

Apoptotic effects of shikonin on human hepatoma cells SMMC-7721

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Abstract: Apoptosis is a process that leads to programmed cell death and also a therapeutic target of cancer. In order to investigate the proliferative and apoptotic effects of shikonin on human hepatoma cell line SMMC-7721, and to explore the mechanisms of shikonin induced apoptosis, this research observed morphological alterations with phase contrast microscopy, confocal microscopy and transmission electron microscopy, detected apoptotic rates, cell cycle progression, mitochondrial transmembrane potential, intracellular calcium homeostasis. The results suggested that typical apoptotic morphological alterations occurred after shikonin treatment. Shikonin exerts a strong inhibitory the proliferation of SMMC-7721 cell line, and induces its apoptosis in a dosage and duration dependent manner. Cell cycle was arrested at G0/G1 phase. Mitochondrial transmembrane potential dropped. Calcium homeostasis was disturbed. It is concluded that shikonin can induce apoptosis of SMMC-7721 via arresting cell cycle progression, reducing mitochondrial transmembrane potential and disturbing intracellular calcium homeostasis. Although our present study is preliminary, shikonin could potentially be a therapeutic agent for the treatment of hepatocellular carcinoma.

[Wang H, Li XC, Zhao RJ. **Apoptotic effects of shikonin on human hepatoma cells SMMC-7721.** *Life Sci J* 2013;10(2):1247-1255] (ISSN:1097-8135). <http://www.lifesciencesite.com>. 173

Keywords: Shikonin; apoptosis; human hepatoma cells; SMMC-7721 cells

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common tumor worldwide and continues to have a poor prognosis. Despite of surveillance efforts, most tumors are diagnosed at late stages. In China, hepatoma is so high incidence that it has been the main threat of people's health and has the front rank of the tumor cancers. One of the most important mechanisms underlying tumorigenesis is uncontrolled proliferation and apoptosis, consequently, the crux of tumor therapy is to inhibit cell division and induce apoptosis. Apoptosis occurs through activation of a cell suicide process regulated by many different intracellular and extracellular events. Apoptosis is governed by several genes, some of which are mutated or dysfunctionally regulated in various of human tumors (Brown and Attardi, 2005). Apoptosis, as programmed cell death, is the most well-defined type of cell death pathway, both morphologically and biochemically. Apoptosis is characterized by shrinkage of the cell, DNA fragmentation, fragmentation into membrane-bound apoptotic bodies and rapid phagocytosis by neighbouring cells.

Apoptosis is a process that leads to programmed cell death and also a therapeutic target of cancer. Induced apoptosis of tumor cells provides a new strategy and was believed to hold profound significance for prevention and cure of neoplastic

transformation. Cancer is a prevailing lethal pathology, and commonly used therapies of hepatoma can hardly achieve satisfactory results. Traditional Chinese medicine, the essence accumulated throughout centuries, attracts more and more attention owing to its remarkable curative effects on cancer. Drug induced apoptosis of malignant cells is presumably a promising tumor therapeutic strategy and emerging evidence is supporting its effectiveness against hepatoma as well as other cancers.

Many compounds purified from plants have revealed anticancer activity. It is well known that chemotherapeutic agents, such as taxol (Bhalla et al., 1993) and camptothecin (Kaufman et al., 1989) can induce cancer cells to differentiate and undergo apoptosis. Some anticancer drugs are known to induce apoptosis via the inhibition of topoisomerase II (Walker et al., 1991). Shikonin is a naphthoquinone compounds. Shikonin is the primary active components isolated from Zicao plants and it's multiple pharmacological actions have been documented (Figure 1A). Based on the long history of its use, shikonin has been extensively characterized (Chen et al., 2002). It is believed that shikonin possesses detoxification properties and it has been used for thousands of years to treat macular eruptions, measles, sore-throat, carbuncles and burns (Wang et al., 1994). Studies have shown that

shikonin has a significant role which Inhibit bacterial and fungal growth, promote tissue healing, anti-inflammatory and antioxidant. Shikonin can inhibit replication of type I human immunodeficiency virus and pathological response that caused by it (Chen et al., 2003), and can Inhibit capillary permeability and treatment of lupus nephritis (Wang et al., 2009). Zicao is a commonly used anticancer herbal medicine in China and medicinal mixtures containing purified shikonin are reported to be safe and effective in the treatment of late stage lung cancer patients (Guo et al., 1991). Shikonin can inhibit the proliferation of many tumor cell lines, and promote apoptosis and inhibit angiogenesis play a role in anti-tumor, these studies provide a lot of experimental basis for shikonin in clinical anti-cancer treatment.

Although shikonin had been shown to induce apoptosis in cancer cells, the exact mechanism is still not clear. Therefore, the biological activity of shikonin on human hepatoma cells SMMC-7721 still needs further investigation. In elucidating the therapeutic values of shikonin, this research investigated its apoptotic effects and mechanism on human hepatoma cells.

2. Material and Methods

2.1 materials

Human hepatoma cells SMMC-7721 was purchased from Peking Union Medical College. Shikonin was purchased from Chinese National Institutes for Food and Drug Control. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) was purchased from Gibco. Annexin V-FITC Apoptosis Detection Kit I was purchased from BD corporation.

2.2 methods

2.2.1 Cell culture and growth dynamics

The cells were cultured in DMEM supplemented with 10% FBS at 37 °C under humidified with 5% CO₂ and 95% air at one atmosphere. Cells at the concentration of 2.0×10⁴ cells/ml were plated into 24-well microplates. Data on cell growth and density were calculated and recorded each day until plateau phase; three wells were counted each time. The growth curve was then plotted and the population doubling time (PDT) was calculated according to this curve.

2.2.2 Drug solution preparation and treatment

Shikonin was dissolved with Dimethyl sulfoxide (DMSO), diluted with DMEM medium, filtered for sterilization, aliquoted.

It should be diluted to the required concentration with DMEM medium prior to treatment, and the final concentration of DMSO

should be 0.05% in experiments. For control specimens, the same volume of 0.05% DMSO without shikonin was added. The experimental cells were logarithmic phase. After treatment with DMEM medium containing shikonin or DMSO, they were cultured to the scheduled time.

2.2.3 Morphological observation

2.2.3.1 Observation by inverted phase contrast microscope

The cells were plated in 6-well plates at a density of 2.0×10⁵ cells/well and grown for 24 h. Different concentrations of shikonin were added and they were grown for 24 h. For the cell morphology experiment, the culture plates were examined under a phase contrast microscope and photographed.

2.2.3.2 Fluorescent observation using confocal microscopy

To visualize the morphological alterations of apoptotic nuclei, acridine orange (AO) and ethidium bromide (EB) fluorescent staining of shikonin treated cells was performed. Cells were treated by shikonin for 24 h. Cell suspension was harvested from each well, and stained with AO, EB solution (both 2 mg/ml in ethanol) of 6 μl, and gently mixed. Incubated at room temperature in the dark for 5min, the samples were then observed using confocal microscopy (Nikon TE-2000-E, Japan) immediately.

2.2.3.3 Observation by transmission electron microscopy

The cells of controls and experimental samples were collected, and fixed with 2.5% (m/v) glutaraldehyde, then washed with 0.1 mol/L phosphate buffered saline (PBS). And subjected to serial dehydration with 30%, 50%, 70%, 80%, 90% and 100% acetone (v/v). The samples were embedded with epoxy resin (SPURR) for polymerization, then slice thin slicer with ultramicrotome (LEICAUC6i), Uranyl acetate and lead citrate for double staining. The samples were observed using transmission electron microscopy (JEM-1230).

2.2.4 Annexin V-FITC/PI double-labeling

The detection of apoptosis using annexin V-FITC/propidium iodide (PI) staining was performed as described previously (Cetindere et al., 2010). The cells of controls and experimental samples were collected and adjusted to the concentration of (1-5)×10⁵ cells/ml in binding buffer. For each sample, 100 μl cell suspension was stained with 5 μl fluorescein isothiocyanate (FITC) and 5 μl propidium iodide (PI) and incubated at room temperature in the dark for 25-30 min. After the addition of 400 μl

binding buffer each sample, and then detected with flow cytometer (FCM) (BD FACSCalibur, USA) within 1 h.

2.2.5 Cell cycle progression

To estimate the proportion of the cells in different phases of the cell cycle and the apoptotic effect of shikonin, the cell DNA contents were measured using FCM. The cells of controls and experimental samples were harvested by centrifugation. The cells were fixed gently (drop by drop) with 70% ethanol (in PBS) in ice overnight and add PI solution (PI 0.05 mg/ml, RNase 0.02 mg/ml, NaCl 0.585 g/ml, sodium citrate 1 mg/ml, pH 7.2-7.6). The cells were analyzed with FCM (BD FACSCalibur, USA) with an argonion laser at 488 nm wave-length and the cell cycle was determined.

2.2.6 Mitochondrial transmembrane potential

Mitochondrial transmembrane potential was determined as described previously (Yang, 2006; Zhang et al., 2006). The cells of controls and experimental samples were collected and adjusted to the concentration of 1.0×10^6 cells/ml, and then spun at 1200 g, 4 °C for 10 min. With supernatant discarded, they were washed twice with prewarmed PBS. After the addition of JC-1 working solution (5 µg/ml, 0.5 ml/sample), the cells were incubated at 37 °C in the dark for 10-15 min, and then were washed twice with prewarmed PBS. After centrifugation at 1200 g, 4 °C for 10 min, the supernatant was discarded. Each sample was resuspended with 0.5 ml PBS, and then detected with FCM (BD FACSCalibur, USA) immediately.

2.2.7 Intracellular calcium homeostasis

The intracellular Ca^{2+} release was assessed by staining with Ca^{2+} -sensitive dye Fluo3-AM (Adachi, 2008). The cells of controls and experimental samples were collected and adjusted to the concentration of $(1-2) \times 10^6$ cells/ml. Fluo-3/Am (Invitrogen, USA) was added to each sample to reach a final concentration of 5-10 µmol/L. Incubated at 37 °C, 5% CO_2 in the dark for 30-60 min, the samples were oscillated several times gently. Prepare negative controls (without Fluo-3/Am) for reference. The cells were centrifuged at 1200 g, 4 °C for 5 min, and washed twice with calcium-free PBS buffer, in order to remove the excessive dye. Each sample was resuspended in 0.5 ml calcium-free PBS, and then detected with FCM (BD FACSCalibur, USA) immediately.

2.2.8 Statistical analysis

Each assay was repeated in triplicate in three independent experiments. Statistical significance of

differences between groups was analyzed by using Student's t test ANOVA analysis. A value of $P < 0.05$ and $P < 0.01$ was thought of as statistically significant.

3 Results

3.1 Growth dynamics

The growth curve of SMMC-7721 cells displayed an obvious "S" shape, and the PDT was approximately 24 h. The cells were in the latent phase in days 1 and 2, and then entered logarithmic phase in days 2 to 5. The concentration reached its peak on day 5, and then the cells entered the plateau phase in day 6, followed by an overall degeneration thenceforth (Figure 1B).

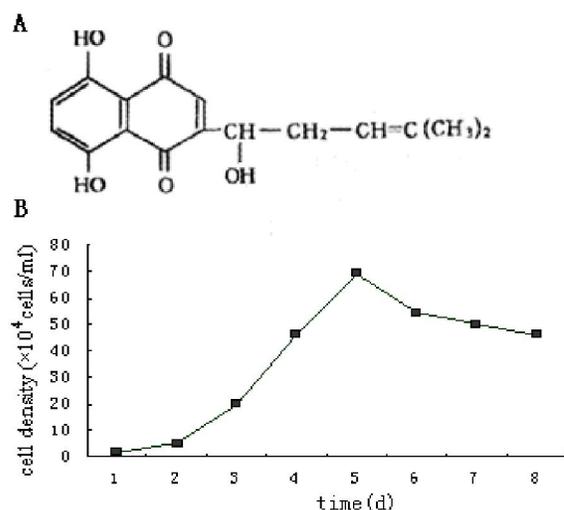


Figure 1. A, the molecular structure of shikonin (chemical formula: $\text{C}_{16}\text{H}_{16}\text{O}_5$, molecular weight: 288.29). B, growth Curve of SMMC-7721 Cells.

3.2 Morphological observation

3.2.1 observation using inverted phase contrast microscope

Under normal circumstances, the adherent cells were elliptical shaped. In logarithmic phase, the passaged cells will gradually adhere and grow. The cells of control showed that cells were closely arranged with, uniform size, as well as good vitality and refractivity (Figure 2A). The shikonin treated cells displayed atrophy and vacuoles (Figure 2B), shrinkage of the cytoplasm, cell number decrease, and cell fragmentation took place (Figure 2C), with blurred contour, the declined in cells connection, and even lysed into small pieces. Apoptotic cells detached from adjacent normal cells, and are obviously different in morphology (Figure 2D).

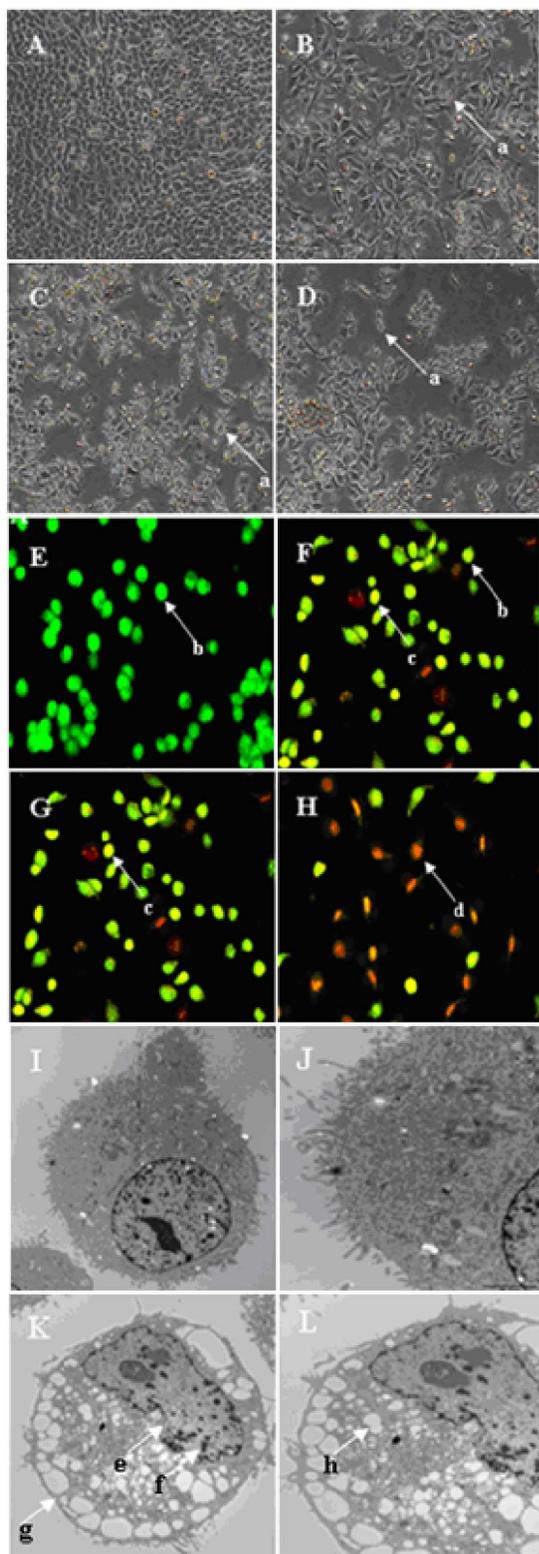


Figure 2. A-D, morphology of SMMC-7721 cells at 24 h after treatment with shikonin (100 \times). (A) control, and SMMC-7721 cells treated with shikonin of (B) 1 μ M, (C) 2 μ M, (D) 4 μ M. Apoptotic cells are

marked with arrow a. E-H, morphology of SMMC-7721 cells at 24 h after treatment with shikonin using AO/EB double staining. (E) control; and SMMC-7721 cells treated with shikonin of (F) 1 μ M, (G) 2 μ M, (H) 4 μ M. Arrow b, normal cells. Arrow c, viable apoptotic cells. Arrow d, Late apoptotic cells. Scale bars=50 μ m. I-L, subcellular observation using transmission electron microscopy. Morphology of SMMC-7721 cells at 24 h after treatment with shikonin. (I,J) control, and SMMC-7721 cells treated with shikonin of (K,L) 2 μ M. (I, 5000 \times . J, 12000 \times . K, 8000 \times . L, 12000 \times .) Arrow e indicate broken nuclear membrane. Arrow f indicate chromatin condensation. Arrow g indicate microvilli disappear. Arrow h indicate vacuoles.

3.2.2 observation using confocal microscopy

AO and EB differ in permeability and fluorescence, distinguishing cells in early apoptosis and late apoptosis. Viable apoptotic cells have intact membrane which prevents EB from entering the interior of cells, possess yellow cytosol and condensed nuclei, with condensation-like or dead-like nuclear chromatin. Membrane of non-viable apoptotic cells is permeable, through which both AO and EB will enter, thus cells will display condensed nuclei and orange fluorescence (Figure 2E-H).

3.2.3 Observation by transmission electron microscopy

The control group cell (Figure 2I-J), cell plasma is homogenous and plump, cell nucleus is large and round, nucleoli is clear and regular, chromatin is Loose. Nucleus, nuclear membrane and subcellular structures are intact. There is a large number of microvilli on cell surface. Cells in the experimental group (Figure 2K-L), vesiculation of cytoplasmic organelles, membrane blebbing, cell nucleus is pyknosis and fragmentation, smaller, Nucleolus is shrink, concentrate or even disappear. cytoskeletal degradation, and rupture of the plasma membrane. Chromatin is aggregate, marginalization, and is lumpish in the inner nuclear membrane, heterochromatin is increase. nuclear membrane is breakdown, microvilli on cell surface is reduce.

3.3 Annexin V-FITC/PI double-labeling

Quantitative analysis of apoptotic effects of shikonin on SMMC-7721 cells, cells by flow cytometry for Annexin V-FITC and PI staining. Annexin V FITC and PI staining serves as a measure of phosphatidylserine externalization. Double staining was used to distinguish between viable, early apoptotic, necrotic and late apoptotic cells. Results interpretation: the first quadrant (FITC⁻/PI⁻), viable cells; the second quadrant (FITC⁻/PI⁺), cell debris,

result from the mechanical factor for cell processing; the third quadrant (FITC⁺/PI⁺), late stage apoptotic cells and some necrotic cells; the fourth quadrant (FITC⁻/PI⁺), early stage apoptotic cells. These results show that the apoptotic effect of dose-dependent and time-dependent (Figure 3A-I). The data of apoptotic rate at 12 h and 36 h not shown.

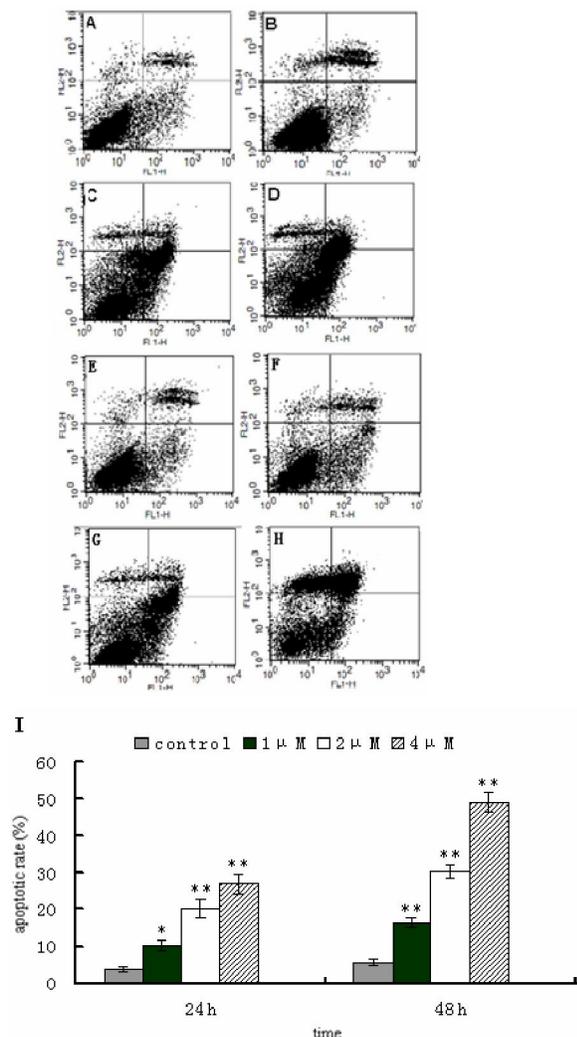


Figure 3. The apoptotic rate of SMMC-7721 cells at 24 h (A-D), 48 h (E-H) treatment with shikonin. A and E, control, and SMMC-7721 cells treated with shikonin of 1 μM (B and F), 2 μM (C and G), 4 μM (D and H). I, statistical significance to control is marked with (*) ($P < 0.05$) and (**) ($P < 0.01$) (n=3).

3.4 cell cycle progression

To test the mechanisms of shikonin induced SMMC-7721 cells apoptosis, cell cycle progression was analyzed by FCM. With the increasing concentration of shikonin, resulted in an accumulation of these cells in the G0/G1 phase of the cell cycle, with a concomitant decrease in the

proportion of those in the S phase, indicating an arrest in G1 phase, and that DNA synthesis was inhibited. The effects on cell cycle were even more significantly with elevated shikonin dose, reflecting a dose dependent correlation (Figure 4A-E). The percentage of apoptotic cells in hypodiploid DNA peak (sub-G1 population) was calculated by sub-G1 population / total cell cycle populations, sub-G1 population (corresponding to apoptotic cells) also showed dose dependent (Figure 4E). Similar results were obtained in all repeated experiments.

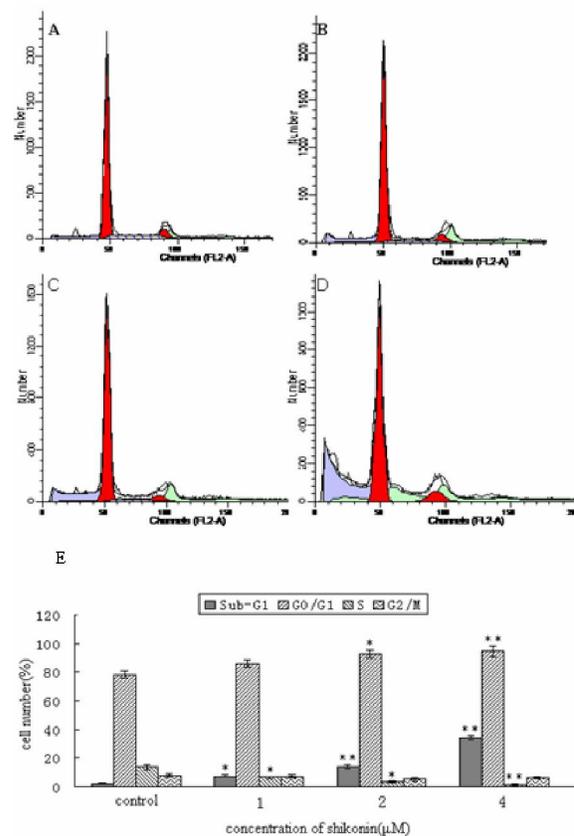


Figure 4. FACS analysis of cell cycle progression and sub-G1 content of SMMC-7721 cells at 24 h after treatment with shikonin. A, control, and SMMC-7721 cells treated with shikonin of 1 μM (B), 2 μM (C), 4 μM (D). E, Distribution of cells in the Sub-G1, G0/G1, S and G2/M phases of the cell cycle. Statistical significance to control is marked with (*) ($P < 0.05$) and (**) ($P < 0.01$) (n=3).

3.5 mitochondrial transmembrane potential

To observe the changes in mitochondrial membrane potential after treatment by shikonin, cells were stained with JC-1 and examined by FCM. JC-1 is a lipophilic, cationic dye that can selectively enter mitochondria and reversibly change color from green

to red as the membrane potential increases. JC-1 dye accumulates as aggregates in the mitochondria in normal cells, which results in red fluorescence, whereas, in apoptotic or necrotic cells, JC-1 exists in monomeric form and stains the cytosol green. The cells number of C gate reflects the change of mitochondrial transmembrane potential, The increase of cells number of C gate means decrease of mitochondrial transmembrane potential (Figure 5a-d). Mitochondrial transmembrane potential significantly dropped after treatment with shikonin, displayed significant differences compared with the control (Figure 5e).

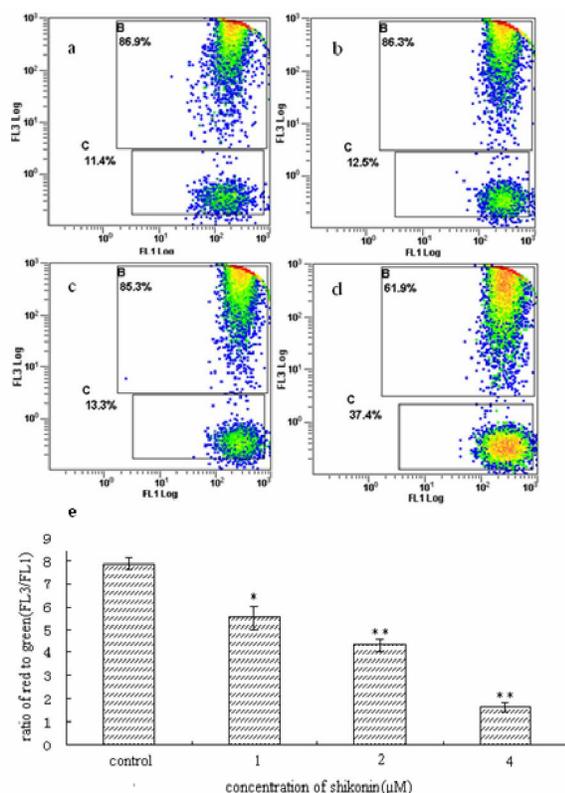


Figure 5. FACS analysis of the mitochondrial membrane potential of SMMC-7721 cells at 24 h after treatment with shikonin. a, control; and SMMC-7721 cells treated with shikonin of 1 μM (b), 2 μM (c), 4 μM (d). e, active mitochondria with high transmembrane potential form JC-1 aggregates, which are red (FL3, 620 nm), whereas in mitochondria with low transmembrane potential, JC-1 remains in a monomeric, green form (FL1, 527 nm). The ratio of red to green (FL3/FL1) reflects the change in mitochondrial membrane potential. Statistical significance to control is marked with (*) ($P < 0.05$) and (**) ($P < 0.01$) ($n = 3$).

3.6 intracellular calcium homeostasis

SMMC-7721 cells at 24 h after treatment with shikonin and were subsequently labelled with the molecular probe Fluo-3/AM. The results show that peak position in the histograms reflects the intracellular Ca^{2+} concentration of shikonin treated SMMC-7721 cells (Figure 6A-D). The Ca^{2+} concentration of experimental samples treated with shikonin displayed significant differences compared with the controls. It was revealed that there is a positive correlation between Ca^{2+} release and shikonin concentration (Figure 6E).

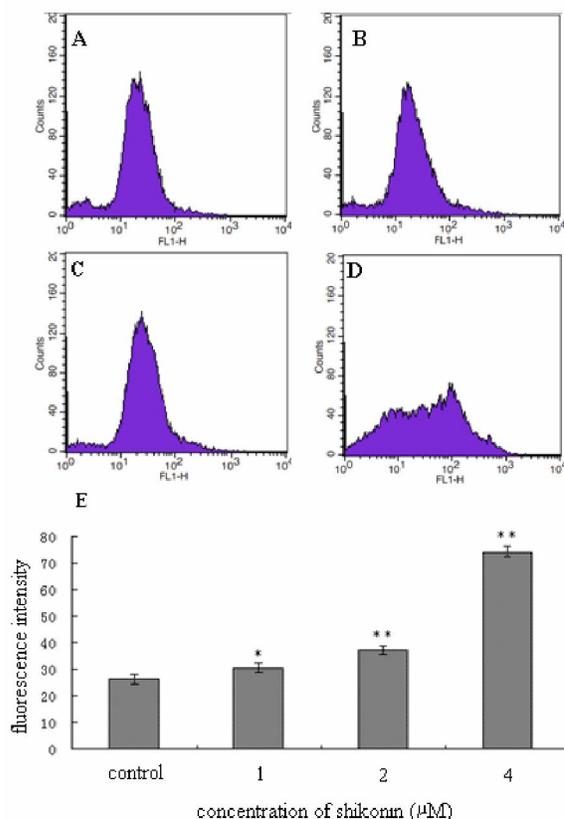


Figure 6. FACS analysis of intracellular calcium homeostasis of SMMC-7721 cells at 24 h after treatment with shikonin. A, control, and SMMC-7721 cells treated with shikonin of 1 μM (B), 2 μM (C), 4 μM (D). Peak moving to the right means an increase of intracellular Ca^{2+} concentration. E, statistical significance to control is marked with (*) ($P < 0.05$) and (**) ($P < 0.01$) ($n = 3$).

4 Discussion

Cancer cells can be induced to differentiate and undergo apoptosis by various chemotherapeutic agents, e.g. taxol (Bhalla et al., 1993), a well known plant-derived anticancer compound. Therefore, chemical agents with potent differentiation inducing or apoptosis-inducing activity, but acceptable toxic

side-effects, have potential as anticancer drugs. A number of studies suggest that shikonin derivatives meet this criterion (Lu et al., 1990).

Shikonin derivatives, which are the active components of the medicinal plant *Lithospermum erythrorhizon*, exhibit many biological effects including apoptosis induction through undefined mechanisms. Shikonin is the enantiomer of alkannin, which has multiple pharmacological actions including anti-bacterial, anti-fungal, anti-inflammatory, anti-thrombotic, anti-tumour, anti-gonadotrophic and anti-human immunodeficiency virus activities (Chen et al., 2002), inhibits angiogenesis in vivo and in vitro (Hsia et al., 1998). Angiogenesis is critical for tumor growth and inflammation reaction.

The growth of new blood vessels, or angiogenesis, plays an important role in the growth of solid tumors (Folkman et al., 1989). In a murine model, angiogenesis induced by TNF- α (100ng) was inhibited when shikonin (1-5 mg) was injected along with TNF- α . Shikonin (0.2 mg) co-injected with B16 melanoma cells strongly inhibited tumor growth and tumor-induced angiogenesis. Shikonin added to the diet (0.02%) significantly inhibited the incidence and average number of intestinal tumors in rats treated with azoxymethane, suggesting that shikonin might be a promising chemopreventive agent for intestinal neoplasia (Yoshimi et al., 1992). Shikonin induced apoptosis in the HL-60 human promyelocytic leukemia cell line (Yoon et al., 1999) and topoisomerase II-mediated DNA cleavage in vitro (Fujii et al., 1992), and stimulated glucose uptake in 3T3-L1 adipocytes via an insulin-independent tyrosine kinase pathway (Kamei et al., 2002).

Our study evaluated a wide variety of apoptotic indices, and definitely proved that a certain concentration of shikonin can inhibit the proliferation of SMMC-7721 cells and induce apoptosis, and hence have anti-tumor effects.

4.1 cell cycle progression

It is generally believed that physiological or pathological apoptotic stimuli are correlated with cell cycle progression (Siegers et al., 1999). Unscheduled proliferation constitutes a key step in canceration, and an altered death to division speed would eventually lead to malignant transformation and neoplastic growth (Frantz et al., 2000). Currently considered, cell cycle arrest would induce apoptosis, and influence proliferation. Many apoptotic signals affect apoptotic machineries as well as cell cycle progression at the same time. Therefore, cell cycle analysis is one of the most important evaluations in apoptotic research. Furthermore, blocking cell cycle

to induce apoptosis now serves as a new target for anticancer drugs.

Shikonins are known to modulate multiple signal transduction pathways, including inhibition of DNA topoisomerases, induction of reactive oxygen species release (Gao et al., 2000), and inhibition of survival pathways involving extracellular signal-regulated kinase, Akt, and nuclear factor κ B activities (Hashimoto et al., 1999). Shikonins could induce apoptosis and the G0/G1 phase cell-cycle arrest of cultured endometrial stromal cells (Nishida et al., 2006).

In this study, we demonstrated that shikonin inhibited cell proliferation by inducing apoptosis and the G0/G1 arrest of the cell cycle of human hepatoma cells SMMC-7721 in vitro. Interference with the cell cycle at the G1-phase and with DNA synthesis, by arresting cells at the G0/G1-phase of the cell cycle and thereby preventing them from entering the M-phase. Cells permeable to PI increased proportionally to the increment of both concentration and incubation time with shikonin, indicating the loss of plasma membrane integrity. Shikonin-treated cells exhibit apoptotic nuclear fragmentation, probably reflecting sub-G1 proportion showed dose dependent.

4.2 Mitochondrial transmembrane potential

The drop of mitochondrial transmembrane potential is considered to be the first event of apoptotic signaling, which occurs before occurrence of apoptotic characteristics in nuclear. The present study of apoptotic mechanism shows that mitochondrion plays a pivotal role in the process of apoptosis. Mitochondrial transmembrane potential will change when apoptosis happens, leading to changes in membrane permeability. Mitochondrial transmembrane potential, the driving force of ATP production, is decreased during apoptosis (Richter et al., 1996). ATP depletion is an important mechanism of apoptosis (Moley and Mueckler, 2005; Nakamura and Wada, 2000). Shikonin caused a loss of mitochondrial membrane potential, which was proportionally correlated with the loss of plasma membrane integrity. When cells were treated with shikonin, cells with low mitochondrial membrane potential and positive PI were significantly reduced.

In this study, cells labeled with JC-1 staining solution were subjected to flow cytometry to detect changes in mitochondrial transmembrane potential. It was found that mitochondrial transmembrane potential decreased in SMMC-7721 cells upon treatment with shikonin, subsequently by the possible suppression of ATP production even leading to ATP depletion and breakdown of mitochondria, activating downstream apoptotic pathways, therefore, the results indicated that shikonin induced apoptosis of SMMC-7721 cells.

is related to mitochondrial pathway, it indicating the critical roles of mitochondrial dysfunction in shikonin-induced apoptosis.

4.3 Intracellular calcium homeostasis

The release of Ca^{2+} from the endoplasmic reticulum into the cytoplasm has been implicated as a key-signalling event in many models of apoptosis and it may sensitize mitochondria to trigger apoptotic cell death. In addition, an increasing number of endoplasmic reticulum proteins have been described to influence apoptosis by either interacting with Bcl-2 family members or altering endoplasmic reticulum Ca^{2+} responses. Also, several endoplasmic reticulum proteins are caspase substrates that may regulate the execution phase of apoptosis (Breckenridge, 2003). Mitochondrion is an intracellular calcium store. The Ca^{2+} uptake depends on mitochondrial transmembrane potential. Mitochondrial Ca^{2+} elevation mechanisms include non-specific leakage and pore formation. With the existence of proper stimulus, Ca^{2+} is released from mitochondrion and endoplasmic reticulum. Mitochondrial calcium overload leads to mitochondrial damage, release of cytochrome C and caspase activation, and subsequent apoptosis. It has been reported that some cellular processes involved in the cell cycle play an important role in the signal transmission pathway of apoptosis and in particular mitochondrial cytochrome c release is an important control point in caspase activation and apoptosis (Solange et al., 2000).

Our data demonstrated when shikonin induced apoptosis of SMMC-7721 cells, cytosolic free Ca^{2+} concentration increased, so that Ca^{2+} homeostasis was disturbed. It indicates appropriate concentration of shikonin has significant effects in inducing apoptosis on SMMC-7721 cells. The result of disturbed calcium homeostasis, taken together with mitochondrial transmembrane potential decrease, presumably links it with endoplasmic reticulum calcium release.

In conclusion, the results of our study offer some information for the treatment of liver cancer. Our results show that shikonin induces apoptosis in hepatoma cells by inhibiting DNA synthesis, reducing mitochondrial membrane potential and interfering calcium homeostasis. These findings may have potential applications in the treatment of liver cancer. The results demonstrate that shikonin might be developed into a new anti-cancer drug. In addition, further studies on hepatocellular carcinoma may contribute to the establishment of more effective and sophisticated treatment strategies.

Acknowledgements:

This research was supported by the Ministry of Agriculture of China for Transgenic Research Program (2011ZX08009-003-006, 2011ZX08012-002-06) and the project National Infrastructure of Animal Germplasm Resources (2013 year).

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2/28/2013