Inhibition of Migration and Invasion in Melanoma Cells by $\beta$-Escin via the ERK/NF-\(\kappa\)B Signaling Pathway

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$\beta$-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 $\beta$-escin, we performed wound healing, invasion, and adhesion assays to examine the effects of $\beta$-escin on cell migration, invasion, and angiogenesis. Our results revealed that $\beta$-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 $\beta$-escin increased TIMP-1, -2 while significantly downregulated phosphorylated extracellular signal-regulated kinase (p-ERK) expression, and suppressing nuclear factor-kappa B (NF-\(\kappa\)B) and inhibitor of nuclear factor-kappa B (1xB) expression. Overall, the data from the current study suggest that $\beta$-escin has the potential for inhibiting both metastatic and angiogenic activities, and are the earliest evidence for the involvement of the NF-\(\kappa\)B/1xB signaling in $\beta$-escin-induced anti-tumor effects.

Key words $\beta$-escin; extracellular signal-regulated kinase; nuclear factor-kappa B (NF-\(\kappa\)B); cell migration; invasion

$\beta$-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. Recent studies have investigated and demonstrated the protective effects of $\beta$-escin in vascular, lung, and liver injuries. Moreover, $\beta$-escin has been found to possess anti-cancer activity because of its ability to inhibit cell growth in several cancer cell lines. However, till date, the effects of $\beta$-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 $\beta$-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 $\beta$-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-xB/IxB) signaling event. Our findings provide the first insight into the signaling-mediated anti-tumor potential of $\beta$-escin, and hence, we strongly suggest that $\beta$-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 $\beta$-escin (95% pure, E1378) used in the experiments were purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). $\beta$-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. For the wound healing assay, cells seeded on a 6-well plate (3×104 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 $\beta$-escin, was filled up in the wells and the cells were further incubated for 24 h at 37°C. As a positive control, 50 µM of dykelic 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 µm, BB Biosciences, NY, U.S.A.) were coated with 100 µL of Matrigel (1 mg/mL) and incubated for 6 h at 37°C. Cells treated with various concentrations of $\beta$-escin were seeded on the Transwell inserts with serum-free medium, and 750 µL 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

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methanol, followed by staining with 0.1% crystal violet. The images of the assayed cells were captured using 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 TRIsol reagent (Life Technology, CA, U.S.A.) according to the commercially available manufacturing protocol as described previously. cDNA was synthesized from 2 μg of total RNA using RT-αGO 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 AGA CC and reverse-AAAGCATCA TCC AC CG GTT TC; mouse timp-2 forward-GAGTGCC CAG ATG TG CAG AA and reverse-CCA TCA AAG GGG AAG CTG GT; mouse matrix metalloproteinase-7 ( mmp-7) forward-GGTT TTT CTT TCC AGC CCA AG and reverse-GGATG CTT CTA TGT CGT CT; mouse mmp-13 forward-TTG ATG GCC AAG GGT GTA CA and reverse-CCA AAT GTG CTG GGG TTA AG; human mmp-2 forward-AGACC CGC CTC TCT TTC CA and reverse-AGG TCC CCT CAG TCC AGA GT; human mmp-2 forward-GACATTGG CTC TCT TTTCCA and reverse-AGG TCC CCT CAG TCC AGA GT; human vegf forward-GACATTGG CTC TCT TTTCCA and reverse-AGG TCC CCT CAG TCC AGA GT; mouse glyceraldehyde 3-phosphate dehydrogenase ( gapdh) forward-ATG TTC CAG TAT GAC TCC AC and reverse-GCCA AAG GGT GTC ATG GGT GA; and human glyceraldehyde 3-phosphate dehydrogenase ( gapdh) forward-ATG TTC CAG TAT GAC TCC AC and reverse-GCCA AAG GGT GTC ATG GGT GA (Bioneer, Daejeon, Korea). The PCR products were detected by 1% agarose gel electrophoresis, and the banding pattern was subsequently visualized using RedSafe™ (nIutron 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 mM dithiothreitol (DTT), 10% bromophenol blue, 50% glycerol, and 5% β-mercaptoethanol), and denatured at 100°C for 5 min. Proteins in the samples (10 μg) 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-κB (SC53744), human anti-IκB (SC945), and human anti-phospho IκB (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±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.13,14 MMPs are a group of proteinases that are primarily responsible for ECM degradation.15 We used wound healing assay revealed that while untreated control monolayers showed complete wound healing within 24h, monolayers treated with 20 μM 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, dykelic 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 μM 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 (21kDa) 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, TIMP-2, and TIMP-3, respectively.
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. 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-κB, anti-IκB, and anti-phospho-IκB antibodies. In β-escin-treated SK-MEL5 cells, phosphorylation of ERK was significantly downregulated compared with the control after 30–120 min 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. 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 demonstrated 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-κB plays a major role in the immune-inflammatory response, and is associated with numerous skin diseases and skin cancer. Activated NF-κB 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. Expression of not only NF-κB but also p-IκB 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-κB while increasing those of IκB. These findings indicate that β-escin not only activates TIMP-1, -2 and p-ERK expression but also inhibits NF-κB and IκB expression, suggesting that β-escin should be considered as a potential candidate for...
future development as an anti-cancer agent.

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Conflict of Interest The authors declare no conflict of interest.

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