Astragaloside IV regulates NF-κB-mediated cellular senescence and apoptosis of hepatic stellate cells to suppress PDGF-BB-induced activation

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Abstract. Activated hepatic stellate cells (HSCs) are the principal effectors during hepatic fibrosis, which is characterized by the accumulation of extracellular matrix. Therefore, present therapies and investigations into hepatic fibrosis mainly focus on the suppression of activated HSCs. Astragaloside IV (ASIV) is an effective constituent extracted from the plant Astragalus membranaceus and has exhibited anti-fibrotic properties in hepatic fibrosis. However, its protective mechanism against hepatic fibrosis is not fully understood. The present study aimed to investigate the mechanistic role of ASIV on rat HSC-T6 cells activated with platelet-derived growth factor (PDGF)-BB. HSC-T6 cells were activated using PDGF-BB and subsequently treated with ASIV (final concentrations of 20 and 40 µg/ml) for 48 h. ASIV treatment decreased the expression of α1 type I collagen, α-smooth muscle actin and fibronectin on mRNA and protein levels, suggesting that ASIV suppresses PDGF-BB-induced HSC-T6 activation. Senescence-associated β-galactosidase activity, p21, high-mobility group AT-hook 1 and p53, common biomarkers of senescence, were upregulated by ASIV treatment. In addition, the expression of telomerase reverse transcriptase was reduced. ASIV promoted apoptosis of PDGF-BB-activated HSC-T6 cells. The NF-κB signaling pathway, which controls cellular senescence and apoptosis, was demonstrated to be stimulated by ASIV by increasing p65, p52, p50 and inhibitor of NF-κB kinase α expression levels, and by suppressing the expression of NF-κB inhibitor α. Taken together, these results demonstrated that ASIV promoted cellular senescence and apoptosis by activating the NF-κB pathway to suppress PDGF-BB-induced HSC-T6 activation; with potential implications for the treatment of hepatic fibrosis.

Introduction

Hepatic fibrosis is a common pathological feature of various chronic liver diseases, including viral hepatitis, alcoholic liver disease and non-alcoholic fatty liver disease (1). If untreated, hepatic fibrosis ultimately develops into liver cirrhosis or hepatocellular carcinoma (2). In the normal liver, most hepatic stellate cells (HSCs) are in a quiescent state to maintain a balance between synthesis and degradation of the fibrillar extracellular matrix (ECM) (3). Hepatic fibrosis is characterized by the progressive secretion and accumulation of ECM by activated HSCs (4). A previous study demonstrated that liver injury or culturing in vitro will result in HSC activation and lead to the excessive accumulation of ECM components, including fibronectin (FN) and various types of collagens (5). In addition, a number of chemokines, cytokines and growth factors have been reported to contribute to HSC activation (6). In particular, the platelet-derived growth factor (PDGF) family is one of the most effective inducers of HSC activation, which further exacerbates hepatic fibrosis (7).

Induction of activated HSC senescence, apoptosis and reversion to the quiescent state has been reported to facilitate the interruption of hepatic fibrosis (8,9). Senescent cells display a large and flat morphology, exhibit upregulated p21, p53 and HMGA1 expression (10), and exhibit enhanced senescence-associated β-galactosidase (SA-β-gal) activity (11). During senescence, p21 expression is increased and activated, which act as an inhibitor to cyclin-dependent kinases, to arrest cell cycle progression (12). In addition,
high-mobility group AT-hook 1 (HMG A) proteins accumulate on the chromatin of senescent fibroblasts to stabilize the senescent state by synergizing with p16 (13). In terms of transcription, p53 is upregulated and cooperates with NF-κB to promote cellular senescence (14). The activation of NF-κB transcription factors serves as a driver for the ageing process (15). Telomerase reverse transcriptase (TERT), the catalytic subunit of telomerase, elongates telomeres and maintains their structures. However, declined activity or expression of TERT shortens telomeres leading to senescence (16). Activated HSCs can be depleted by the activation of programmed cell death. Apoptotic cells undergo various morphological changes, including cell shrinkage and convolutions, pyknosis and karyorrhexis (17). Phosphatidylserine translocation to the cell surface is another apoptotic feature that has been exploited for the detection of apoptosis (18). Activated HSCs can also be reverted to a deactivated intermediate semi-quiescent state to reduce ECM accumulation (19). Therefore, investigations into determining the cell fate of activated HSCs have become a focus of intense research for alleviating or reversing hepatic fibrosis.

Astragalus membranaceus is one of the most commonly used Traditional Chinese Medicine for hepatic fibrosis treatment in China (20). Astragaloside IV (ASIV) is the major active constituent of A. membranaceus. At present, the degree of ASIV content is an important parameter for the evaluation of A. membranaceus quality (21). Previous in vitro and in vivo studies have indicated that ASIV possesses a variety of cytoprotective properties, including anti-oxidative (22,23) and anti-cancer (24,25), in addition to some anti-fibrotic roles in pulmonary (26,27), cardiac (28,29), renal (30,31) and hepatic fibrosis (32). However, the molecular mechanism of ASIV-mediated anti-fibrosis remains poorly understood.

The present study aimed to investigate the protective mechanisms of ASIV on activated HSCs by using a PDGF-BB-activated rat HSC-T6 cell line as a model. Reverse transcription-quantitative PCR (RT-qPCR), western blotting and immunofluorescence staining were performed to evaluate the expression of fibrotic and senescence markers, and components of the NF-κB signal pathway. SA-β-gal staining was implemented to determine the degree of senescence at various concentrations of ASIV. Flow cytometry was used for the analysis of apoptosis in activated HSCs. In conclusion, it was demonstrated that ASIV treatment suppressed PDGF-BB-induced HSC activation by activating the NF-κB signaling pathway and promoting cellular senescence and apoptosis. These data may provide new insights into the cell fate determination of PDGF-BB-activated HSCs and implicated ASIV as a potential treatment strategy for hepatic fibrosis.

Materials and methods

Reagents. DMEM, FBS, trypsin, penicillin-streptomycin (100X) were purchased from Biological Industries. ASIV was purchased from Beijing Solarbio Science & Technology Co., Ltd. (purity ≥98%, HPLC). The chemical structure of ASIV (C_{37}H_{44}O_{14}; molecular weight, 784.97) is illustrated in Fig. 1. Rat PDGF-BB was purchased from GenScript. Gene-specific primers were synthesized by TsingKe Biological Technology Co., Ltd. DMSO was purchased from Sigma-Aldrich (Merck KGaA).

Cell culture and ASIV treatment. The rat HSC line HSC-T6 was obtained from Procelf Life Science & Technology Co., Ltd. The cells were cultured using DMEM supplemented with 10% (v/v) FBS and 100 µg/ml penicillin-streptomycin at 37°C under 5% CO₂. HSC-T6 cells were activated using PDGF-BB at a final concentration of 20 ng/ml, as previously reported (33). ASIV powder was first dissolved in DMSO and subsequently diluted in DMEM to a final concentration of 0, 20 and 40 µg/ml (34). The final concentration of DMSO was not in excess of 0.1% (v/v).

Immunofluorescence staining. HSC-T6 cells were seeded onto coverslips placed in 24-well plates at 2x10⁵ cells/well at 37°C for 12 h prior to treatment with PDGF-BB and ASIV for 48 h. Following drug treatment, the coverslips were rinsed three times with 1X PBS for 5 min each, before fixation in 4% paraformaldehyde for 30 min at room temperature. Then, 0.2% Triton X-100 (Beijing Solarbio Science & Technology Co., Ltd.) was used to permeabilize cells for 10 min at room temperature. After blocking with 5% BSA (Beyotime Institute of Biotechnology) for 30 min at room temperature, the coverslips were subsequently treated with primary antibodies (1:100) for 2 h at room temperature. The primary antibodies used were as follows: Collagen type I α1 (COL1A1; cat. no. ab6308; Abcam), α-smooth muscle actin (α-SMA; cat. no. MAB1420; R&D Systems, Inc.) and fibronectin (FN; cat. no. AF5335; Affinity Biosciences). Cells were washed three times with 1X PBS, and the coverslips were incubated with Cy3-conjugated secondary antibodies (1:200; cat. no. E031620-01; EarthOx Life Sciences) for 1 h at room temperature. After another rinsing three times with 1X PBS, cells were finally stained using 10 µg/ml DAPI (Beijing 4A Biotech Co., Ltd.) for 30 min at room temperature for microscopic analysis using upright fluorescence microscopes at x10 eyepiece and x20 objective lens.

RNA isolation and RT-qPCR. HSC-T6 cells were seeded in 12-well plates at a density of 5x10⁴ cells/well. Following a 12-h culture, cells were treated with PDGF-BB and ASIV for 48 h. Total RNA was extracted from HSC-T6 cells using RNAiso Plus reagent (Takara Bio, Inc.), according to manufacturer’s protocols. A total of 500 ng total RNA was reverse-transcribed into cDNA using PrimeScript™ RT Master Mix (Takara Bio, Inc.). RT reaction steps consisted of 37°C for 15 min and 85°C for 5 sec. Gene-specific primers were designed using NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast) and are listed in Table I. qPCR analysis was performed in a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.) using TB Green™ Premix Ex Taq™ (Takara Bio, Inc.). The qPCR cycling conditions were as follows: 30 sec at 95°C, and 40 cycles of 10 sec at 95°C, 30 sec at 60°C and 20 sec at 72°C. Relative gene expression was quantified using the 2^(-ΔΔCq) method (35). GAPDH was used as internal control.

Western blot analysis. HSC-T6 cells were seeded in 6-well plates at a density of 2x10⁶ cells/well. Following a 12-h culture, cells were treated with PDGF-BB and ASIV for 48 h.
Total cellular protein from HSC-T6 cells was extracted using RIPA lysis buffer (Beyotime Institute of Biotechnology), and protein concentration was determined using bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology). Proteins (40 µg/lane) were separated using 10% SDS-PAGE, and subsequently transferred to PVDF membranes. The membranes were blocked for 2 h at room temperature with 5% skimmed milk powder dissolved in Tris-buffered saline containing 0.1% Tween-20. Following blocking the membranes were incubated with primary antibodies (1:1,000) in 5% skimmed milk overnight at 4˚C. The primary antibodies used were as follows: \( \alpha \)-smooth muscle actin (\( \alpha \)-SMA; cat. no. MAB1420; R&D Systems, Inc.), FN (cat. no. AF5335; Affinity Biosciences), GAPDH (cat. no. E021060-03; EarthOx Life Sciences), HMGA1 (cat. no. ab129153; Abcam), p21 (cat. no. ab109199; Abcam), p65 (cat. no. AP6387; Affinity Biosciences), p52 (cat. no. AP6373; Affinity Biosciences) and NF-\( \kappa \)B inhibitor \( \alpha \) (I\( \kappa \)B\( \alpha \); cat. no. NB100-56507; Novus Biologicals, Ltd.). Following primary antibody incubation, the membranes were washed five times with Tris-buffered saline containing 0.1% Tween-20. Then membranes were subsequently incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin (Ig)G (cat. no. E030120-01; 1:10,000; EarthOx Life Sciences) and HRP-conjugated goat anti-mouse IgG (cat. no. GB23301; 1:4,000; Wuhan Goodbio Technology Co., Ltd.) in 1% skimmed milk at room temperature for 1 h, then the membranes were washed three times with Tris-buffered saline containing 0.1% Tween-20. Finally, the protein bands were visualized using UltraSignal Maximum Sensitivity ECL Substrate (Beijing 4A Biotech Co., Ltd.) and detected using an imaging system (Odyssey Fc Imaging System; Gene Company Ltd.). GAPDH was used as the internal control.

**SA-\( \beta \)-gal staining.** HSC-T6 cells were seeded into 6-well plates at 6x10⁴ cells/well prior to treatment with PDGF-BB and ASIV for 48 h. SA-\( \beta \)-gal activity, a marker of senescence, was evaluated using the SA-\( \beta \)-gal Staining kit (Beyotime Institute of Biotechnology), according to the manufacturer's protocols.

**Flow cytometry analysis of apoptosis.** Apoptosis analysis was performed using Annexin V/PI assay kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to manufacturer's protocols. Briefly, HSC-T6 cells treated with 20 ng/ml PDGF-BB and ASIV (0 or 40 µg/ml) for 48 h, and then cells were collected for cell counting. A total of 1x10⁶ cells were rinsed once using ice-cold PBS and then with 1X Annexin V binding buffer, before being subsequently suspended in 100 µl binding buffer containing 5 µl Annexin V binding buffer, followed by incubation at room temperature for 15 min. Finally the cells were detected using a flow cytometer no more than 30 min after the incubation. The data were analyzed by FlowJo V10 (Expert Cytometry).

**Statistical analysis.** All data were presented as mean ± standard deviation. All statistical analyses were performed using GraphPad Prism software 5.0 (GraphPad Software, Inc.). A two-tailed t-test was performed to assess differences between two data groups. One-way ANOVA followed by Newman-Keuls’ post hoc test was used to compare differences between >2 data groups. \( p < 0.05 \) was considered to indicate a statistically significant difference.
Results

**ASIV suppresses ECM expression in PDGF-BB-activated HSC-T6.** Hepatic fibrosis is characterized by the excessive secretion and accumulation of ECM by activated HSCs (36). ECM components, mainly including α-SMA, COL1A1 and FN, were used as the marker for HSC activation. Therefore, the present study aimed to investigate the effects of ASIV on HSC activation. PDGF-BB-activated HSC-T6 were treated with varying concentrations of ASIV (0, 20 or 40 µg/ml) for 48 h. The expression levels of COL1A1, α-SMA and FN were subsequently measured using RT-qPCR, western blotting or immunofluorescence staining. The relative mRNA expression levels of COL1A1, α-SMA and FN mRNA were significantly reduced following ASIV treatment in a dose-dependent manner compared with untreated cells (Fig. 2A-C). On the protein level, data from western blot analysis and immunofluorescence showed that ASVI treatment downregulated the expression of COL1A1, α-SMA and FN in HSC-T6. (Figs. 2D and 3).

**ASIV promotes cellular senescence in PDGF-BB-activated HSC-T6.** Induction of cellular senescence in activated HSCs would be beneficial to the prevention of hepatic fibrosis (37). Therefore, the activity of SA-β-gal, a common biomarker of senescence, was measured in activated HSCs in the presence of ASIV. Following ASIV treatment, SA-β-gal activity was
revealed to be significantly enhanced in PDGF-BB-activated HSC-T6 (Fig. 4A and B). Supporting this, mRNA of senescence markers, p21, p53 and HMGA1 was significantly upregulated (Fig. 4C-E), while the protein expression of p53 and HMGA1 were markedly upregulated (Fig. 4G). Conversely, the corresponding mRNA levels of TERT were significantly reduced by ASIV treatment (Fig. 4F). In addition, all the observations made in this analysis appeared to display a dose-dependent trend compared with the untreated control (Fig. 4). These results suggest that ASIV treatment promoted senescence in activated HSC-T6.

ASIV induces apoptosis in PDGF-BB-activated HSC-T6. Apoptosis of activated HSCs contributed to the resolution of hepatic fibrosis (38). To investigate the effect of ASIV on the survival of PDGF-BB-activated HSC-T6, apoptosis was measured using flow-cytometry. The percentage of early and late apoptotic cells was revealed to be 2-3 times higher in the ASIV treatment group compared with the control group (Fig. 5A-C). At the same time, the result from flow-cytometry indicated that ASIV did not affect the necrotic cell death in PDGF-BB-activated HSC-T6 (Fig. 5A). These data support the notion that ASIV treatment accelerated apoptosis in addition to promoting cellular senescence in activated HSC-T6.

ASIV regulates the NF-κB signaling pathway in PDGF-BB-activated HSC-T6. The NF-κB signaling pathway is involved in significant physiological and biochemical processes including cellular senescence and apoptosis (39,40). To investigate the relationship between NF-κB signaling and PDGF-BB-activated HSC-T6 in the presence of ASIV, expression levels of p65, p52 and p50, components of the NF-κB protein family, were analyzed. On mRNA level, p65, p52 and p50 were significantly upregulated by ASIV treatment in a dose-dependent manner (Fig. 6A-C), whereas RelB and c-Rel mRNA expression were not detectable in HSC-T6 using RT-qPCR. Western blot analysis appeared to support the upregulation of p65 and p52 on protein level in PDGF-BB-activated HSC-T6 (Fig. 6F). To investigate the effect of ASIV on the NF-κB signaling pathway further, the expression levels of IκKα and IκBα, upstream regulatory components of the signaling pathway, were measured. RT-qPCR results revealed that ASIV treatment increased IκKα mRNA expression and decreased IκBα mRNA expression (Fig. 6D and E, respectively). ASIV also reduced IκBα protein expression, according western blot analysis (Fig. 6F).

Discussion

The activation of HSCs marks a significant event in the hepatic fibrosis process. Activated HSCs adversely disrupt the balance between ECM production and proteolysis due to decreased expression of matrix metalloproteinases and increased expression of tissue inhibitors of metalloproteinases (37). Large amounts of collagen (types I, III, IV, V and VI), α-SMA and FN are deposited by activated HSCs. If the suppression of HSC activation can be achieved, the degree of hepatic fibrosis could be considerably reduced or even reversed (41). Therefore, investigations into inhibiting HSC activation have become hotspots of research into hepatic fibrosis. In the present study, ASIV treatment downregulated the expression of fibrosis markers COL1A1, α-SMA and FN to suppress PDGF-BB-induced HSC-T6 activation. Indeed, previous studies demonstrated that ASIV inhibited collagen synthesis in activated HSCs by regulating p38 MAPK pathway (32,34). Evidence presented above demonstrated that ASIV can suppress a variety of factors leading to HSCs activation. As the principal mediator of hepatic fibrosis, data in the present study indicated ASIV could be used as a potentially therapeutic agent for hepatic fibrosis treatment.

Induction of cellular senescence and apoptosis are the most effective methods of inhibiting HSC activation and alleviating hepatic fibrosis (37). During the senescence process, cell proliferation and ECM expression become suppressed in activated HSCs (36). Further evidence exists demonstrating...
that cellular senescence is involved in the upregulation of p53 (42). In particular, HSCs in p53⁻/⁻ mice exhibited senescence inhibition (8), and the knockdown of p53 in activated HSCs suppressed the expression of p21, which leads to cellular senescence (43). In the present study, ASIV significantly increased the expression of p21 and p53 in PDGF-BB-activated HSC-T6, which indicated that cell senescence was induced. In addition, ASIV enhanced the expression of the senescence marker HMGA1 and activity of SA-β-gal, which have been reported to result from cellular senescence (11,13). By contrast, telomere shortening is a driving force behind replicative senescence (44), and TERT is involved in the maintenance of telomere length (45). ASIV treatment suppressed the mRNA expression of TERT in PDGF-BB-activated HSC-T6 in the present study. Taken together, these findings suggest that ASIV upregulated the expression of senescence markers, concomitant with downregulating fibrosis markers in activated HSC-T6 to attenuate fibrosis.

Apoptosis is involved in the resolution of hepatic fibrosis through a variety of intracellular signaling pathways (46). A large number of experimental and clinical chemotherapeutic agents including the active components of Traditional Chinese Medicines have been applied to inhibit hepatic fibrosis by inducing HSC apoptosis (47–49). ASIV has been demonstrated to induce apoptosis in osteosarcoma (50) and vascular smooth muscle cells (51). Data from the present study demonstrated that ASIV promoted apoptosis but did not affect necrotic cell death in PDGF-BB-activated HSC-T6. Crosstalk between cellular senescence and apoptosis contributes to the inhibition of HSCs activation (52). Therefore, these observations suggested that ASIV suppressed the activation of HSC-T6 by promoting cellular senescence and apoptosis.
A previous study demonstrated that NF-κB signaling contributes to senescence, whereas decreasing the expression of NF-κB components can bypass senescence (14). The main protein components that makes up the NF-κB transcription factor include RelA/p65, RelB, c-Rel, p52 (NF-κB2) and p50 (NF-κB1) (53). The present study revealed that ASIV treatment upregulated the expression of p65, p52 and p50 in PDGF-BB-activated HSC-T6; RelB and c-Rel were undetectable possibly because of low expression. This result suggested that ASIV increased NF-κB activity by upregulating the expression of NF-κB components. In unstimulated cells, inactive NF-κB heterodimers are sequestered into the cytoplasm by associating with IκB proteins. In stimuli-activated cells, the IKK complex induces the phosphorylation and degradation of IκB, causing NF-κB heterodimers to be released and translocated into the nucleus (54). In the present study, ASIV treatment upregulated the expression of IKKα and downregulated IκBα. This further indicated that ASIV promoted HSCs senescence by upregulating the NF-κB signaling pathway. In addition, ASIV induced apoptosis in activated HSCs, which indicated a positive association between apoptosis and NF-κB signaling activation in this cell type. This observation is consistent with previous studies, where compounds including flavokawain B (55), daunorubicin (55), sodium benzoate (56) and nicotine (57) have been reported to induce apoptosis by activating NF-κB signaling. The present study hypothesized

Figure 5. Analyzing apoptosis of platelet-derived growth factor-BB-activated HSC-T6 treated with ASIV. (A) Apoptosis of activated-HSC-T6 treated with 0 and 40 µg/ml ASIV was stained with Annexin V-APC/PI and analyzed using flow cytometry. (B and C) histogram quantifying the percentage of (B) early and (C) late apoptotic cells from (A). *P<0.05 and ***P<0.001. APC, allophycocyanin; ASIV, astragaloside IV; PI, propidium iodide.
that ASIV promoted cellular senescence and apoptosis through regulating expression of factors in NF-κB signaling pathway.

PDGF-BB, a mitogen and fibrogenic cytokine, activates HSCs effectively through intracellular signaling pathways including Ras/Raf/MEK, PI3K/Akt, STATs, Rho/ROCK and NF-κB (58). A number of preclinical and clinical investigations were performed to suppress hepatic fibrosis by targeting PDGF signaling. In particular, silybin has been demonstrated to exert anti-fibrotic effects by inhibiting the phosphorylation of Raf, MEK and ERK (59), whereas celecoxib promoted HSC apoptosis by suppressing Akt activation (60). Astragaloside IV, extracted from the plant *A. membranaceus*, significantly enhanced the NF-κB signaling pathway. Results from the present study provided useful indications that *A. membranaceus* may be capable of alleviating hepatic fibrosis or cirrhosis in China (20). However, its molecular anti-fibrotic mechanism remains unclear. ASIV, extracted from the plant *A. membranaceus*, significantly enhanced the NF-κB signaling pathway. Results from the present study provided useful indications that *A. membranaceus* may be capable of alleviating hepatic fibrosis or cirrhosis possibly by activating the NF-κB signaling pathway, resulting in HSC senescence and apoptosis. Therefore, it is worth exploring the comprehensive anti-fibrotic mechanism of ASIV to design combination therapies using ASIV and clinical medicine in treating hepatic fibrosis or cirrhosis.

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**Availability of data and materials**

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

**Authors' contributions**

ZC performed the experiments. ZC, YL and WC designed the study and wrote the manuscript. GW collected the samples. LY, ZP, SP, JC and JW analyzed the data and interpreted the results. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

Not applicable.

**Patient consent for publication**

Not applicable.
Compelling interests
The authors declare that they have no competing interests.

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