Apoptotic effects of shikonin on human hepatoma cells HepG-2

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Abstract: In order to investigate the proliferative and apoptotic effects of shikonin on human hepatoma cell line HepG-2, this research observed morphological alterations with phase contrast microscopy, detected apoptotic rates, cell cycle progression, mitochondrial transmembrane potential. The results suggested that typical apoptotic morphological alterations occurred after shikonin treatment. Shikonin exerts a strong inhibitory the proliferation of HepG-2 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. It is concluded that shikonin can induce apoptosis of HepG-2 via arresting cell cycle progression, reducing mitochondrial transmembrane potential. Although our present study is preliminary, shikonin could potentially be a therapeutic agent for the treatment of hepatocellular carcinoma.
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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 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. 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. 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. 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 HepG-2 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 HepG-2 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 Cell culture and growth dynamics

The cells were cultured in DMEM supplemented with 10% FBS at 37 ℃ under humidified with 5% CO2 and 95% air at one atmosphere. Cells at the concentration of 2.0×10^4 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.

2.3 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.

2.4 Observation by inverted phase contrast microscope

The cells were plated in 6-well plates at a density of 2.0×10^5 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.

2.5 Annexin V-FITC/PI double staining

Cells upon shikonin treatment were collected, and then spun at 1200 rpm, 4℃ for 10min. With supernatant discarded, they were washed with precooled PBS twice, 10^6 cells of which were then resuspended with 500 μl Binding Buffer and stained with 5 μl AnnexinV-FITC (20mg/L) and 5 μl PI (20mg/L) in the dark for 15min at room temperature. The samples were analysed with FCM (BD FACSCalibur, USA) within 1 h.

2.6 Cell cycle analysis

Cells upon shikonin treatment were collected, and then spun at 1200 rpm, 4℃ for 10min. With supernatant discarded, they were washed with precooled PBS twice, resuspended in ice-cold 70% ethanol (v/v), and kept at 4℃ overnight. Pelleted and washed twice with PBS, the samples were stained with 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) at 4℃ for 30min. The cells were filtered using 400-mesh sieve, and then detected with FCM (BD FACSCalibur, USA) immediately.

2.8 Detection of mitochondrial transmembrane potential

Cells upon shikonin treatment were collected, and then spun at 1200 rpm, 4℃ for 10min. With supernatant discarded, they were washed with prewarmed PBS twice. The cells were stained with Rhodamine 123 (5 μg/ml in PBS, pH 7.4) and incubated at 37 ℃ in the dark for 1 h. Washed thrice with prewarmed PBS (Zhang et al., 2006), they were resuspended and analysed using FCM (BD FACSCalibur, USA) immediately.

3 Results

3.1 Growth dynamics

The growth curve of HepG-2 cells displayed an obvious "S" shape, and the PDT was proximately 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 1).

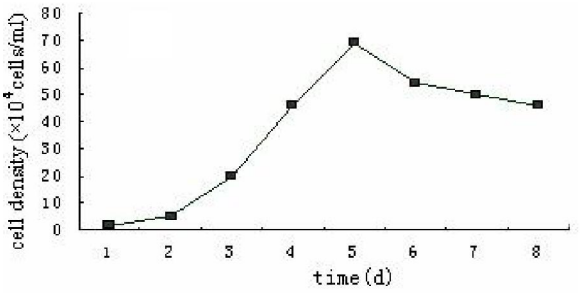

Figure 1. The growth curve of HepG-2 cell line

3.2 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. The shikonin treated cells displayed atrophy and vacuoles, shrinkage of the cytoplasm, cell number decrease, and cell fragmentation took place, 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 2).

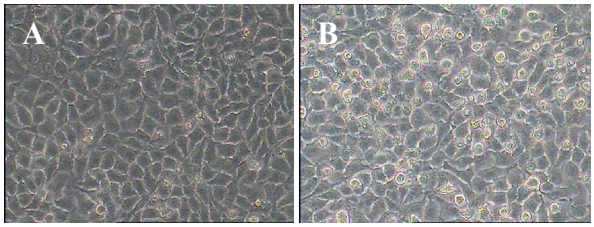

Figure 2. Morphological alterations of HepG-2 cell with shikonin treatement for 24 h (100×) A. control; B. 4 μM

3.4 Annexin V-FITC/PI double-labeling

Quantitative analysis of apoptotic effects of shikonin on HepG-2 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. These results show that the apoptotic effect of dose-dependent and time-dependent (Table 1). The data of apoptotic rate at 12 h, 36 h and 48 h not shown.

Table 1 Apoptosis rates of HepG-2 cell line upon shikonin treatment for 24 h ($\overline{X} \pm SD$, n=3)

group	apoptotic rate (%)
control	1.4±0.11
1 μM	13.23±0.84**
2 μM	35.84±3.53**
4 μM	45.77±4.87**

Statistical significance to control is marked with (*) (P<0.05) and (**) (P<0.01)

3.4 Cell cycle progression

To test the mechanisms of shikonin induced HepG-2 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 (Table 2).

Table 2 Cell cycle analysis of HepG-2 cells treated with shikonin for 24 h ($\overline{X} \pm SD$, n=3)

phase	G1(%)	S (%)	G2 (%)
control	38.24±4.46	45.07±4.59	9.57±1.27
1 μg/ml	46.76±5.48*	33.25±3.08*	16.50±2.46**
2 μg/ml	49.89±5.67**	30.12±3.39**	14.43±1.88**
4 μg/ml	56.70±5.72**	36.35±4.45	1.15±0.08**

Statistical significance to control is marked with (*) (P<0.05) and (**) (P<0.01)

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. Mitochondrial transmembrane potential significantly dropped after treatment with shikonin, displayed significant differences compared with the control (Table 3).

Table 3 Mitochondrial transmembrane potential of HepG-2 cell with shikonin treatement for 24 h ($\overline{X} \pm SD$, n=3)

group	(ΔΨ)
control	43.39±4.67
1 μM	31.06±4.48**
2 μM	30.23±4.36**
4 μM	25.77±4.57**

Statistical significance to control is marked with (*) (P<0.05) and (**) (P<0.01)

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 manybiological 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 premyelocytic leukemia cell line (Yoon et al., 1999) and topoisomerase IImediated DNA cleavage in vitro (Fujii et al., 1992), and stimulated glucose uptake in 3T3-L1 adipocytes via an insulinindependent 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 HepG-2 cells and induce apoptosis, and hence have huge 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 signalregulated kinase, Akt, and nuclear factor κB activities (Hashimoto et al.,1999). Shikonins could induce apoptosis and the G0/G1 phase cell-cycle arrest of cultured endometriotic 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 HEPG-2 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 of and incubation time with shikonin, indicating the loss of plasma membrane integrity. Shikonin-treated cells exhibit apoptotic nuclear fragmentation, probably reflects 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. ATP depletion is an important mechanism of apoptosis (Moley, 2005). 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 detect changes in mitochondrial transmembrane potential. It was found mitochondrial transmembrane potential decrease in HepG-2 cells upon treatment with shikonin, subsequently by the possible suppression of ATP production even lead to ATP depletion and breakdown of mitochondria, activating downstream apoptotic pathways, therefore, The results indicated that shikonin induced apoptosis of HepG-2 cells is related to mitochondrial pathway, it indicating the critical roles of mitochondrial dysfunction in shikonin-induced apoptosis.

Our data demonstrated when shikonin induced apoptosis of HepG-2 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 HepG-2 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 interferencing

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.

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