

***Astragalus* saponins induce apoptosis via an ERK-independent NF- κ B signaling pathway in the human hepatocellular HepG2 cell line**

KATHY KA-WAI AUYEUNG, PUI-CHING LAW and JOSHUA KA-SHUN KO

Pharmacology and Toxicology Laboratory, School of Chinese Medicine,
Hong Kong Baptist University, Hong Kong, SAR, P.R. China

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Abstract. *Astragalus membranaceus* has been used to ameliorate the side effects of anti-neoplastic drugs. We recently reported that total *Astragalus* saponins (AST) possess anti-tumor properties in human colon cancer cells and tumor xenografts. Nevertheless, the precise mechanism of action has not been fully elucidated. The present study aimed to unveil the anti-carcinogenic potential of AST in HepG2 human hepatocellular carcinoma (HCC) cells and to clarify the signaling pathway. We demonstrated here that AST down-regulated expression of the HCC tumor marker α -fetoprotein and suppressed HepG2 cell growth by inducing apoptosis. AST also caused caspase activation, poly(ADP-ribose) polymerase (PARP) cleavage, nuclear chromatin condensation, with downregulation of the anti-apoptotic proteins bcl-2 and bcl-x_L and decreased nuclear factor-kappa B (NF- κ B)/DNA-binding activity. Concomitantly, expression of the phosphorylated form of the extracellular signal-regulated protein kinase (ERK) was prominently increased. Nevertheless, pre-treatment of ERK inhibitor PD98059 did not attenuate AST-induced PARP cleavage. Taken together, these results exemplify that AST induced growth inhibition and promoted apoptosis in HepG2 cells through modulation of an ERK-independent NF- κ B signaling pathway.

Introduction

Hepatocellular carcinoma (HCC) is one of the most frequently occurring malignancies in Southeast Asia and Africa, and ranks third among the causes of cancer-related mortality in Hong Kong. The increasing incidence of HCC appears to be

associated with the dissemination of hepatitis B and C viral infections (1). Prognosis of HCC is usually poor, with the 5-year post-surgical survival rate being limited to 25-49% (2). In addition, conventional therapies such as transcatheter arterial embolization, percutaneous ethanol injection therapy and microwave coagulation therapy are generally not prescribed for advanced HCC due to low efficacy and potential complications (3). In some clinical trials, combination chemotherapy using interferon- α and 5-fluorouracil have demonstrated outstanding anti-tumor effects in patients with advanced HCC (4). Unfortunately, the response of HCC to current chemotherapy remains inconsistent, with many associated side effects (e.g. neutropenia and immunosuppression). Hence, the discovery of novel anti-carcinogenic agents with high efficacy, low toxicity and known mechanisms of action is crucial.

α -fetoprotein is only present in fetal liver cells or HCC, thus it can be a unique indicator of HCC progression. It has been identified as the downstream target of nuclear factor-kappa B (NF- κ B) (5). Aberrant NF- κ B activity results when there is overexpression of the proto-oncogene *c-myc* in HCC. The *c-myc* protein eventually activates transcription of the growth-related genes *cyclin A*, *cyclin D1* and *cyclin E*, which in turn increase expression of their respective nuclear proteins. In spite of this, in the cell-cycle regulation of active HCC, negative modulators such as p53 and p27^{Kip1} (both tumor suppressor genes) are less expressed, which is in contrast to overexpression of the cyclins and their respective kinases. p27 protein is in fact a cyclin-dependent kinase inhibitor that inhibits progression of the cell cycle (6). A combination of these events drives the hepatocytes into active proliferation (7), which contributes to further development of HCC (8).

NF- κ B was found to be involved in the regulation of cell proliferation and survival during hepatic regeneration and development in liver epithelium. Several models were established that support the role of NF- κ B in suppressing liver cell apoptosis (9). Other studies also demonstrated that inhibition of NF- κ B in hepatocyte cell lines blocks tumor necrosis factor (TNF)-induced proliferation and sensitizes these cells to undergo apoptosis (10,11). The enhancer elements of NF- κ B were found to be located at the promoter region of inducible nitric oxide synthase (iNOS), thus NF- κ B can induce iNOS gene expression, which is consistent with its role in regulating inflammatory events. In most tumors, there is higher NF- κ B expression and activity when compared to the

Correspondence to: Dr Joshua Ka-shun Ko, Pharmacology and Toxicology Laboratory, School of Chinese Medicine, Hong Kong Baptist University, 7 Baptist University Road, Kowloon Tong, Hong Kong SAR, P.R. China
E-mail: jksko@hkbu.edu.hk

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adjacent normal tissues, but such expression strongly depends on the tumor cell types and stage of development (12).

Astragalus membranaceus (Fischer) Bge. var. *mongolicus* is a key herb in many traditional Chinese medicinal formulations. According to its contemporary use, it shows an immunostimulatory effect by enhancing phagocytosis, increasing T-killer cell activity and immunoglobulin concentration, as well as promoting sperm motility and cardiogenic effects. It also exhibits anti-inflammatory, anti-hypertensive, anti-ulcerative and anti-aging effects, plus improvement in hematopoiesis and blood pressure regulation (13). The interleukin-2 (IL-2)-inducing activity of the root of the *Astragalus* species might be involved in its immunomodulatory effects (14). Discovery and investigation of the anti-cancer effects of *Radix Astragali* have been undergoing since the last decade, but the mode of action is still poorly understood. According to the literature, the crude herb extract inhibits the growth of hepatoma (HepA) and Sarcoma 180 (S180) cells in mice (15). The bioactive compounds obtained from *Radix Astragali* include various polysaccharides, saponins and flavonoids (16). It was reported that hepatocarcinogenesis was prevented in rats fed with the aqueous *Astragalus* extract, which is mainly composed of polysaccharides (17). On the other hand, the total saponins of *Radix Astragali* (AST), one of the most active components of the herb, have shown prominent pharmacological effects. Key constituents of AST include astragalosides I-VII, acetyl-astragaloside I and isoastragaloside I, II, IV. Despite the fact that some *Astragalus* saponins such as astragaloside IV possess immunomodulating effects by increasing B and T cell proliferation (18), we recently reported that AST exhibit anti-carcinogenic effects in HT-29 human colon cancer cells and tumor xenografts. The anti-tumor effects were comparable to that of conventional chemotherapeutic drug 5-FU, with fewer side effects (19). However, the precise anti-carcinogenic mechanism of AST remains unclear.

In the present study, we aimed to unveil the pro-apoptotic and growth-inhibitory potential of AST in human HCC cells, and to further elaborate its correlation with the NF- κ B signaling pathway. Our key findings demonstrated that AST exert cytotoxicity and induce apoptosis in HepG2 cells involving extracellular signal-regulated protein kinase (ERK)-independent NF- κ B inactivation.

Materials and methods

Materials. The enhanced chemiluminescence (ECL) detection kit was purchased from Amersham Biosciences (Piscataway, NJ). The mouse monoclonal anti-bcl-2 and the mouse anti-bcl-x_L were from Zymed Laboratories Inc. (San Francisco, CA). The mouse monoclonal anti-caspase 3 and anti-procaspase 9 were from Upstate (Charlottesville, VA), and the rabbit anti-phospho-p44/p42 MAP kinase (Thr²⁰²/Tyr²⁰⁴) antibody and the rabbit anti-p44/p42 MAP kinase antibody were from Cell Signaling Technology (Danvers, MA). The mouse monoclonal anti-PARP was from Becton Dickinson (San Jose, CA), and the mouse monoclonal anti- α -fetoprotein and anti- β -actin were from Sigma-Aldrich (St. Louis, MO). 3-[4,5-Dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide (MTT) and other chemicals were also obtained from Sigma-Aldrich unless specified.

Preparation of total *Astragalus* saponin extract. *Radix Astragalus membranaceus* (Fisch.) Bunge var. *mongolicus* was obtained from the province of Shanxi, P.R. China. The authenticity and quality of the crude herb were tested in the Quality Assurance Laboratory of the School of Chinese Medicine, Hong Kong Baptist University by microscopic and chromatographic analyses as well as DNA finger-printing. A voucher specimen was kept at the herbarium centre for future reference to ensure consistency among batches. *Astragalus* saponins were extracted according to the method of Ma *et al* (16) with slight modifications. In brief, 500 g of crude herb was refluxed with 2% potassium hydroxide in methanol for 1 h. Butan-1-ol was added to the reconstituted residue from above for phase separation to obtain total saponins. The dried and lyophilized AST powder (~0.6% w/w) was reconstituted in ultrapure water to form a 10-mg/ml stock and stored at -20°C.

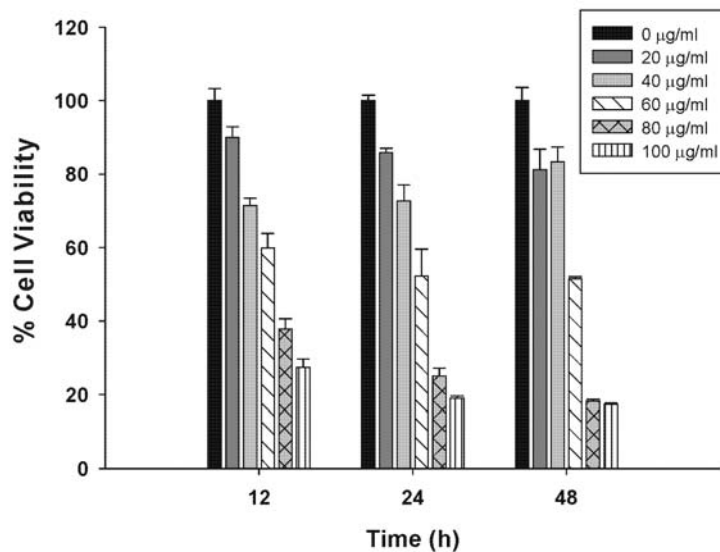
Cell culture. HepG2 cells (HB-8065), originating from human liver tissues with HCC, were obtained from American Type Culture Collection (Rockville, MD). Cells were cultured in Dulbecco's modified Eagle's medium (D-MEM; Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum plus penicillin (100 U/ml) and streptomycin (100 μ g/ml) (Gibco). The cells were subcultured every 2-4 days according to the previous subculture ratio.

ERK inhibitor test. To examine the role of the ERK signaling pathway in AST-induced apoptosis, a MAP kinase inhibitor PD98059 (Calbiochem, San Diego, CA), was used in the present study. PD98059 was dissolved in dimethyl sulfoxide (DMSO) to prepare a 50-mM stock. The final concentration of PD98059 used throughout the present study was 20 μ M.

Viability assay. Cell viability was measured by the MTT assay. HepG2 cells were seeded at a density of 2×10^3 cells/well in 96-well plates. After treatment with various concentrations of AST for 12-48 h, cells in each well were incubated with 30 μ l of MTT at 37°C for 3 h. The culture medium was then refreshed, and the intracellular formazan product was dissolved in 100 μ l of DMSO. Absorbance was determined spectrophotometrically at 540 nm. Six independent experiments were conducted for each test.

Western immunoblotting. Cells were seeded in 100-mm petri dishes at a density of 3.0×10^5 cells/dish and were treated with various concentrations of AST. After treatment for 48, 72 or 96 h, cells were lysed in RIPA buffer containing 50 mM Tris, 150 NaCl, 0.5% deoxycholate, 0.1% SDS, 2 mM EDTA, 0.1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin and 5 μ g/ml pepstatin A. After the insoluble materials were removed by centrifugation at 14,000 \times g for 10 min at 4°C, protein content was quantified using Coomassie Plus Protein Assay Reagent Kit (Pierce, Rockford, IL). The cellular levels of AFP, procaspase 3, procaspase 9, bcl-2, bcl-x_L, ERK and poly(ADP-ribose) polymerase (PARP) were then determined by Western immunoblotting. Total cellular proteins (20-40 μ g) in the cell lysate were separated by 8-15% SDS polyacrylamide gel electrophoresis. The proteins on the gel were transferred onto a nitrocellulose membrane and then probed with the respective

(A)



(B)

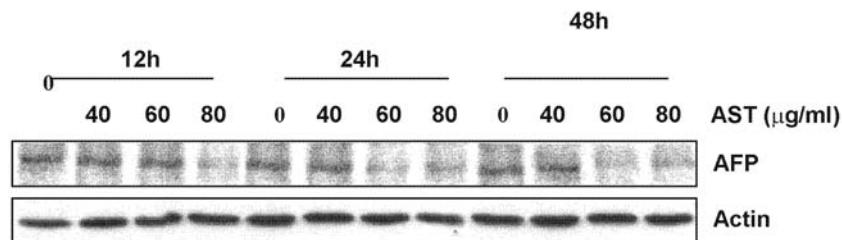


Figure 1. Growth-inhibitory effect of AST in HepG2 cell culture. (A) Cells were incubated for 12, 24 or 48 h in graded concentrations of AST, and viability was determined by MTT assay. Data show an increase in the number of non-viable cells by increasing concentrations of the drug. (B) AST downregulated α -fetoprotein expression in HepG2 cells. Cells were treated with 0 (medium control), 40, 60 or 80 μ g/ml of AST for 12, 24 and 48 h. Expression of α -fetoprotein was determined by Western immunoblotting. Representative immunoblots from four independent experiments are shown.

primary antibodies at a 1:1000 dilution, followed by incubation with the corresponding secondary antibodies conjugated with horseradish peroxidase. Protein bands were visualized using ECL reagents and exposed to Kodak X-Omat Blue XB-1 film (Rochester, NY). Results were analyzed by using the Quantity One version 4.4.1 Basic software (BioRad, Hercules, CA). The membrane was then stripped by stripping buffer containing 100 mM β -mercaptoethanol, 2% SDS and 62.5 mM Tris-HCl at 50°C for 30 min for quantification and normalization with β -actin (1:10000).

Electrophoretic mobility shift assay. Nuclear proteins were isolated from the cells, and electrophoretic mobility shift assay (EMSA) was performed to determine NF- κ B/DNA-binding activity. Nuclear proteins (10 μ g) were incubated with the reaction buffer for 15 min, followed by incubation with 32 P end-labeled oligonucleotide containing a sequence for the NF- κ B/DNA-binding site (5'-AGT TGA GGG GAC TTT CCC AGG C-3'). The reaction mixture was separated in a non-denaturing 6% polyacrylamide gel that was later exposed to radiographic film at -80°C.

Hoechst nuclear staining. HepG2 cells were grown on glass coverslips. Drug-treated cells were washed with phosphate-buffered saline (PBS) three times and then fixed in 4% paraformaldehyde solution for 10 min. After fixation, cells were washed with PBS three more times and then permeabilized with methanol at -20°C for 10 min. Cells were finally stained with 10 μ M of the membrane-permeable DNA-binding dye benzimide Hoechst 33342 trihydrochloride (H33342) at room temperature for 15 min. Cells were examined under a fluorescence microscope (magnification, x40) to visualize cell and nuclear morphology.

Statistical analysis. Data were expressed as the mean \pm SEM. Statistical significance of at least $P < 0.05$ was determined by one-way analysis of variance (ANOVA) followed by a *post-hoc* Tukey's test using the SPSS version 10.0 software.

Results

AST induce cytotoxicity and downregulates α -fetoprotein expression in HepG2 cells. HepG2 cells were treated with

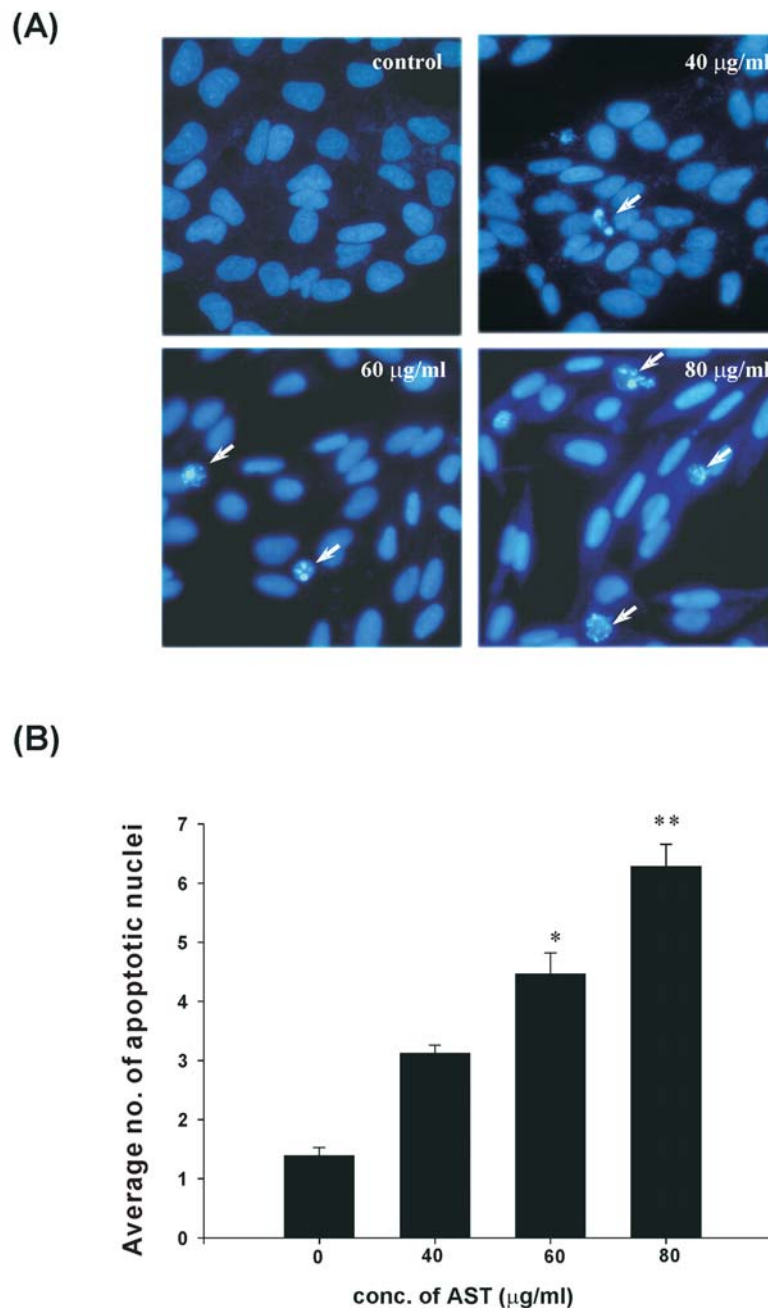


Figure 2. HepG2 cells were cultured in the absence (0 $\mu\text{g/ml}$) or presence of AST (40–80 $\mu\text{g/ml}$) for 48 h. H33342 was used to stain the nucleus, in which nuclear chromatin condensation was observed as a marker of apoptosis. (A) Control cells were round and present with homogeneous staining (upper left panel), while nuclear chromatin condensation was observed after the treatment of AST at various concentrations. Representative images were obtained from three separate experiments. Apoptotic cells are indicated by arrows. (B) Data were summarized as the number of apoptotic nuclei. Results are expressed as the average number of cells \pm SEM of four independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. medium control.

AST for 12–48 h, which resulted in a concentration-dependent growth-inhibitory effect (Fig. 1A). Cell viability was decreased by $\sim 80\%$ after treating the cells with the highest concentration of AST (80 $\mu\text{g/ml}$) for 24–48 h. In addition, the median inhibitory concentration (IC_{50}) of AST following 48 h of AST treatment was found to be ~ 60 $\mu\text{g/ml}$, while the 25 and 75% inhibitory concentrations (IC_{25} and IC_{75}) were 40 and 80 $\mu\text{g/ml}$, respectively. These concentrations were used to demonstrate the anti-carcinogenic effects of AST throughout the rest of the study. α -fetoprotein is an oncofetal glycoprotein which is frequently expressed in HCC. It can be used as a tumor marker for liver cancer. Downregulation of its expression

indicates that the process of carcinogenesis was modulated by the therapeutic regimen. In our study, expression of α -fetoprotein in HepG2 cells was significantly downregulated following treatment with AST (Fig. 1B). α -fetoprotein expression was decreased after cells were treated with 80 $\mu\text{g/ml}$ for 48 h. These results provide evidence regarding the anti-carcinogenic potential of AST.

AST promote apoptotic cell death without inducing cell cycle arrest in HepG2 cells. A disturbance in the balance between the rate of cell proliferation and incidence of apoptosis may lead to the development of tumor cells. The main purpose of

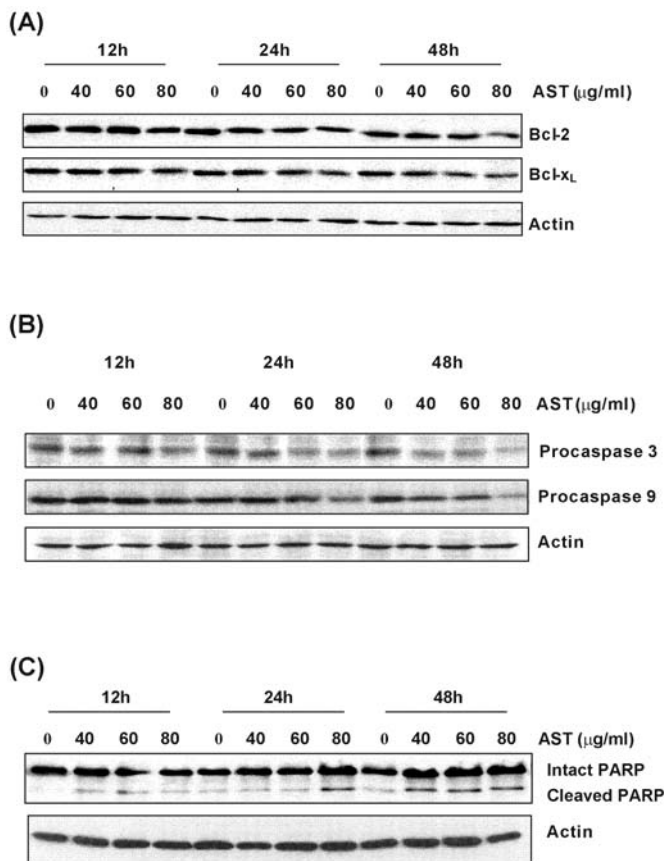


Figure 3. HepG2 cells were treated with 0 (medium control), 40, 60 or 80 $\mu\text{g/ml}$ of AST for 12, 24 and 48 h. Specific antibodies against (A) bcl-2 and bcl-x_L, (B) procaspase 3 and procaspase 9 and (C) PARP were used in Western immunoblotting to detect their protein expression following corresponding treatments. Data shown are representative immunoblots from four independent experiments with similar findings.

cancer chemotherapy is to regulate such equilibrium (20). In order to unveil the mechanism underlying the growth-inhibitory activity of AST, we investigated whether AST induced the apoptotic cell death of HepG2 cells using H33342 immunofluorescence staining. As shown in Fig. 2A, no morphological change was observed in control HepG2 cells, which appeared to be round and manifested homogeneous staining. In the AST-treated cells, the cell size was reduced, while nuclear chromatin condensation was clearly visible, with the presence of granular bodies. The data were summarized as the average number of apoptotic nuclei in AST-treated HepG2 cells for 48 h. In accordance with the decline in cell viability, the number of apoptotic nuclei increased in a dose-dependent manner (Fig. 2B). A most significant change was observed when cells were treated with 80 $\mu\text{g/ml}$ AST. In addition, the cell cycle response of HepG2 cells to AST was also investigated by using flow cytometry. However, no significant change in cell distribution at different phases of the cell cycle was observed (data not shown).

AST modulate expression of apoptosis-related proteins in HepG2 cells. NF- κB has been proposed to be involved in the regulation of genes which control apoptotic cell death. Activation of NF- κB in cancer cells is correlated with the inhibition of apoptosis that leads to increased expression of

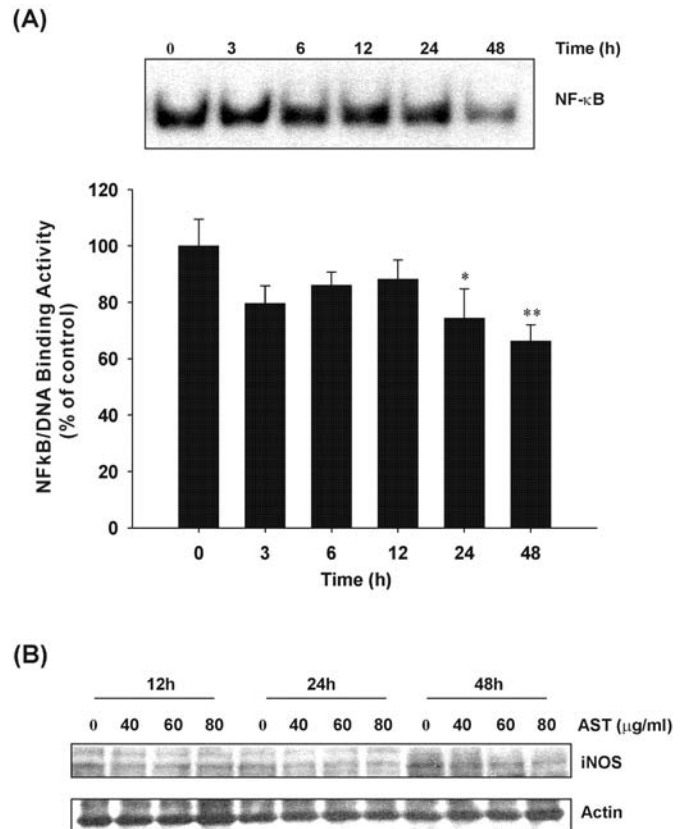


Figure 4. (A) Time-course study of NF- κB /DNA-binding activity following AST (80 $\mu\text{g/ml}$) treatment (3-48 h) in HepG2 cells was performed. Nuclear proteins were isolated, and EMSA was performed to determine the NF- κB /DNA-binding activity. Data are summarized in the lower panel as percentage of medium control (0 $\mu\text{g/ml}$ AST) at the respective time. Results are expressed as means \pm SEM of three independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. time 0 h (before AST was added). (B) Cells were treated with 0 (medium control), 40, 60 or 80 $\mu\text{g/ml}$ of AST for 12, 24 and 48 h. Specific antibody against iNOS was used in Western immunoblotting to detect the protein expression following corresponding treatments. Data shown are representative immunoblots from four independent experiments with similar findings.

anti-apoptotic proteins. The processes of apoptosis involve a cascade of caspase proteolytic activity and members of the bcl-2 family of proteins. Several key anti-apoptotic proteins were assessed in the present study. Incubation of AST in the HepG2 cell culture for 48 h significantly decreased the expression of bcl-2 and bcl-x_L (Fig. 3A). This concentration-dependent downregulation of the bcl-2 members of proteins was found to be working in concert with activation of the executional caspase 3 (Fig. 3B). Furthermore, reduced protein expression of procaspase 9 was also observed in AST-treated HepG2 cells (Fig. 3B), designating possible involvement of the mitochondrion-dependent apoptotic pathway. As a downstream target of active caspase 3 during the induction of apoptosis, PARP was cleaved into two fragments. In the present study, expression of cleaved PARP increased markedly when cells were treated with 80 $\mu\text{g/ml}$ AST (Fig. 3C). Such cleavage of PARP would subsequently lead to defective DNA repair and resulted in apoptotic cell death.

AST inhibit NF- κB activation and reduce iNOS protein expression in HepG2 cells. As mentioned previously, NF- κB

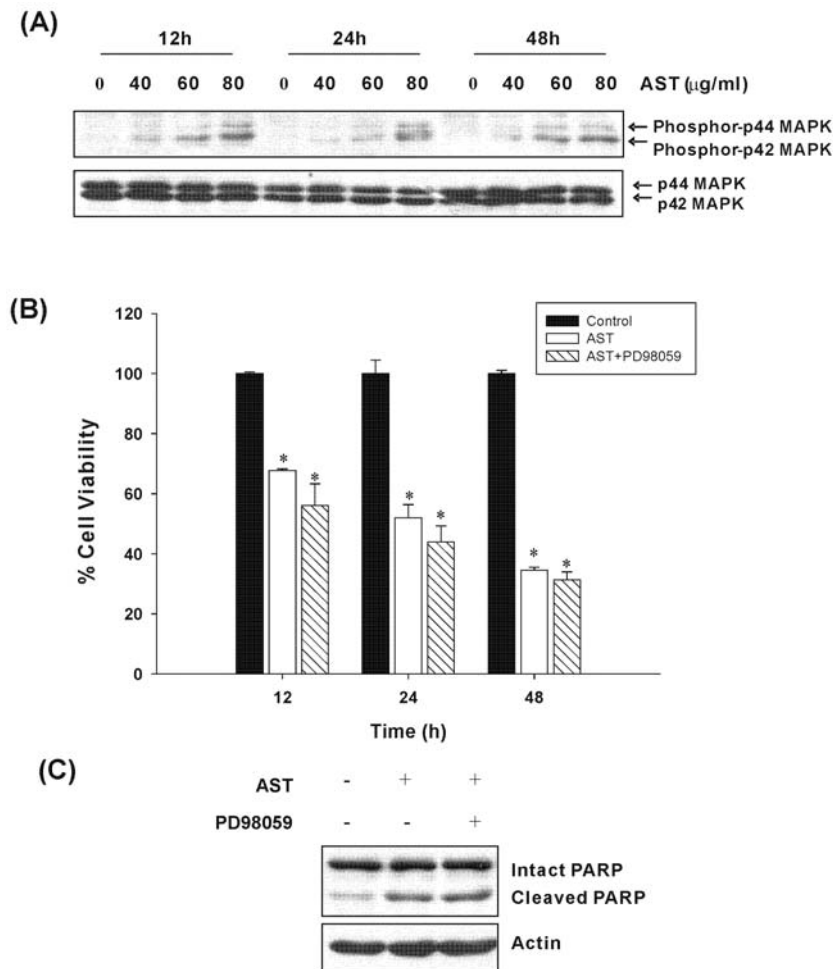


Figure 5. HepG2 cells were treated with 0 (medium control), 40, 60 or 80 $\mu\text{g/ml}$ of AST for 12, 24 and 48 h. (A) Specific antibodies against anti-phospho-p44/p42 MAP kinase and anti-p44/p42 MAP kinase were used in Western immunoblotting to detect protein expression following corresponding treatments. Membranes were re-probed with anti-p44/p42 MAP kinase to detect the total p44/p42 MAP kinase. (B) Cells were treated with 80 $\mu\text{g/ml}$ of AST in the absence or presence of PD98059 for 12, 24 and 48 h, and cell viability was determined by MTT assay. * $P < 0.05$ vs. medium control. (C) Specific antibody against PARP was used in Western immunoblotting for detection of its protein expression following corresponding treatments. Data shown are representative immunoblots from four independent experiments with similar findings.

is involved in the regulation of cell proliferation and survival during hepatic regeneration and development in liver epithelium. In addition, contemporary studies have also indicated that NF- κB signaling could be involved in tumor development (21,22). After AST was applied for 3–48 h, the NF- κB /DNA-binding activity was reduced in a time-dependent manner after 12 h, with the lowest activity being reached following 48 h of AST incubation (Fig. 4A). iNOS expression was shown to prevent apoptosis in TNF- α/β -treated hepatocytes (23). In most tumors, there is higher iNOS expression and activity when compared to the adjacent normal tissues. Studies actually showed that cytokine-induced iNOS expression in human liver and lung epithelial cell lines is dependent upon NF- κB activation (12). Thus, iNOS protein expression was determined in AST-treated HepG2 cells. Results indicated that iNOS protein expression was significantly downregulated following 24–48 h of AST treatment (Fig. 4B).

Activation of ERK signaling pathway is not associated with AST-induced apoptosis and growth inhibition. The ERK-

dependent pathway has been proposed to be involved in the induction of cell death by 2'-hydroxycinnamadehyde in TNF-treated colon cancer cells (24). In this study, the expression of total ERK remained unchanged in both control and AST-treated cells. Nevertheless, phosphorylated ERK expression was elevated concentration-dependently following AST treatment (Fig. 5A), implicating a possible involvement of ERK activation in AST-induced apoptosis. We then used a specific inhibitor of ERK, PD98059, to confirm the reversibility of the growth-inhibitory and pro-apoptotic effects brought about by AST. Our results demonstrated that ERK inhibition neither reversed the AST-induced growth-inhibitory effects (Fig. 5B) nor reduced PARP cleavage (Fig. 5C).

Discussion

In the present study, we explicitly demonstrated that AST induced cytotoxicity in HepG2 HCC cells. Other than growth inhibition, AST also promoted apoptotic cell death. Exposure of HepG2 cells to AST resulted in an extensive down-regulation of α -fetoprotein, signifying the successful

prevention of tumor progression. In addition, inhibition of NF- κ B signaling and concurrent activation of ERK were observed. However, the use of ERK inhibitor, PD98059, did not reduce the cleavage of PARP. These results suggest that ERK signaling may not be involved in AST-induced apoptosis. Nonetheless, several controversies regarding the role of ERK in apoptosis have yet to be resolved; one previous report stated that the strong and persistent activation of ERK might lead to cell death while transient activation may be associated with cell proliferation (25). Therefore, the detailed mechanisms by which AST act on the ERK-dependent or ERK-independent pathway need further evaluation. It was suggested that NF- κ B regulates expression of the gene that encodes the tumor-associated α -fetoprotein. Downregulation of α -fetoprotein following adenoviral-mediated inhibition of NF- κ B was found to be essential for enhancing the sensitization of HCC cells to TNF- α cytotoxicity (5). Our findings indicate that AST inhibit NF- κ B/DNA-binding activity along with α -fetoprotein downregulation in HepG2 cells that subsequently leads to apoptotic cell death. Yet, NF- κ B is normally sequestered in the cytoplasm by its interaction with I κ B, a family of inhibitory proteins of NF- κ B, and remains inactive. Under circumstances of viral infection, DNA damage, exposure to pro-inflammatory cytokines or partial hepatectomy, the inhibitor of I κ B kinase (IKK) complex is activated, while I κ B becomes phosphorylated and undergoes degradation via the proteasome, thereby releasing NF- κ B into the nucleus for transcription of target genes (26). A super-repressor form of I κ B containing serine-to-alanine mutations at amino acids 32 and 36 has been identified, which inhibits the signal-induced phosphorylation of I κ B. The use of this super-repressor has been implicated in a variety of chemotherapeutic agents through inhibition of NF- κ B and induction of apoptosis (27). Further study is required to delineate the role of I κ B in AST-mediated suppression of NF- κ B.

Nitric oxide (NO) is cytotoxic to a number of human cells while at the same time protects other cell types including splenocytes (28), endothelial cells (29) and hepatocytes (30) from apoptosis. Inducible NO synthase (iNOS) is one of three key isoenzymes that generates NO from the precursor amino acid L-arginine. Overexpression of iNOS produces a sustainable amount of NO, which is a common phenomenon during chronic inflammatory conditions which precede carcinogenesis. One proposed mechanism for the inhibition of apoptosis by NO is the S-nitrosylation of caspase which subsequently inhibits caspase activity. NF- κ B enhancer elements have been found on the iNOS promoter gene. Therefore, it is believed that NF- κ B is responsible for the induction of iNOS protein expression, which was confirmed by our data in HepG2 cells. iNOS protein downregulation resulted from the inhibition of NF- κ B/DNA-binding activity which leads to apoptosis. Furthermore, it is well known that the bcl-2 family of proteins is critical for determining the fate of cells in the apoptotic pathway. Bcl-2 and its homologs including bcl-x_L and Mcl-1 prevent cell death, while Bax and Bak promote apoptosis (31). Among these, bcl-x promoter activation is dependent on the NF- κ B/DNA-binding site, and its protective activity is correlated with that of c-Rel. Another study showed that bcl-x_L suppresses TNF- α -induced cell death under the condition of suppressed NF- κ B activity by dominant-

negative I κ B (32). These results suggest that bcl-x_L might play a role as death antagonist in the NF- κ B signaling pathway for cell survival. Our results demonstrated that bcl-x_L expression in HepG2 cells was decreased after AST treatment. It is possible that the downregulation of NF- κ B/DNA-binding activity and a decreased protein level together trigger apoptosis in AST-treated HepG2 cells.

In response to drug-induced DNA damage, mammalian cells activate different cell cycle checkpoints. The accumulation of cells in the S and G2/M phases could prevent them from facilitating mitosis. Our previous findings showed that AST caused human colonic HT-29 cells to accumulate in the S phase and to arrest at the G2/M phase, with concomitant overexpression of the cyclin-dependent kinase inhibitor p21 (19). However, similar effects of AST were not observed in HepG2 cells as demonstrated from flow cytometric analysis (data not shown). We propose that AST may not lead to universal cell cycle arrest in all cell types, but are rather specific to certain cell lines. Alternatively, it appears that AST mainly act by regulating the apoptotic mechanism in HepG2 cells. Cells that undergo apoptosis appear to be the result of a shift in the balance between anti- and pro-apoptotic factors. One of the hallmarks of apoptosis is the cleavage of chromosomal DNA into nucleosomal units. Nuclear condensation was observed in the AST-treated cells, which indicates an 'irreversible death' or apoptosis in HepG2 cells. In this study, we focused on the mitochondrial-dependent apoptotic pathway. The degradation of DNA in the nuclei of apoptotic cells is in fact accomplished following activation of caspases. This could be due to the initial release of cytochrome *c* from mitochondria, which in turn facilitates changes in the Apaf-1 structure to allow procaspase 9 recruitment and processing. As a result, procaspase 9 is converted to caspase 9, leading to subsequent activation of caspase 3. Our results showed that procaspase 9 and procaspase 3 expression was down-regulated following AST treatment, along with the inhibitory effects on the two anti-apoptotic proteins bcl-2 and bcl-x_L. These modulations control the gateway for cytochrome *c* release, which determines the onset of apoptosis through the mitochondrial-dependent pathway (20). Furthermore, previous studies have identified another important regulator of apoptosis, Smac/DIABLO, which is a mitochondrial protein released into cytosol in response to apoptosis (33,34) which promotes cytochrome *c*-dependent caspase activation by neutralizing the inhibitor of apoptosis proteins (IAPs). A recent study showed that Smac/DIABLO release is required in p53-induced apoptosis (35). On the other hand, X-linked inhibitor-of-apoptosis protein (XIAP) interacts with caspase 9 and inhibits its activity, whereas Smac/DIABLO relieves this inhibition through interaction with XIAP. The binding of Smac/DIABLO to XIAP antagonizes caspase-XIAP interaction and hence releases caspases to trigger apoptosis (36). If this is the case, prospective study is needed to determine whether AST induce overexpression of Smac/DIABLO and promote apoptosis by increasing the release of cytochrome *c* to facilitate formation of a cytochrome *c*/Apaf-1/procaspase 9 apoptosome complex.

In summary, AST induced cytotoxicity and promoted apoptosis in HepG2 cells. These effects were mediated through an ERK-independent NF- κ B signaling pathway.

Decreased iNOS and bcl-x_L protein expression may also be responsible for the induction of apoptosis in AST-treated cells. This study provides several lines of evidence to support the potential use of AST as a cancer chemotherapeutic adjuvant. Data generated in this study could facilitate the establishment of a novel effective regimen with specific cellular and molecular targets for the treatment of human hepatocellular carcinoma.

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