-ATG TTCCAG TAT GACTCCAC and reverse-GCC AAA GTT GTC ATG GAT GA; and human glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) forward-ATG TTCCAG TAT GACTCCAC and reverse-GCC AAA GTT GTC ATG GAT GA (Bioneer, Daejeon, Korea). The PCR products were detected by 1% agarose gel electrophoresis, and the banding pattern was subsequently imaged using a ChemiDOC™ XRS⁺ Molecular Imager (Bio-Rad, Hercules, CA, U.S.A.). The band density was measured using Image Lab software (5.0 version; Bio-Rad). The mRNA levels were normalized to those of the housekeeping genes, glyceraldehyde 3-phosphate dehydrogenases. The resulting PCR products were analyzed by 2% agarose gel electrophoresis and visualized using RedSafe™ (iNtRON Biotechnology, Sungnam, Korea).

Western Blot Analysis B16F10 and SK-MEL5 cell lysates were prepared using a standard protocol, mixed with sample buffer (250 mM Tris-HCl at pH 6.8, 0.5 M dithiothreitol (DTT), 10% bromophenol blue, 50% glycerol, and 5% β -mercaptoethanol), and denatured at 100°C for 5 min. Proteins in the samples (10 μ g) were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Following electrotransfer to nitrocellulose membranes (Whatman, Dassel, Germany), the membranes were incubated overnight with the following specific primary antibodies-human anti-extracellular signal-regulated kinase (ERK) 1/2 (ADI-KAP-MA001), human anti-phospho-ERK 1/2 (BML-SA275), human anti-NF- κ B (SC53744), human anti-I- κ B (SC945), and human anti-phospho I- κ B (SC7977) (Santa Cruz Biotechnology, CA, U.S.A.). Anti-rabbit immunoglobulin G (IgG)-horseradish peroxidase (HRP) (Santa Cruz Biotechnology) was used as the secondary antibody. The antigen-antibody reaction was detected using an ECL solution

system (ChemiDoc™ XRS⁺, BIO-RAD).

Statistical Analyses The experiments were performed in triplicate, and data were expressed as the mean $\pm$ standard deviation (S.D.). Statistical significance was determined by one-way ANOVA followed by Dunnett's test using the GraphPad Prism 5. The critical level for significance was set at * $p < 0.05$ or ** $p < 0.01$.

RESULTS AND DISCUSSION

We initially examined the motility of β -escin-treated B16F10 and SK-MEL5 cells. We used wound healing and invasion assays to test the *in vitro* effect of β -escin on cell migration, which is an important facet of cell motility, especially studied in cancer. Metastasis is a complex phenomenon that involves discrepancies in cell adhesion, migration, and invasion. Mounting evidences demonstrated the pivotal role of matrix metalloproteinases (MMPs) and the extracellular matrix (ECM) in cancer invasion and metastasis.^{11,12)} It has been observed that ECM degradation by extracellular proteinases leads to tumor progression, invasion, and metastasis.¹³⁾ MMPs are a group of proteinases that are primarily responsible for ECM degradation *in vivo*.¹⁴⁾ Our wound healing assay revealed that while untreated control monolayers showed complete wound healing within 24 h, monolayers treated with 20 μ M β -escin showed clear wound width, suggesting that β -escin might inhibit cell migration in both B16F10 and SK-MEL5 cell lines in a dose-dependent fashion (Fig. 1A). Interestingly, dykellic acid-treated monolayers showed a strong cell growth-inhibitory pattern similar to that of the controls, suggesting that β -escin might inhibit cell migration with or without a cell proliferation inducer. Similarly, the invasion assay revealed that β -escin inhibits the invasion of cells in a concentration-dependent manner up to 73% in SK-MEL5 cells and approximately 30% in B16F10 cells (Fig. 1B). From the above results, we can conclude that β -escin inhibits wound healing and invasion *in vitro* in a dose-dependent manner. We further analyzed whether β -escin inhibits cell adhesion in both cell lines. The results showed that 20 mM β -escin confers an inhibition rate of 55% in SK-MEL5 cells and 17% in B16F10 cells (Fig. 1C). Subsequently, to determine the association of β -escin with metastasis-related markers, RT-PCR analysis was performed to detect the transcriptional expression of *MMPs*, *TIMPs*, and *vascular endothelial growth factor (VEGF)*. MMPs are proteinases that are crucial for malignant cells in the proteolytic degradation of the basement membrane and ECM, for migration of the malignant cells and invade to the surrounding tissues.¹⁵⁾ Among them, MMP-2 and MMP-9, owing their type IV collagenase activity, have been reported to play a critical role in cancer cell migration and invasion by contributing to the degradation of the ECM and cancer progression.¹⁶⁾ It has been found that TIMP-2 (21 kDa) exists on the cell surface and is associated with pro-MMP-2. This inhibitor suppresses tumor angiogenesis especially in melanoma and mammary carcinomas. When we studied the involvement of β -escin in angiogenesis, *TIMP-2* expression increased by 37 and 74% in the β -escin-treated cells of B16F10 and SK-MEL5, respectively. *TIMP-1* expression also increased slightly with β -escin treatment in both cell lines but the increase did not surpass that of *TIMP-2*. However, significant changes in the expression of *TIMP-1*, *MMP-7*, *-13* in B16F10 cells, and that of *TIMP-1*,

Fig. 1. β -Escin Prevents Cell Migration, Invasion and Adhesion in Murine/Human Melanoma Cells

(A) Inhibition of migration was measured by wound healing assay in B16F10 and SK-MEL5 cells. After wounding, cells were treated with dykellic acid or β -escin for 24h. The image was captured by ECLIPSE TE2000-U at 0, 12, and 24h, respectively. (B) Inhibition of invasion was measured by the Transwell invasion assay. The upper lane is of B16F10 cells and bottom lane is of SK-MEL5 cells. Transwell inserts were coated with 100 μ L Matrigel (1 mg/mL), and β -escin-treated cells were seeded in the Matrigel-coated Transwells for 24h. (C) Inhibition of cell adhesion by β -escin. * $p < 0.05$ and ** $p < 0.01$ denote significant difference between control and β -escin-treated cells in (B) or (C).

MMP-2, and *VEGF* in SK-MEL5 cells were not observed (Fig. 2A).

Mitogen-activated protein kinases (MAPKs), including c-Jun N-terminal kinase (JNK), p38, and ERK have been demonstrated to be crucial for cell migration.^{17,18)} To check whether β -escin has the potential for MAPK protein phosphorylation in SK-MEL5 cells, Western blot analysis was performed using anti-ERK 1/2, anti-phospho-ERK 1/2, anti-NF- κ B, anti-I κ B, and anti-phospho-I κ B antibodies. In β -escin-treated SK-MEL5 cells, phosphorylation of ERK was significantly downregulated compared with the control after 30–120min stimulation (Fig. 2B, left), and as expected, this effect also suppressed in a dose-dependent manner (Fig. 2B, right). It has been observed that suppression of ERK expression contributes to silibinin-inhibited cell migration and invasion in human osteosarcoma MG-63 cell lines.¹⁹⁾ Fisetin was also found to inhibit the migration and invasion of A549 cells through ERK1/2 inhibition. Furthermore, to investigate whether the anti-tumor effect of escin was attributed to ERK signaling suppression, the effect of U0126 (ERK inhibitor) on SK-MEL5 cell migration with or without escin was examined. As shown in Fig. 2C, the result of wound healing assay dem-

onstrated that the escin-induced inhibition of wound healing was significantly enhanced by using an ERK inhibitor. Based on these observations and our results, it can be inferred that β -escin significantly suppressed the ERK1/2 signaling pathway, thereby enhancing the inhibitory effects of β -escin on cell migration and invasion. It has been reported that NF- κ B plays a major role in the immune-inflammatory response, and is associated with numerous skin diseases and skin cancer. Activated NF- κ B translocates to the nucleus to bind to the promoter or enhancer regions of specific genes and then induces the expression of relevant genes, including various MMPs.^{17,18)} Expressions of not only NF- κ B but also p-I κ B were inhibited by β -escin in a concentration-dependent manner in SK-MEL5 cells (Fig. 2D). In contrast, β -escin did not show any other MAPK protein phosphorylation in B16F10 cells.

We, herein, show for the first time that β -escin inhibits cell migration and motility in B16F10 and SK-MEL5 cell lines in a dose-dependent manner, and thereby decreasing the levels of NF- κ B while increasing those of I κ B. These findings indicate that β -escin not only activates *TIMP-1*, -2 and *p-ERK* expression but also inhibits NF- κ B and I κ B expression, suggesting that β -escin should be considered as a potential candidate for

Fig. 2. Comparison of mRNA and Protein Expressions of β -Escin-Treated Melanoma Cells

(A) mRNA expression of B16F10 or SK-MEL5 melanoma cells analyzed by RT-PCR. RNA was isolated from β -escin-treated cells using TRIzol. cDNA was synthesized from 2 μ g of total RNA and PCR amplified with specific primers. The mRNA levels were normalized to that of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*gapdh*). (B) Western blot analysis of MAPK protein expression in SK-MEL5 cells. Cell lysates were prepared using a standard protocol, mixed with sample buffer and denatured at 100°C for 5 min. Proteins in the samples (10 μ g) were separated by 10% SDS-PAGE. Following electrotransfer to nitrocellulose membranes, the membranes were incubated overnight with the primary antibodies. The antigen-antibody reaction was detected using an ECL solution system. Statistical analysis of the Western blotting results. * $p < 0.05$ and ** $p < 0.01$ vs. the control group. (C) Inhibition of extracellular signal-regulated kinase 1/2 signaling enhances the anti-metastatic effect of escin in SK-MEL5 cells. Cells were treated with escin (20 μ M) and/or U0126 (20 μ M) for 24 h, and then assessed by a wound healing assay. Statistical analysis of the Western blotting results. * $p < 0.05$ vs. the control group. E: β -escin, U: U0126 (D) Western blot analysis of NF- κ B signaling in SK-MEL5 cells. Statistical analysis of the Western blotting results. ** $p < 0.01$ vs. the control group.

future development as an anti-cancer agent.

Acknowledgment We thank Dr. Hyung-U Son for his critical advice during the experiment.

Conflict of Interest The authors declare no conflict of interest.

REFERENCES

- 1) Fu F, Hou Y, Jiang W, Wang R, Liu K. Escin: inhibiting inflammation and promoting gastrointestinal transit to attenuate formation of postoperative adhesions. *World J. Surg.*, **29**, 1614–1620, discussion, 1621–1622 (2005).
- 2) Guillaume M, Padieau F. Veinotonic effect, vascular protection, anti-inflammatory and free radical scavenging properties of horse

- chestnut extract. *Arzneimittelforschung*, **44**, 25–35 (1994).
- 3) Xin W, Zhang L, Fan H, Jiang N, Wang T, Fu F. Escin attenuates acute lung injury induced by endotoxin in mice. *Eur. J. Pharm. Sci.*, **42**, 73–80 (2011).
- 4) Liu ZP, Hu P, Li CH, Wang XL, Xiao X. Therapeutic value of aescine in traumatic acute lung injury. *J. Traum. Surg.*, **15**, 497–499 (2013).
- 5) Patlolla JM, Raju J, Swamy MV, Rao CV. Beta-escin inhibits colonic aberrant crypt foci formation in rats and regulates the cell cycle growth by inducing p21(waf1/cip1) in colon cancer cells. *Mol. Cancer Ther.*, **5**, 1459–1466 (2006).
- 6) Piao S, Kang M, Lee YJ, Choi WS, Chun YS, Kwak C, Kim HH. Cytotoxic effects of escin on human castration-resistant prostate cancer cells through the induction of apoptosis and G2/M cell cycle arrest. *Urology*, **84**, 982.e1–982.e7 (2014).
- 7) Lee HS, Hong JE, Kim EJ, Kim SH. Escin suppresses migration and invasion involving the alteration of CXCL16/CXCR6 axis in human gastric adenocarcinoma AGS cells. *Nutr. Cancer*, **66**, 938–945 (2014).
- 8) Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods*, **65**, 55–63 (1983).
- 9) Sullivan RM, Stone M, Marshall JF, Uberall F, Rotenberg SA. Photo-induced inactivation of protein kinase calpha by dequalinium inhibits motility of murine melanoma cells. *Mol. Pharmacol.*, **58**, 729–737 (2000).
- 10) Yao Z, Che XC, Lu R, Zheng MN, Zhu ZF, Li JP, Jian X, Shi LX, Liu JY, Gao WY. Inhibition by tyrosinleutide (YSL) on the invasion and adhesion of the mouse melanoma cell. *Mol. Med.*, **13**, 14–21 (2007).
- 11) Stallings-Mann M, Radisky D. Matrix metalloproteinase-induced malignancy in mammary epithelial cells. *Cells Tissues Organs*, **185**, 104–110 (2007).
- 12) Kessenbrock K, Plaks V, Werb Z. Matrix metalloproteinase: Regulators of the tumor microenvironment. *Cell*, **141**, 52–67 (2010).
- 13) Liotta LA, Stetler-Stevenson WG. Tumor invasion and metastasis: An imbalance of positive and negative regulation. *Cancer Res.*, **51** (Suppl. S18), 5054s–5059s (1991).
- 14) Yoon WH, Jung YJ, Kim TD, Li G, Park BJ, Kim JY, Lee YC, Kim JM, Park JI, Park HD, No ZS, Lim K, Hwang BD, Kim YS. Gabexatemesilate inhibits colon cancer growth, invasion, and metastasis by reducing matrix metalloproteinases and angiogenesis. *Clin. Cancer Res.*, **10**, 4517–4526 (2004).
- 15) Gialeli C, Theocharis AD, Karamanos NK. Roles of matrix metalloproteinases in cancer progression and their pharmacological targeting. *FEBS J.*, **278**, 16–27 (2011).
- 16) Overall CM, Dean RA. Degradomics. Systems biology of the protease web. Pleiotropic roles of MMPs in cancer. *Cancer Metastasis Rev.*, **25**, 69–75 (2006).
- 17) Hsieh YS, Chu SC, Yang SF, Chen PN, Liu YC, Lu KH. Silibinin suppresses human osteosarcoma MG-63 cell invasion by inhibiting the ERK-dependent c-Jun/AP-1 induction of MMP-2. *Carcinogenesis*, **28**, 977–987 (2007).
- 18) Liao YC, Shih YW, Chao CH, Lee XY, Chiang TA. Involvement of the ERK signaling pathway in fisetin reduces invasion and migration in the human lung cancer cell line A549. *J. Agric. Food Chem.*, **57**, 8933–8941 (2009).
- 19) Shih YL, Chou HM, Chou HC, Lu HF, Chu YL, Shang HS, Chung JG. Casticin impairs cell migration and invasion of mouse melanoma B16F10 cells via PI3K/AKT and NF- κ B signaling pathways. *Environ. Toxicol.*, **32**, 2097–2112 (2017).