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Expression of angiotensinogen during hepatic fibrogenesis and its effect on hepatic stellate cells

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: The liver renin-angiotensin system (RAS) plays an important role in promoting the development of hepatic fibrogenesis. Angiotensinogen (AGT) is an important precursor in tissue RAS. This study aimed to investigate the expression and cellular source of AGT in hepatic fibrogenesis and its effect on proliferation and collagen metabolism of hepatic stellate cells.

Material/Methods: In a rat carbon tetrachloride (CCl_4)-induced liver fibrosis model the mRNA expression of AGT was determined by real-time PCR and the cellular source of AGT was determined by immunohistochemical staining. *In vitro* HSC-T6 cells were transfected with AGT, and the expression plasmid, AGT shRNA plasmid and negative shRNA plasmid were constructed. Real-time PCR and ELISA were applied to determine the mRNA expressions and contents of TIMP-1, TGF-β1, type I collagen and type III collagen of the cells or in the supernatants.

Results: Compared to normal liver, the AGT and α-SMA mRNA expressions increased at the early stage of hepatic fibrosis and decreased in hepatic cirrhosis. The expressions of AGT and α-SMA mRNA were correlated with the hepatic fibrosis (r=0.915, P=0.03). Immunohistochemistry demonstrated the activated HSCs were the main source of AGT due to colocalization of AGT and α-SMA expressions. The mRNA and protein of TGF-β1, TIMP-1, type I collagen and type III collagen were markedly up-regulated.

Conclusions: ACEI and angiotensin II type 1 receptor antagonist (AT1RA) could attenuate the progression of hepatic fibrosis in the early stage. Direct inhibition of AGT from aHSCs may become an effective antifibrotic anti-liver fibrosis strategy.

key words: **angiotensinogen (AGT) • Hepatic stellate cells (HSCs) • Liver fibrosis • RNA interfere**

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Background

Accumulating evidence indicates that tissue renin-angiotensin system (RAS) plays an important role in promoting hepatic fibrogenesis. Angiotensinogen (AGT) is a renin substrate generating a decapeptide, angiotensin-I (Ang I), which is then converted to angiotensin II (Ang II) after removal of a C-terminal dipeptide by angiotensin-converting enzyme (ACE), a zinc-dependent metallopeptidase. Ang II exerts effects by interaction with specific receptors (subtype 1 [AT1] and subtype 2 [AT2]) [1]. Hepatic stellate cells (HSCs) become activated, and this process is pivotal in hepatic fibrosis [2]. Activated HSCs (aHSCs) express AT1 receptors and *de novo* generate Ang II [3]. Local Ang II can stimulate the proliferation and migration of aHSCs as well as collagen synthesis through autocrine and/or paracrine, improve the expressions of TGFβ-1 and TIMP1, and inhibit collagen degradation, leading to hepatic fibrosis [4–6]. Angiotensin-converting enzyme inhibitor (ACEI) and angiotensin II type 1 receptor antagonist (AT1RA) can markedly attenuate hepatic fibrosis in different animal models [7–9]. Preliminary clinical studies on chronic hepatitis C and non-alcoholic steatohepatitis (NASH) suggest that RAS blocking agents may have beneficial effects on progression of fibrosis [10–12].

In healthy individuals, AGT is produced in hepatocytes [13] demonstrated in bile duct-ligated rats that AGT protein is detected in abundance in the region with active fibrosis by immunohistochemistry, and the mRNA expression of AGT in the fibrotic liver is increased without statistical significance (P=0.12). Does the expression of AGT increase at the early stage of hepatic fibrosis? The cellular source of AGT in the process of hepatic fibrosis (hepatocytes or HSCs) is still unknown. Clarifying these problems is beneficial for determining when to initiate therapy with RAS-blocking agents during fibrosis progression and can also provide a basis for the prevention and treatment of hepatic fibrosis by selectively suppressing AGT secretion by aHSCs. As the main cells generating extracellular matrix (ECM) during hepatic fibrosis, HSCs have become the target cells in antifibrotic therapy [14,15]. Direct inhibition of AGT, a precursor of Ang II, from aHSCs may reduce the systemic effects of ACEI and AT1RA, particularly the hemodynamic effects, and become an effective antifibrotic strategy.

In the present study, Sprague-Dawley (SD) rats were subcutaneously injected with carbon tetrachloride (CCl_4) to induce hepatic fibrosis. Based on the hepatic fibrosis score, the mRNA expression of AGT was dynamically detected by real-time polymerase chain reaction (PCR) and the cellular source of AGT determined by immunohistochemistry. Subsequently, AGT expression plasmids and the AGT shRNA plasmids were constructed successfully, and the effects of AGT on the expression and secretion of TGF-β1, TIMP-1, type I collagen and type III collagen by HSC-T6 cells and on the HSC-T6 proliferation were also detected.

Material and Methods

Animal model and sample collection

Six-week-old male SD rats weighing 250±30 g were purchased from the Shanghai Laboratory Animal Center of the Chinese Academy of Science. They were housed in a 12/12 h dark/light cycle and given *ad libitum* access to water and standard food. Seventy-two rats were randomly divided into 2 groups. Rats in the normal control group (NC, n=12) were killed using an overdose of subcutaneous ketamine after 2 weeks of accommodation; those in the fibrosis group (n=60) were treated with 40% CCl_4 (3 ml/kg, CCl4/olive oil=2: 3) twice weekly and the first dose was doubled. Four weeks later, 12 rats were sacrificed every week.

Animals were killed and the liver was obtained. The left lobe (0.5×0.5×0.5 cm) was fixed in 4% paraformaldehyde and embedded in paraffin for immunohistochemical examination. The right lobe (200 mg) was snap-frozen in liquid nitrogen and then stored at –80°C for subsequent real-time PCR.

Histological examination

Liver tissues were sectioned consecutively for hematoxylin-eosin (H&E) staining. Two pathologists independently performed blinded evaluation of the slides under a light microscope (Olympus, Tokyo, Japan). The hepatic fibrosis was scored with the Knodell's scoring system, which is a 4-grade system derived from the Metavir's scoring system. The area with fibrosis was measured with an image analysis system. The histopathological scores of hepatic fibrosis were as follows: perivenular and/or pericellular fibrosis (S_1); septal fibrosis (S_2); incomplete cirrhosis (S_3); and complete cirrhosis (S_4) [16]. Five fields were randomly selected and the inflammatory activity grade and fibrosis stage recorded followed by averaging.

Real-time RT-PCR of AGT and α-SMA

Total RNA was extracted from the liver by using Trizol reagent. Total RNA (2 μg) was reversely transcribed into cDNA in 20 μl of mixture using MMLV Reverse Transcriptase according to the manufacturer's instructions. Real-time quantitative PCR was performed with an MX 3000P Sequence Detection System (Stratagene, USA) and SYBR Green PCR kit (Applied Biosystems, CA, USA). The specific primers for each gene were synthesized by Invitrogen Technologies (Shanghai, China). AGT: Forward 5'-CATCTTCCCTCGCTCTCTG-3'; Reverse: 5'-GCCTCTCATCTTCCCTTGG-3' (175 bp); α-SMA: Forward 5'-TGAACCCTAAGGCCAACCG-3'; Reverse 5'-TCCAGAGTCCAGCACAATACCA-3 (140 bp), β-actin: Forward 5'-CTGTCCCTGTATGCCTCTG-3', Reverse 5'-TGTCACGCACGATTTCC-3' (217 bp). Conditions for PCR included a pre-denaturation at 95°C for 10 min, and 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 45 s. The housekeeping gene β-actin was used as an internal reference, and mRNA expression of target genes was normalized by that of β-actin.

Immunohistochemical staining of AGT and α-SMA

The expressions of AGT and α-SMA were detected with the streptavidin peroxidase (SP) method. Briefly, after being deparaffinized and serially dehydrated in ethanol, liver tissues were treated with 3% hydrogen peroxide in methanol for 10 min to inactivate the endogenous peroxidase. Following 10 min of blocking with PBS containing 10% normal goat serum, the sections were incubated with mouse anti-rat AGT monoclonal antibody (1:500; Swant Biotechnology,

Table 1. Primers for real-time PCR.

Genes	Sequences	Expected size
TGF-β1	Forward 5′-TCCTGTGACAGCAGGGATAA-3′	248bp
	Reverse 5′-GGCAGAAGTTGGCATGG TAG-3′	
TIMP-1	Forward 5′-GACCTGGTCATAAGGGCTAAA-3′	219bp
	Reverse 5′-GCCCGTGATGAGAAACTTCACT-3′	
α1(I)procollagen	Forward5′-CCAATCTGGTTCCTCCC-3′	212bp
	Reverse 5′-AGGTTGAATGCACTTTTGG-3′	
α1(III)procollagen	Forward 5′-GTACAGCTGGCTTCCTCAG-3′	255bp
	Reverse 5′-GGCCTTGCCTCTTTCATATT-3′	
β-actin	Forward 5′-CTGTCCCTGTATGCCTCTG-3′	217bp
	Reverse 5′-TGTCACGCACGATTTCC-3′	

Bellinzona, Switzerland) or α-SMA monoclonal antibody (1:200; Sigma, CA, USA) for 1 h at 37°C. After treatment with HRP-conjugated goat anti-mouse IgG and 50 μl of streptavidin-peroxidase solutions for 30 min each at 37°C, the sections were developed with DAB and counterstained with hematoxylin. The sections treated with PBS instead of primary antibody served as negative controls, and the known positive sections were used as the positive controls. Immunohistochemical analysis of AGT and α-SMA was performed with an image analysis system (Imagepro Plus 6.0). Five fields were randomly selected (×200), and the percentage of cells positive for both α-SMA and AGT was calculated, which represents the proportion of activated HSCs expressing AGT during hepatic fibrosis.

Cell culture, *in vitro* transfection and AGT detection

Rat hepatic stellate cell line (HSC-T6) cells were purchased from the Institute of Liver Disease, Shanghai University of Traditional Chinese Medicine (Shanghai, China). The HSC-T6 cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO_2 in air. The HSC-T6 cells were incubated in culture plates. After overnight incubation, the cells were transfected with different plasmids (control shRNA, AGT expression plasmids, and AGT shRNA plasmid) using Lipofectamine™ 2000 (Invitrogen Life Technologies, CA, USA) according to the manufacturer's instructions. The medium was supplemented with 10% fetal calf serum (FCS) 4 h post-transfection. One day after transfection, the transfection efficiency was analyzed under a fluorescence microscope by detecting EGFP expression. Two days after transfection, cells were screened by G418 (400 mg/L) (Sigma, CA, USA) for 4 weeks, and the positive colonies with more than 60% of cells expressing EGFP were used for the subsequent experiments.

Total RNA was extracted from the cells above by using Trizol reagent and the mRNA and protein expressions of AGT were determined by real-time quantitative PCR and Western blot assay. The contents of Ang I and Ang II in the supernatants were measured with enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions.

Effect of AGT on the expression and secretion of TGF-β1, TIMP-1, type I collagen and type III collagen in HSC-T6 cells

Total RNA was extracted from the cells above by using Trizol reagent, and 2 μg of total RNA were reversely transcribed into cDNA in 20 μl of mixture using MMLV Reverse Transcriptase according to the manufacturer's instructions. The mRNA expressions of TGF-β1, TIMP-1, α1(I) procollagen and α1(III) procollagen were determined by real-time quantitative PCR. The specific primers for each gene were synthesized by Invitrogen Technologies (Shanghai, China) and are shown in Table 1.The supernatants were harvested and contents of TGF-β1 and type I collagen in the supernatants were measured using the specific ELISA kits (R&D Systems, Inc., Minneapolis, MN). The content of PC III collagen in the supernatants was detected through radioimmunoassay (Naval Institute of Biomedical Sciences, Shanghai, China).

Effect of AGT on HSC-T6 cell proliferation

The proliferation of HSC-T6 cells was measured by MTT assay. The HSC-T6 cells were seeded in a 96-well plate at a density of 2×10^3 cells/well and incubated at 37°C for 24 h. Then, 25 μl of MTT solution (5 mg/ml in PBS) was added followed by incubation for 4 h. The medium was removed and 200 μl of DMSO were added to each well. The absorbance was measured at 490 nm, with a microplate reader (MK3 Thermo Labsystem, Finland) representing the metabolic activity of these cells.

Statistical analysis

Data are expressed as means ±standard deviation (SD) and statistical analysis was performed with the SPSS version 11.0 statistical software. Comparisons were done by T test and a value of $P<0.05$ was considered statistically significant.

Results

Dynamic expression of AGT and α-SMA in rat fibrotic liver

According to the fibrosis scores, the fibrotic rats were divided into 4 groups: S_1 group (n=14), S_2 group (n=15), S_3 group

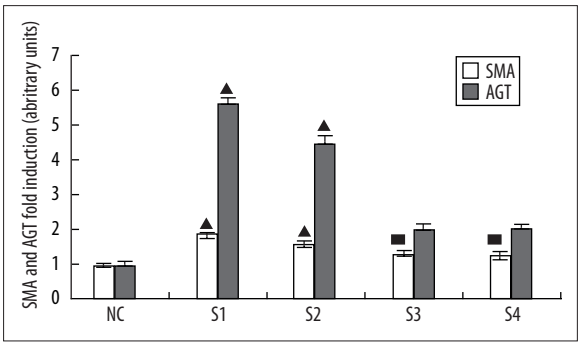

Figure 1. mRNA expression of AGT and α-SMA in rat fibrotic livers. Rats were subcutaneously injected with carbon tetrachloride (40%) to induce hepatic fibrosis. According to the hepatic fibrosis scores after the hematoxylin-eosin (H-E) staining, the rats with hepatic fibrosis were divided into 4 groups including S_1 group (n=14), S_2 group (n=15), S_3 group (n=16) and S_4 group (n=15). The livers were collected and total RNA extracted for PCR. The mRNA expressions of AGT and α-SMA were normalized by those of β-actin. Data are presented as means ± standard error (SE), *vs.* NC group, ■ P<0.05, ▲ P<0.01.

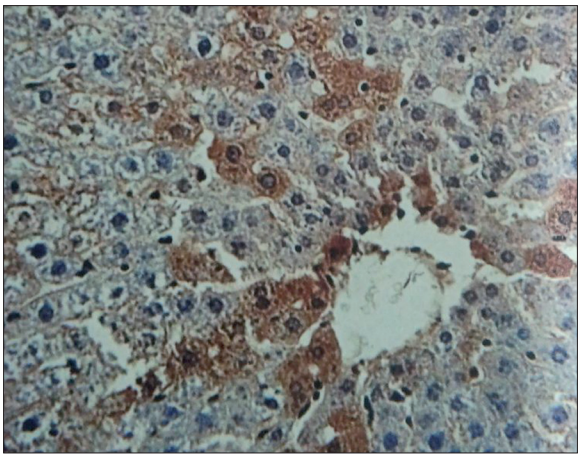

Figure 2. Immunohistochemistry for AGT in the liver. Immunohistochemical examination showed AGT positive cells (brown) in the normal liver are mainly hepatocytes around the central veins. Hepatocytes showed a strong cytoplasmic AGT immunoreactivity.

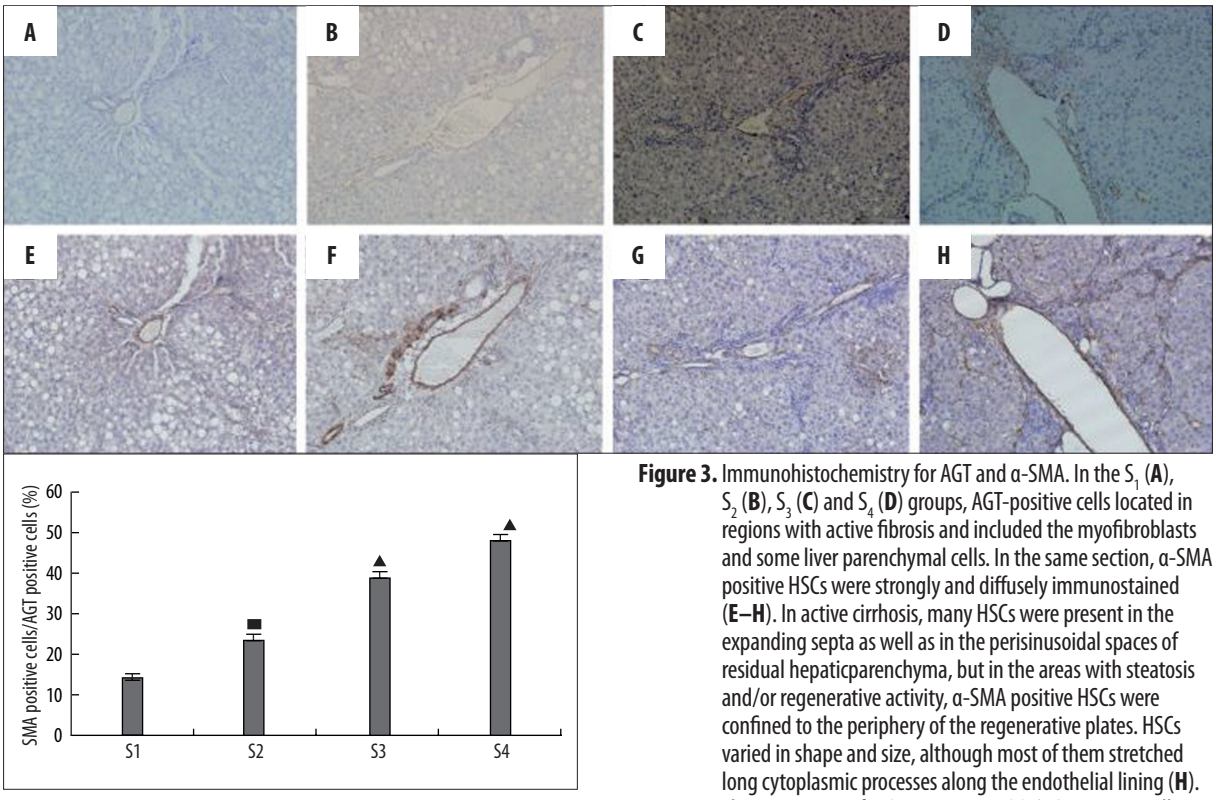

Figure 3. Immunohistochemistry for AGT and α-SMA. In the S_1 (**A**), S_2 (**B**), S_3 (**C**) and S_4 (**D**) groups, AGT-positive cells located in regions with active fibrosis and included the myofibroblasts and some liver parenchymal cells. In the same section, α-SMA positive HSCs were strongly and diffusely immunostained (**E–H**). In active cirrhosis, many HSCs were present in the expanding septa as well as in the perisinusoidal spaces of residual hepaticparenchyma, but in the areas with steatosis and/or regenerative activity, α-SMA positive HSCs were confined to the periphery of the regenerative plates. HSCs varied in shape and size, although most of them stretched long cytoplasmic processes along the endothelial lining (**H**). The percentage of α-SMA positive HSCs/AGT-positive cell was up-regulated gradually (S_1:13.67%, S_2:22%, S_3:36.33%, S_4:47.67%) in the fibrotic liver.

(n=16) and S_4 group (n=15). As shown in Figure 1, the mRNA expression of AGT was significantly higher in the S_1 and S_2 groups (0.5493±0.0938 and 0.4065±0.0726, respectively) than that in the NC group (0.09662±0.0184). When compared with the NC group (0.1162±0.0187), the mRNA expression of α-SMA was significantly elevated in 4 fibrosis groups (0.2093±0.0175, 0.1658±0.0155, 0.1507±0.0014, 0.1453±0.0025, respectively; P<0.05). The expression of α-SMA and AGT mRNA had a correlation with the hepatic fibrosis (r=0.915, P=0.03).

Cellular source of AGT in the fibrotic liver

AGT is synthesized in the liver and secreted into the circulation. Immunohistochemical examination (Figure 2) showed AGT positive (brown) granules in the normal livers were mainly located in the hepatocytes surrounding the central vein.

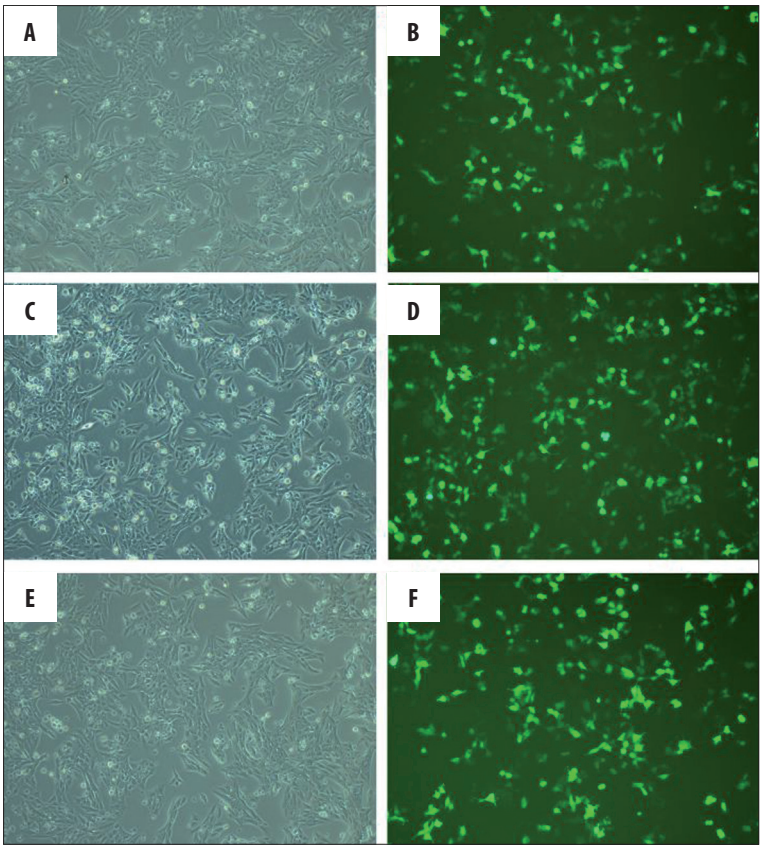

Figure 4. Transfection efficiency after G 418 screening. HSC- T6 were transfected with one of the plasmids (A: control shRNA group, B: AGT expression group, C: AGT shRNA group). Forty-eight hours later, the AGT positive cells were screened using G418 (400 mg/L). The colonies with more than 60% EGFP positive cells were used for the following experiments 4 weeks later.

AGT- positive cells were mainly found in the active fibrotic regions in the fibrotic liver (Figure 3A–D). In the same section, HSCs were also strongly positive for α-SMA and were diffusely distributed (Figure 3E–H). Our results show the distribution of AGT-positive cells and α-SMA-positive HSCs in the same region. The percentage of α-SMA-positive HSCs/AGT-positive cells was up-regulated gradually (S_1:13.67%, S_2:22%, S_3:36.33%, S_4:47.67%) in the fibrotic livers. Our findings show aHSCs can express AGT during hepatic fibrosis.

AGT expression after transfection in HSC-T6 cells

HSC-T6 cells are one of the hard-to-transfect cell types. Approximately 10% of the HSCs were transfected, demonstrated by counting EGFP positive cells. HSC-T6 cells with stable transfection were selected with G418 48 h later. As shown in Figure 4, 4 weeks later, the positive colonies with more than 60% of cells expressing EGFP were used for the following experiments (Figure 4). The mRNA and protein expressions of AGT were determined by quantitative real-time RT-PCR and Western blot assay, respectively. As shown in Figure 5A–C, in the HSC-T6 cells transfected with pEGFP-AGT, the mRNA expression of AGT was enhanced (0.3158±0.0753, P<0.01) and the AGT shRNA transfection significantly inhibited the mRNA expression of AGT (0.0011±0.0007, P<0.05) when compared with the negative control group (0.1152±0.0798). The mRNA levels in the AGT expression groups were increased by about 174%, whereas a 99% reduction was found in the AGT shRNA group when compared with the negative control group. Furthermore, the protein expressions of AGT were determined by Western blot analysis (Figure 5B, C). To evaluate the effects of AGT over-expression or AGT shRNA gene silencing on the protein expressions of Ang I and Ang II, the supernatants were collected and the contents of Ang I and Ang II detected in the 3 groups by ELISA after transfection (Figure 6). Results suggest that over-expression or silencing of AGT expression significantly alters the expressions of Ang I and Ang II in HSC-T6 cells. Therefore, the shRNA-mediated interference plasmid has been successfully constructed to silence the expression of the AGT gene to a certain extent.

TGF-β1 and TIMP-1 expression after transfection in HSC-T6 cells

The mRNA levels of TGF-β1 in the HSC-T6 cells transfected with pEGFP-AGT or pEGFP-AGT shRNA were compared with those in cells in the control shRNA group (Figure 7A). When compared with the control shRNA group, the mRNA expression of TGF-β1 was significantly increased in the AGT expression group (5.0778±0.5465 *vs.* 1.0982±0.016), and dramatically decreased in the AGT shRNA group (0.2896±0.0299 *vs.* 1.0982±0.016). The protein expressions of TGF-β1 in the supernatants had similar trends in the 3 groups (Figure 7B) (1529.50±146.95 *vs.* 720.28±69.38, P<0.01; 429.11±88.23 *vs.* 720.28±69.38, P<0.05, respectively). The mRNA expressions of TIMP-1 in the 3 cells groups were also detected by PCR (Figure 7C). When compared with the control shRNA group (data), mRNA expression of TIMP-1 was significantly increased in the AGT expression group (5.7498±1.0337), and markedly decreased in the AGT shRNA group (0.0122±0.0026). These findings suggested that over-expression or silencing of AGT expression significantly changed the expressions of TGF-β1 and TIMP-1 in HSC-T6 cells.

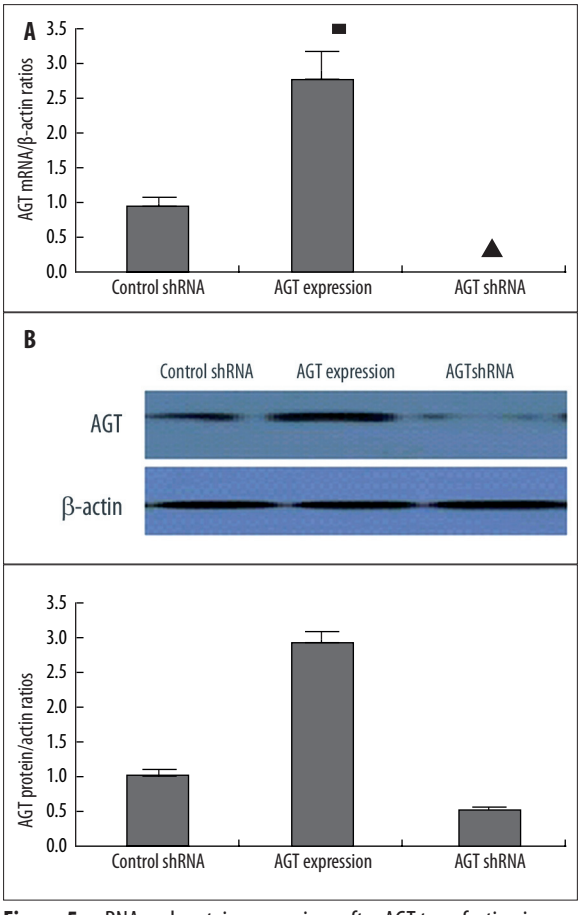

Figure 5. mRNA and protein expressions after AGT transfection in HSC T6? The AGT positive HSC-T6 cells were screened using G418 for 4 weeks and cells with stable transfection were collected and total RNA and protein were extracted for PCR and western blot assay. (**A**) mRNA expressions of AGT in the three groups. (**B**) protein expressions of AGT in the three groups. The mRNA and protein expressions of AGT were determined by densitometric analysis and normalized by those of β-actin. Data are presented as the means ± SE, *vs.* control shRNA group, ■ P<0.05, ▲ P<0.01.

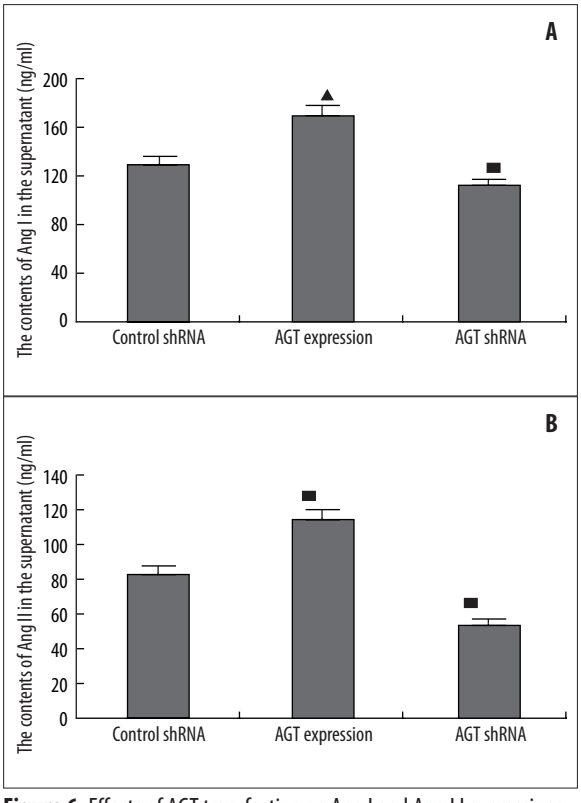

Figure 6. Effects of AGT transfection on Ang I and Ang I I expressions in HSC T6. The supernatants were collected and the protein expressions of Ang I and Ang II were detected by ELISA. (**A**) Protein expressions of Ang I in the supernatants of three groups. (**B**) Protein expressions of Ang II in the supernatants of three groups. Data are presented as the means ± SE, *vs.* control shRNA group, ■ P<0.05, ▲ P<0.01.

Effects of AGT on collagen synthesis and extracellular matrix secretion in HSC-T6 cells

The mRNA levels of collagen I and III transcripts in cells transfected with pEGFP-AGT were dramatically increased as compared with cells in the control shRNA group (Figure 8A, C). The quantitative analysis revealed the mRNA expressions of collagen I and III were increased by over 316% and 424%, respectively, by AGT transfection (Figure 8A, C). Simultaneously, inhibitory effects of AGT shRNA transfection on the expressions of collagen I and III were also noted in HSC-T6 cells (Figure 8A, C). Therefore, AGT shRNA-mediated silencing of AGT expression significantly inhibited the *in vitro* synthesis of collagen I and III in HSCs.

The levels of collagen I and PC III in the supernatants were determined by ELISA and radioimmunoassay, respectively. As shown in Figure 8B and d, the levels of collagen I and PC III in the supernatants of the AGT expression groups were dramatically increased as compared with cells in the control shRNA group. Over-expression of AGT increased the contents of collagen I and PC III in the supernatants by 27% and 69%, respectively. However, silencing of AGT expression by AGT shRNA transfection significantly reduced the secretion of ECM, evidenced by the fact that the levels of collagen I and PC III in the supernatants of cells transfected with pEGFP-AGT shRNA were decreased by 26% and 28%, respectively.

Effects of AGT on proliferation of HSC-T6 cells

The absorbance was measured at 490 nm (OD_{490}) in MTT assay. The cell growth curves were delineated after transfection (Figure 9). When compared with the control shRNA group, OD_{490} was significantly increased in the AGT expression group and remarkably decreased in the AGT shRNA group (P<0.05). These results suggest that AGT transfection can induce cell proliferation, and silencing of AGT expression inhibited the proliferation of HSC-T6 cells. The growth curves show the cell proliferation reached a peak level on the 4th day and then declined on the 5th day.

Discussion

Hepatic fibrosis represents the hepatic cirrhosis at the middle stage developed from various types of chronic hepatic diseases, and is a result of substantial accumulation of ECM. HSCs are the multifunctional non-parenchymal cells in the

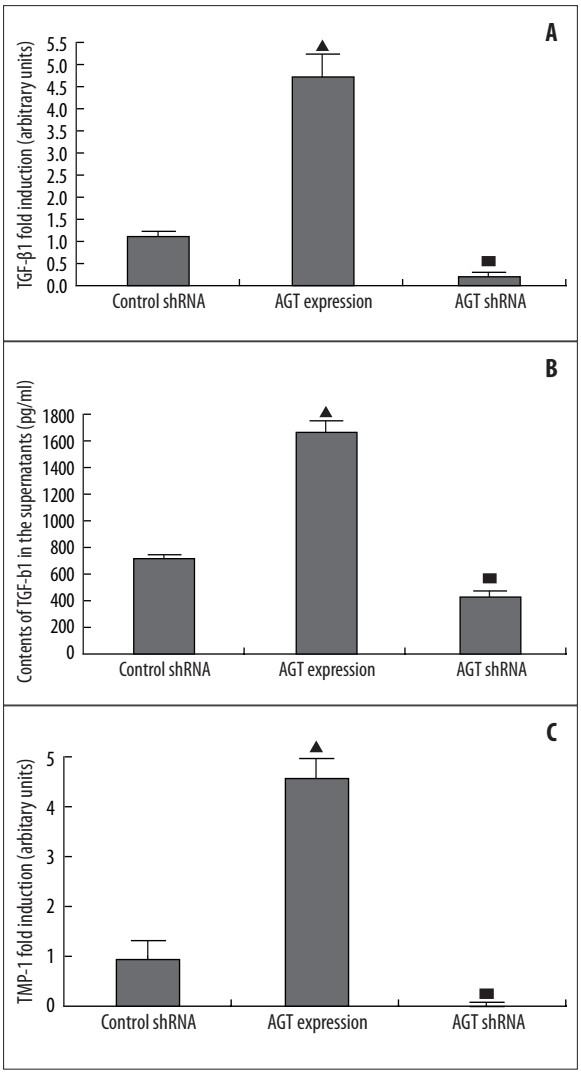

Figure 7. Effects of AGT transfection on expressions of TGF-β1 and TIMP-1 in HSC T6. Cells with stable transfection were obtained and total RNA extracted for PCR. The supernatants were collected for the detection TGF-β1 protein expressions by ELISA. (**A**) TGF-β1 mRNA expressions in the three groups. (**B**) TGF-β1 protein expressions in the supernatant of three groups. (**C**) TGF-β1 mRNA expressions in the three groups. The mRNA and protein expressions of TGF-β1 and TIMP-1 were determined by densitometric analysis and normalized that of β-actin. Data are presented as the means ± SE, *vs.* control shRNA group, ■ P<0.05, ▲ P<0.01 (which is the protein expression).

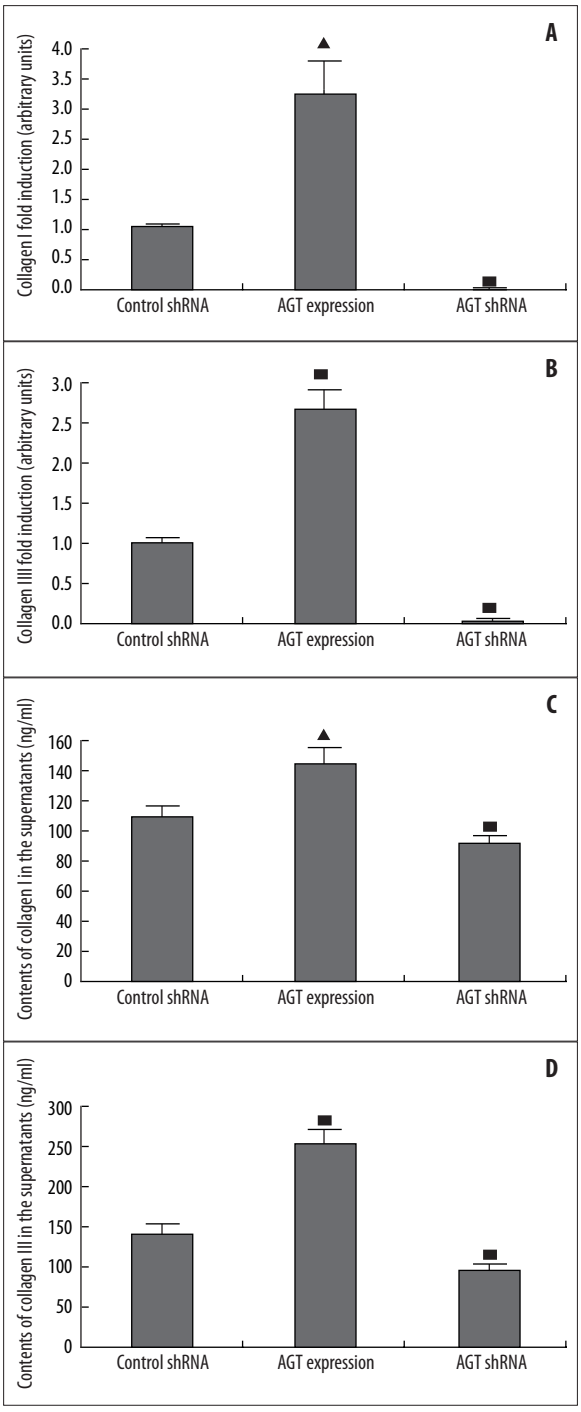

Figure 8. Effects of AGT transfection on expressions of type I and III collagen and on ECM secretion in HSC-T6 cells. Cells with stable transfection were obtained and total RNA were extracted for PCR. The supernatants were collected for the detection of protein contents of type I and III collagen by ELISA and radioimmunoassay, respectively. (**A**) α1 (I) procollagen mRNA expressions in the three groups. (**B**) type I collagen protein expressions in the supernatants of three groups. (**C**) α1 (III) procollagen mRNA expressions in the three groups. (**D**) type III collagen protein expressions in the supernatants of three groups. The mRNA and protein expressions of type I and III collagen were determined by densitometric analysis and normalized by that of β-actin. Data are presented as the means ± SE *vs.* control shRNA group, ■ P<0.05, ▲ P<0.01.

liver [17]. In the presence of chronic liver injuries, HSCs become activated and transform to proliferative myofibroblast-like cells, playing a pivotal role in hepatic fibrosis [18]. In the present study, the expressions of AGT in the liver of rats with CCl_4-induced hepatic fibrosis were detected, and results show the aHSCs can express AGT, a precursor in RAS, in the process of hepatic fibrosis. Then, the bioeffects of AGT on HSC-T6 cells were further investigated.

In the normal liver, quiescent HSCs are mainly located in the perisinusoidal space of Disse between the fenestrated endothelium of sinusoids and the hepatocytes [19]. Quiescent HSCs

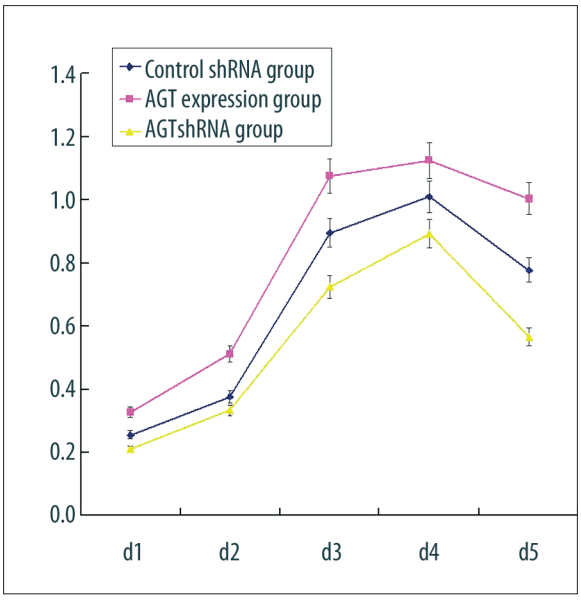

Figure 9. Effect of AGT transfection on proliferation of HSC-T6 cells. Cell culture was performed for 5 consecutive days, and the absorbance was measured at 490 nm in MTT assay. Then, the growth curves were delineated. Data are presented as the means ± SE *vs.* control shRNA group, ■ P<0.05, ▲ P<0.01.

show strong cytoplasmic desmin immunoreactivity, but do not express α-SMA. In the presence of chronic liver injuries, HSCs become activated (aHSCs) and have strong cytoplasmic desmin and α-SMA immunoreactivity. Therefore, α-SMA has been used as a marker of aHSCs. α-SMA-positive cells, which are aHSCs and possibly proliferating HSCs, are increased after injury and localized in the area with collagen deposition. The increase in α-SMA-positive cells is correlated with the mRNA expressions of α-SMA [20]. At the early stage of hepatic fibrosis (S_1-S_2), many HSCs are activated, and the expressions of α-SMA increase rapidly. When compared with S_1 and S_2, the mRNA levels of α-SMA decrease at the late stage of hepatic fibrosis (S_3) and the early stage of hepatic cirrhosis (S_4). These findings show the number of aHSCs decreased when compared with S_1 and S_2, which is consistent with previous reports [21].

In the process of hepatic fibrosis, there was a correlation between the mRNA expressions of α-SMA and AGT (r=0.915, P=0.03). *In vivo* immunohistochemical examination showed, in the normal liver, AGT positive cells (brown) were mainly hepatocytes and were only located around the central veins. In the presence of hepatic injury, AGT-positive cells existed in regions with active fibrosis in the fibrotic liver. In the same slice, α-SMA-positive HSCs were strongly and diffusely immunostained. Our results show the distribution of AGT-positive cells and α-SMA-positive HSCs in the same region. In hepatic fibrogenesis, the percentage of α-SMA-positive HSCs/AGT-positive cells was up-regulated gradually in the fibrotic liver. These findings showed that, in the hepatic fibrogenesis, AGT was mainly generated by aHSCs. Therefore, it may be an effective strategy in treatment of hepatic fibrosis through decreasing the autocrine of AGT from aHSCs.

Compared to the NC group, the mRNA expression of AGT increased in the S_1 and S_2 groups. These findings supported the fact that ACEI and AT1RA can attenuate the progression of rat hepatic fibrosis at the early stage. Jonsson et al. [22] investigated the effects of an ACEI (captopril) on the progression of hepatic fibrosis in a bile duct ligation (BDL) rat model. Captopril treatment was initiated 1 or 2 weeks after BDL. Results showed captopril could decrease the hepatic hydroxyproline levels, the mean fibrosis score, and the steady state messenger RNA levels of TGF-β1 and procollagen α1 (I), and these effects were more obvious after 1 week of treatment. This may be explained by the observation that the AGT expression mainly increased after liver injuries and at the early stage of hepatic fibrosis, and induced an array of fibrogenic actions. The AGT expression in aHSCs increased, but the absolute number of aHSCs declined at the late stage of hepatic fibrosis. AGT expression in the hepatocytes may decrease in the presence of liver injuries. The mRNA expression of AGT is indifferent at the late stage of hepatic fibrosis (S_3, S_4) when compared with the NC group.

Although AGT is the only component of RAS expressed in the normal rat liver, ACE and AT1 are widely expressed in the fibrotic rat livers. In humans, ACE and chymase are up-regulated in livers with evident fibrosis, whereas AT1 receptor expression is shifted to fibrotic areas [23–25]. In other tissues (e.g., heart), myofibroblasts accumulating in the areas with remodeling express all components of RAS and generate angiotensin II, which participates in the process of tissue repair. In the human liver, quiescent HSCs neither express the RAS components nor secrete angiotensin II [3]. In contrast, after activation *in vitro* and *in vivo*, myofibroblastic HSCs express key components of RAS and generate mature angiotensin II. Our results show *in vivo* aHSCs expressed AGT, a precursor molecule for Ang II synthesis at the early stage of hepatic fibrosis.

The reaction between renin and AGT is an initial and rate-limiting step in the synthesis of angiotensin II. Inhibition of AGT transcription results in a reduction in plasma AGT level [26].Various molecular variants of *AGT* have been reported. It has been suggested that *AGT* might be a determinant of hepatic fibrosis. Powell et al. [27] reported a striking relationship between TGF-β1 and AGT genotypes and the development of progressive hepatic fibrosis (OR: 0.080, P=0.019) [28] also found that the combination of high AGT and TGF-β1-producing polymorphisms were associated with advanced hepatic fibrosis in obese patients with NAFLD (OR: 5.7, 95% CI 1.5–21.2, P<0.05). Local angiotensinogen also increases the TGF-β1 mRNA expression [29]. Therefore, we speculated that reduction of AGT expression could reverse the hepatic fibrosis. Thereafter, the mechanisms underlying the effects of AGT on hepatic fibrosis were further explored.

The rat HSC-T6 cells, activated HSCs, have been a useful tool in studying hepatic fibrogenesis [30]. In our study, the expression plasmids targeting AGT and the AGT shRNA plasmids were successfully constructed and transfected into HSC-T6 cells. After G418 screening for 4 weeks, the mRNA and protein expressions of AGT and the contents of Ang I and Ang II in the supernatants were determined. Results indicated the transfection was effective. Our results suggest that at the cell level AGT can regulate the mRNA and protein expressions of TGF-β1, TIMP-1, procollagen α1(I) and

procollagen α1(III). The intrahepatic RAS is expressed in chronically damaged livers, and angiotensin II can induce an array of fibrogenic actions in HSCs, including increased collagen synthesis and elevated secretion of inflammatory mediators. RAS inhibition is associated with a decrease in the expression of TGF-β1, a key effector mediating the fibrogenic effects of angiotensin II in other tissues, in the injured liver [31]. Our results reveal that AGT can regulate the expression of TGF-β1 and affect the metabolism of ECM.

Two major events occur following HSC activation and then promote the fibrogenic effects in these cells. First, HSCs become directly fibrogenic by increasing the synthesis and deposition of ECM. Second, the fibrogenic cells proliferate, which dramatically increases the number of fibrogenic cells in the liver. Effective treatment regimens aiming to reduce or inhibit either the fibrogenic or proliferative response of HSCs may reduce the detrimental effects on HSC during the progression of hepatic fibrosis. Our results indicate AGT plays an important role in regulating proliferation of HSCs. These results provide evidence useful in preventing and treating hepatic fibrosis by selectively suppressing AGT expression by aHSCs.

Conclusions

In summary, AGT is mainly produced in the hepatocytes in healthy rats, but mainly in the aHSCs at the early stage of hepatic fibrosis. Application of ACEIs and AT1RAs at the early stages may be beneficial for the prevention and treatment of hepatic fibrosis. aHSCs are the major cellular source of AGT. It is effective and specific to selectively inhibit AGT expression by aHSCs in the treatment of hepatic fibrosis.

Conflict of interest

The authors declare no conflicts of interests.

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