Induction of apoptosis in human hepatocarcinoma SMMC-7721 cells *in vitro* by flavonoids from *Astragalus complanatus*

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ABSTRACT

Aim of the study: Flavonoids extracted from the seeds of *Astragalus complanatus* R.Br. reduce the proliferation of many cancer cells. The present study was carried out to evaluate the effects of these flavonoids from *Astragalus complanatus* (FAC) on human hepatocarcinoma cell viability and apoptosis and to investigate its mechanisms of action in SMMC-7721 cells.

Materials and methods: Cell viability was measured using the MTT assay. To detect apoptotic cells, SMMC-7721 cells treated with FAC were stained with Hoechst 33258 and subjected to agarose gel electrophoresis. Quantitative detection of apoptotic cells was performed by flow cytometry. The effects of FAC on apoptosis and cell cycle regulatory genes and proteins in SMMC-7721 cells were examined using an S series apoptosis and cell cycle gene array and Western blot analysis.

Results: The growth of SMMC-7721 and HepG2 cells was inhibited by treatment with FAC. Cell death induced by FAC was characterized by nuclear condensation and DNA fragmentation. Moreover, the cell cycle was arrested in the G0/G1 and S phases in FAC-treated SMMC-7721 cells. A sub-G1 peak with reduced DNA content was also formed. The activity of caspase-3 was significantly increased following FAC treatment. Microarray data indicated that the expression levels of 76 genes were changed in SMMC-7721 cells treated with FAC: 35 genes were up-regulated and 41 were down-regulated. Western blot analysis showed that caspase-3, caspase-8, Bax, P21, and P27 protein levels in SMMC-7721 cells were increased after 48 h of FAC treatment, while cyclinB1, cyclinD1, CDK1, and CDK4 protein levels were decreased.

Conclusions: These results suggest that FAC may play an important role in tumor growth suppression by inducing apoptosis in human hepatocarcinoma cells via mitochondria-dependent and death receptor-dependent apoptotic pathways.

1. Introduction

A wide variety of biological activities from medicinal plants have recently been reported, in addition to their traditional medicinal effects. Herbal medicines have attracted considerable interest as alternative cancer remedies because of their low toxicity and costs. Liver cancer rates are continuously increasing in Asia due to persistent high incidences of hepatic diseases. A number of effective prevention measures have been introduced in attempts to substantially reduce both the incidence of and mortality due to liver cancer. The search for new anti-cancer compounds in foods or in plant medicines is one realistic and promising approach to prevention. Chinese medicine provides a rich pool of novel and efficacious agents for cancer prevention and treatment. The seeds of *Astragalus complanatus* R.Br. (AC) provide a traditional Chinese medicine, which is described in the 2005 version of the "Chinese Pharmacopoeia". In China, AC has been extensively used for clinical treatment of liver and kidney complaints, including tumors. Many reports on the biological activities of AC indicate that it shows protective effects against hepatic injury, anti-peroxidation of lipids, inhibition of platelet aggregation, reduction of serum lipids, anti-inflammatory effects and immune enhancement through specific and non-specific immunity (Huang, 1992). Phytochemical studies on the components of AC have revealed the presence of flavonoids, triterpenes, organic acids, polysaccharides, polypeptides, proteins and sterols (Cui et al., 1992a,b; Huang, 1992).

We previously reported that AC had a protective effect on the liver (Liu et al., 2002). Our further investigation has revealed that the flavonoids extracted from AC (FAC) were useful in preventing rat liver fibrosis induced by dimethylnitrosamine (DMN) and carbon tetrachloride (CCl$_4$) (Liu et al., 2005a,b). It was recently demonstrated in our laboratory that FAC showed antiproliferative activities in the human carcinoma cell lines, HL-60, BGC-823, A549, HeLa, and SMMC-7721, and had anticancer effects on human liver cancer

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subcutaneously transplanted into nude mice (Liu et al., 2007a,b). However, the mechanisms of FAC in human liver cancer cells remained unknown. Therefore, the present study was carried out to evaluate the effects of FAC on human hepatocarcinoma cell viability using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and to investigate the effects of FAC on apoptosis and cell cycle regulatory genes and proteins.

2. Materials and methods

2.1. Plant material

Dried seeds of *Astragalus complanatus* R.Br. (Family: Leguminosae) were obtained from Shanxi Weinan Pharmaceutical Corporation (Shanxi, China) in December 2004 and identified by chief pharmacist, Wu Yin-Sheng (Suzhou Drug Administration Bureau). The voucher specimen (No. SAC/200504) was deposited at the Herbarium of the College of Pharmacology, Suzhou University, China.

2.2. Preparation of flavonoids from Astragalus complanatus (FAC)

Dry seeds of *Astragalus complanatus* were pulverized in an electric grinder and the fine powder was defatted using ligarine in a Soxhlet apparatus. The defatted portion was taken out, dried, and extracted twice with 80% ethanol (EtOH). The solvents were evaporated to dryness under vacuum, and the residue was diluted in distilled water. After separating the precipitate by filtration, the remaining solution was further separated on an adsorptive resin (ZTC-5, Nankai University, Tianjin, PR China) column and eluted with water, 50% EtOH and 95% EtOH to produce three fractions. The 50% EtOH fraction was dried under vacuum, recrystallized with EtOH and a yellow powder (1.1%, w/w) was obtained containing the

flavonoid component of *Astragalus complanatus* (FAC). The chemical profile of FAC extracts was analyzed using high-pressure liquid chromatography (HPLC) and is shown in Fig. 1. Briefly, FAC was analyzed by reverse phase (RP)-HPLC using a Shimadzu DGU-20A3 HPLC system equipped with a diode array detector. The separation was performed on a Shim-pack VP-ODS analytical column (4.6 mm × 150 mm, 5 μm). FAC was dissolved in acetonitrile, filtered through a 0.45 μm filter and the filtrate (20 μL) was injected into the HPLC column. Compounds were separated using an acetonitrile–1% acetic acid gradient (acetonitrile from 12 to 40%) with a flow rate of 1 mL/min and UV detector set at 266 nm. Complanatuside A accounted for about 10% of FAC, and total flavonoids for 78%, determined by UV spectroscopy at 266 nm, with complanatuside A as a standard. The purity of complanatuside A separated from FAC was more than 99.0%, as shown by RP-HPLC with DAD, and its structure was determined by IRMS, ^{13}C NMR and ^{1}H NMR spectroscopy.

2.3. Cell culture

The human hepatocarcinoma cell lines SMMC-7721 and HepG2 were obtained from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, PR China). Cells were cultured in a nutrient medium (RPMI 1640) (GIBCO Invitrogen, Grand Island, NY), supplemented with heat-inactivated 10% fetal bovine serum (GIBCO Invitrogen), 100 U/mL penicillin, 100 mg/mL streptomycin in a water-saturated atmosphere with 5% CO_2 at 37 °C. The culture medium was changed every 2 days.

2.4. Cell viability assay

Cell viability was measured using the MTT assay. SMMC-7721 or HepG2 cells were plated onto 96-well plates at a cell density of 50,000 cells per well in 100 μL of RPMI 1640 and allowed to grow
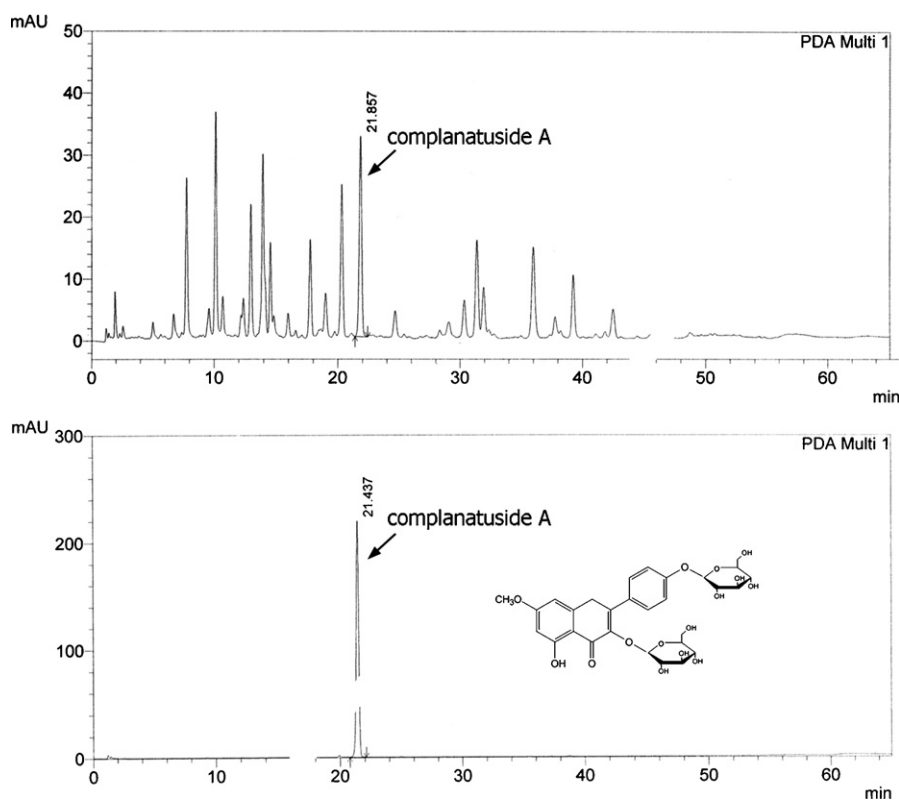

Fig. 1. High-pressure liquid chromatography fingerprinting of flavonoid extract from *Astragalus complanatus* seeds (FAC). Mode: gradient, increasing the organic phase acetonitrile from 12% to 40% over 65 min; flow rate: 1.0 mL/min; injected volume: 20 μL; detection wavelength: 266 nm.

in a CO_2 incubator for 24 h. The medium was then removed and replaced by fresh medium containing different concentrations of FAC (from 25 to 400 μg/mL) for 48 h. Cultures were incubated in 100 μL of medium with 10 μL of 5 mg/mL MTT solution for 3 h at 37 °C. The medium with MTT was removed, and 100 μl of DMSO was added to each well to dissolve the formazan. Absorbance was read at 570 nm (630 nm as a reference) on a microplate reader (BIORAD-Benchmark, California, USA)

2.5. Morphological assessment of apoptotic cells

To detect apoptotic cells, SMMC-7721 cells were stained with the DNA-binding dye, Hoechst 33258 (Dojindo, Kumamoto, Japan). After the cells were exposed to FAC for 12 h, 24 h and 48 h, they were fixed with 2% paraformaldehyde in phosphate buffered saline (PBS) for 10 min at 4 °C, and then washed with PBS. To stain the nuclei, cells were incubated for 10 min with 20 mg/mL Hoechst 33258. After washing with PBS, the cells were observed using an Olympus Microscope with a WU excitation filter (Tokyo, Japan). Cells exhibiting condensed chromatin and fragmented nuclei were scored as apoptotic cells. A minimum of 200 cells was scored from each sample.

2.6. Detection of DNA fragmentation

Cells were treated with FAC (0, 200, 400 μg/mL) for 48 h and the fragmented DNA was isolated using the sodium dodecyl sulfate (SDS)/Proteinase K/RNase A extraction method, which allows the isolation of only fragmented DNA, without contaminating genomic DNA (Reddy et al., 2003). DNA fragmentation was analyzed by 1.5% agarose gel electrophoresis. DNA bands were observed and recorded using a Multi-Imager (Bio-Rad, California, USA). The presence of apoptosis was indicated by the appearance of a ladder of oligonucleosomal DNA fragments of approximately 180–200 bp multiples on the agarose gel.

2.7. Flow cytometry analysis of cell cycle and apoptosis

SMMC-7721 cells were seeded into 50-mL culture flasks and, after treatment with 0 μg/mL, 200 μg/mL, and 400 μg/mL FAC for 24 h, 48 h and 72 h, cellular DNA content was detected by flow cytometry via determination of propidium iodide. Briefly, the cells were trypsinized, washed twice with ice-cold PBS and fixed with 70% EtOH. After overnight refrigeration at 4 °C and subsequent rehydration in PBS for 30 min at 4 °C, cells were stained at 37 °C for 20 min with 50 μg/mL propidium iodide and 100 units/mL RNase.
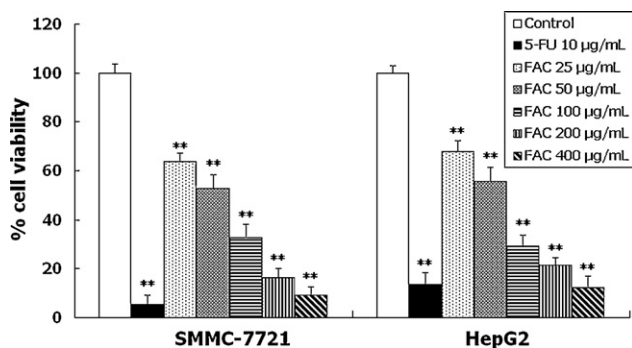
Analysis was performed with the aid of an EPICS XL flow cytometer (BeckmanCoulter, Miami, FL), equipped with the Multicycle AV program for cell cycle analysis (Phoenix Flow Systems, San Diego, CA).

2.8. Caspase-3 activity assay

The activity of caspase-3 was determined using the Caspase-3 activity kit (Beyotime Institute of Biotechnology, Haimen, PR China) according to the manufacturer's protocol. The assay is based on spectrophotometric detection of the chromophore, p-nitroanilide (pNA), after its cleavage from the labeled substrate, acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA). Briefly, SMMC-7721 cells were lysed after treatment with FAC (100, 200, 400 μg/mL) for 12 h, 18 h, 24 h and 30 h. Assays were performed on 96-well microtiter plates by incubating 10 μL protein of cell lysate per sample in 80 μL reaction buffer (1% NP-40, 20 mM Tris–HCl (pH 7.5), 137 mM Nad, and 10% glycerol) containing 10 μL caspase-3 substrate (Ac-DEVD-pNA) (2 mM). Lysates were incubated at 37 °C for 4 h. Samples were measured with an enzyme-linked immunosorbent assay (ELISA) reader at an absorbance of 405 nm and the caspase activities were expressed as percentage of enzyme activity compared with control. All the experiments were carried out in triplicate.
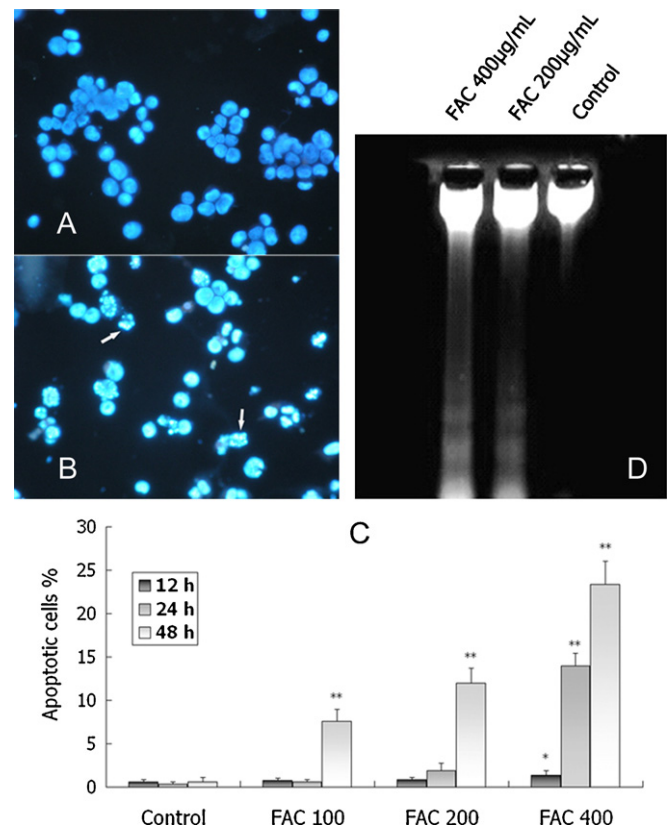

Fig. 3. Time course of appearance of apoptotic nuclei and DNA fragmentation in *Astragalus complanatus* flavonoid (FAC)-treated SMMC-7721 cells. SMMC-7721 cells were treated without or with FAC (100, 200, 400 μg/mL) for 12 h, 24 h, and 48 h. Cells were stained with Hoechst 33258 and observed under a fluorescence microscope. Arrows indicate the formation of apoptotic bodies, condensed nuclei and membrane blebbing as evidence of FAC-induced apoptosis. The number of apoptotic cells was determined by counting cells that had nuclear condensation and fragmentation, confirmed by Hoechst 33258 staining. Values are expressed as mean ± S.D. of four independent experiments (*$p < 0.05$, **$p < 0.01$ vs control group). (A) Cells without FAC treatment. (In controls, the majority of cells had uniformly stained nuclei.) (B) Cells treated with 200 μg/mL FAC for 48 h. (After exposure to 200 μg/mL FAC for 48 h, cells showed morphological changes typical of apoptosis, i.e. nuclear fragmentation with condensed chromatin.) (C) Time course of appearance of apoptotic nuclei. (D) Detection of DNA fragmentation by agarose gel electrophoresis in SMMC-7721 cells.

Fig. 2. Effect of *Astragalus complanatus* flavonoids (FAC) on proliferation of SMMC-7721 and HepG2 cells. The cells were cultured in 10% FBS medium and treated with FAC (25, 50, 100, 200, 400 μg/mL) for 48 h. 5-Fluorouracil (5-Fu) (10 μg/mL) was used as a positive control. The percent cell viability was calculated in comparison to control, which was taken as 100%. Values are expressed as mean ± S.D. of three independent experiments (**$p < 0.01$ vs control group).

2.9. Microarrays

2.9.1. RNA isolation

After treatment with 400 μg/mL FAC for 48 h, total RNA was extracted from SMMC-7721 cells using TRIzol (Invitrogen, Rockville, MD), following the manufacturer's instructions. The quality of total RNA was determined by measuring the absorbance at 260 nm ($A260$) and 280 nm ($A280$) in a spectrophotometer. The $A260/A280$ ratio of the samples was 1.9–2.1.

2.9.2. Synthesis of cDNA probes (Zhu et al., 2002)

Total RNA was used as a template for biotinylated probe synthesis using a GEArray Kit (SuperArray, Inc., Bethesda, MD). Five micrograms of total RNA was annealed with GEAprimer Mix at 70 °C for 3 min and cooled to 42 °C. The RNA was then labeled with labeling cocktail (GEAlabeling Buffer, biotin-16-dUTP, RNase inhibitor and reverse transcriptase) at 42 °C for 90 min. The reaction was stopped, denatured, and neutralized using specific solutions from SuperArray, Inc. The resulting cDNA probe was ready to be used for hybridization.

2.9.3. Hybridization and chemiluminescence detection

An S series apoptosis and cell cycle gene array (267 genes) (SuperArray Bioscience, Frederick, MD) was used, according to the manufacturer's protocol. The hybridized membranes were incubated with chemiluminescent substrate and exposed to X-ray film. The image was scanned as a grayscale TIFF file and converted into numerical data using the free ScanAlyze software. Signals
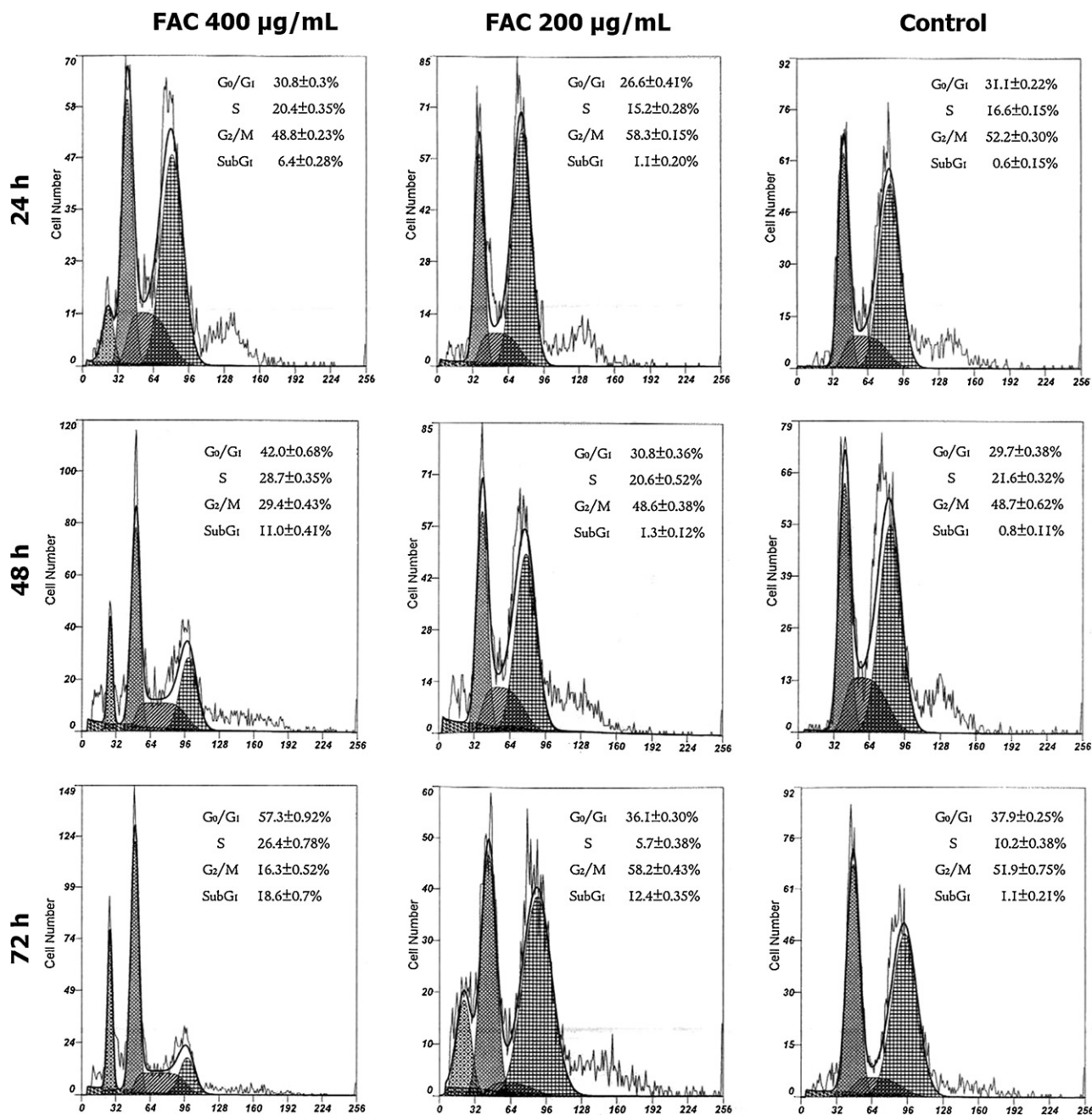

Fig. 4. Flow cytometry analysis of the DNA content of SMMC-7721 cells treated with *Astragalus complanatus* flavonoids (FAC). SMMC-7721 cells were treated with 0 μg/mL, 100 μg/mL, 200 μg/mL and 400 μg/mL FAC for 24 h and 48 h. The sub-G1 peak formed by cells with reduced DNA content represents the presence of apoptotic cells. The two major peaks represent the G0/G1 and G2/M phases of the cell cycle. .

were quantitated using GEArray Analyzer software for background subtraction and data normalization. Each GEArray™ S Series membrane was spotted with negative controls (pUC18 DNA and blanks) and housekeeping genes, including β-actin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), cyclophilin A and ribosomal protein L13a. Signals were quantitated by scanning the film and converting the image to a grayscale TIFF file. The intensity of the spots was analyzed using the "Image-Pro Plus" software (Media Cybernetics). β-Actin and GAPDH were used as positive controls, and the bacterial plasmid (pUC18) was used as a negative control.

2.10. Western blot analysis

SMMC-7721 cells were treated with 100 μg/mL, 200 μg/mL and 400 μg/mL FAC for 48 h. Both adherent and floating cells were collected and frozen at $-80\,^\circ$C. The protein expression of cyclinB1, cyclinD1, CDK1, CDK4, caspase-3, caspase-8, Bax, bcl-2, P21, and P27 in SMMC-7721 cells was detected by Western blot analysis. Briefly, the cell pellets were resuspended in lysis buffer, including HEPES 50 mmol/L, pH 7.4, Triton-X 100 1%, sodium orthovanadate 2 mmol/L, sodium fluoride 100 mmol/L, edetic acid 1 mmol/L egtazic acid 1 mmol/L, phenylmethylsulfonyl fluoride (PMSF) 1 mol/L, aprotinin 0.1 g/L, leupeptin 0.01 g/L, then lysed at $4\,^\circ$C for 1 h. After centrifugation at $12,000 \times g$ for 10 min, the protein content of the supernatant was determined using Bio-Rad protein assay reagent (Bio-Rad, California, USA). Equal amounts of protein lysates (50 μg) were separated by electrophoresis in 12% SDS–polyacrylamide gels and blotted onto nitrocellulose membranes. Protein expression was detected by rabbit polyclonal anti-cyclinB1, -cyclinD1, -CDK1, -CDK4, -caspase-3, -caspase-8, -Bax, -bcl-2, -P21, and -P27 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and secondary antibody conjugated with peroxidase (goat anti-rabbit IgG) (Santa Cruz Biotechnology). The immunoreactive proteins on the membrane were visualized with an enhanced chemiluminescence detection system (Applygen Technologies Inc., Beijing, PR China). The images were scanned and analyzed semiquantitatively using SigmaScan Pro 5 software (SPSS Inc., Chicago, IL). Experimental values were normalized to β-actin reactivity.

2.11. Statistical analysis

All data are presented as the mean ± standard deviation (S.D.). Differences between groups were evaluated using a two-tailed Student's t-test. Mean values were considered to be statistically significant at p values < 0.05.

3. Results

3.1. Cytotoxicity of FAC

Cytotoxic activity of FAC was evaluated by MTT assay. As shown in Fig. 2, the cytotoxicity of FAC in SMMC-7721 and HepG2 cells was dose-dependent. When SMMC-7721 and HepG2 cells were treated for 48 h, the IC_{50} values of FAC were 48 μg/mL and 53 μg/mL, respectively. These results suggest that FAC can dramatically inhibit the growth of SMMC-7721 and HepG2 cells *in vitro*.

3.2. Morphology of apoptotic cells

SMMC-7721 cells treated with FAC were stained with Hoechst 33258 and the appearance of chromatin condensation and fragmentation of nuclei was monitored. Morphological observation of SMMC-7721 cells showed that control SMMC-7721 cells had regular and round-shaped nuclei revealed by nuclear staining with Hoechst

33258 (Fig. 3A and B). By contrast, condensation and fragmentation of nuclei characteristic of apoptotic cells were evident in SMMC-7721 cells treated with FAC, especially at a dose of 400 μg/mL for 48 h. The percentage of apoptotic cells was significantly increased in cells exposed to FAC (200, 400 μg/mL) in a time-dependent manner (Fig. 3C). These results indicate that FAC induced apoptotic cell death in SMMC-7721 cells.

3.3. FAC-induced DNA fragmentation in SMMC-7721 cells

DNA extracted from SMMC-7721 cells treated with FAC at indicated concentrations (200, 400 μg/mL) for 48 h revealed a progressive increase in the 180–200 bp ladder fragments. Such a pattern corresponds to inter-nucleosomal cleavage, which is characteristic of apoptosis. Control cells did not exhibit any such DNA fragmentation (Fig. 3 D).

3.4. SMMC-7721 apoptosis and cell cycle inhibition by FAC

Quantitative detection of apoptotic cells and analysis of cell cycle kinetics were performed by flow cytometry. The sub-G1 peak formed with reduced DNA content represented the presence of apoptotic cells. The two major peaks represented the G0/G1 and G2/M phases of the cell cycle. After treatment with FAC at a dose of 400 μg/mL for 24 h, 48 h and 72 h, the proportions of apoptotic cells were 6.39%, 10.98% and 18.59%, respectively. After treatment with FAC at a dose of 200 μg/mL for 72 h, the proportion of apoptotic cells was 12.37%. G0/G1 phase and S phase cells represented 37.89% and 10.17%, respectively, in the control group after 72 h. However, treatment with FAC at 400 μg/mL for 72 h increased the proportions of G0/G1 phase and S phase cells to 57.34% and 26.35%, respectively. These results show that treatment with FAC (400 μg/mL) induced a time-dependent increase in the apoptotic rate and marked accumulation in the G0/G1 phase and S phase of the cell cycle in SMMC-7721 cells (Fig. 4).

3.5. Effects of FAC on activation of caspase-3

The cellular pathway of FAC-induced cell death was examined by assessing caspase-3 activity, which plays a critical role in apoptosis. Following 12 h, 18 h, 24 h and 30 h treatment of SMMC-7721 cells with various concentrations of FAC, caspase-3 activities were measured and compared with control cells (Fig. 5A). As shown, SMMC-7721 cells treated with FAC for 0–30 h showed significant
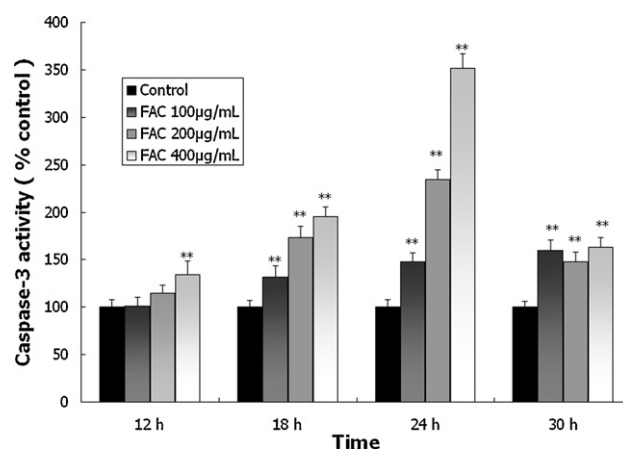
Fig. 5. Effects of *Astragalus complanatus* flavonoids (FAC) on caspase-3 activation in SMMC-7721 cells. The cells were incubated with FAC at 0 μg/mL, 100 μg/mL, 200 μg/mL and 400 μg/mL for 0–30 h. Data are the mean ± S.D. *$p < 0.05$, **$p < 0.01$ *vs* control group. Three separate experiments were performed and the caspase activities are expressed as percentage of enzyme activity compared with control.
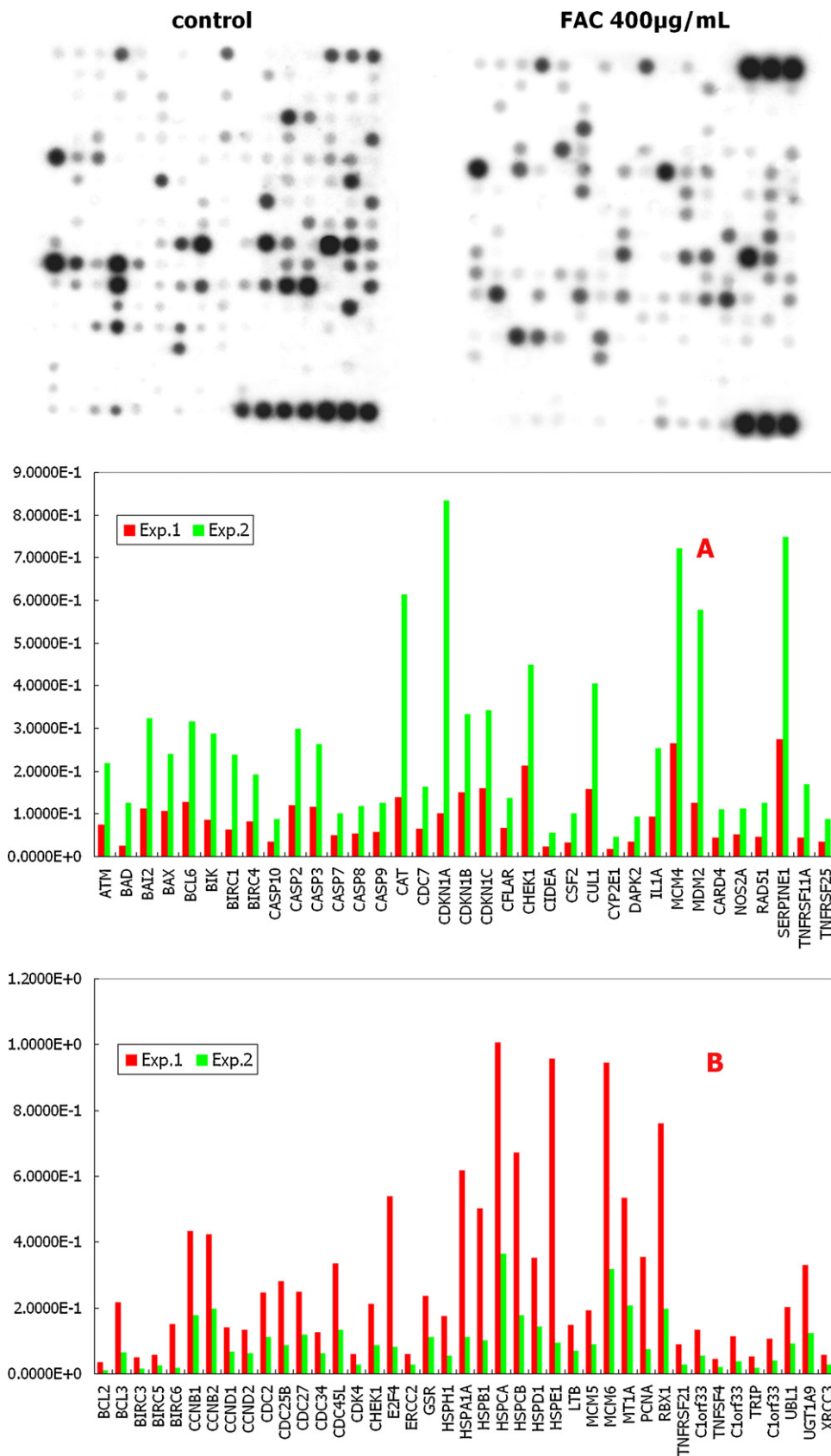
Fig. 6. The regulated genes and scanned SuperArray images in SMMC-7721 cells. Total RNA was isolated after treatment with *Astragalus complanatus* flavonoids (FAC) for 48 h. RNA was analyzed using a GEArray S series kit. The relative expression of apoptosis and cell cycle genes was normalized to average signal of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin expression. Plasmid DNA (pUC18) was used as a negative control for background subtraction. (A) Up-regulated genes; (B) down-regulated genes.
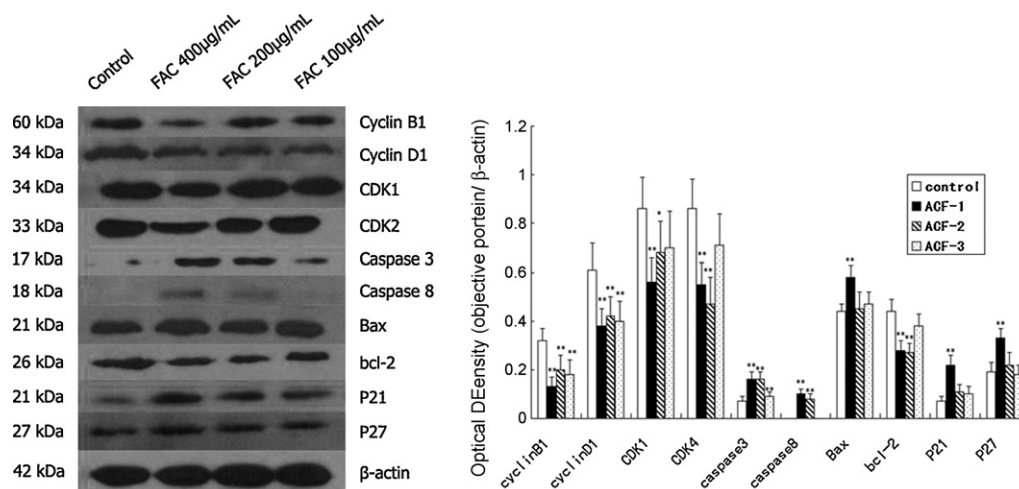
Fig. 7. Effects of *Astragalus complanatus* flavonoids (FAC) on cyclinB1, cyclinD1, CDK1, CDK4, bcl-2, caspase-3, caspase-8, Bax, P21, and P27 protein expression in SMMC-7721 cells. SMMC-7721 cells were treated with 100 μg/mL, 200 μg/mL and 400 μg/mL FAC for 48 h. The protein levels were monitored by Western blot analysis. The signal was normalized using actin as an internal standard. Three separate experiments were done. $^*p < 0.05$, $^{**}p < 0.01$ *vs* control group.

time- and concentration-dependent increases in caspase-3 activity. These results suggest that FAC-induced apoptosis occurs through the activation of common executors of apoptosis, such as caspase-3.

3.6. Effect of FAC on expression of apoptosis and cell cycle-related genes

To investigate the effects of FAC on apoptosis and cell cycle regulatory molecules, total RNA was harvested from synchronized SMMC-7721 cells, either untreated or treated with FAC 400 μL/mL for 48 h. The results of a representative experiment using an S series apoptosis and cell cycle gene array are shown in Fig. 6. The gene array analysis indicated that the expression levels of 76 genes were changed: 35 genes were up-regulated and 41 were down-regulated. The functions of the affected genes were related to apoptosis, cell cycle, stress and toxicity.

3.7. Effect of FAC on expression of cell cycle and apoptosis-related proteins

To further examine the effects of FAC on cyclinB1, cyclinD1, CDK1, CDK4, caspase-3, caspase-8, Bax, bcl-2, P21, and P27, their protein levels were evaluated using Western blot analysis. Fig. 7 shows that caspase-3, caspase-8, Bax, P21, and P27 protein levels increased after 48 h of treatment with FAC, while cyclinB1, cyclinD1, CDK1, and CDK4 protein levels decreased.

4. Discussion and conclusions

The seeds of *Astragalus complanatus* are a well-known traditional Chinese medicine for liver and kidney complaints. Our previous investigation revealed that FAC had a protective effect on the liver both *in vivo* and *in vitro* (Liu et al., 2002). The experimental results *in vitro* showed that FAC increased the activity of primary cultures of rat liver cells. In the present study, we demonstrated that FAC dramatically inhibited the growth of both SMMC-7721 and HepG2 cells *in vitro*. These results suggest that the action of FAC is specific to hepatocarcinoma cells.

Cancer develops when the balance between cell proliferation and cell death is disturbed, and aberrant cell proliferation leads to tumor growth. Apoptosis is a form of physiological cell death essential to normal tissue development and homeostasis (Vaux and Korsmeyer, 1999). After receiving an apoptotic death stimulus, cells

first enter a signaling phase followed by the final degradation phase, in which apoptosis is identifiable by chromatin condensation, cell shrinkage, caspase activation, membrane lipid rearrangement, DNA fragmentation, and cell fragmentation, through the formation of 'apoptotic bodies' (Jacobson et al., 1997). Apoptosis and its related signaling pathways have a profound effect on the progression of cancer (Lowe and Lin, 2000). Induction of apoptosis is therefore a highly desirable goal of preventive strategies for cancer control (Reed and Pellecchia, 2005). Flavonoids are naturally occurring molecules that are abundant in fruits, vegetables, nuts, seeds, and beverages such as tea and wine. There have been reports of flavonoids inducing apoptosis in cancer cells (Wang et al., 1999; Park et al., 2008). The results of the present study clearly demonstrate that FAC suppressed SMMC-7721 cell viability through inducing apoptosis. After treatment with FAC, nuclear condensation and DNA fragmentation were clearly observed. These results suggest that SMMC-7721 cells treated with FAC undergo typical apoptosis. Furthermore, flow cytometric analysis after propidium iodide staining validated FAC-induced apoptosis in SMMC-7721 cells.

It is now well known that apoptosis occurs through two main pathways, namely extrinsic and intrinsic apoptosis signaling (Budihardjo et al., 1999; Chen and Wang, 2003; Twomey and McCarthy, 2005). The extrinsic or cytoplasmic pathway is triggered through the Fas death receptor, a member of the tumor necrosis factor (TNF) receptor superfamily. The intrinsic or mitochondrial pathway leads, when stimulated, to the release of cytochrome *c* from the mitochondria and activation of the death signal. The intrinsic or mitochondrial pathway of apoptosis is regulated by the Bcl-2 family of proteins, which is involved in positive and negative regulation of apoptotic cell death (Vander Heiden and Thompson, 1999). Among the antiapoptotic members, Bcl-2, Bcl-XL, Bcl-w, Bfl-1, Bag-1, Mcl-1, and A1 are known for protecting against cell death, whereas others, such as Bax, Bak, Blk, Bad, and Bid promote or accelerate cell death. Antiapoptotic Bcl-2 and proapoptotic Bax are two of the major members of the Bcl-2 family (Wolter et al., 1997). An elevated intracellular Bax/Bcl-2 ratio occurs during increased apoptotic cell death (Zha and Reed, 1997). Caspases, the cytoplasmic aspartate-specific cysteine proteases, also play an important role in apoptosis (Thornberry, 1998). The death receptor-dependent apoptotic pathway is triggered at the cell surface and requires activation of caspase-8, whereas the mitochondrion-dependent pathway is initiated by the release of mitochondrial cytochrome

c into the cytoplasm and requires activation of caspase-9. Subsequently, caspase-8 or -9 can activate caspase-3, which in turn targets and degrades specific and vital cellular proteins, ultimately resulting in nuclear DNA degradation and apoptotic death of the cells (Krammer and Debatin, 2004). It is obvious that activation of caspases is central to the execution of apoptosis (Thornberry and Lazebnik, 1998; Nagane et al., 2001).

In the present study, an S series apoptosis and cell cycle gene array was used to identify changes in gene expression in human hepatoma SMMC-7721 cells in response to FAC treatment. We investigated the activity of caspase-3, which is considered to play a central role in many types of stimulus-induced apoptosis (Nicholson and Thornberry, 1997; Salvesen and Dixit, 1997; Shi, 2004). The results indicated that the expression levels of 76 genes were changed; 35 were up-regulated and 41 were down-regulated. Analysis of the gene array demonstrated that apoptosis induction in the SMMC-7721 cells treated with FAC was related to the regulation of genes involved in apoptosis signaling pathways and the cell cycle. The gene expression level of Bcl-2 was decreased, while that of Bax, Bad and bik were increased. Furthermore, the gene expression levels of caspase-2, -3, -7, -8, -9, and -10 were increased. Western blotting showed that FAC was able to inhibit the protein expression of Bcl-2 and stimulate the protein expression of Bax, caspase-3, and caspase-8, and significantly increase the Bax/Bcl-2 ratio. These results were consistent with those from the gene expression studies. SMMC-7721 cells treated with FAC showed a significant time- and concentration-dependent increase in caspase-3 activity. These results suggest that FAC could activate caspase-8 via the mitochondria-dependent pathway, and caspase-8 could then activate the downstream effector caspase-3, which in turn cleaves cytoskeletal and nuclear proteins, finally inducing apoptosis. Simultaneously, FAC could induce apoptosis in SMMC-7721 cells via the death receptor-dependent apoptotic pathway, by increasing the ratio of Bax and Bcl-2 and up-regulating the gene expression of caspase-9. However, further studies are needed to confirm the detailed mechanisms of FAC activity.

Cell cycle progression and apoptosis are two pivotal signaling mechanisms used to maintain homeostasis in healthy tissues. Many anti-cancer agents and DNA-damaging agents arrest the cell cycle at the G0/G1, S, or G2/M phase and then induce apoptotic cell death (Kessel and Luo, 2000; Purohit et al., 2000; Tanaka et al., 2004; Harakeh et al., 2008;). Cell proliferation is governed by the cell cycle, which is tightly regulated by a number of cyclin-dependent kinases (CDK) and cyclins (Buecher et al., 2003) The assembly and disassembly of CDKs drive progression through the cell cycle. CDK activity is further regulated by other classes of proteins such as the Cip/Kip family (including p21/WAF1, p27/Kip1 and p57/Kip2). A shift in favor of proliferative signals, for example, over-activation of cyclin/CDK, often leads to uncontrolled cell division and malignancy. Arrest in the G1 phase results in an accumulation in the sub-G1 phase, which has been identified as a hallmark of apoptosis. In the present study, we found that FAC arrested SMMC-7721 cells in the G0/G1 and S phases, to different degrees. The results of gene array and Western blot analyses in this study showed that P21 and P27 were up-regulated, while cyclinB1, cyclinD1 and the associated kinases CDK1 and CDK2 were down-regulated in the FAC-treated SMMC-7721 cells. These findings suggest that the antiproliferative effect of FAC in SMMC-7721 cells may reflect changes in the expression levels of several genes related to the cell cycle and apoptosis induction.

Our results show that FAC demonstrates significant cytotoxicity in SMMC-7721 and HepG2 cells. The cytotoxic mechanisms of FAC relate to its effects on apoptosis and cell cycle arrest. FAC could induce apoptosis through mitochondria-dependent and death receptor-dependent apoptotic pathways. These results suggest that FAC deserves further study as a potential anti-cancer drug.

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