Astragaloside IV Induces Apoptosis, G₁-Phase Arrest and Inhibits Anti-apoptotic Signaling in Hepatocellular Carcinoma

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Abstract. *Background/Aim:* Hepatocellular carcinoma (HCC) is a primary malignancy of the liver and the third leading cause of cancer death worldwide. Although multiple chemotherapies options are available for HCC, chemo-induced toxicity is inevitable during clinical treatment. Therefore, identifying possible adjuvant agents with both liver-protective and antitumor effects is critical. Herbal medicines have chemopreventive and anti-HCC effect, such as Juzen taiho-to and Sho-saiko-to. Astragaloside IV is a compound extracted from the Chinese medical herb *Astragalus membranaceus* (Fisch.) Bge. with liver protection potential. However, whether astragaloside IV may also possess tumor-inhibitory capability and its underlying mechanism is remaining unknown. *Materials and Methods:* Viability analysis, cell-cycle analysis, apoptosis analysis, western blotting analysis and invasion trans-well assay were performed to identify tumor-inhibitory potential of astragaloside IV on HCC cells (SK-Hep1 and Hep3B cells). *Results:* We found that astragaloside IV may induce cytotoxicity and extrinsic/intrinsic apoptosis effect, but also trigger G₁ arrest in HCC cells. The expression of anti-

apoptotic proteins of HCC were all reduced by astragaloside IV. Additionally, astragaloside IV also suppressed HCC cell invasion ability. *Conclusion:* Astragaloside IV effectively suppressed HCC cell proliferation, invasion and anti-apoptosis *in vitro*.

Hepatocellular carcinoma (HCC) is the type of primary liver malignancy with highest incidence rates in the United States, Asia, and Africa (1, 2). Viral hepatitis infection and long-term exposure to chemical carcinogens or plant toxins can elicit malignant transformation of hepatocytes resulting in development of HCC (3). Preclinical and clinical studies showed herbal medicines may act as chemoprotective agents to effectively prevent hepatitis C virus or toxicity agent-induced HCC formation. In addition to chemoprevention, herbal medicines also demonstrated to possess anti-HCC potential (4-6).

Juzen taiho-to (TJ-48), a herbal formulation, was found not only to alleviate hepatocarcinogenesis through reduction of pro-inflammatory cytokines and oxidative stress, but also to prolong cumulative disease-free survival in patients with HCC (7). Sho-saiko-to (TJ-9) and Stronger-Neo Minophagen C (SNMC, 0.2% glycyrrhizin combined with 2.0% glycine and 0.1% cysteine in physiological solution) was shown to reduce risk of HCC in patients with cirrhosis and chronic hepatitis C, respectively (8, 9). Furthermore, sho-saiko-to and glycyrrhizin inhibited tumor cell growth through induction of apoptosis and G₁-phase arrest in HCC *in vitro* and *in vivo* (10, 11).

Astragaloside IV, the bioactive compound derived from *Astragalus membranaceus*, has been indicated to suppress acetaminophen-, and cisplatin-induced liver injuries in animal models (12, 13). Astragaloside IV was also shown to

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down-regulate metastatic activity through blockage of epithelial–mesenchymal transition in HCC (14). However, the anti-HCC mechanism of astragaloside IV have not yet been elucidated. Therefore, the aim of the present study was to verify the effects of astragaloside IV on cell-cycle progression and survival in HCC *in vitro*.

Materials and Methods

Chemicals and reagents. Astragaloside IV, propidium iodide and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical (St. Louis, MO, USA). Culture medium Dulbecco's modified Eagle's medium (DMEM) with additional 10% fetal bovine serum and 1% L-glutamine were purchased from Gibco BRL (Grand Island, NY, USA). Trypsin-EDTA was obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). Active caspase-3, -8, and -9 activity assay kit for flow cytometric assay was from Biovision (Mountain View, CA, USA). NP40 Cell lysis buffer and Bio-Rad assay kit were purchased from Thermo Fisher Scientific (Fremont, CA, USA).

Cell culture of SK-Hep1 and Hep3B cells. The human hepatocellular carcinoma cell lines SK-Hep1 and Hep3B were obtained from Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC). SK-Hep1 and Hep3B cells were both maintained in a DMEM plus 10% fetal bovine serum, 1% L-glutamine, 100 U/ml of penicillin G and 100 µg/ml of streptomycin. The cells were kept in an incubator at 37°C under 5% CO₂ and 95% air.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assay. SK-Hep1 and Hep3B cells were plated on a 96-well plate at a density of 3×10^4 cells/well and grown overnight. Astragaloside IV (0, 100, 150, 200, 300 and 400 µM) was added to cells DMSO (0.1%) served as a solvent control. Cells were maintained with drugs at 37°C under 5% CO₂ and 95% air for 48 h. The medium was then replaced by fresh medium with MTT reagents (DMEM:MTT=9:1) and incubated for another 4 h at 37°C. DMSO was finally used to dissolve MTT reagents for absorbance measurement at 570 nm by SpectraMax iD3 (Molecular Device, San Jose, CA, USA) (16).

Colony formation assay. Clone formation assay was used for testing cell proliferation. SK-Hep1 and Hep3B cells were plated on a 6-well plate overnight and treated with astragaloside IV (0, 200, and 400 µM) for 48 h. Treated cells were digested with 0.25% trypsin (Sigma-Aldrich), counted, resuspended in a 10 cm dish and maintained at 37°C for 2 weeks. Then cultured cells were washed in phosphate-buffered saline (PBS) twice, fixed in paraformaldehyde (Sigma-Aldrich) for 20 min and stained in 0.2% crystal violet (Solarbio, Beijing, PR China) for 10 min. Stained cells were washed in PBS and photographed using an Olympus XZ-1 camera (17). Quantitation of colonies was performed by Image J 1.490 software (National Institutes of Health, Bethesda, MD, USA).

Cell-cycle analysis. SK-Hep1 and Hep3B cells were plated on a 6-well plate at a density of 5×10^5 cells/well and grown overnight. Cells were then treated with astragaloside IV 200 µM for 0, 6, 12, 24, and 48 h, respectively. After treatment, cells were harvested, fixed by 70% ethanol and storage at -20°C overnight. Cells were finally centrifuged and stained by propidium iodide staining solution

(BD Pharmingen™, Franklin Lakes, NJ, USA) in the dark at 37°C for 30 min. The cell-cycle distribution was then acquired by flow cytometry (FACSCalibur; Becton-Dickinson, Franklin Lakes, NJ, USA) and quantified by FlowJo 7.6.1 system (18).

Annexin-V staining. SK-Hep1 and Hep3B cells were plated on a 6-well plate at a density of 5×10^5 cells/well to grown overnight and treated with astragaloside IV (0, 200, and 400 µM) for 48 h. Cells were finally centrifuged and stained by annexin-V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Vazyme Biotech Co. Ltd, Nanjing, PR China). Annexin-V expression pattern was then acquired by flow cytometry (FACSCalibur; Becton-Dickinson) and quantified by FlowJo 7.6.1 system as previously described (19).

Analysis of caspase-3, caspase-8, and caspase-9 activities. Flow cytometry was used to measure the activities of cleaved caspase-3, -8, and -9 in SK-Hep1 and Hep3B cells after exposure to astragaloside IV. SK-Hep1 and Hep3B cells were plated on a 6-well plate at a density of 5×10^5 cells/well overnight and treated with astragaloside IV (0, 200, and 400 µM) for 48 h. Cells were collected and re-suspended in 300 µl phosphate-buffered saline of 1 µl substrate solution containing FITC-Asp(OCH₃)-Glu(OCH₃)-Val-Asp(OCH₃)-fluoromethyl ketone (DEVD-FMK) for caspase-3, or containing sulforhodamine-Ile-Glu-Thr-Asp-fluoromethyl ketone (Red-IETD-FMK) for caspase-8 or containing FITC-Leu-Glu-His-Asp-fluoromethyl ketone (LEHD-FMK) for caspase-9 activities for 30 min at 37°C with 5% CO₂. All samples were finally isolated, washed with phosphate-buffered saline, and were measured for caspase-3, -8, and -9 activities by flow cytometry as described previously (20).

Western blot. SK-Hep1 and Hep3B cells (2×10^6 cells/dish) were maintained in 10-cm dishes with culture medium overnight and then were incubated with 0, 200, or 400 µM astragaloside IV for 48 h. Cell were isolated and lysed by NP-40 cell lysis buffer. Lysate of cells was centrifuged at $21,500 \times g$ for 30 min at 4°C, followed by quantitated total protein using Bio-Rad assay kit using as a protein control. Proteins were separated by 10-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electro transferred onto a polyvinylidene difluoride membrane (Thermo Fisher Scientific). This was followed by incubation with primary antibodies and then washed followed by secondary antibodies. Finally, the membrane was visualized by enhanced chemiluminescence kit (Thermo Fisher Scientific) as described previously (21, 22). Primary antibodies listed below were used and purchased from various companies: X-linked inhibitor of apoptosis protein (XIAP; Thermo Fisher Scientific), myeloid cell leukemia 1 (MCL1; BioVision), FADD-like interleukin 1β-converting enzyme-inhibitory protein (c-FLIP; Cell signaling Technology, Danvers, MA, USA), survivin (Abcam Plc., Cambridge, UK) and β-actin (Thermo Fisher Scientific). Secondary horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG antibodies were purchased from Jackson Immuno-Research Laboratories, Inc. (West Grove, PA, USA).

Invasion assay. SK-Hep1 and Hep3B cells (2×10^6 cells/dish) were maintained in 10-cm dishes with culture medium overnight and then were incubated with 0, 200, or 400 µM astragaloside IV for 48 h. Apical chamber was coated with 50 µl matrigel one day before the experiment. Cells were then trypsinized, collected, counted, re-suspended by 100 µl serum-free media onto apical chamber of tranwell as 1×10^6 cells/well. After 48-h incubation, cells were then fixed and stained by 0.5% crystal violet solution. Cells were finally

Figure 1. Viability and proliferation of hepatocellular carcinoma cells were suppressed by astragaloside IV. SK-Hep1 and Hep3B cells were treated with astragaloside IV (0-400 μM) for 48 h and MTT and colony formation assay were performed. Viability results of SK-Hep1 (A) and Hep3B (B) cells are displayed. C: Representative images of colonies from each group are displayed. D: Quantification of colony numbers of each group are displayed as a bar chart. Significantly different at * $p < 0.05$, and ** $p < 0.01$ vs. 0 μM astragaloside IV.

photographed using a Nikon ECLIPSE Ti-U light microscope at $\times 100$ and quantified with ImageJ software version 1.50 (National Institutes of Health, Bethesda, MD, USA) (20).

Statistical analysis. Data are presented as the mean $\pm$ standard. A Student *t*-test (one-tailed) was used to compare between groups. Values of $p < 0.05$ was considered as a statistically significant difference. Analyses were carried out using GraphPad Prism version 7.0 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Astragaloside IV induced cytotoxicity and inhibited proliferation of HCC cells. To identify whether astragaloside IV may induce toxicity and suppress proliferation of HCC cells, we treated SK-Hep1 and Hep3B cells with increasing

doses of astragaloside IV from 0-400 μM for 48 h. As indicated in Figure 1A and B, viability of SK-Hep1 and Hep3B cells were reduced by astragaloside IV in a dose-dependent manner. In addition, the colony-formation assay was used to validate proliferation inhibition by astragaloside IV. In Figure 1C, the formation of colonies was effectively reduced by astragaloside IV treatment. Higher concentrations of astragaloside IV led better of proliferation inhibition in SK-Hep1 and Hep3B cells (Figure 1D). To sum up, viability and proliferation of SK-Hep1 and Hep3B cells were both markedly suppressed by astragaloside IV treatment.

Astragaloside IV induced G_1 phase arrest and apoptosis of HCC cells. Next, we investigated the effect of astragaloside IV on cell-cycle distribution and apoptosis by flow cytometry.

Figure 2. G_1 -Phase arrest and apoptosis were triggered by astragaloside IV in hepatocellular carcinoma cells. SK-Hep1 and Hep3B cells were treated without or with 200 or 400 μM astragaloside IV for 0-48 h and validated by cell-cycle analysis, annexin-V and active caspase-3 staining. Cell-cycle distribution of SK-Hep1 (A) and Hep3B (B) cells after 200 μM astragaloside IV treatment. Significantly different at $*p<0.05$, $**p<0.01$ vs. 0 μM astragaloside IV. C: Annexin-V expression pattern after 48 h astragaloside IV treatment and quantification of annexin V assay results. D: Cleaved caspase-3 expression pattern and quantification of cleaved caspase-3 assay results after 48 h astragaloside IV treatment. Significantly different at $**p<0.01$ vs. 0 μM astragaloside IV; $##p<0.01$ vs. 200 μM astragaloside IV.

Figure 3. Cleavage of caspase-8 and -9 were both induced by astragaloside IV on hepatocellular carcinoma cells. SK-Hep1 and Hep3B cells were treated with astragaloside IV 0, 200 or 400 μM for 0-48 h and validated by active caspase-8 and -9 staining. A: Cleaved caspase-8 activation pattern after astragaloside IV treatment and quantification of results. B: Cleaved caspase-9 activation pattern after astragaloside IV treatment and quantification of results. Significantly different at ** $p < 0.01$ vs. 0 μM astragaloside IV; ## $p < 0.01$ vs. 200 μM astragaloside IV.

Astragaloside IV effectively triggered G_1 arrest of SK-Hep1 and Hep3B cells (Figure 2A and B, respectively). In addition, apoptosis (annexin-V) of HCC cells was also triggered by astragaloside IV treatment in a dose-dependent manner (Figure 2C). The cleavage of caspase-3, recognized as an apoptosis marker, was significantly increased by astragaloside IV (Figure 2D). The percentage of cleaved caspase-3 was increased on 48 h treatment with 200 μM astragaloside IV and on treatment with 400 μM astragaloside IV (Figure 2D) in SK-Hep1 and Hep3B cells. Taken together, these data show astragaloside IV may not only trigger SK-Hep1 and Hep3B cells to arrest at G_1 phase but also promote apoptosis.

Astragaloside IV activated caspase-8 dependent extrinsic and caspase-9 dependent intrinsic apoptosis signaling of HCC cells. We further investigated whether apoptosis

triggered by astragaloside IV was mediated by extrinsic or intrinsic apoptosis signaling. Here, we evaluated activation of related markers, including extrinsic apoptosis-dependent caspase-8 and intrinsic apoptosis-dependent caspase-9. As indicated in Figure 3A, the cleavage of caspase-8 was significantly increased by astragaloside IV in both SK-Hep1 and Hep3B cells. Furthermore, the activation of cleaved caspase-9 was also massively increased by astragaloside IV (Figure 3B). Thus, astragaloside IV-induced apoptosis was mediated by both extrinsic and intrinsic apoptosis signaling.

Astragaloside IV effectively suppressed expression of anti-apoptotic proteins. After validating the apoptotic effect of astragaloside IV, we further investigated whether the effects of astragaloside IV may also involve expression of anti-apoptosis and proliferation-related factors by western blot.

Figure 4. Anti-apoptotic proteins were reduced by astragaloside IV on HCC cells. SK-Hep1 and Hep3B cells were treated with astragaloside IV (0, 200 or 400 μM) for 0-48 h and expression of X-linked inhibitor of apoptosis protein (XIAP), myeloid cell leukemia 1 (MCL1), FADD-like interleukin-1 β -converting enzyme-inhibitory protein (c-FLIP) and survivin proteins were validated by western blot (left panel) and quantified (right panel) in SK-Hep1 (A) and Hep3B (B). Significantly different at * $p < 0.05$, ** $p < 0.01$ vs. 0 μM astragaloside IV; ## $p < 0.01$ vs. 200 μM astragaloside IV.

In Figure 4 (left panels), the expression of XIAP, MCL1, C-FLIP and survivin were all reduced by astragaloside IV in a dose-dependent manner in both SK-Hep1 and Hep3B cells. Through quantification analysis, relative expression of anti-apoptotic-related proteins was found to be reduced in astragaloside IV treated groups (Figure 4, right panels). In conclusion, astragaloside IV successfully suppressed the expression of proteins related to anti-apoptotic and proliferative effects in HCC cells.

Astragaloside IV markedly inhibited invasion of HCC cells. To identify whether astragaloside IV may also inhibit invasion capacity of HCC cells, we performed a transwell invasion assay. The number of invading SK-Hep1 and Hep3B cells were significantly suppressed by astragaloside IV as shown in transwell membranes (Figure 5). Quantification indicated dramatic suppression of HCC invasion by astragaloside IV.

Discussion

Apoptosis is modulated by apoptotic signaling cascades. Anticancer agents trigger severe deoxyribonucleic acid (DNA) injury and interaction with cell surface membrane death receptors/death receptor ligands initiates apoptosis through intrinsic and extrinsic apoptotic pathways (23). Caspase family members are essential components of the apoptotic response. Cleaved-caspases-9 and -8 as the activator up-regulates downstream activation of caspases in intrinsic and extrinsic apoptotic pathways, respectively. Cleaved-caspase-3 as executioner participates in formation of apoptotic DNA fragmentation and cleavage of DNA repair protein poly (ADP-ribose) polymerase-1 (PARP1) (24). Our data demonstrated astragaloside IV significantly induced apoptosis and increased cleaved-caspase-3, -8, and -9 activation in HCC SK-Hep1 and Hep3B cells (Figures 2D and 3).

Figure 5. Invasive ability of hepatocellular carcinoma cells was suppressed by astragaloside IV. SK-Hep1 and Hep3B cells were treated with 0, 200 or 400 μM for 0–48 h astragaloside IV before performing transwell invasion assay. A: Images of transwell membranes of SK-Hep1 and Hep3B cells after astragaloside IV treatment. B: Quantification of invasion cells calculated using five different images from each group. Significantly different at * $p<0.05$, ** $p<0.01$ vs. 0 μM astragaloside IV; ## $p<0.01$ vs. 200 μM astragaloside IV.

Evasion of apoptosis and aberrant cell-cycle progression contribute to tumor progression. Overexpression of anti-apoptotic proteins (XIAP, MCL1, C-FLIP, and Survivin) has the capacity to attenuate therapeutic efficacy of anticancer agents *via* blocking intrinsic and extrinsic pathway-mediated apoptosis. The reduced expression of anti-apoptotic proteins was associated with favorable prognosis in patients with HCC (18, 25). Cyclin-dependent kinases (CDK)4/6 inhibitors disrupt cell-cycle progression by inducing G_1 phase arrest in different types of cancer (15). Palbociclib, an inhibitor of cyclin-dependent kinase 4 and 6 approved for patients with breast cancer, was demonstrated to inhibit tumor cell growth and promote G_1 phase arrest in HCC (26). Our results indicated astragaloside IV patently attenuated expression of anti-apoptotic proteins (XIAP, MCL-1, C-FLIP, and survivin) (Figure 4) and induced G_1 -phase arrest in HCC SK-Hep1 and Hep3B cells (Figure 2A and B).

In conclusion, astragaloside IV not only triggers apoptosis through intrinsic/intrinsic pathways and G_1 -phase arrest but also diminishes cell invasion (Figure 5) and anti-apoptosis mechanisms in HCC.

Conflicts of Interest

The Authors declare no conflicts of interest in regard to this study.

Authors' Contributions

Data curation: Chun-Min Su, Hsiao-Chia Wang, Fei-Ting Hsu; Funding acquisition: Chun-Min Su; Methodology: Chun-Min Su, Chun-Hui Lu and Chien-Kai Lai; Validation: Jing-Gung Chung and Yu-Cheng Kuo; Writing – original draft: Fei-Ting Hsu and Yu-Cheng Kuo; Writing – review and editing: Jing-Gung Chung and Yu-Cheng Kuo.

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