Shikonin induces apoptosis of HaCaT cells via the mitochondrial, Erk and Akt pathways

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Abstract. Shikonin, which is a major ingredient of the traditional Chinese herb Lithospermum erythrorhizon, possesses various biological functions, including antimicrobial, anti-inflammatory, and antitumor activities. The present study aimed to determine the molecular mechanisms underlying the effects of shikonin on HaCaT cell apoptosis. Treatment with shikonin significantly inhibited the viability of HaCaT cells in a dose- and time-dependent manner, and promoted cell cycle arrest at G0/G1 phase and apoptosis. In addition, shikonin treatment reduced the mitochondrial membrane potential and induced reactive oxygen species generation. The results of a western blot analysis demonstrated that shikonin significantly activated caspase 3 expression, downregulated B-cell lymphoma 2 (Bcl-2) expression, and upregulated Bcl-2-associated X protein and Bcl-2 homologous antagonist killer expression in a dose-dependent manner in HaCaT cells. Furthermore, shikonin decreased extracellular signal-regulated kinase (Erk) and Akt phosphorylation. These results indicated that shikonin may exert its anti-proliferative effects by inducing apoptosis via activation of the mitochondrial signaling pathway and inactivation of the Akt and Erk pathways in HaCaT cells. Therefore, the present study suggested that shikonin may have potential as a component of therapeutic strategies for the treatment of skin diseases.

Introduction

Psoriasis is a complex inflammatory skin disease that is characterized by inflammatory cell infiltration, increased dermal vascularity, and keratinocyte proliferation (1). The HaCaT cell line is an immortalized line of human epidermal keratinocytes, which has previously been used in experiments examining the effects of therapeutic drugs on keratinocytes (2). A series of clinical studies have indicated that certain Chinese herbs are effective in psoriasis treatment (3,4). Shikonin, which is a napthoquinone isolated from the Chinese herbal plant Lithospermum erythrorhizon, has long been used in traditional medicine to treat hemorrhoids, burns, infected wounds, anal ulcers, external wounds, and psoriasis (5,6).

Shikonin is known to possess several medicinal properties, including promotion of wound healing, and antibacterial, anti-inflammatory and antitumor effects (5). In addition, shikonin has been shown to exert inhibitory effects on tumor necrosis factor-α-induced angiogenesis, tumor cell-induced angiogenesis, and normal programmed developmental angiogenesis (7). Shikonin also inhibits angiogenesis in inflammatory skin diseases, such as psoriasis (8). Shikonin treatment has been reported to activate the caspase pathway, in order to induce cellular apoptosis in HL-60 leukemia cells (9) and human colorectal cancer cells (10). Shikonin induces apoptosis via the reactive oxygen species (ROS)/extracellular signal-regulated kinase (Erk) pathway in osteosarcoma cells (11). Furthermore, it inhibits cell proliferation by decreasing Erk activities in human epidermoid carcinoma cells (12), and triggers apoptosis through the ROS/Akt and nuclear factor-xB (NF-xB) pathways in hepatocellular carcinoma cells (13). Shikonin also elevates ROS generation to induce apoptosis in human glioma cells (14), and is able to increase intracellular ROS generation during the early phase of apoptotic progression, alongside a disturbance in mitochondrial transmembrane potential, in SK-Hep-1 hepatoma cells (15).

Two apoptotic pathways have been established: The cell death receptor pathway and the mitochondria-initiated pathway.
pathway (16). Members of the B-cell lymphoma 2 (Bcl-2) family are key regulators of mitochondria-initiated apoptosis. When anti-apoptotic members of the Bcl-2 family are inhibited and/or proapoptotic members are activated, mitochondrial integrity is disrupted and cytochrome c is released into the cytosol (17), thus activating caspase 9 and caspase 3, and subsequently leading to cell apoptosis (18). The present study aimed to investigate whether shikonin was able to induce mitochondrial-initiated apoptosis in HaCaT cells, in order to inhibit cell proliferation.

Materials and methods

Chemicals and reagents. Shikonin was purchased from Shanghai PureOne Biotechnology Co., Ltd. (Shanghai, China), and its purity was determined to be ~99.5% using high-performance liquid chromatography. Cell culture medium (RPMI-1640), trypsin, 3-(4,5-dimethyl-2-thiazolyl) -2,5-diphenyl-2H-tetrazolium bro tide (MTT), Hoechst 33258 and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). RNase, propidium iodide (PI), Annexin V-fluorescein isothiocyanate (FITC), ROS and 5,5′,6,6′-tetrachloro-1,1,3,3′-tetracythylbenzimidazolylcarbocyanine iodide (JC-1) were purchased from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China). Fetal bovine serum (FBS) was purchased from National Hyclone (Lanzhou) Bio-engineering Co., Ltd. (Lanzhou, China). Rabbit polyclonal antibodies against caspase 3 (9662), Akt (9272), phosphorylated (p)-Akt (9271), Erk1/2 (9102) and p-Erk1/2 (9101) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibodies against cyclin B1 (55004-1-AP), cyclin D1 (60186-1-Ig), cyclin E (11554-1-AP), Bcl-2 (12789-1-AP), Bcl-2-associated X protein (Bax; 50599-2-Ig) and Bcl-2 homologous antagonist killer (Bak; 14673-1-AP) were purchased from Proteintech Group, Inc. (Rosemont, IL, USA), and antibodies against glyceraldehyde 3-phosphate dehydrogenase (GAPDH; sc-365062) and β-actin (sc-47778) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (H+L) secondary antibodies (A0208) were purchased from Beyotime Institute of Biotechnology, Nanjing, China).

Cell culture. The HaCaT normal human epidermal keratinocyte cell line was obtained from the Chinese Academy of Sciences (Kunning, China). The cells were cultured in RPMI-1640 supplemented with 10% FBS and 1% penicillin-streptomycin (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) at 37°C in an atmosphere containing 5% CO₂.

Cell viability assay. Cell viability was determined using the MTT colorimetric assay. Exponentially growing cells were seeded in 96-well plates in culture medium at density of 2x10⁴ cells/well. Following a 24 h incubation, the cells were treated with various concentrations of shikonin between 0-20 μM for 24 and 48 h. Subsequently, the medium was discarded, and 200 μl MTT (0.5 mg/ml) was added to each well and incubated for 4 h at 37°C. The medium was then removed, and the formazan salt was dissolved in 150 μl DMSO. Optical density of the cells was determined using a Bio-Rad Model 680 microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at a wavelength of 490 nm. Cell viability was expressed as a percentage of the control. Three replicate wells were used for each analysis.

Cell cycle analysis using flow cytometry. Exponentially growing cells were seeded in 6-well culture plates in culture medium at a density of 4x10⁴ cells/well. Following treatment with 1, 2 and 4 μM shikonin for 24 h, both adherent and floating cells were collected, washed in ice-cold phosphate-buffered saline (PBS), and fixed with ice-cold 70% ethanol overnight. After fixation, the ethanol was removed via centrifugation, and the cells were suspended in 0.1 ml RNase solution at 37°C for 30 min. Subsequently, 0.4 ml PI was added and incubated at 4°C for 30 min in the dark. Stained cells were analyzed using a FACS Canto flow cytometer (BD Biosciences, San Jose, CA, USA). Data acquisition and analyses were performed using WinMDI 2.9 software (BD Biosciences).

DNA morphological observation using Hoechst staining assay. To visualize apoptotic cell death and nuclear morphology, the cells were stained with Hoechst 33258. Briefly, the treated cells were collected, washed twice in PBS, and fixed in 4% formaldehyde for 10 min. The cells were then washed and stained with Hoechst 33258 for 20 min at room temperature, after which they were examined under a Nikon Eclipse TE2000-PFS inverted fluorescence microscope (Nikon Corporation, Tokyo, Japan) at 340 nm. The number of apoptotic cells was measured by calculating the percentage of cells displaying chromatin condensation compared with the total number of cells.

Detection of apoptosis using flow cytometry. Cellular apoptosis was detected using an Annexin V-FITC/PI Apoptosis Detection kit. Briefly, HaCaT cells were treated with 1, 2 and 4 μM shikonin for 24 h, and collected via centrifugation. The cells were then washed in PBS and resuspended in binding buffer, and the apoptotic cell death rate was examined using Annexin V-FITC and PI double staining (incubation with 5 μl Annexin V-FITC and 10 μl PI for 15 min in the dark), according to the manufacturer’s protocol. Subsequently, the cells stained with Annexin V-FITC/PI were detected using a FACS Canto flow cytometer (BD Biosciences). Data acquisition and analyses were performed using WinMDI 2.9 software. All experiments were performed in triplicate.

Measurement of the mitochondrial membrane potential (Δψm). The Δψm was assessed as previously described (19). Briefly, the HaCaT cells were treated with 1, 2 and 4 μM shikonin for 4 h, and were harvested and collected via centrifugation. The cells were resuspended in PBS and were then incubated with 10 μM JC-1 for 15 min at room temperature in the dark. The fluorescently labeled cells were washed in PBS and analyzed using a BD FACS Calibur flow cytometry system (excitation, 485 nm; emission, 530/590 nm; BD Biosciences). The 590 nm/530 nm fluorescence ratio was used to quantify the Δψm. Data acquisition and analyses were performed using WinMDI 2.9 software.
Intracellular ROS assay. The ROS generation assay was performed as described in our previous study (20). Briefly, the HaCaT cells were treated with 1, 2 and 4 µM shikonin for 24 h, and were harvested and collected via centrifugation. The cells were resuspended in PBS and were then incubated with 10 µM DCF-DA (Nanjing KeyGen Biotech Co., Ltd.) for 30 min at room temperature in the dark. The fluorescently labeled cells were washed in PBS and analyzed using a BD FACSCalibur flow cytometry system (excitation, 485 nm; emission, 538 nm; BD Biosciences). Data acquisition and analyses were performed using WinMDI 2.9 software.

Western blot analysis. HaCaT cells, treated with 1, 2 and 4 µM shikonin for 24 h, were lysed in radioimmunoprecipitation assay lysis buffer containing a protease and phosphatase inhibitor cocktail on ice for 30 min. Then lysate was then collected and centrifuged at 56,000 x g for 15 min at 4°C. Protein concentration was determined using the Bicinchoninic Acid Protein Quantification kit (Pierce Biotechnology, Inc., Rockford, IL, USA), according to the manufacturer's protocol. Protein lysates were then denatured for 10 min at 95°C and 50 µg protein per lane was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA). After immunoblotting, the PVDF membranes were blocked with 5% skimmed milk for at least 2 h, washed and incubated with anti-caspase 3, anti-Akt, anti-p-Akt, anti-Erk 1/2, anti-p-Erk 1/2, anti-cyclin B1, anti-cyclin D1, anti-cyclin E, anti-Bcl-2, anti-Bax, anti-Bak, anti-GAPDH and anti-β-actin primary antibodies at 4°C overnight (all 1:1,000). Subsequently, the membranes were washed three times with Tris-buffered saline with Tween 20 and incubated with the HRP-conjugated goat anti-rabbit IgG (H+L) secondary antibodies (1:10,000) at 37°C for 1 h. Chemiluminescence detection was assayed using an enhanced chemiluminescence detection kit (Pierce Biotechnology, Inc.). Results were analyzed using Quantity One software (version 4.4.0.36; Bio-Rad Laboratories, Inc.), in order to obtain the optical density ratio of the target protein to GAPDH and β-actin.

Statistical analysis. All of the data, which were obtained from at least three independent experiments, were expressed as the mean ± standard deviation for each group. Statistical analyses, including Student's t-test, one-way analysis of variance and regression analysis, were performed using GraphPad Prism 4.0 software (GraphPad, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Shikonin inhibits the growth of HaCaT cells. The chemical structure of shikonin is shown in Fig. 1A (21). The effects of shikonin on the growth of HaCaT cells were evaluated using the MTT assay. Human HaCaT cells were treated with various concentrations of shikonin (0-20 µM) for 24 and 48 h. As shown in Fig. 1B, the proliferation of shikonin-treated HaCaT cells was markedly suppressed at 24 and 48 h compared with the control group. These results suggest that shikonin may exhibit dose- and time-dependent inhibitory effects on the viability of HaCaT cells. The calculated half maximal inhibitory concentration (IC50) values for shikonin were 6.34 and 2.43 µM at 24 and 48 h, respectively. Based on these IC50 values, doses of 1, 2 and 4 µM shikonin were selected for use in subsequent experiments to assess HaCaT cell growth inhibition. Notably, concentrations of DMSO, which was used to dissolve shikonin, were maintained at <0.2% (v/v).

Shikonin induces a cell cycle arrest at G0/G1 phase in HaCaT cells. To investigate the mechanisms underlying shikonin-induced inhibition of cell proliferation, changes in cell cycle progression were detected after shikonin treatment using flow cytometry. The percentage of cells that accumulated in G0/G1 phase was significantly increased following treatment with 2 and 4 µM shikonin for 24 h, as compared with the control group (P<0.05 and P<0.01, respectively; Fig. 2A and B). These results indicate that shikonin may partially mediate HaCaT cell growth inhibition by inducing G0/G1 phase cell cycle arrest.

Following a 24 h treatment with 1, 2 or 4 µM shikonin, the expression levels of cell cycle regulatory proteins were examined in HaCaT cells using western blot analysis. As shown in Fig. 2C, cyclin D1 expression was significantly increased following treatment with 2 and 4 µM shikonin, as compared with the control group (P<0.01 and P<0.001, respectively); whereas cyclin B1 and cyclin E expression levels were decreased in a dose-dependent manner (Fig. 2C and D).
Shikonin induces apoptosis of HaCaT cells. To assess whether shikonin-induced cell growth inhibition was associated with cell apoptosis, the effects of shikonin on apoptosis were evaluated by flow cytometry using Annexin V-FITC/PI double staining. As shown in Fig. 3A, the percentage of dead cells (Annexin V-positive, PI-positive) was increased in a dose-dependent manner. The percentage of apoptotic HaCaT cells following a 24 h treatment with 2 or 4 µM shikonin was 22.0 and 33.6%, respectively; therefore, the percentage of apoptotic cells were significantly increased, as compared with the control group (P<0.05 and P<0.01, respectively; Fig. 3A and B). These results correspond to the addition of values that are shown in the higher and lower right quadrants of each panel, which indicate the early and late stages of apoptosis, respectively.

Nuclear fragmentation is an important characteristic of apoptosis, which is easily distinguished by Hoechst staining. As shown in Fig. 3C, marked nuclear condensation or nuclear fragmentation was induced following treatment of the cells with 2 or 4 µM shikonin for 24 h. These results clearly indicate that inducing cellular apoptosis is a primary mechanism underlying the inhibitory effects of shikonin on HaCaT cell growth. In addition, shikonin-induced apoptosis occurred in a dose-dependent manner.

Shikonin decreases the Δψm and induces ROS generation. To evaluate whether the mitochondrial pathway was responsible for shikonin-induced apoptosis, the effects of shikonin on the Δψm after 4 h were examined using the mitochondria-specific dye, JC-1. The percentage of cells with depolarized Δψm significantly increased following treatment of HaCaT cells with 1, 2 or 4 µM shikonin for 4 h, as compared with the untreated cells, and this trend occurred in a dose-dependent manner (P<0.05 and P<0.01, respectively; Fig. 4A and B). Depolarization of the Δψm is a characteristic event of early apoptosis.

Since intracellular ROS generation is considered to be associated with mitochondrial dysfunction, the present study further...
examined whether shikonin could stimulate ROS generation in HaCaT cells. A significant increase in ROS generation was observed in the HaCaT cells treated with shikonin compared with the untreated cells (P<0.001; Fig. 4C and D).

**Shikonin regulates Bcl-2 family members and activates caspase family proteins.** Bcl-2 family proteins are known to be involved in the apoptotic process, due to their ability to form membrane channels in mitochondria. The Bcl-2 proteins control cytochrome c release into the cytosol, which activates caspase 3, subsequently leading to cell apoptosis (22). Therefore, the present study examined the expression levels of Bcl-2 family proteins (Bcl-2, Bax and Bak), and caspase 3 in HaCaT cells treated with 1, 2 or 4 µM shikonin for 24 h using western blotting. The effects of shikonin on the expression levels of Bcl-2 family proteins are presented in Fig. 5A and B. Shikonin significantly decreased the expression levels of the anti-apoptotic Bcl-2 protein, and increased the expression levels of the proapoptotic Bax and Bak proteins in HaCaT cells, as compared with the untreated control cells. A dose-dependent significant increase in the Bax/Bcl-2 ratio was observed (P<0.001). The present study also verified the induction of apoptosis via caspase 3 activation using western blotting. The results revealed that treatment with shikonin significantly increased caspase 3 cleavage, as compared with the untreated cells (P<0.001; Fig. 5C and D). These results indicate that shikonin-induced HaCaT cell apoptosis is mediated via a mitochondria-dependent pathway.

**Erk and Akt pathways are associated with shikonin-induced HaCaT cell apoptosis.** The mitogen-activated protein kinases (MAPKs) and phosphatidylinositol 3-kinase (PI3K)/Akt pathways have essential roles in regulating cell proliferation, cell
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survival and apoptosis. To evaluate whether shikonin is capable of modulating the PI3K/Akt and MAPK signaling pathways in order to induce the inhibition of HaCaT cell proliferation, the effects of shikonin on the phosphorylation of signaling molecules in these two pathways were detected. As shown in Fig. 5E and F, treatment of HaCaT cells with 1, 2 or 4 µM shikonin for 24 h led to a significant reduction in the expression levels of p-Erk1/2 and p-Akt, as compared with the total protein expression levels of Erk1/2 and Akt (P<0.001). These results suggest that shikonin may downregulate the phosphorylation of these proteins in a dose-dependent manner.

Discussion

HaCaT cells have been used extensively as in vitro models of psoriasis (2,23,24). Apoptotic inhibition occurs in psoriatic lesional keratinocytes (25), resulting in keratinocyte hyperproliferation, which induces psoriasis. Therefore, the present study hypothesized that effective therapeutic agents for the treatment of psoriasis should inhibit keratinocyte hyperproliferation and induce apoptosis.

The results of the present study revealed that shikonin significantly decreased HaCaT cell viability and induced a G0/G1 phase cell cycle arrest. These results indicated that cell cycle arrest may be partially responsible for shikonin-induced HaCaT cell growth inhibition. Phosphatidylserine is translocated from the inner to the outer leaflet of the plasma membrane in apoptotic cells. In the present study, Annexin V-FITC/PI staining was used to determine whether apoptosis had occurred. Compared with untreated cells, the fluorescence intensity of HaCaT cells treated with shikonin was significantly increased in a dose-dependent manner, which was indicative of apoptosis. These findings are similar to those from a previous report, which demonstrated that apoptosis of HaCaT human keratinocytes can be induced by celastrol, which is a triterpenoid isolated from Celastrus orbiculatus, via inhibition of NF-κB activity (24).

Figure 4. Effects of shikonin on the mitochondrial membrane potential (Δψm) and induction of reactive oxygen species (ROS) generation. (A and B) Δψm was assessed using flow cytometry following treatment of the HaCaT cells with 1, 2 or 4 µM shikonin for 24 h. (C and D) ROS generation was assessed by flow cytometry following treatment of the HaCaT cells with 1, 2 and 4 µM shikonin for 24 h. Data are presented as the mean ± standard deviation obtained from three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 vs. the control group. DCF, dichlorofluorescein.

Figure 5. Effects of shikonin on the phosphorylation of Erk1/2 and Akt. (A) Western blotting was used to detect the expression of the p-Erk1/2 and p-Akt proteins in HaCaT cells treated with 1, 2 or 4 µM shikonin for 24 h. (B) Quantification of the Western blot images showed a significant reduction in the expression levels of p-Erk1/2 and p-Akt (P<0.001) compared with the expression levels of total Erk1/2 and Akt.
Apoptosis is a highly regulated process leading to programmed cell death, which is regulated by several signaling pathways, including the caspase and MAPK pathways (26). The Bcl-2 protein family has an important role in the mitochondrial apoptotic pathway, which results in the release of mitochondrial cytochrome c, leading to caspase 9 activation and subsequent caspase 3 activation (27). The present study examined the effects of shikonin on mitochondrial function. The results demonstrated that the ΔΨm was significantly decreased, which was accompanied by an increase in ROS generation, indicating that mitochondrial dysfunction had occurred. Subsequently, an increase in caspase 3, Bax and Bak protein expression, and a decrease in Bcl-2 protein expression was observed in the shikonin-treated HaCaT cells, thus suggesting that shikonin-induced apoptosis occurred via the mitochondrial apoptotic pathway.
Previous studies have demonstrated that shikonin potently inhibits cell growth and induces cell apoptosis in various types of cells via its effects on several molecular targets, including members of the MAPK family (28), Akt/apoptosis signal-regulating kinase 1p38 (29) and NF-κB (30). The present study evaluated the effects of shikonin on the phosphorylation of signaling molecules in these pathways in HaCaT cells. Treatment with shikonin resulted in marked reductions in the expression levels of p-Erk1/2 and p-Akt in a dose-dependent manner, thus suggesting that shikonin may dose-dependently downregulate the phosphorylation of these proteins.

In conclusion, in HaCaT human epidermal keratinocyte cells, shikonin is able to exert its anti-proliferative activity by inducing cellular apoptosis via the mitochondrial apoptotic pathway. The mechanism underlying these effects is associated with inactivation of the Akt and Erk pathways. Therefore, the present study suggested that shikonin may have potential as a component of therapeutic strategies for the treatment of skin diseases.

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