Original article

Phosphorylated extracellular signal-regulated kinase up-regulated p53 expression in shikonin-induced HeLa cell apoptosis

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Keywords: shikonin · apoptosis · HeLa cells · extracellular signal-regulated kinase · p53

Background The role of extracellular signal-regulated kinase 1/2 (ERK1/2) in shikonin-induced HeLa cells apoptosis remains vague. This study was to investigate the activation of caspase pathways and the role of ERK1/2 in human cervical cancer cells HeLa by shikonin.

Methods The inhibitory effect of shikonin on the growth of HeLa cells was measured by MTT assay. Fluorescent microscopic analysis of apoptotic cells stained with 4', 6'-oliiamiclino-2-phenylindole C (DAPI) and Hoechst 33258 was carried out. Caspase-3 and -8 activities were detected using caspase-3 substrate and caspase-8 substrate as substrates respectively. The protein levels of ERK, p53 and p-ERK were determined by Western blot analysis.

Results Shikonin inhibited cell growth in a time- and dose-dependent manner. Caspase-3 and caspase-8 were activated in the apoptotic process and caspase inhibitors effectively reversed shikonin-induced apoptosis. Phosphorylation of ERK resulted in up-regulation of p53 expression, which was blocked by mitogen-activated protein kinase (MEK), inhibitor PD 98059.

Conclusion Shikonin induces HeLa cell apoptosis through the ERK, p53 and caspase pathways.

Chin Med J 2005; 118 (8): 671-677

Apoptosis is a biological process that removes cancerous or virally infected cells. Induction of apoptosis is a viable and important new therapeutic approach to control cancer. The development of cancer is associated with several key events including deregulated cell growth in response to oncogene activation that sensitizes cells to apoptosis. Apoptosis is executed by caspases, a family of intracellular aspartate-specific cysteine proteases which amplify the apoptotic signal and proteolytically process numerous cellular target molecules with different functions.[1] Once apoptosis was initiated, activation of upstream caspase-8 or caspase-10 induced by various stimuli led to activation of downstream caspase-3, -6 or -7, regulating proteins participating in these signal transduction pathways.[2,3]

The extracellular signal regulated kinase (ERK), one of the mitogen-activated protein kinase (MAPK) family, is an important mediator of signal transduction processes that serve to coordinate the cellular responses to a variety of extracellular stimuli. Three major mammalian MAPK subfamilies, ERKs, the c-Jun N-terminal kinases (JNK) and the p38 kinase, were activated through a specific phosphorylation cascade.[4] ERK pathway is activated in a variety of cell types by diverse extracellular stimuli and participates in a wide range of cellular programs including proliferation, differentiation and movement.[5,6] Activation of the ERKs was also associated with apoptosis of tumor cells induced by various stimuli.[7,8] Natural products regulate cell growth in response to oncogene

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activation that induces cell cycle arrest and apoptosis in tumor cell lines.[9] Shikonin, a naphthoquinone compound isolated from the ground rhizome of the Lithospermum erythrorhizon Sieb. et Zucc, which has been used in East Asia for treating burns, has antiinflammatory[10,11] and antitumor effects.[12] It was reported that shikonin inhibited cell growth in tumor cell lines.[13] Although shikonin-induced apoptosis in human premyelocytic leukemia HL60 cells involved in activation of caspase-3 mechanism,[14] identification of upstream mediators and their targets remains vague. The signal transduction pathway of shikonin-induced HeLa cell apoptosis is unclear. In the present study, we demonstrated that phosphorylated ERK contributed to p53 expression in shikonin-induced apoptosis, and amplified the activation of caspase-8 cascade, resulting in apoptosis in HeLa cells.

METHODS

Materials

Shikonin was purchased from National Institute for the Control Pharmaceutical and Biological Products (Beijing, China). Detection kit for caspase-3 and -8, rabbit polyclonal antibodies against ERK1, ERK2, p38, JNK, p-JNK, p-p38 and mouse monoclonal antibodies against p-ERK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal antibody against p53 was obtained from Oncogene Research Products (Boston, MA, USA). Z-Ile-Glu (OMe)-Thr-Asp (OMe)-fluoromethylketone (Z-IETD-FMK) were purchased from ICN (Aurora, OH, USA). Inhibitor of mitogen-activated protein kinase (MEK) (PD98059), benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone (Z-DEVD-FMK) and benzyloxy-carbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-FMK) were from Calbiochem (La Jolla, CA, USA). Hoechst 33258, wortmannin, 4', 6'-diamidino-2-phenylindole (DAPI), RNase A, proteinase K and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Cell lines and cell culture

The human cervical cancer cells (HeLa #CCL-2) were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in RPMI 1640 (GIBCO, NY, USA) supplemented with 10% fetal bovine serum (Dalian Biological Reagent Factory, Dalian, China), L-glutamine (2 mmol/L, GIBCO, USA), penicillin (100 U/ml) and streptomycin (100 μg/ml), and maintained at 37℃ in 4% CO_2 in a humidified atmosphere.

Viability assay

The inhibitory effect of shikonin on the growth of HeLa cells was measured by MTT assay. 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 hours incubation, they were treated with various concentrations of shikonin for the indicated time periods. The cells were incubated with inhibitors for 1 hour 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.

$$\text{Viability}(\%) = 100 - [(A_{490,\ control} - A_{490,\ shikonin}) / A_{490,\ control} \times 100\%]$$

Apoptosis assay

The apoptotic effect of shikonin on HeLa cells was analyzed by DNA staining. HeLa cells were placed on glass coverslips in the wells of a 6-well plate. After a 12-hour cell culture, they were treated with shikonin for the indicated time periods. The cells were washed by PBS and fixed in 3.7% formaldehyde for 12 hours and centrifugated at 2500 r/min at 4℃ for 10 minutes, then stained with Hoechst 33258 or DAPI and fixed on glass microscope slides. Apoptotic cells were identified as cells with condensed and fragmented nuclei. The fraction of apoptotic cells was determined by counting from 400 to 500 cells for each cell population.

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 centrifugated at 1500 r/min for 5 minutes. 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 minutes. Reaction buffer containing 5 μl DDT, 5 μl of IETD-AFC (or DEVD-AFC) substrate and 380 μl H_2O was added to each aliquot of cell lysate. Mixtures were incubated at 37℃ for 1 hour. 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 corresponded to the activity that cleaved the respective substrate in 1 min / mg protein at 37℃.

Lactate dehydrogenase (LDH) activity-based cytotoxicity assay

LDH activity was measured in both floating dead cells and viable adherent cells. 100 μl (1×10^5 cells/ml) of a cell suspension was placed in the wells of 96-well plates. They were cultured in the incubator at 37℃ for 12 hours. After treatment with shikonin, floating cells were collected from culture medium by centrifugation at 2500 r/min at 4℃ for 10 minutes. 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 μl) at 37℃ for 30 minutes and centrifugated at 2500 r/min at 4℃ for 10 minutes. 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 μl) at 37℃ for 30 minutes and centrifugated at 2500 r/min at 4℃ for 10 minutes. 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:

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

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

Western blot analysis

HeLa cells were harvested and lysed for 1 hour on ice in lysis buffer [50 mmol/L 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (pH 7.4), 1% Triton X-100, 2 mmol/L sodium orthovanadate, 100 mmol/L sodium fluoride, 1 mmol/L EDTA, 1 mmol/L ethylene glycol tetracetic acid, 1 mmol/L phenylmethanesulfonyl fluoride (PMSF)], supplemented with proteinase inhibitors 100 μg/ml aprotinin, 10 μg/ml leupeptin, 100 μg/ml pepstatin. The lysate was centrifugated at 16 000 r/min at 4℃ for 10 minutes. Equal amounts of total protein were mixed in 2× loading buffer [50 mmol/L Tris-HCl (pH 6.8), 2% sodium clodecyl sulfate, 10% 2-mercaptoethanol, 10% glycerol, and 0.002% bromphenol blue], boiled 5 minutes and run on a 12% SDS-polyacryamide gel electrophoresis. Equivalent loading was confirmed by Bio-Rad (USA) protein assay. Proteins were electrotransferred onto nitrocellulose membranes. After being blocked with Tween 20-Tris-buffered saline [50 mmol/L tris-HCl (pH 7.5), 150 mmol/L NaCl, and 0.02% Tween 20] containing 5% non-fat milk at room temperature. The membranes were incubated for 2 hours at room temperature with the primary antibodies at 1: 1000 dilution in washing buffer. After being washed three times for 10 minutes each in Tris-buffered saline, the membrane was incubated with a diluted horseradish peroxidase-labeled secondary antibody (1:500) in washing buffer at room temperature for 1 hour. After further three washes, proteins were detected by chemiluminescence according to the manufacturer's instructions (Bio-Rad, Hercules, CA, USA).

Statistical analysis

All data represent at least three independent experiments and are expressed as mean ± SD unless otherwise indicated. Statistical comparisons were performed by Students' s t test. A P value less than 0.05 was considered as statistically significant.

RESULTS

Inhibitory effects of shikonin on the growth of HeLa cells

To evaluate the inhibitory effects of shikonin on the growth of HeLa cells, the cells were treated with 5 – 80 μmol/L of shikonin for 12, 24 and 48 hours, and the cell viability was determined by MTT assay. Shikonin inhibited the growth of HeLa cells in a time- and dose-dependent manner. IC_{50} of 48 hours time course was 18.8 ± 1.2 μmol/L (Fig. 1).

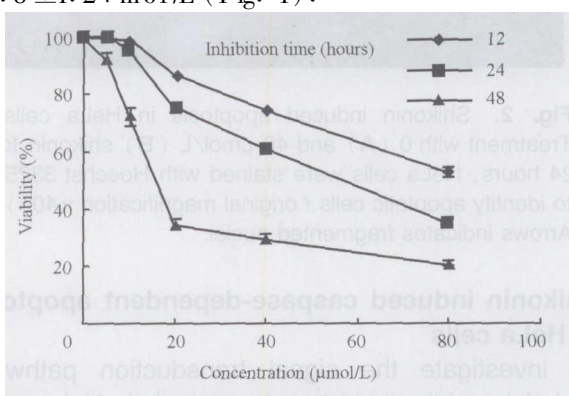

Fig. 1. Inhibitory effects of shikonin on the growth of HeLa cells. HeLa cells were treated with various concentrations of shikonin for 12, 24 and 48 hours. The viability was determined by the MTT assay. Data of the three independent experiments are expressed as mean ± SD.

Shikonin induced HeLa cell apoptosis

To confirm whether HeLa cell death induced by shikonin

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was caused by apoptosis or necrosis. Microscopic assessment and LDH activity-based cytotoxicity assay were carried out. HeLa cells treated with 40 μmol/L shikonin showed that the apoptotic cells had characteristic condensed nuclei stained by Hoechst 33258 (Fig. 2). After exposure to 40 μmol/L shikonin for 24 hours, the proportion of apoptotic cells markedly increased (Fig. 3A), while a negligible increase in necrotic cell death was detected, 14% compared with 10% of the control (Fig. 3B). These results demonstrated that main cause of HeLa cell death induced by shikonin was apoptosis.

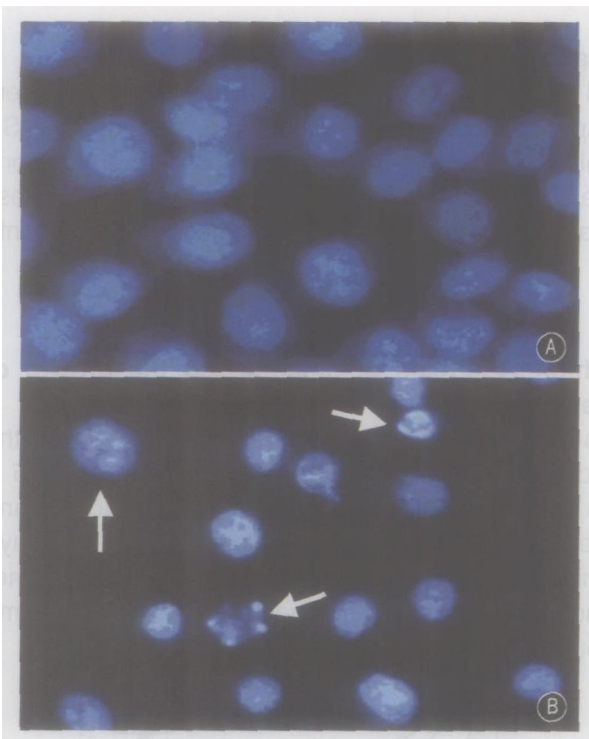

Fig. 2. Shikonin induced apoptosis in HeLa cells. Treatment with 0 (**A**) and 40 μmol/L (**B**) shikonin for 24 hours. HeLa cells were stained with Hoechst 33258 to identify apoptotic cells (original magnification×400). Arrows indicates fragmented nuclei.

Shikonin induced caspase-dependent apoptosis in HeLa cells

To investigate the signal transduction pathways underlying shikonin-induced apoptosis in HeLa cells, caspase inhibitors were applied and the enzyme activities were measured. As shown in Fig. 4, shikonin-induced apoptosis was blocked by pan-caspase inhibitor (20 μmol/L Z-VAD-FMK), indicating that caspase family proteinases play a role in this signaling pathway. 20 μmol/L caspase-3 inhibitor (Z-DEVD-FMK) and 20 μmol/L caspase-8 inhibitor (Z-IETD-FMK) significantly reversed

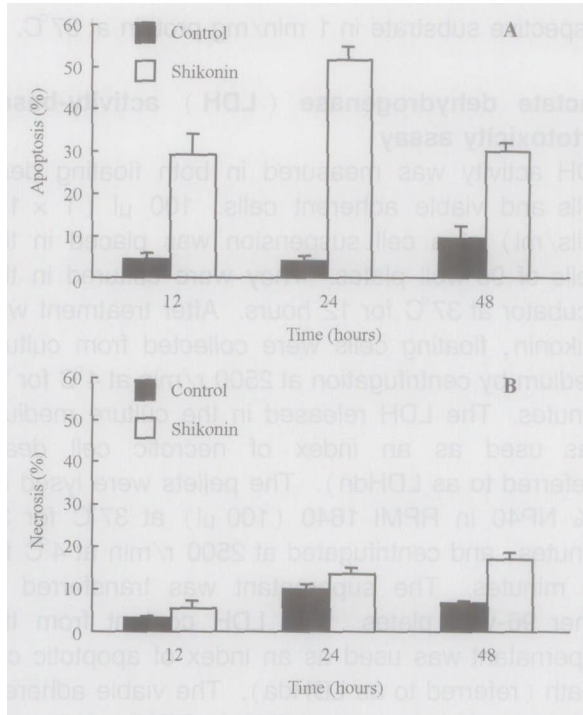

Fig. 3. Characterization of cell death induced by shikonin in HeLa cells. HeLa cells were treated with 40 μmol/L shikonin for 12, 24 and 48 hours. The extent of cell death was assessed by LDH activity based assays. The data are express as mean ± SD of the results for three independent experiments.

40 μmol/L shikonin-induced apoptosis, indicating that caspase-3 and caspase-8 were activated in this apoptotic pathway. Upon treatment of HeLa cells with 40 μmol/L shikonin, caspase-3 activities increased markedly within 12 hours after the drug treatment, then continued to reach maximum at 24 hours (Fig. 5). Simultaneously, the shikonin-treated HeLa cells underwent moderate activation of caspase-8. It suggested that shikonin induced cell apoptosis via capase-dependent mechanism.

Shikonin-induced phosphorylation of ERKs and p53 expression

To investigate the features of cell growth inhibition by shikonin, Western blot analysis was performed to examine ERK1, ERK2 and phosphorylated ERK (p-ERK) during this process. Shikonin of 40 μmol/L produced a marked increase in ERKs phosphorylation with dominant phosphorylation of ERK2 (42 KDa) in Hela cells (Fig. 6). Maximal phosphorylation was observed in 6 hours and then declined towards basal level. In contrast, shikonin-treated HeLa cells resulted in no observable change of the ERKs expression. Exposure of HeLa cell to shikonin for 12 hours up-regulated p53 expression.

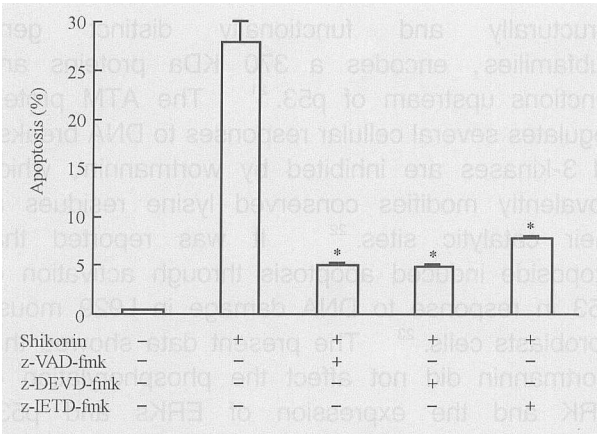

Fig. 4. Effects of various caspase inhibitors on shikonin induced HeLa apoptosis. HeLa cells were pretreated with 20 μmol/L caspase inhibitors for 1 hour and then treated with 40 μmol/L shikonin for 24 hours. The fraction of apoptotic cells was determined by counting 400 to 500 cells for each cell population. The cells were counter stained with DAPI to visualize the nuclei. Data are expressed as mean±SD ($n=3$, $^*P<0.05$ vs shikonin alone group).

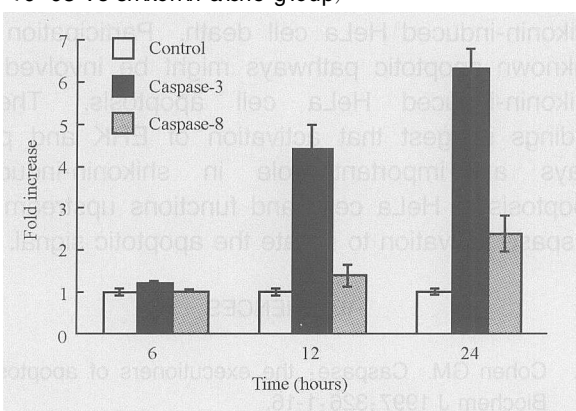

Fig. 5. Activities of csapase-3 and caspase-8 in shikonin treated HeLa cells. HeLa were incubated with or without 40 μmol/L shikonin (control) and caspase-3 and -8 activities (units/mg protein) were measured. Data are expressed as mean±SD.

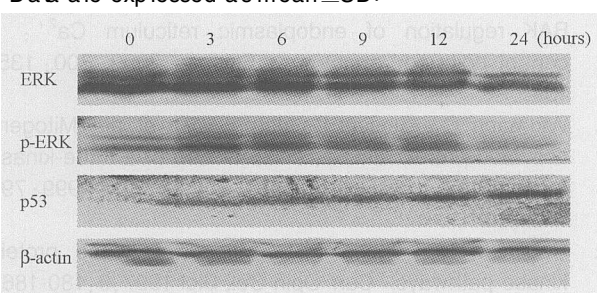

Fig. 6. Effects of shikonin on the expression of ERKs, p-ERK and p53. The cells were treated with 40 μmol/L shikonin for 0, 3, 6, 9, 12 and 24 hours. Cell lysates were separated by 12% SDS-PAGE electrophoresis and ERK1, ERK2, p-ERK and p53 protein bands were detected by Western blot analysis.

These results suggested that shikonin induced activation of ERKs, which resulted in activation of p53.

MEK inhibitor prevented phosphorylation of ERKs and p53 expression

Activated ERKs were responsible for p53 accumulation in a mouse JB6 epidermal cell line.[15] Therefore, effects of MEK inhibitor PD 98059 on phosphorylation of ERKs and the expression of p53 protein were examined (Fig. 7). 20 μmol/L PD 98059 effectively inhibited phosphorylation of ERKs in 40 μmol/L shikonin-treated HeLa cells for 6 hours, whereas the total level of ERKs in all the samples showed no changes. Simultaneously, the inhibitor reversed shikonin-induced p53 expression, indicating that phosphorylation of ERKs contributed to p53 activation. In addition, wortmannin, an inhibitor of the catalytic subunit of the PI 3-kinase family of enzymes, failed to block shikonin-induced phosphorylation of ERKs and p53 expression. Taken together, these data suggested a possible role of ERKs on acting upstream of p53 in this apoptotic pathway.

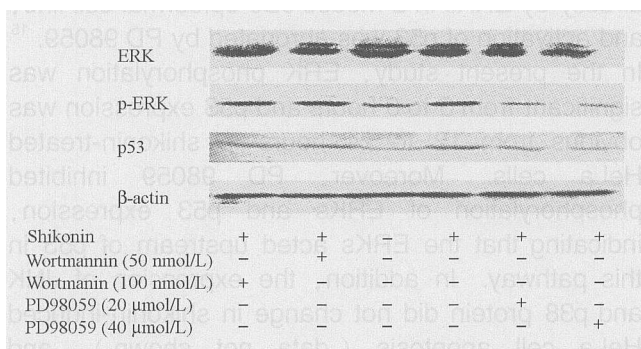

Fig. 7. PD 98059 prevents the phosphorylation of ERK and p53 expression. Western blot analysis for ERK1, ERK2, p-ERK and p53 protein expression in HeLa cells were treated with shikonin (40 μmol/L) for 6 hours. The cells were incubated with PD 98059 and wortmannin for 1 hour prior to the administration of shikonin.

DISCUSSION

In this study, it showed that shikonin inhibited the growth of HeLa cells in time- and dose-dependent manner and activation of caspase-3 and caspase-8 contributed to apoptosis in response to shikonin administration. Morphological changes of nuclei by staining Hoechst 33258 suggested that shikonin induced HeLa cell death involved in a mechanism of apoptosis. We found that this apoptosis was blocked by pan-caspase inhibitor, indicating that caspase family proteinases play a role in

this apoptotic process. Since caspase-3 inhibitor (Z-DEVD-FMK) and caspase-8 inhibitor (Z-IETD-FMK) effectively reduced the shikonin-induced HeLa cell apoptosis, initiator caspase-8 got involved in the apoptotic cell death.

Activation of the ERK signaling pathway protects cells from a variety of cellular stress.[16] Blockade of the ERK pathway by treatment with MEK inhibitor PD 98059 suppressed the growth of tumor cells.[17] The inhibition of MEK/ERK enhanced paclitaxel-induced apoptosis in tumor cell lines.[7] On the contrary, ERK activation plays an active role in mediating apoptosis in other cells. Cisplatin-induced HeLa cell apoptosis was involved in a requirement for ERK activation.[8] Growing evidence showed that activation of ERKs and MEK induced by various stimuli was observed in a relatively large number of tumor cells. The precise cause of constitutive activation of the ERK pathway remains vague. It was reported that Ultraviolet B irradiation-induced phosphorylation of p53 at Serine 15 was mediated directly by ERKs in a mouse JB6 epidermal cell line, and activation of p53 was abrogated by PD 98059.[15] In the present study, ERK phosphorylation was significant from 3 to 6 hours and p53 expression was obvious from 12 to 24 hours in shikonin-treated HeLa cells. Moreover, PD 98059 inhibited phosphorylation of ERKs and p53 expression, indicating that the ERKs acted upstream of p53 in this pathway. In addition, the expression of JNK and p38 protein did not change in shikonin-induced HeLa cell apoptosis (data not shown), and activation of JNK and p38 was not observed in this process.

p53 protein is a regulator of cell cycle progression and mediator of apoptosis in many cell lines. Phosphorylation of p53 protein at serine 15 plays a critical role in the stabilization and functional activation of p53 during cellular stress.[18,19] Bax, a proapoptotic protein, is transcriptionally regulated by p53. Bax acts in the mitochondria to cause the release of cytochrome c, which leads to the activation of caspase-9, subsequently activates downstream caspase-3.[20]

Members of the phosphoinositide-3 kinase-related kinase (PIK) superfamily function in regulating diverse processes including DNA repair. The ataxia telangiectasia mutated (ATM), one of three structurally and functionally distinct gene subfamilies, encodes a 370 KDa proteins and functions upstream of p53.[21] The ATM protein regulates several cellular responses to DNA breaks. PI3-kinases are inhibited by wortmannin, which covalently modifies conserved lysine residues at their catalytic sites.[22] It was reported that etoposide induced apoptosis through activation of p53 in response to DNA damage in L929 mouse fibroblasts cells.[23] The present data showed that wortmannin did not affect the phosphorylation of ERK and the expression of ERKs and p53, suggesting the signal transduction pathway might be different from this DNA damage pathway.

Caspases play a crucial role in the apoptotic progression, and they may play signaling role in the activation of other proteases involved in other pathways. 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. Participation of unknown apoptotic pathways might be involved in shikonin-induced HeLa cell apoptosis. These findings suggest that activation of ERK and p53 plays an important role in shikonin-induced apoptosis of HeLa cells and functions upstream of caspase activation to initiate the apoptotic signal.

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(*Received May 14, 2004*)
Edited by LIU Hui and ZHOU Ling-ni