Original Article
Withaferin A inhibits hepatoma cell proliferation through induction of apoptosis and cell cycle arrest

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Abstract: Withaferin A (WFA) is an active compound from Withania somnifera and has been reported to exhibit a variety of pharmacological activities such as anti-inflammatory and anti-cancer activities. In the present study, we investigated the anti-proliferative effect of WFA on human hepatocellular carcinoma (HCC) cells and the molecular mechanism underlying the cytotoxicity of WFA. We found that treatment with WFA obviously inhibited HCC cells proliferation in a dose and time-dependent manner. Moreover, the apoptosis rate of HCC cells was significantly increased in the presence of WFA. Exposure to WFA also resulted in G1 phase cell cycle arrest in HCC cells. On the molecular level, our results showed that WFA treatment increased the expression of p53 and bax, and decreased the expression of bcl-2. In addition, WFA administration led to a significant up-regulation of p21 and down-regulation of CDK2 and cyclin D1. Taken together, the present study demonstrated that WFA might be a potential therapeutic candidate for HCC treatment through regulation of proteins related to apoptosis and cell cycle arrest.

Keywords: Withaferin A, human hepatocellular carcinoma, proliferation, apoptosis, cell cycle arrest

Introduction

Hepatocellular carcinoma (HCC) is one of the most malignant cancers, ranking the third cause of cancer-related mortality in the world and causing more than 600,000 deaths each year [1, 2]. Current treatments for HCC include chemotherapy, radiotherapy, and surgical operation. Systematic chemotherapy plays a critical role in HCC treatment especially for patients with advanced stage [3, 4]. However, accumulating studies have shown that the resistance of HCC to conventional drugs has becoming great challenges, indicating the necessity of developing new therapeutic strategies for HCC therapy [5-7].

Natural products are a rich source of compounds with enormous structural diversity and have great potential application in the field of drug discovery. Especially, the search for anti-cancer drugs from natural products represents an area of interest worldwide and a large number of different sources of natural compounds are shown to present anti-tumor properties, such as some natural products Rg3 may increase the efficacy of cancer chemotherapy, possibly through inhibitory effects on NF-κB and AP-1 activity, and downregulation of angiogenesis associated with VEGF expression [8-10]. Withania somnifera, a well known medicinal plant, has long been used to prevent dermatological disorders and infectious wounds [11]. Withaferin A (WFA) is a steroidal lactone derived from Withania somnifera and considered to be a bioactive compound. It has drawn much attention from researchers due to its anti-inflammatory, anti-carcinogenic and pro-apoptotic properties [12]. Increasing studies have demonstrated that WFA is involved in the
regulation of many molecules associated with cancer initiation and progression including NFκB [13], heat shock proteins [14], Akt [15], and the estrogen receptor [16]. In addition, it has been suggested that WFA exerts promising anti-tumor efficacy in many types of cancers such as prostate, breast, melanoma, ovarian, and lung cancer models [17-20]. However, the effect of WFA on human liver cancer cells has never been investigated. Therefore, our present study aimed to investigate the anti-proliferative effect of WFA on hepatocarcinoma cells, and explored the mechanism of action under the anti-tumor property on HCC.

Materials and methods

Reagents

Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS) and antibiotics were purchased from Gibco (Invitrogen, Carlsbad, CA, USA). WFA was purchased from Sigma (St. Louis, MO, USA) and dissolved in DMSO.

Cell culture

Human HCC cell lines Hep3B and Huh7, HepG2 and normal liver cell line L-02 were obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM supplemented with 10% fetal bovine serum, streptomycin (100 mg/mL) and penicillin (100 U/mL). Cultured cells were maintained at 37°C and 5% CO₂ in a humid environment and passaged when the confluency reached 80%.

Cell viability assay

Cell viability was determined by MTT assay. To be brief, cells were grown in 96-well plates at a density of 5×10³/well over night. Then cells were washed with fresh medium and treated with different concentrations of WFA for indicated time points. 100 μL of 1 mg/mL MTT was added into the medium and incubated in the dark for 2 h. Finally, the absorbance at 490 nm was measured using a microplate reader (Biorad, USA).

Flow cytometry

HCC cells were exposed to WFA at indicated concentrations for 24 h. Then cells were washed twice with PBS, detached with trypsin and harvested. Apoptosis cells were detected with annexin V-FITC/PI according to the instruction of Annexin V-FITC cell Apoptosis Detection Kit (BD, USA).

Cell cycle distribution

HCC cells were seeded at the density of 1.0×10⁶ cells and treated with WFA for 24 h. Cells were collected by centrifugation and fixed in ice-cold 70% ethanol at -20°C overnight. Then, cells were stained with 100 μl PI staining solution for 30 min in the dark followed by cell cycle analysis (BD, USA).

Western blot

Cells were harvested by trypsinization, lysed in buffer and prepared for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After immunoblotting, the membranes were blocked in PBS/0.1% Tween-20 with 5% nonfat dry milk, and primary antibodies including anti-p53, bax, bcl-2, p21, CDK2, cyclin D1 and GAPDH were incubated in PBS/0.1% Tween-20 with 0.1%-5% nonfat dry milk (Santa Cruz, CA, USA). The membrane was incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h and visualized using the ECL detection kit (Pierce Chromatography Cartridges, USA). GAPDH was used as a loading control.

Statistical analysis

Data were presented as means ± SD and treated for statistics analysis by SPSS 16.0. Comparison between groups was made using ANOVA and statistically significant difference was defined as P<0.05.

Results

WFA inhibits the proliferation of HCC cells

To determine the effect of WFA on human liver cancer cell growth, three human HCC cell lines including Hep3B and Huh7 and HepG2 were treated with WFA (Figure 1A) at different concentrations (1 μM, 2 μM, 3 μM and 4 μM) for indicated time points (12 h, 24 h and 48 h) and cells viability was determined by MTT methods. We found that the growth rate of Hep3B (Figure 1B), Huh7 (Figure 1C) and HepG2 (Figure 1D) cells was inhibited by WFA in a dose- and time-dependent manner but not in normal liver cell line L-02 (Figure 1E).
Anti-proliferative effect of WFA on human HCC cells

WFA induces dose-dependent apoptosis and cell cycle arrest in HCC cells

We further confirmed the induction of apoptosis by WFA by flow cytometry. Hep3B and Huh7 and HepG2 cells were incubated with WFA at different concentrations (1 μM, 2 μM and 3 μM) for 24 h. Annexin V and PI staining indicated that the apoptosis rates of Hep3B (Figure 2A), Huh7 (Figure 2B) and HepG2 (Figure 2C) was significantly increased (10%, 15%, 20% apoptosis) compared to the control group (5% apoptosis) (P<0.05). But, the apoptosis in L-02 cell was not significantly increased (Figure 2D). In addition, we examined the effect of WFA on cell cycle distribution in liver cancer cells. Hepatocarcinoma cells were treated with different concentrations of WFA (1 μM, 2 μM, 3 μM and 4 μM) for different time points (12 h, 24 h and 48 h). Results showed that WFA incubation significantly increased the proportions from 50% to 70% of cells in G1 phase and decreased the number from 40% to 20% of cells in S phase in Hep3B cells (Figure 3A and 3B). Similar results were also observed in Huh7 (Figure 3C).
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and 3D) and HepG2 (Figure 3E and 3F) cells treated with different doses of WFA. However, WFA did not affect the cell cycle in normal liver cell L-02 (Figure 3G, 3H). Collectively, these results demonstrate that WFA induces apoptosis and cell cycle arrest in human HCC cells but not in normal liver cell.

**WFA regulates the expression of apoptosis- and cell cycle-related proteins in HCC cells**

To reveal the molecular basis for WFA-induced apoptosis and cell cycle arrest in HCC cells, we examine the expression levels of apoptosis and cell cycle-associated proteins by western blot. We found that the protein level of tumor suppressor gene p53 was dose-dependently elevated in Hep3B cells treated with WFA (Figure 4A and 4B). Moreover, WFA administration showed an increase in bax expression (Figure 4C) and a decrease in bcl-2 (Figure 4D) in a dose-dependent manner. Meanwhile, the bax/bcl-2 ratio was remarkably increased following treatment with WFA (Figure 4E). Furthermore, the apoptotic protein cleaved caspase3 was significantly increased following the WFA treatment (Figure 4F).

Furthermore, WFA incubation significantly elevated the expression of p21 (Figure 5A and 5B). By contrast, the G1-phase cell cycle regulatory proteins, such as CDK2 and Cyclin D1, showed a markedly decrease in dose-dependent manners after treatment with WFA (Figure 5C and 5D). Collectively, these results demonstrated that WFA modulated the expression of pro/anti-apoptotic proteins and critical cell cycle regulators, leading to apoptosis and cell cycle arrest.

**Discussion**

Nowadays, HCC has become a serious public health threat in many countries and brought much financial burden to the patients, making it a necessity to search more chemicals that...
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Figure 3. WFA treatment induces cell cycle arrest in HCC cells. Cells were treated with WFA at different concentrations (1 μM, 2 μM and 3 μM) for 24 h. Cell cycle distributions at G1 and S phase of Hep3B (A and B), Huh7 (C and D), HepG2 (E and F) and L-02 (G and H) were analyzed by flow cytometry with PI staining. *P<0.05; **P<0.01.
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may exhibit therapeutic potential in this disease [21]. WFA is a phytochemical which has received considerable attention due to its in vitro and in vivo anti-cancer properties [22, 23]. In the present study, we evaluated the effects of WFA on proliferation, apoptosis and cell cycle distribution of HCC cells.

Abnormal cell proliferation plays an important role in human diseases and it is suggested that WFA inhibits the growth of renal carcinoma, non-small cell lung cancer, and breast cancer [20, 22, 24]. In our study, we demonstrated that WFA treatment suppresses HCC cells proliferation in a dose- and time-dependent manner whereas no remarkable anti-proliferative effect was detected in normal liver cells. These data suggest that WFA exhibits inhibitory effect on the proliferation of hepatocarcinoma cells.

Program cell death, or apoptosis, plays a critical role in maintaining cell functions by remov-
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Figure 5. Effect of WFA on the expression of cell cycle-related proteins. (A) Hep3B cells were treated with or without WFA for 24 h and cell lysates were extracted for western blot analysis. Relative protein levels including p21 (B), CDK2 (C) and Cyclin D1 (D) versus control were quantified and standardized to GAPDH. *P<0.05; **P<0.01.

We further investigated the molecular mechanism underlying the anti-tumor effect by examination of apoptosis- and cell cycle-related proteins expression. Tumor suppressor p53 is a cell cycle regulator with a short half-life. The function of p53 is achieved by increasing p53 transcription and post-translational stabilization to escape ubiquitin-dependent degradation. Apoptosis mediated by p53 is associated with Bcl2 and Bax, a pro-apoptotic member of the Bcl2 family. To examine the involvement of the mitochondrial pathway in WFA-induced apoptosis, we examined the levels of Bcl2, Bax, and p53 [15]. WFA has been shown to induce p53-dependent apoptosis by regulating the expression of tumor suppressor proteins in human cervical cancer cells [23]. In addition to p53, the pro-apoptotic bax and anti-apoptotic bcl-2 are well documented downstream molecules of p53, which regulate mitochondrial physiology and thus induce cell apoptosis. Our study demonstrated that WFA remarkably ele-

ing unnecessary cells [25]. In most types of cancers, the tumor cells are characterized by the acquired ability to resist apoptosis [6, 7]. Cell cycle is controlled by cell cycle checkpoint which ensures the fidelity of cell division. Exogenous or endogenous stimulating factors can induce cell cycle arrest and resulted in the breakdown of cell division, cell death and apoptosis [26]. Various studies demonstrate that WFA achieves the anti-tumor effect through induction of apoptosis and cell cycle arrest in several types of cancer cells. For example, WFA exhibits antiproliferative activity against the head and neck squamous cell carcinoma by inducing apoptosis and cell death as well as cell-cycle arrest [27]. It is also suggested that WFA inhibits cell proliferation, shifts cell cycle arrest, and induces apoptosis in uveal melanoma through suppression of Akt and c-MET activation [28]. In the present study, we observed that WFA treatment resulted in apoptosis and cell cycle arrest in human HCC cells.

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vated the protein expression of p53 in liver cancer cells. Moreover, WFA treatment resulted in an increase in bax and cleaved caspase3 expression and a decrease in bcl-2 in a dose-dependent manner. The bax/bcl-2 ratio was also dose-dependently increased in HCC cells after incubation with WFA. The protein p21 is a universal inhibitor of cell cycle progression. It is well documented that p21 negatively regulates the G1/S phase transition and reinforces cell cycle arrest in G1 phase upon induction [29]. CDK2 and Cyclin D1 belong to the cyclin-dependent kinase family and play crucial roles in the G1/S phase progression [30, 31]. WFA-treated cancer cells clearly demonstrated concentration-dependently increase in p21 levels. Meanwhile, WFA incubation led to decreased expression levels of cyclin D1 and CDK2.

Taken together, our study demonstrated that the modulation of these proteins by WFA partially account for the induction of apoptosis and cell cycle arrest.

In conclusion, the present study shows that WFA inhibits the proliferation of liver cancer cells through induction of apoptosis and cell cycle arrest. Therefore, our findings suggest that WFA could be a potential therapeutic candidate for treatment of HCC in the future.

Disclosure of conflict of interest

None.

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