Shikonin regulates HeLa cell death *via* caspase-3 activation and blockage of DNA synthesis

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SHIKONIN REGULATES HᴇLᴀ CELL DEATH *VIA* CASPASE-3 ACTIVATION AND BLOCKAGE OF DNA SYNTHESIS

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Shikonin, isolated from the plant *Lithospermum erythrorhizon* Sieb. Et Zucc, inhibited tumor cell growth and induced cell death in various tumor cells, with 50% growth inhibition of human cervical cancer cells, HeLa, at $18.9 \pm 1.1\,\mu\mathrm{mol\,L}^{-1}$. Treated with $40\,\mu\mathrm{mol\,L}^{-1}$ shikonin, HeLa cells underwent marked apoptotic morphological changes such as a round shape, membrane blebbing and apoptotic bodies derived from the fragmented nuclei. Another hallmark of apoptosis, DNA fragmentation, was observed by gel electrophoresis. Shikonin ($10\,\mu\mathrm{mol\,L}^{-1}$) significantly blocked the transition from G1 to S phase in the HeLa cell cycle. Pan-caspase inhibitor (Z-VAD-FMK), caspase-3 inhibitor (Z-DEVD-FMK) or caspase-8 inhibitor (Z-IETD-FMK) effectively inhibited shikonin-induced cell death, while caspase-1 inhibitor (Ac-YVAD-CMK) and caspase-9 inhibitor (Z-LEHD-FMK) failed to affect cell death. Caspase-3 activity significantly increased within 12 h after shikonin treatment. Reduced expression of inhibitor of caspase-activated deoxyribonuclease (ICAD) after exposure to shikonin for 12 h suggests the resultant activation of caspase-activated deoxyribonuclease (CAD), leading to apoptosis.

Keywords: Shikonin; Apoptosis; HeLa cells; Caspases

INTRODUCTION

Shikonin (Fig. 1) is a naphthoquinone that has been isolated from the ground rhizome of *Lithospermum erythrorhizon* Sieb. Et Zucc, which has been used as herbal medicine in East Asia [1]. Shikonin has anti-inflammatory [2,3], antifungal [4], antitumor effects and inhibits angiogenesis *in vivo* and *in vitro* [5,6]. It has been also reported that shikonin derivatives inhibit DNA topoisomerases and induce human premyelocytic leukemia HL60 cell apoptosis [7–9]. Topoisomerase inhibitors, widely used in cancer therapy, induce apoptosis in various tumor cells. Etoposide, a topoisomerase II inhibitor, induced apoptosis in HL60 cells [10].

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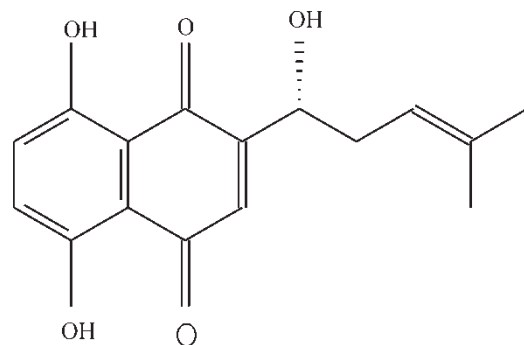
FIGURE 1   Structure of shikonin.

Apoptosis, or programmed cell death, is an important process in biological systems, including normal cell turnover, immune system, embryonic development, metamorphosis and endocrine-dependent tissue atrophy. Apoptosis is characterized by a decrease in cell volume, condensation and fragmentation of nuclear chromatin and dilatation of the endoplasmic reticulum. The cells undergoing apoptosis execute the death program by activating caspases. Caspases, a family of at least 14 related cysteine proteases, are mediators of apoptotic processes caused by various inducers such as Fas ligand, TNFα, etoposide and actinomycin D [11]. Caspase-3 plays a crucial role in the apoptotic progression. As an executor it is activated from an inactive precursor by upstream caspases, and activated caspase-3 then cleaves ICAD, leading to activation of a latent cytosolic endonuclease, CAD, which cleaves DNA into oligonucleosomal fragments, a hallmark of apoptotic cell death [12,13].

Recently, many natural products have been reported to exert their antitumor effects through various apoptotic pathways [14,15]. In the present study, human cervical epithelial cancer cells, HeLa, were used to examine the molecular mechanisms involved in the apoptotic signal transduction pathways.

RESULTS AND DISCUSSION

Inhibition of Cell Growth by Shikonin

Inhibitory effects of shikonin on the growth of four tumor cell lines were detected by MTT assay. The IC_{50} for A375-S2 cells, MCF-7 cells and L929 cells were 7.1 ± 0.9, 22.1 ± 2.5 and $14.5 \pm 1.2\,\mu\text{mol}\,L^{-1}$, respectively (Table I). Shikonin ($2.5–80\,\mu\text{mol}\,L^{-1}$) induced HeLa cell death in a time- and dose-dependent manner, as measured by MTT assay. The IC_{50} of a 48 h time course was $18.9 \pm 1.1\,\mu\text{mol}\,L^{-1}$ (Fig. 2).

TABLE I   Inhibitory effects of shikonin on the growth of various tumor cells[a]

Cell lines	IC_{50} ($\mu\text{mol}\,L^{-1}$)
HeLa	18.9 ± 1.1
A375-S2	7.1 ± 0.9
MCF-7	22.1 ± 2.5
L929	14.5 ± 1.2

[a] Cells were incubated with shikonin for 48 h. Percentage viability was determined by the MTT assay. Each result represents three independent experiments.
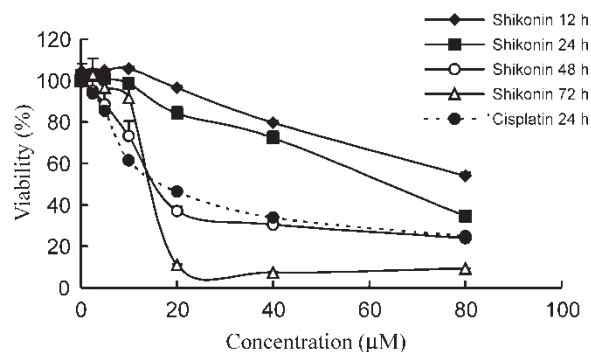
FIGURE 2   Inhibitory effects of shikonin on the growth of HeLa cells. HeLa cells were treated with various concentrations of shikonin. Treatment of HeLa cells with cisplatin for 24 h was used as positive control. The viability was determined by the MTT assay. Data are presented as the mean $\pm$ SD of the results for three independent experiments.

Shikonin Induces Apoptotic Cell Death in HeLa Cells

To determine whether the features of HeLa cell death induced by shikonin were caused by apoptosis or necrosis, cellular morphology and DNA fragmentation were examined. The cells underwent marked morphological changes (Fig. 3A, right) upon treatment with $40 \, \mu mol \, L^{-1}$ shikonin compared with the untreated control (Fig. 3A, left). The HeLa cells became round, and granular apoptotic bodies were observed. Hoechst 33258-stained HeLa cells showed that the apoptotic cells had characteristic condensed nuclei (Fig. 3B, right).
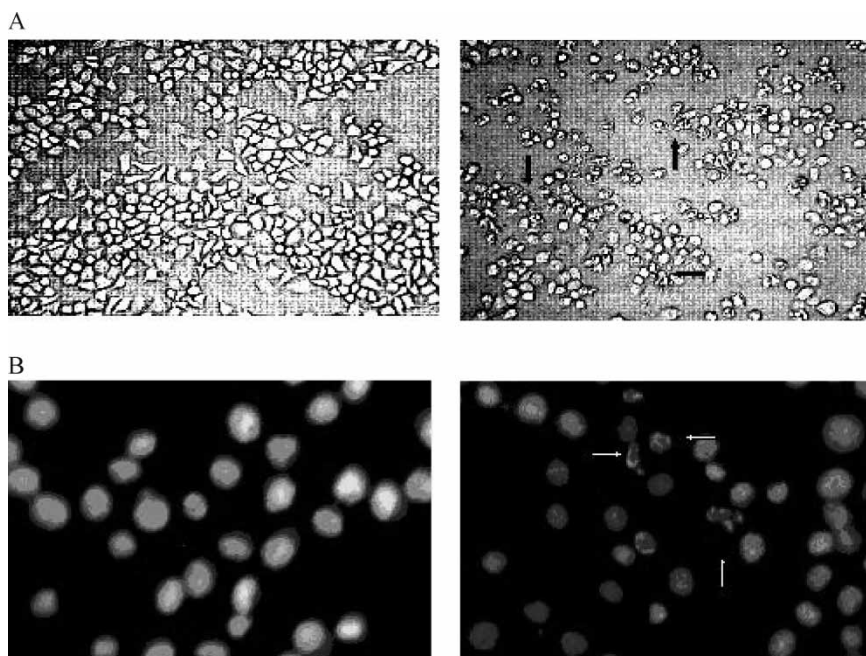
FIGURE 3   Shikonin-induced apoptosis in HeLa cells. (A) Morphological changes of HeLa cells treated with shikonin were observed in the absence (left) and presence (right) of shikonin ($40 \, \mu mol \, L^{-1}$) at 24 h with $\times 200$ magnification. Arrows indicate multiblebbing cells and apoptotic bodies. (B) Morphological changes of HeLa cell nuclei were observed by fluorescence microscopy (left) medium control (right) treated with shikonin ($40 \, \mu mol \, L^{-1}$) for 24 h. Cells were stained with Hoechst 33258 to identify apoptotic cells with $\times 400$ magnification; arrows indicate fragmented nuclei.

A   B   C   D

FIGURE 4   Internucleosomal DNA fragmentation induced in HeLa cells by shikonin. HeLa cells were treated with shikonin for 24 h (lane A, marker, and lane B, 0; lane C, 20; lane D, 40 μmol L^{-1}).

Treatment with 20 and 40 μmol L^{-1} shikonin for 24 h resulted in typical DNA fragmentation in agarose electrophoresis, a hallmark of apoptosis (Fig. 4). These results demonstrated that the cause of some HeLa cell death induced by shikonin was apoptosis.
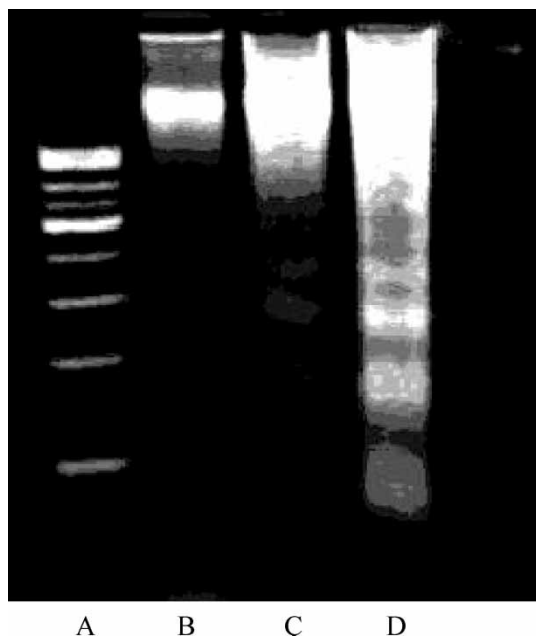
Cell death induced by various stimuli occurs by either of two distinct mechanisms, necrosis or apoptosis. There are some reports that both modes of cell death can be found in the same cell [16]. The ratio of LDH released from viable cells, floating dead cells and the culture medium was used to distinguish the number of apoptotic and necrotic cells. After exposure to 40 μmol L^{-1} shikonin for 24 h, the proportion of apoptotic cells reached a maximum (54.37%) at 24 h, then started to decline, whereas the proportion of necrotic cells increased time dependently (Fig. 5A). Higher doses (80 μmol L^{-1}) of shikonin caused earlier induction of necrosis, compared with the lower dose (Fig. 5B). These results indicate that, in early stages, the major cause of HeLa cell death was apoptosis; however, after 48 h, necrosis plays a greater part in the cell death.

Flow cytometric analysis of 40 μmol L^{-1} shikonin-treated HeLa cells showed that ratio of the apoptotic DNA fragmentation increased in a time-dependent manner (Fig. 6).

Effects of Shikonin on the Cell Cycle

Some natural products reportedly induced cell cycle arrest and apoptosis. The alcohol extract of *Ganoderma lucidum* can inhibit cell growth by preventing the transition from G1 to S phase in HeLa cells and induce cell cycle arrest at the G1 phase in MCF-7 human breast cancer cells [17,18]. Tetrandrine induced cell cycle arrest at the G1 phase and apoptosis in A549 human lung carcinoma cells, which was associated with the induction of p21 [19]. When HeLa cells were treated with 2.5 mmol L^{-1} thymidine (an agent that blocks cell cycle transition from G1 to S phase), the cells were effectively blocked at the G1 phase (Fig. 7a). After further treatment with 0, 0.1, 1 and 10 μmol L^{-1} shikonin for 12 h, the numbers of G2/M phase cells were detected by fluorescence flow cytometry. 10 μmol L^{-1} shikonin
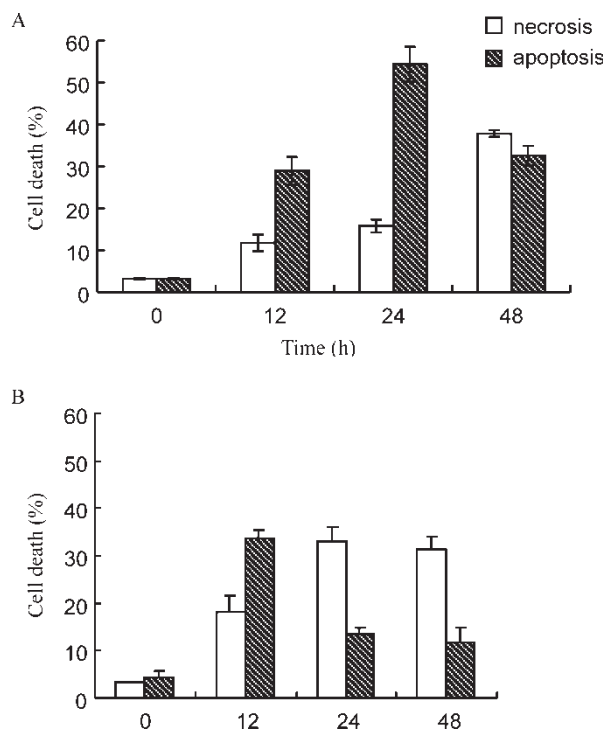
FIGURE 5   Characterization of cell death induced by shikonin in HeLa cells. HeLa cells were treated with (A) 40 and (B) 80 μmol L^{-1} shikonin. The extent of cell death was assessed by LDH activity-based assays. Data are the mean ± SD of the results for three independent experiments.

significantly blocked the transition from G1 to S and S to G2/M phase (Fig. 7e). The proportion of cells in G2/M was 12.22% compared with 55.33% of the shikonin-free culture (Fig. 7b). However, cell cycle blockage was not observed in HeLa cells treated with lower
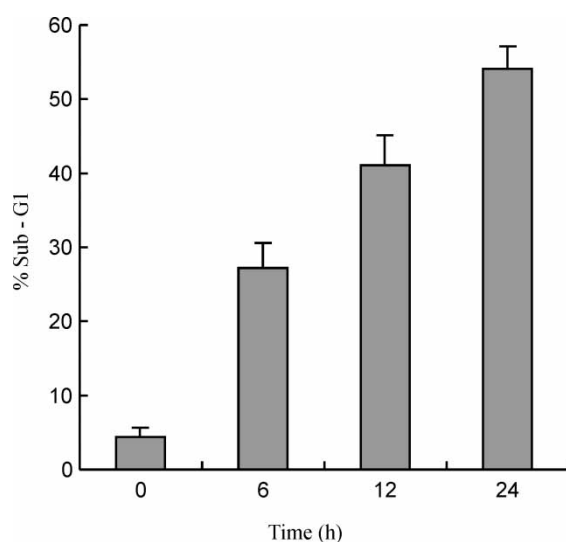
FIGURE 6   Effects of shikonin on the induction of apoptosis by DNA damage in HeLa cells. HeLa cells (1×10^6 cells) were treated with shikonin (40 μmol L^{-1}) and the DNA content after PI staining was analyzed by fluorescence flow cytometry.
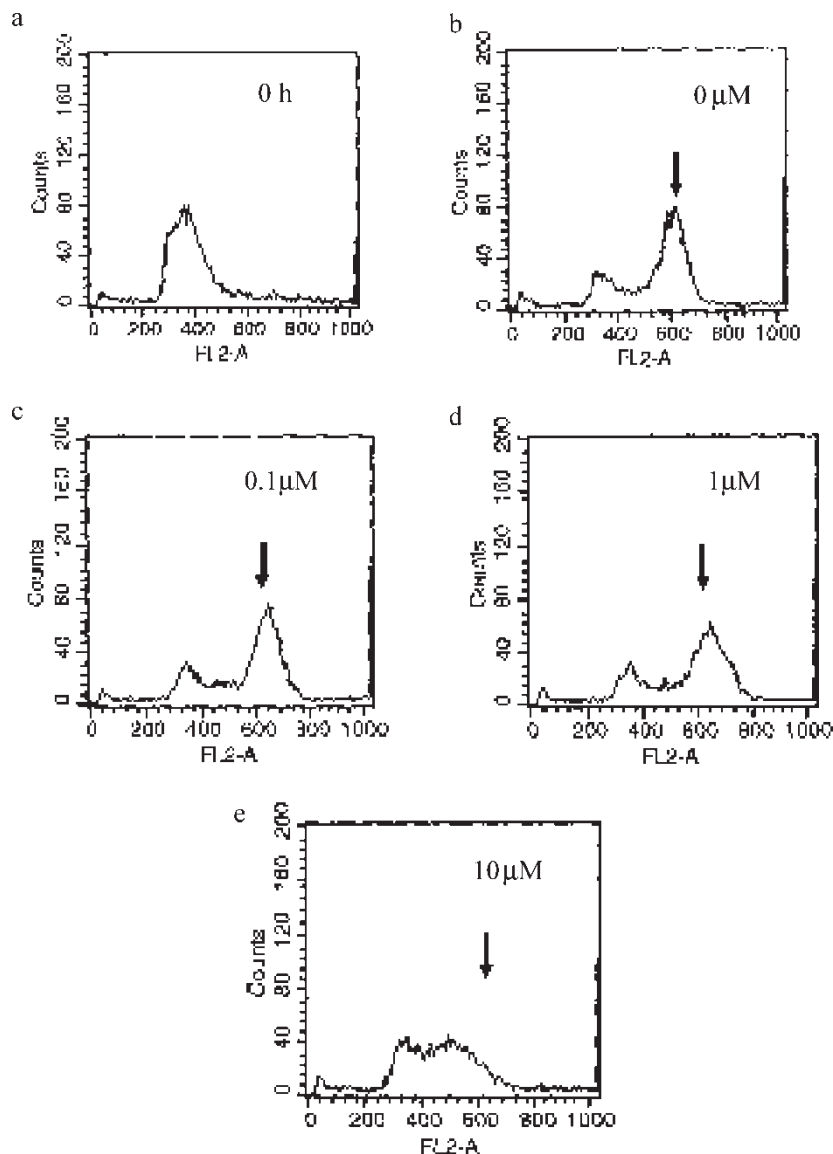
FIGURE 7   Effects of shikonin on the cell cycle. After treatment with $2.5 \, mmol \, L^{-1}$ thymidine (a), HeLa cells were further untreated (b) and treated with 0.1 (c), 1 (d) and $10 \, \mu mol \, L^{-1}$ (e) shikonin for 12 h. Cells were washed with PBS and stained with PI followed by flow cytometric analysis. Arrows indicate cells at the G2/M phase. Cells were blocked at the G1 phase after thymidine treatment (a).

doses (0.1 and $1 \, \mu mol \, L^{-1}$) of shikonin (Fig. 7c,d). It is suggested that shikonin induced HeLa cell apoptosis by blocking the DNA synthesis.

Effects of Caspase Inhibitors on Shikonin-induced Cell Death

To assess the participation of caspases in the shikonin-induced cell death, five caspase inhibitors were applied. Shikonin-induced apoptosis was blocked by pan-caspase inhibitor ($20 \, \mu mol \, L^{-1}$, Z-VAD-FMK), indicating that caspase family proteinases play a role in shikonin-induced HeLa cell apoptosis. Caspase-3 inhibitor ($20 \, \mu mol \, L^{-1}$)

FIGURE 8   Effects of various caspase inhibitors on shikonin-induced HeLa cell death. HeLa cells (1×10^4 cells) were pretreated with Z-VAD-FMK, Z-DEVD-FMK, Z-IETD-FMK, Ac-YVAD-CMK and Z-LEHD-FMK for 1 h and then treated with shikonin ($40 \, \mu mol \, L^{-1}$) for 24 h. Data are presented as the mean $\pm$ SD of the results for three independent experiments ($n = 3$, $^*p < 0.05$, $^{**}p < 0.01$ *vs.* shikonin alone group).
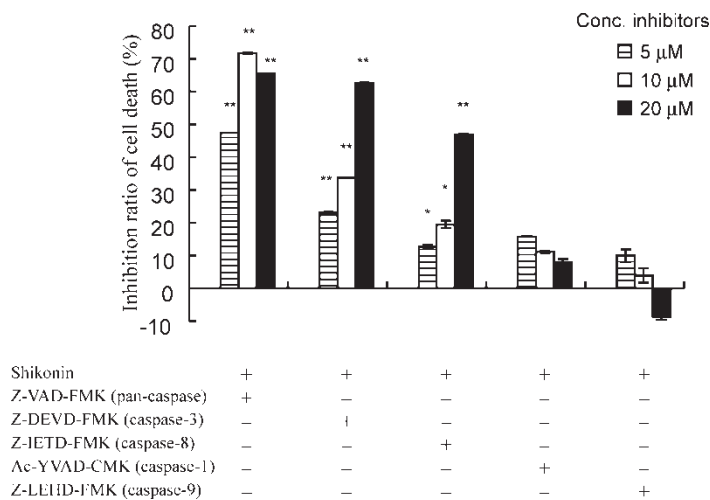
(Z-DEVD-FMK) and caspase-8 inhibitor ($20 \, \mu mol \, L^{-1}$) (Z-IETD-FMK) significantly reversed $40 \, \mu mol \, L^{-1}$ shikonin-induced cell death, whereas caspase-1 inhibitor (Ac-YVAD-CMK) at any doses failed to inhibit cell death (Fig. 8). These results indicate participation of capase-8 activity in shikonin-induced HeLa cell death. It was reported that activation of *c-myc* led caspase-8 to participate in the mitochondrial pathway, which amplified the caspase-8-dependent receptor-interacting protein (RIP) death signal [20]. Therefore, the effects of caspase-9 inhibitor on shikonin-induced HeLa cell death were examined. Three doses of caspase-9 inhibitor (5, 10 and $20 \, \mu mol \, L^{-1}$), Z-LEHD-FMK, failed to reverse the shikonin-induced cell death. Unexpectedly, a high dose of caspase-9 inhibitor rendered the cells even more sensitive to shikonin and the high dose of caspase-1 inhibitor also tended to weakly augment the cell death (Fig. 8). This sensitization might be correlated to a higher production of reactive oxygen radicals [21]. To further confirm that the Apaf-1/caspase-9 apoptosome was not involved in this death pathway, we examined the expression of cytochrome *c* protein by Western blotting assay. However, cytochrome *c* was not released to cytosol from mitochondria in the apoptotic progression (data not shown). These results suggest that shikonin might induce HeLa cell death *via* caspase-9-independent pathways, and apoptotic synergy between c-myc and caspase-8 pathway was not involved in this pathway.

Shikonin Triggers the Activation of Caspase-3 and Caspase-8 in HeLa Cells

To confirm the involvement of caspases in shikonin-induced HeLa cells apoptosis, the activities of caspase-3 and caspase-8 proteinases were measured. Upon treatment of HeLa cells with $40 \, \mu mol \, L^{-1}$ shikonin, caspase-3 activity increased markedly within 12 h after the drug treatment (about 4.4 times that of the control), then continued to reach 6.3 times the control level at 24 h (Fig. 9). Simultaneously, the shikonin-treated HeLa cells underwent relative activation of upstream caspase-8 (about 1.4–2.4 times to the control). This signal transduction pathway resembled the Fas and TNF pathways. Activation of caspase-8 might be mediated by several death receptors including Fas, a plausible mechanism in which the expression of Fas-L would be induced by shikonin in HeLa cells. However, our preliminary
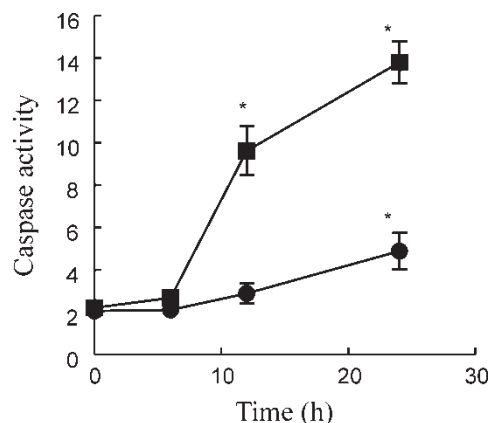
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FIGURE 9　Activities of caspase proteases during apoptosis induced by shikonin. HeLa cells (1×10^6 cells) were incubated with or without shikonin ($40\,\mu\mathrm{mol\,L}^{-1}$). Caspase-3 (square) and caspase-8 (circle) activities (units per mg of protein) were measured. Data are presented as the mean $\pm$ SD of the results for three independent experiments ($n = 3$, $^*p < 0.05$ *vs.* without shikonin group).

data showed the effect of shikonin-induced apoptotic HeLa cell death was not decreased in the presence of competitive antagonistic anti-Fas antibody (data not shown).

Shikonin-induced ICAD Expression in HeLa Cells

To further examine the role of downstream caspase-3 in shikonin-induced HeLa cells apoptosis, we performed Western blot analysis to examine ICAD expression during cell death. ICAD was expressed as two isoforms, a M_r 45,000 isoform (ICAD-L/DFF45) and a M_r 35,000 isoform (ICAD-S/DFF35). ICAD-L/DFF45 was reported to be functional because can act as a molecular chaperone for the nuclease to ensure its correct folding [22]. The activation of CAD is attributed to the cleavage of ICAD, which is specifically cleaved by caspase-3 or -7 [14]. Caspase-3 or -7 initiated apoptotic DNA fragmentation by proteolytical inactivation of ICAD, resulting in the release of active CAD [13]. Exposure of HeLa cells to shikonin for 12 h reduced the expression of inactivating ICAD; this decline was effectively prevented by caspase-3 inhibitor (Z-DEVD-FMK) after 24 h (Fig. 10). Thus, these experiments provide additional evidence of caspase-3 activation in the shikonin-induced apoptotic pathways in HeLa cells.

Pan-caspase inhibitor Z-VAD-FMK, casepase-3 inhibitor Z-DEVD-FMK and caspase-8 inhibitor Z-IETD-FMK did not completely inhibit shikonin-induced HeLa cell death. Consequently, an unknown apoptotic pathways might participate in shikonin-induced HeLa cell death.
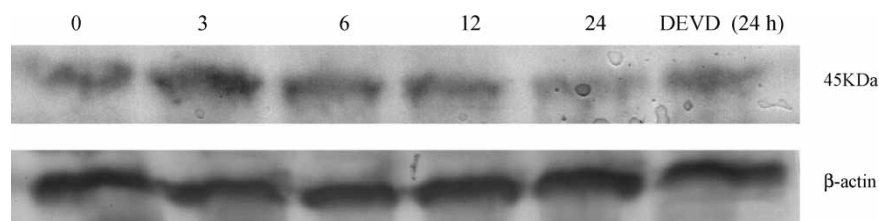

FIGURE 10　Effects of shikonin on the expression of ICAD. Western blotting analysis for ICAD protein expression in HeLa cells treated with shikonin ($40\,\mu\mathrm{mol\,L}^{-1}$) for various time periods.

In conclusion, shikonin, in part, activated caspase-8 in HeLa cells, which triggers mitochondrial pathway-independent caspase cascades, leading to activation of caspase-3 and DNA fragmentation, simultaneously, which blocked the G1 to S transition.

EXPERIMENTAL

Chemicals

Shikonin was obtained from the Beijing Institute of Biologic Products (Beijing, China). The caspase-8 apoptosis detection kit, caspase-3 apoptosis detection kit and polyclonal rabbit antibodies to human ICAD for Western blot analysis were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Ac-Val-Ala-Asp-CMK (Ac-YVAD-CMK), Z-Ile-Glu (OMe)-Thr-Asp (Ome)-FMK (Z-IETD-FMK), Z-LEHD-FMK were purchased from Enzyme Systems (CA, USA). Benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone (Z-DEVD-FMK) and benzyloxy-carbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-FMK) were purchased from Calbiochem (CA, USA). Hoechst 33258, propidium iodide (PI), RNase A, proteinase K and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemicals (St. Louis, MO). An antagonistic anti-Fas antibody (UB2) was obtained from the Medical & Biological Laboratories (Osaka, Japan). Cisplatin was purchased from the Jinzhou Pharmaceutical Factory (Jinzhou, China).

Cell Lines and Cell Culture

Human cervical cancer cells (HeLa #CCL-2), human malignant melanoma cells (A375-S2 #CRL-1872) and mouse fibrosarcoma cells (L929 #CRL-2148) were obtained from the American Type Culture Collection (ATCC Manassas, VA). The cells were cultured in RPMI 1640 (GIBCO, USA) supplemented with 10% fetal bovine serum (Dalian Biological Reagent Factory, Dalian, China), L-glutamine ($2\,mmol\,L^{-1}$, GIBCO), penicillin ($100\,U\,mL^{-1}$) and streptomycin ($100\,\mu g\,mL^{-1}$) and maintained at 37°C with 4% CO_2 in a humidified atmosphere.

Cytotoxicity Assay

The cytotoxic effect of shikonin on HeLa cells was measured by MTT assay as described previously [23]. The cells were dispensed in 96-well flat bottom microtiter plates (NUNC, Roskilde, Denmark) at a density of 1×10^4 cells per well. After 12 h incubation, they were treated with various concentrations of shikonin for the indicated time periods. The cells were incubated with inhibitors for 1 h prior to the administration of shikonin. Cell growth was measured with an ELISA reader (TECAN SPECTRA, Wetzlar, Germany) by MTT assay at the indicated time points.

$$\text{Cell death}\,(\%) = (A_{490,\text{control}} - A_{490,\text{shikonin}})/A_{490,\text{control}} \times 100\%$$

Morphological Changes

HeLa cells were placed in the wells of a six-well plate. After 12 h cell culture they were treated with shikonin for the indicated time periods. The cellular morphology was observed by means of photomicroscopy (Motic Incorporation Ltd, Hong Kong). After cells were treated with shikonin, they were washed by PBS and fixed in 3.7% formaldehyde overnight.

The cells were centrifuged and washed, then stained with Hoechst 33258. Apoptotic cells were identified as cells with condensed and fragmented nuclei.

DNA Fragmentation Assay

HeLa cells (1×10^6 cells) were collected by centrifugation at $150g$ for 5 min, and washed with Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS). The cells were pelleted and suspended in $10 \, mmol \, L^{-1}$ Tris (pH 7.4), $10 \, mmol \, \mu mol \, L^{-1}$ EDTA (pH 8.0), 0.5% Triton X-100 and kept at $4°C$ for 10 min. The supernatant was then incubated with $20 \, mg \, m \, L^{-1}$ of RNase A ($2 \, \mu L$) and $20 \, mg \, m \, L^{-1}$ of proteinase K ($2 \, \mu L$) at $37°C$ for 1 h, and then kept in $0.5 \, M$ NaCl ($20 \, \mu L$) and isopropanol ($120 \, \mu L$) at $-20°C$ overnight, and centrifuged at $15,000g$ for 15 min. DNA was dissolved in TE buffer [$10 \, mmol \, L^{-1}$ Tris (pH 7.4), $10 \, mmol \, L^{-1}$ DETA (pH 8.0)] and subjected to 2% agarose gel electrophoresis at 50 V for 40 min, and stained with ethidium bromide.

LDH Activity-based Cytotoxicity Assay

LDH activity was measured in both floating dead cells and viable adherent cells [22]. One hundred μL ($1 \times 10^5 \, cells \, mL^{-1}$) of a cell suspension were placed in the wells of 96-well plates. They were then cultured in an incubator at $37°C$ for 12 h. After treatment with shikonin, floating cells were collected from the culture medium by centrifugation at $240g$ at $4°C$ for 10 min. The LDH released in the culture medium was used as an index of necrotic cell death (referred to as LDHdn). The pellets were lysed by 1% NP40 in RPMI 1640 ($100 \, \mu L$) for 30 min at $37°C$, and centrifuged at $240g$ at $4°C$ for 10 min. The supernatant was transferred to other 96-well plates. The LDH content from the supernatant was used as an index of apoptotic cell death (referred to as LDHda). The viable adherent cells were lysed by 1% NP40 in RPMI 1640 ($100 \, \mu L$) at $37°C$ for 30 min, and centrifuged at $240g$ at $4°C$ for 10 min. The LDH present in the adherent viable cells was used as an index of viable cells (LDHv). The percentage of apoptotic and necrotic cell death was calculated as follows:

$$\% \text{ apoptosis} = LDHda/(LDHdn + LDHda + LDHv) \times 100; \text{ and}$$

$$\% \text{ necrosis} = LDHdn/(LDHdn + LDHda + LDHv) \times 100.$$

Caspases Activity Assay

HeLa cells (1×10^6 cells) were incubated with or without shikonin. Harvested cells at various time points were washed with PBS and centrifuged at $150g$. Supernatant was aspirated off and cell lysis buffer (provided) was added to an Eppendorf centrifuge at 0.5 mL per 1×10^6 cells. Cells in lysis buffer were incubated on ice for 10 min. Reaction buffer containing DDT ($5 \, \mu L$), of IETD-AFC ($5 \, \mu L$) (or DEVD-AFC) substrate and H_2O ($380 \, \mu L$) was added to each aliquot of cell lysate. The mixtures were then incubated at $37°C$ for 1 h. The fluorescence of the cleaved substrates was determined with a spectrofluorometer set at 400 nm (excitation wavelength) and at 505 nm (emission wavelength). The unit of enzyme activity corresponds to the activity that cleaves the respective substrate in 1 min per mg of protein at $37°C$.

Flow Cytometric Analysis

HeLa cells (1×10^6 cells) were harvested and washed once in cold PBS. Cell pellets were fixed in 70% ethanol and washed in cold PBS. Then the pellets were suspended

in propidium iodide (PI) solution (1 mL) containing $50 \, \mu g \, mL^{-1}$ of PI, 0.1% (w/v) sodium citrate, 0.1% (v/v) Triton X. Cell samples were incubated at 4°C in the dark for at least 15 min, and analyzed by a FACScan flow cytometer (Becton Dickinson).

Cell Cycle Analysis

HeLa cells (1×10^6 cells) were incubated with $2.5 \, mmol \, L^{-1}$ thymidine (cell cycle blocker from G1 to S phase) for 18 h, and collected by centrifugation at 100g for 5 min. The cells were suspended in RPMI 1640 medium supplemented with 10% fetal bovine serum and cultured for 15 h. They were then treated with $2.5 \, mmol \, L^{-1}$ thymidine for 15 h again, centrifuged at 100g for 5 min, washed with PBS, then stained with PI and analyzed by fluorescence flow cytometry. The cells were further cultured with different concentrations of shikonin for 12 h, then collected and centrifuged at 100g for 5 min, and analyzed by fluorescence flow cytometry after staining with PI.

Western Blot Analysis

HeLa cells were harvested and lysed for 1 h on ice in lysis buffer [$50 \, mmol \, L^{-1}$ HEPES (pH 7.4), 1% Triton X-100, sodium orthovanadate ($2 \, mmol \, L^{-1}$), sodium fluoride ($100 \, mmol \, L^{-1}$), EDTA ($1 \, mmol \, L^{-1}$), EGTA ($1 \, mmol \, L^{-1}$), phenylmethanesulfonyl fluoride (PMSF) ($1 \, mmol \, L^{-1}$)], supplemented with proteinase inhibitors aprotinin ($100 \, \mu g \, mL^{-1}$), leupeptin ($10 \, \mu g \, mL^{-1}$), pepstatin ($100 \, \mu g \, mL^{-1}$). The protein concentration was determined by the Folin assay. The lysate was centrifuged at 16,000g at 4°C for 10 min. Equal amounts of total protein were mixed in 2 × loading buffer [$50 \, mmol \, L^{-1}$ Tris-HCl (pH 6.8), 2% SDS, 10% 2-mercaptoethanol, 10% glycerol, and 0.002% bromphenol blue], boiled 5 min, and run on a 12% SDS–polyacrylamide gel electrophoresis. Proteins were electrotransferred onto nitrocellulose membranes. After being blocked with Tween 20–Tris-buffered saline [$50 \, mmol \, L^{-1}$ Tris–HCl (pH 7.5), $150 \, mmol \, L^{-1}$ NaCl, and 0.02% Tween 20] containing 5% nonfat milk at room temperature, the membranes were incubated for 2 h at room temperature with the primary antibodies at 1:200 dilution in blotting buffer. After being washed three times, for 10 min each time, in Tris-buffered saline, the membrane was incubated with a diluted horseradish peroxidase-labeled secondary antibody (1:500) in blotting buffer at room temperature for 1 h. After a further three washes, proteins were detected by chemiluminescence, according to the manufacturer's instructions (Bio-Rad, Hercules, CA).

Statistical Analysis

All data represent at least three independent experiments and are expressed as the mean ± SD unless otherwise indicated. Statistical comparisons were made by Student's t-test; significance was considered as $p < 0.05$.

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