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Ginsenoside-Rg1 from *Panax notoginseng* prevents hepatic fibrosis induced by thioacetamide in rats

JiaWei Geng [a,1], Wei Peng [a,1], YouGuang Huang [b], Hong Fan [a], ShuDe Li [b,*]

[a] Department of Digestive Diseases, The First People's Hospital of Yunnan Province, Kunming, Yunnan 650032, China
[b] Faculty of Basic Medicine, Kunming Medical University, Kunming, Yunnan 650031, China

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ABSTRACT

Panax notoginseng saponins have recently been reported to suppress liver fibrosis. Since ginsenoside-Rg1 is the most abundant component of *P. notoginseng* saponins, we investigated the effect of ginsenoside-Rg1 on experimental liver fibrosis in rats. Histological analysis revealed that ginsenoside-Rg1 significantly improved the extent of liver fibrosis in rats induced by thioacetamide. Ginsenoside-Rg1 markedly suppressed the serum levels of fibrotic markers and hepatic hydroxyproline content in rats treated with thioacetamide. Ginsenoside-Rg1 also reduced the serum levels of alanine transaminase, aspartate transaminase and alkaline phosphatase. Finally, ginsenoside-Rg1 attenuated the levels of thiobarbituric acid reactive substances in livers of rats treated by thioacetamide. In cultured hepatic stellate cells, ginsenoside-Rg1 markedly inhibited cell proliferation, activation and formation of reactive oxygen species stimulated by platelet-derived growth factor-BB (PDGF-BB). Additionally, ginsenoside-Rg1 down-regulated the expression of PDGF receptor-β by reducing the nuclear factor-κB activity, which was required for the gene expression. These results suggest that ginsenoside-Rg1, which exhibits its antioxidant and antifibrotic properties, may be of potential therapeutic value in protecting the liver fibrosis.

1. Introduction

Liver fibrosis is the main complication of chronic liver diseases. During the development of liver fibrosis, activated hepatic stellate cells (HSCs) are able to synthesize and deposit excessive extracellular matrix components into the liver parenchyma (Friedman, 2003). Advanced liver fibrosis leads eventually to cirrhosis and liver failure, for which no effective medical treatments are available except for liver transplantation. Therefore, prevention of liver fibrosis is a critical step for protecting the liver against the occurrence of cirrhosis and failure.

Although interferon has been widely used to treat chronic viral hepatitis, the effect of the therapy for liver fibrosis has not always been satisfactory. Herbal medicines have been reported to show protective effects from liver fibrosis and injury (Shimizu et al., 1999a; Cyong et al., 2000; Sun et al., 2007; Lee et al., 2007; Hsieh et al., 2008) For instance, Sho-saiko-to, a herbal medicine, is a common drug to treat chronic liver diseases in Asia (Oka et al., 1995). Sho-saiko-to has been proven to effectively suppress liver fibrosis in various rat fibrosis models, induced by dimethylnitrosamine (Shimizu et al., 1999a), pig serum (Shimizu et al., 1999a), carbon tetrachloride (CCl_4) (Inao et al., 2004), and thioacetamide (TAA) (Imanishi et al., 2004).

Panax notoginseng (Burk.) F. H. Chen, another herbal medicine, was originally used for treatment of trauma and bleeding in Asia, due to its therapeutic abilities to stop haemorrhages and promote blood circulation (Park et al., 2005). The major active components in the root of *P. notoginseng* are *P. notoginseng* saponins (Dong et al., 2003). Previous studies have shown that *P. notoginseng* saponins attenuate liver injury in rats induced by D-galactosamine (Yoshikawa et al., 2003), lipopolysaccharide (Yoshikawa et al., 2003), and ethanol (Lin et al., 2003). *P. notoginseng* saponins can provide strong protection for hepatocytes from ischemic reperfusion injury in the early stage of transplantation (Deng et al., 2001). A recent study revealed that *P. notoginseng* saponins suppress liver fibrosis in Long–Evans rats with cinnamon coat color (Park et al., 2005). These rats have an abnormality of the copper transport protein; eventually develop hepatocyte necrosis and hepatic fibrosis.

There are over 20 different saponins identified in *P. notoginseng* root, including ginsenosides, notoginsenosides, and gypenosides. Among these saponins, ginsenoside-Rg1 is the most abundant components found in the root of *P. notoginseng* (Dong et al., 2003). In this study, we evaluated the effect of ginsenoside-Rg1 on established hepatic fibrosis in rats, induced by TAA. We currently demonstrated that ginsenoside-Rg1 markedly suppressed liver fibrogenesis in rats.

Abbreviations: HSC, hepatic stellate cell; TAA, thioacetamide; HA, hyaluronic acid; PCIII, type III procollagen; ALT, alanine transaminas; AST, aspartate transaminase; AKP, alkaline phosphatase; HE, hematoxylin and eosin; PDGF, platelet-derived growth factor; PDGFRβ, PDGF receptor-β.

* Corresponding author. Tel.: +86 871 5338911; fax: +86 871 5332390.
E-mail address: shudeli006@vip.sina.com (S. Li).

[1] These authors contributed equally to this study.

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2. Materials and methods

2.1. Animals and experimental design

Ginsenosideginsenoside-Rg1 (molecular weight 800, purity 98%) was obtained from Prof. Er-Yi Zhang (Kunming Medical College, China). Adult Sprague Dawley rats were obtained from Baiyao Pharmacological Co. (Kunming, China). The animals were housed at a constant temperature of 22 °C, with a 12 h light/dark cycle of 12:12 h. The protocol of the experiments was approved by the Animal Care and Use Committee of Yunnan University. Rats were given intraperitoneal injection of TAA (200 mg/kg body weight) (solution in saline) twice a week for six weeks. At the same time, some of these rats were given ginsenoside-Rg1 by a subcutaneous injection every day. Thus, five groups each including ten animals could be distinguished. Control group, saline water alone; TAA group, TAA with saline water; low-dose ginsenoside-Rg1 group, TAA withginsenoside-Rg1 (15 mg/kg body weight); middle-doseginsenoside-Rg1 group, TAA withginsenoside-Rg1 (50 mg/kg body weight); high-doseginsenoside-Rg1 group, TAA withginsenoside-Rg1 (100 mg/kg body weight).

2.2. Histopathological examination

After development of liver fibrosis, the animals were anesthetized with phenobarbital. A part of the liver was immediately removed and quickly frozen in liquid nitrogen. The remaining part of liver was fixed in formalin, dehydrated in graded ethylic alcohol, and embedded in paraffin. All slides were evaluated at random with sections stained with hematoxylin and eosin (HE) and Masson's Trichrome. The extent of liver fibrosis was graded with the METAVIR scale, which grades fibrosis on a five-point scale (0, no fibrosis; 1, portal fibrosis without septa; 2, portal fibrosis with few septa; 3, numerous septa without cirrhosis; and 4, cirrhosis) (Bedossa and Poynard, 1996). To avoid sampling errors, the evaluation of the extent of liver fibrosis was performed by an independent pathologist blinded to the treatment protocol.

2.3. Measurement of hydroxyproline content

Hydroxyproline content in fresh liver samples was determined according to the method described previously (Lee et al., 2007). Liver samples were homogenized and hydrolyzed in 6 M HCl for measurement of hydroxyproline content. Protein concentration was determined by the Bradford reagent (Bio-Rad) using bovine serum albumin as the standard. Results were normalized by protein concentration.

2.4. Measurement of thiobarbituric acid reaction substrate in liver

Lipid peroxidation in the liver was determined by measuring the level of thiobarbituric acid reaction substrate (Fraga et al., 1988). The fluorescence of the samples was detected at an excitation wavelength of 515 nm and an emission wavelength of 555 nm in a F4500 fluorescence spectrophotometer (Hitachi, Japan). Results were normalized by protein concentration.

2.5. Serum markers of hepatic fibrosis and function

Serum hyaluronic acid (HA), and type III procollagen (PCIII) were determined by radioimmunoassay according to the manufacturer's instructions (Navy Medical Institute, Shanghai, China). Measurements of alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (AKP) were performed by an automated biochemistry analyzer (Hitachi 7060, Japan).

2.6. Isolation of hepatic stellate cells

HSCs were isolated from the livers of male SD rats by sequential digestion of the liver with 0.1% pronase E (Merck, Darmstadt, Germany) and 0.3% collagenase (Sigma, St. Louis, MO), as previously described (Shimizu et al., 1999b). The HSCs were used in their activated phenotype (passages 3–7). The immortalized rat HSC line HSC-T6 (a gift from Dr. L-M Xu, Shanghai University of Chinese Traditional Medicine, China), which exhibits characteristics compatible with those of rat HSCs (Vogel et al., 2000), was also used in this study. When cells were 60–80% confluent, the culture medium was changed to phenol-red free M199 (Gibco) lacking FBS. Then cells were preincubated with ginsenoside-Rg1 before the addition of test agents.

2.7. Determination of cell growth and [^{3}H]-thymidine incorporation assay

HSCs were grown in phenol-red free M199 supplemented 10% FBS and maintained at 37 °C. When cells were 60–80% confluent, the culture medium was changed to phenol-red free M199 lacking FBS. Then cells were preincubated with ginsenoside-Rg1 for 24 h before the addition of PDGF-BB (PeproTech, Rocky Hill, NJ) (10 ng/ml). Cells were counted in triplicate in a hemocytometer after 48 h of incubation with PDGF-BB.

After pretreated with ginsenoside-Rg1 for 24 h, HSCs were incubated with PDGF-BB (10 ng/ml) for 18 h. Then, 50 μl of serum-free medium containing [^{3}H]-thymidine (Atom High-Tech Co., Beijing, China) at 1 μCi/ml were added to cells in each well. After incubation for an additional 6 h, cells were washed with PBS three times. [^{3}H]-thymidine incorporation was determined according to the method reported by Zou et al. (2009).

2.8. Measurement of reactive oxygen species

Formation of reactive oxygen species, mainly H_2O_2, in cells was measured by using the method described previously (Zou and Banerjee, 2003), but with some modifications. HSC-T6 cells were pretreated with 2′, 7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Molecular Probes, Junction City, OR, USA) for 15 min and then with ginsenoside-Rg1 for 30 min. After incubated with PDGF-BB(10 ng/ml) for an additional 1 h, the cells were washed twice, harvested by trypsinization, and then resuspended in 1 ml of PBS. The cell suspension (20 μl) was mixed with PBS (980 μl). DCF fluorescence was measured by a Shimadzu RF551S fluorometer (Japan) with excitation at 490 nm and emission at 520 nm and a slit width of 5 nm for both excitation and emission. The fluorescence values of tested drug treatment groups were normalized by the mean value of control group.

2.9. Quantitative real-time PCR analysis

Total RNA from liver tissues was isolated using Trizole reagent (Invitrogen Corp., Carlsbad, CA). Random-primed cDNAs were generated by reverse transcription of total RNA samples with SuperScript II (Invitrogen). A real-time-PCR analysis was performed with the ABI Prism 7000 Sequence Detection system (Applied Biosystems, Foster City, CA) using SYBR® Premix-Ex Tag™ (Takara, Dalian, China). All results were standardized to the levels of GAPDH. The primers used for PCR were as follows: PDGFRβ: 5′-CATCTGTGAGGGCAAGTTGGTC-3′ (F), 5′-TCAAGGTGGTGTAGAGGCTGTT-3′ (R); GAPDH: 5′-CATGTTCCAGTATGACTCAACTC-3′ (F), 5′-AGTGTCACCCCATT TGATGT -3′ (R).

2.10. Western blotting

Cells were lysed on ice for 30 min in lysis buffer (containing 0.15 M NaCl, 30 mM Tris, 1 mM phenylmethanesulfonyl fluoride, 1% Triton X-100, 1 mM EDTA, 10 μg/ml leupetin, 2 μg/ml pepstatin, 2 μg/ml

aprotinin). Total protein of cell lysates (25 μg) was loaded per well and separated on a 10% SDS polyacrylamide gel. Proteins were then transferred to PVDF membranes. Primary antibodies were: anti actin, anti-α-smooth muscle actin (α-SMA), anti-type I collagen antibodies (Sigma, St. Louis, MO), and anti-PDGFRβ antibodies (Boster Biol Tech). The secondary antibody was a peroxidase-coupled anti-rabbit or mouse IgG (Amersham Biosciences, Piscataway, NJ). The membrane was exposed to ECL Hyperfilm (Amersham Biosciences), and the film was developed.

2.11. Transfection and luciferase assays

The full-length PDFGR-β reporter plasmid pβ12 was a gift from Dr. Keiko Funa (Ludwig Institute for Cancer Research, Uppsala, Sweden). In this plasmid, a fragment, containing 1366 base pair nucleotides upstream from the start codon of the mouse PDFGR-β gene, was subcloned into a luciferase expression vector pGL2 (basic) (Ballagi et al., 1995). Transient transfection in HSC-T6 cells was performed using Lipofectamine 2000 Reagent in OPTI-MEM medium (Invitrogen) according to the manufacturer's specifications. Cotransfection experiments with an expression vector for β-galactosidase reporter, pSV-β-gal (1 μg of DNA/well) (Promega) was performed. Forty-eight hours following transfection, the HSC-T6 cells were incubated with ginsenoside-Rg1 for 24 h. For luciferase activity determinations, cells were harvested, and cellular proteins were extracted using reporter lysis buffer (400 μl/well) according to the manufacturer's instructions (Promega Corp., Madison, WI). The cell lysate was centrifuged for 5 min at 12,000×rpm. From an aliquot of the supernatant (50 μl), luciferase enzymatic activity (in light units) was measured by adding luciferase substrate (Promega), and measuring in a Cytofluor II fluorescent plate reader (Millipore Corp., Bedford, MA). β-Galactosidase activity was measured using the Galacto-Star System (Applied Biosystems, Bedford, MA).

2.12. Electrophoretic mobility shift assay(EMSA)

Primary HSCs were incubated with serum-free medium M199 medium in the presence of ginsenoside-Rg1 (10 μM) for 6 h. Nuclear extracts were prepared from primary HSCs by the method described previously (Dignam et al., 1983). The nuclear factor-κB (NF-κB) site at −759 to −736 in the PDGFRβ promoter (5′-TGG TAA AGG GAG GCT CCA TTT ACA-3′) were synthesized as complementary oligodeoxyribonucleotide strands for EMSA (Chen and Zhang, 2003). EMSA was performed using a light-shift chemiluminescent EMSA kit (Pierce, Rockford, IL) according to the manufacturer's standard protocol. Binding reactions were performed with 5 μg of nuclear extracts.

2.13. Statistical analysis

Data were expressed as mean ± S.D. Statistical difference between the groups was analyzed using one-way ANOVA, followed by Student–Newman–Keuls' test.

3. Results

3.1. Histological findings

The rat livers treated with TAA for six weeks developed hepatic fibrosis. Histological analysis by HE and Masson's trichrome staining showed distorted tissue architecture with bundles of collagen surrounding the lobules as well as large fibrous septa (Fig. 1AB). Co-administration of ginsenoside-Rg1 significantly attenuated the deposition of collagen fibers. In the TAA group, 8 of 10-rats presented liver fibrosis of score-3. In contrast, in the high-dose ginsenoside-Rg1 groups, there were no rats showing liver fibrosis of score-3 and 6 of 10-rats showed liver fibrosis of score-1 (Table 1).

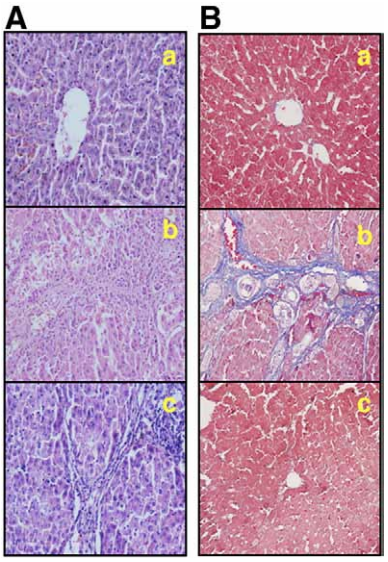

Fig. 1. Photomicrographs of liver sections from rats treated with TAA. (A) HE staining; (B) Masson's trichrome staining. (a), control group, rats were treated with saline water for six weeks; (b), TAA group, rats were treated with TAA for six weeks; (c), ginsenoside-Rg1 group, rats were treated with TAA and ginsenoside-Rg1 (100 mg/kg) for six weeks. Original magnification ×400.

3.2. Body and relative liver weight

The administration of ginsenoside-Rg1 (100 mg/kg body weight) alone did not influence the body weight or the relative liver weight of rats. However, a sharp decline in body weight was observed in rats after treatment with TAA for six weeks. The mean body weight of rat in the TAA-treated group was approximately 61% that of the control group (Table 2). However, co-administration of ginsenoside-Rg1 improved the decrease in body weight of those rats treated with TAA. A significant increase in the relative liver weight was detected in TAA-treated rats (Table 2). Ginsenoside-Rg1 treatment prevented the increase of the relative liver weight induced by TAA.

3.3. Serum markers of liver fibrosis

HA and PCIII in serum have been shown to reflect the extent of liver fibrosis (Ponomarenko et al., 2002). We thus evaluated the serum levels of HA and PCIII. TAA caused three and five-fold increases in serum HA and PCIII levels, respectively, compared with the control group (Fig. 2AB). Ginsenoside-Rg1 significantly attenuated the TAA-induced increase in serum HA and PCIII levels in a dose-dependent manner (Fig. 2AB).

Table 1
Grading of liver fibrosis for rats in different treatment groups.

Score	0	1	2	3	4	Mean	S.D.
Control	10	0	0	0	0	0.00	0.000
TAA treatment	0	0	2	8	0	2.80	0.421
Rg1 (15 mg/kg) [a]	0	2	5	3	0	2.10	0.738
Rg1 (50 mg/kg) [b]	0	4	6	0	0	1.60	0.516
Rg1 (100 mg/kg) [b]	1	6	3	0	0	1.20	0.632

Control group, rats were treated with PBS for six weeks; TAA group, rats were treated with TAA alone for six weeks;ginsenoside-Rg1 treatment Groups, rats were treated with TAA andginsenoside-Rg1 for six weeks. The extent of liver fibrosis was graded by 5-grade scores.

[a] $P<0.05$.

[b] $P<0.001$ versus TAA group.

Table 2
Effect of ginsenoside-Rg1 on body weight and relative liver weight of TAA-treated liver fibrosis in rats ($n=10$; mean ± S.D.).

Groups	Body weight (g)	Relative liver weight (%)
Control	251.2 ± 2.1	3.8 ± 0.18
Rg1 (100 mg/kg)	262.6 ± 3.4	3.7 ± 0.14
TAA treatment	154.4 ± 5.7 [a]	5.4 ± 0.21
TAA + Rg1 (15 mg/kg) [a]	177.3 ± 3.1	5.1 ± 0.15
TAA + Rg1 (50 mg/kg) [b]	207.2 ± 4.6[b]	4.4 ± 0.18[b]
TAA + Rg1 (100 mg/kg) [b]	215.3 ± 2.7 [b]	4.3 ± 0.20[b]

[a] $P<0.05$ compared with group.
[b] $P<0.05$ compared to TAA treatment.

3.4. Effect of ginsenoside-Rg1 on hepatic hydroxyproline content

Hepatic hydroxyproline content is also an index of liver fibrosis. In this study, elevated hydroxyproline levels were observed in the hepatic homogenate of TAA-treated rats (Fig. 3). There was a significant decrease in hepatic hydroxyproline content with ginsenoside-Rg1 administration in the TAA-treated rats (Fig. 3).

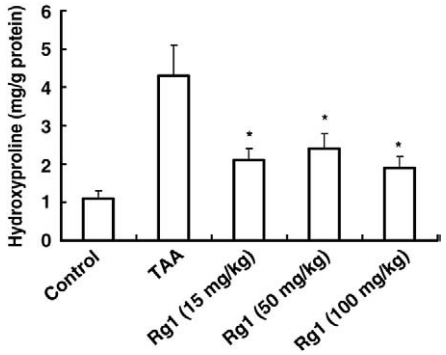

Fig. 3. Effect of ginsenoside-Rg1 on hepatic hydroxyproline content. Each group consists of 8 rats. *$P<0.05$ versus TAA group.

3.5. Effects of ginsenoside-Rg1 on liver damage and the levels of thiobarbituric acid reaction substrate

We next investigated whether ginsenoside-Rg1 has the protective effect on liver damage induced by TAA. As shown in Fig. 4ABC, rats treated with TAA increased the levels of serum AST, ALT, and AKP. Co-administration of ginsenoside-Rg1 was associated with a significant reduction in the serum levels of AST, ALT, and AKP. The data suggested that ginsenoside-Rg1 protects hepatocytes against TAA-induced damage in rats.

It has been shown that *P. notoginseng* saponins suppress liver fibrosis and lipid peroxidation in Long–Evans rats with cinnamon coat color (Park et al., 2005). We thus tested whether ginsenoside-Rg1 could inhibit lipid peroxidation in the livers of TAA-treated rats. Increased levels of thiobarbituric acid reaction substrate, as indicators of lipid peroxidation, were observed in rats treated with TAA (Fig. 5).

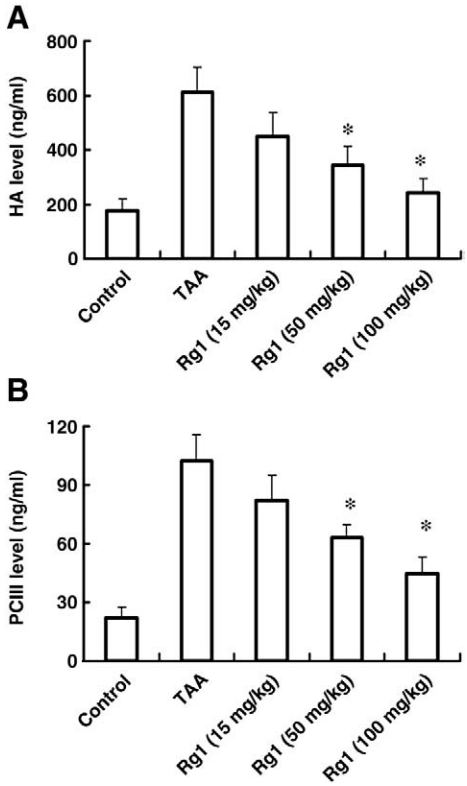

Fig. 2. Effect of ginsenoside-Rg1 on serum markers of liver fibrosis. (A) and (B), TAA treatment. Serum levels of hyaluronic acid (HA) and type III procollagen (PCIII) were determined by radioimmunoassay. *$P<0.05$ versus TAA group ($n=10$).

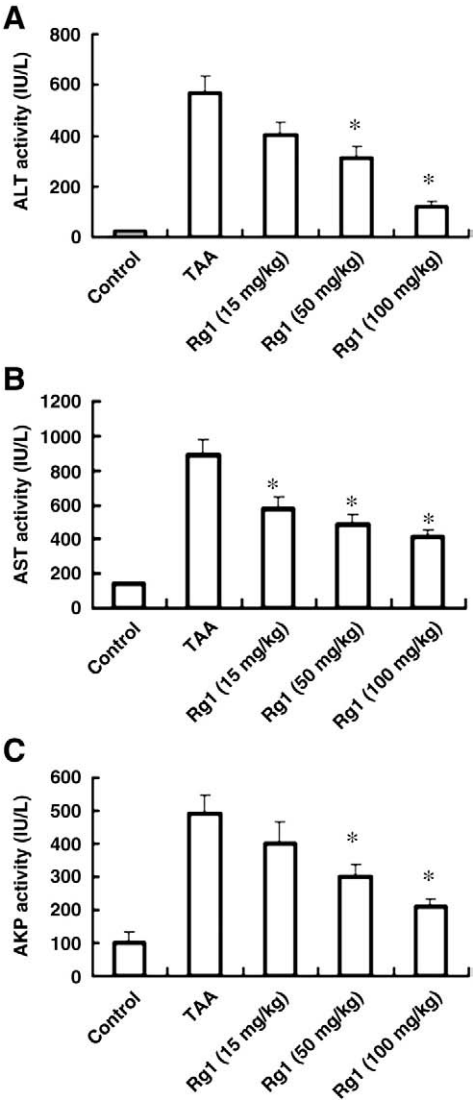

Fig. 4. Effect of ginsenoside-Rg1 on liver function. (A) (B) (C), TAA treatment. Enzymatic activities of alanine transaminas (ALT), aspartate transaminase (AST) and alkaline phosphatase (AKP) were determined by an automated biochemistry analyzer. *$P<0.05$ versus TAA group ($n=10$).

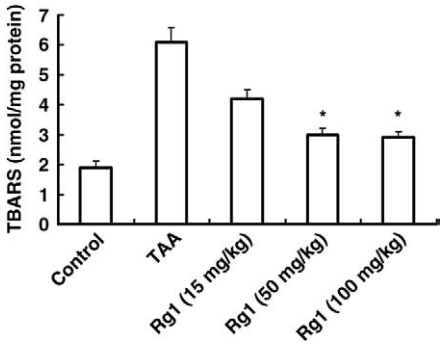

Fig. 5. Effect of ginsenoside-Rg1 on thiobarbituric acid reaction substrate levels in livers. Each group consists of 8 rats. *$P<0.05$ versus TAA group.

Co-administration of ginsenoside-Rg1 markedly suppressed the levels of thiobarbituric acid reaction substrate.

3.6. Ginsenoside-Rg1 inhibits PDGF-induced proliferation and activation in HSCs

Since PDGF-BB is the most potent proliferating cytokine for HSCs (Olaso and Friedman, 1998), we examined the effect of ginsenoside-Rg1 on PDGF-BB-induced HSC proliferation. Before addition of PDGF-BB, we made HSCs quiescent by incubating them in serum-free medium for 24 h. [^{3}H]-thymidine incorporation was then determined to assess the rate of DNA synthesis after incubation with PDGF-BB for 24 h. As shown in Fig. 6A, PDGF-BB (10 ng/ml) significantly promoted [^{3}H]-thymidine incorporation in HSCs. Ginsenoside-Rg1 at 0.1, 1, and 10 μM markedly inhibited [^{3}H]-thymidine incorporation in HSCs by 24%, 42%, 54%, respectively. Meanwhile, ginsenoside-Rg1 suppressed the proliferation of HSCs induced by PDGF-BB as also indicated by cell numbers. By 48 h, ginsenoside-Rg1 at 0.1, 1, and 10 μM reduced cell number by 12%, 30%, and 42%, respectively (Fig. 6B). In this study, we found that ginsenoside-Rg1 (100 μM) did not cause a significant lactate dehydrogenase leakage (an index of cell injury) for 72 h treatment in HSCs (data not shown).

α-SMA is the marker of HSC activation. We thus investigated whether ginsenoside-Rg1 exhibits antifibrotic effect by suppressing the activation of HSCs. Western blot analysis indicated that ginsenoside-Rg1 significantly inhibited the protein levels of α-SMA by PDGF-BB in HSCs (Fig. 6C). Since the activated HSCs could promote the production of collagen, we also tested the effect of ginsenoside-Rg1 on the expression of type I collagen. As shown in Fig. 6C, ginsenoside-Rg1 also markedly reduced the protein levels of type I collagen.

3.7. Ginsenoside-Rg1 inhibits PDGF-induced ROS formation in HSCs

It has been shown that ROS play a critical role in the pathogenic process of fibrosis (Adachi et al., 2005). ROS, which produces via activation of NAD(P)H oxidases in response to PDGF-BB, can stimulate the HSC proliferation (Adachi et al., 2005). To elucidate the possible mechanisms underlying the inhibition of HSC proliferation by ginsenoside-Rg1, we examined the effect of ginsenoside-Rg1 on formation of ROS induced by PDGF-BB using a fluorescence probe, H_2DCFDA. A 2.9-fold in DCF fluorescence production was observed in HSCs after treatment with PDGF-BB (10 ng/ml) for 1 h (Fig. 7). ginsenoside-Rg1 markedly suppressed DCF fluorescence stimulated by PDGF-BB at a dose-dependent manner (Fig. 7).

3.8. Down-regulation of PDGFR-β expression by ginsenoside-Rg1

To further elucidate molecular mechanisms by which ginsenoside-Rg1 inhibits PDGF-mediated cell proliferation, we evaluated the effect

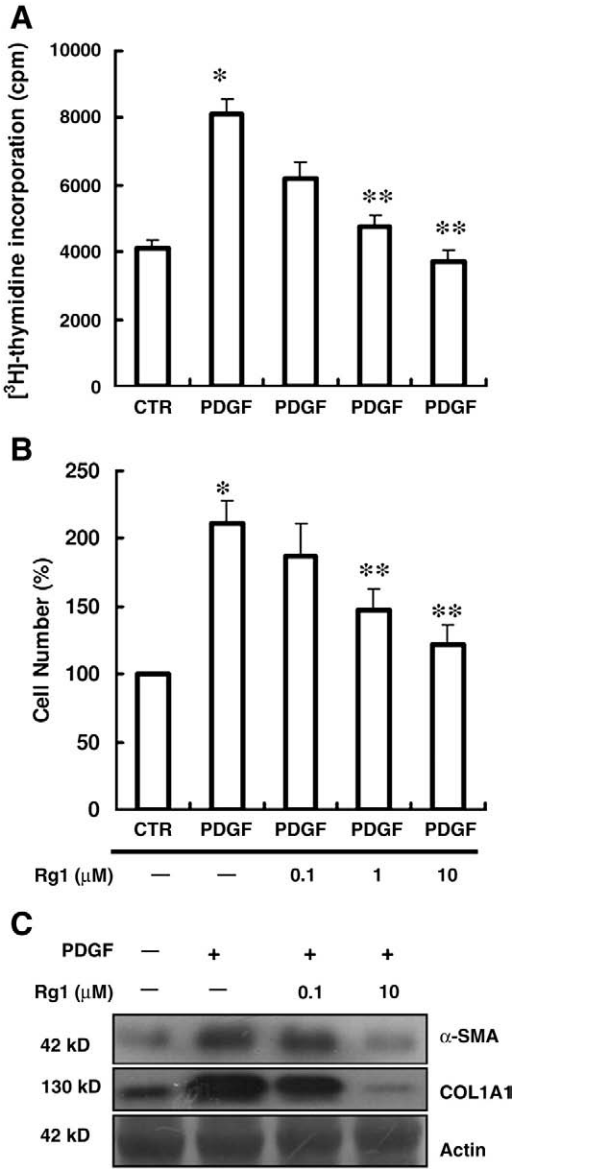

Fig. 6. Effect of ginsenoside-Rg1 on prolieration and activation in HSCs. (A) Primary HSCs were cultured in serum-free medium containing ginsenoside-Rg1 at the indicated doses for 1 h. Then these cells were treated with PDGF-BB (10 ng/ml) for another 24 h. DNA synthesis by the cells was evaluated by [^{3}H]-thymidine incorporation into DNA. Data represent mean ± S.D. of five separate experiments. *$P<0.05$ versus control; **$P<0.05$ versus PDGF alone. (B) The cells in serum-free medium were preincubated with ginsenoside-Rg1 for 1 h at the indicated doses before treatment with PDGF-BB (10 ng/ml) for another 48 h. Cells were collected and counted in a hemocytometer. Data represent mean ± S.D. of five separate experiments. *$P<0.05$ versus control; **$P<0.05$ versus PDGF alone. (C) The cells in serum-free medium were preincubated with ginsenoside-Rg1 for 1 h at the indicated doses before treatment with PDGF-BB (10 ng/ml) for another 24 h. The protein levels were detected by Western blotting. The blot is typical of five experiments. COL1A1, type I collagen.

of ginsenoside-Rg1 on the expression of PDFGR—in HSCs. As shown in Fig. 8, ginsenoside-Rg1 treatment resulted in a decrease in the mRNA and protein levels of PDFGR-β in a dose-dependent manner in HSCs. Thus, the antifibrotic effect of ginsenoside-Rg1 is probably due to inhibition of PDGFR-β expression.

3.9. Ginsenoside-Rg1 inhibits PDGFR-β promoter activity and NF-κB binding activity

To further confirm effect of ginsenoside-Rg1 on PDGFR-β gene transcription, an immortalized rat liver stellate cell line, HSC-T6, was

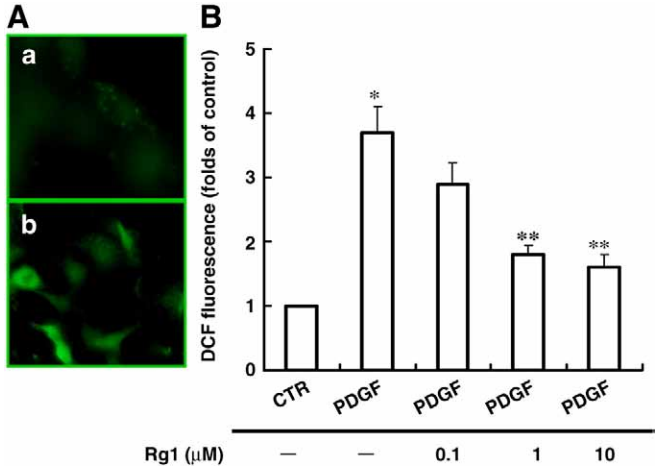

Fig. 7. Ginsenoside-Rg1 inhibits PDGF-induced ROS formation in HSCs. (A) Primary HSCs were cultured in serum-free medium for 1 h. After PDGF-BB (10 ng/ml) treatment for 1 h, formation of ROS in primary HSCs was observed under a fluorescence microscope. (a) Control; (b) PDGF-BB. Magnification, ×400. (B) HSCs were preincubated with ginsenoside-Rg1 for 1 h and then with H_2DCFDA (10 μg/ml) for 15 min. After PDGF-BB (10 ng/ml) treatment for 1 h treatment for 10 min. The fluorescence values for various tested drug treatment were normalized by the value of control. Data represent mean ± S.D. of five separate experiments. *P<0.05 versus control; **P<0.05 versus PDGF alone.

used to examine the activity of PDGFR-β promoter. HSC-T6 cells were transiently transfected with the full-length PDGFR-β reporter plasmid pβ12 fused to a luciferase reporter gene. After 48 h transfection, HSC-T6 cells were incubated with ginsenoside-Rg1 for 24 h. As shown in Fig. 9A, ginsenoside-Rg1 treatment resulted in a significant decrease in luciferase activity.

PDGFR-β gene promoter contains a consensus binding site for NF-κB binding. To confirm the role of NF-κB in the expression of PDGFR-β, we tested NF-κB DNA binding activity using EMSA. As shown in Fig. 9B, ginsenoside-Rg1 significantly suppressed NF-κB DNA binding activity.

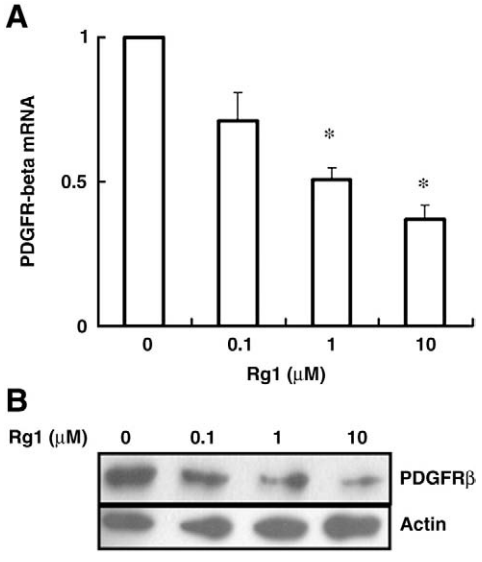

Fig. 8. Down-regulation of PDGFR-beta by ginsenoside-Rg1. (A) Primary HSCs were cultured in serum-free medium containing ginsenoside-Rg1 (10 μM) for 6 h. Total RNA was extracted and subjected to real-time PCR. All results are standardized to the levels of GADPH and are the means ± S.D. of five experiments. *P<0.05 versus control (without ginsenoside-Rg1). (B) The protein levels were detected by Western blotting. The blot is typical of five experiments.

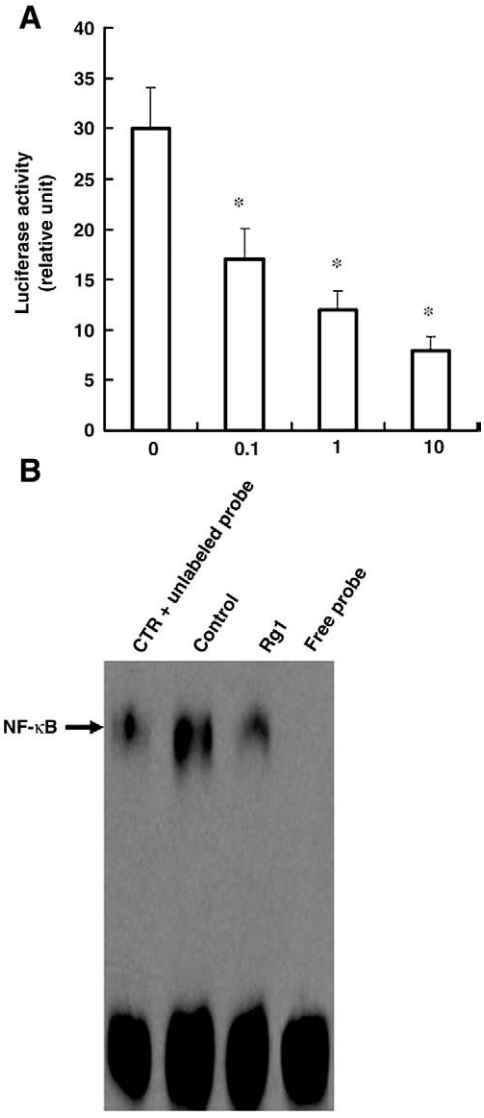

Fig. 9. Ginsenoside-Rg1 inhibits PDGFR-β promoter activity and NF-κB binding activity. (A) HSC-T6 cells were transfected with the full-length PDFGR-β reporter plasmid pβ12. After transfection for 48 h, the cells were incubated with ginsenoside-Rg1 for 6 h. Luciferase assays were performed as described under Materials and methods. Data are the mean and S.D. of five independent experiments performed in triplicate. *P<0.05 versus control (without ginsenoside-Rg1). (B) After primary HSCs were cultured in serum-free medium in the presence of ginsenoside-Rg1 (10 μM) for 6 h. The nuclear proteins were extracted for EMSA. A typical experiment is shown. CTR, control; unlabeled probe, 100-fold excess unlabeled probe.

4. Discussion

A previous study has shown that *P. notoginseng* saponins can suppress hepatic fibrosis in Long–Evans rats with cinnamon coat color (Park et al., 2005). Currently, over 20 different saponins, including ginsenosides, notoginsenosides, and gypenosides, have been identified in *P. notoginseng* root. Among these saponins, ginsenosides-Rg1 is the most abundant components found in the root of *P. notoginseng* (Dong et al., 2003). Like the majority of other traditional Chinese medicines, the efficacy and safety of *P. notoginseng* are mainly based on historical experience rather than on scientific and clinical evidence. Currently, we observed that ginsenoside-Rg1 administered alone (200 mg/kg) had no effect on clinical or biological parameters (data not shown). We have evaluated the effect of ginsenoside-Rg1 on the extent of hepatic fibrogenesis in a rat model of liver fibrosis. We demonstrated for the first time that a single component of

P. notoginseng saponins, ginsenoside-Rg1, significantly inhibited hepatic fibrogenesis induced by TAA.

In this study, histological findings showed that liver fibrogenesis in rats given TAA was ameliorated by administration of ginsenoside-Rg1 (Fig. 1). This improvement was paralleled by a decrease in the serum levels of HA and PCIII as well as the hepatic hydroxyproline content, which are the indexes for liver fibrosis. These results indicate that suppression of activated hepatic stellate cells contributes to the anti-fibrotic effect of ginsenoside-Rg1.The administration of ginsenoside-Rg1 significantly decreased the serum levels of ALT, AST, and AKP in rats given TAA. Previous studies demonstrated that *P. notoginseng* saponins protect rats against hepatic injury induced by D-galactosamine, lipopolysaccharide as well as ethanol (Yoshikawa et al., 2003; Lin et al., 2003). Liver fibrosis is not only the result of derangements in the synthesis and degradation of matrix, but also the result of liver injury. Thus, these data implicates that the improvement of TAA-induced hepatic fibrosis by ginsenoside-Rg1 may result partially from attenuation of hepatocyte injury.

Although all hepatic cell types contribute to fibrogenesis, HSCs play the most important role in fibrotic progression. It has been shown that HSC proliferation and activation are the crucial steps for hepatic fibrosis (Olaso and Friedman, 1998). In this report, we demonstrated that ginsenoside-Rg1 significantly suppressed DNA synthesis and the protein expression of α-SMA and type I collagen in HSCs treated by PDGF-BB. These results indicate that ginsenoside-Rg1 exerts its antifibrotic action by inhibiting HSC proliferation and activation. PDGF-BB is the most potent mitogen for HSC proliferation *in vivo* and *in vitro* (Olaso and Friedman, 1998). During liver injury, the expression of its receptor PDGFRβ is mainly up-regulated along with HSC activation (Kinnman et al., 2003). Our results demonstrated that ginsenoside-Rg1 inhibited the expression of PDGFRβ in HSCs. Thus, the antifibrotic effect of ginsenoside-Rg1 is partially due to inhibition of HSC proliferation through down-regulation of PDGFRβ gene. To elucidate the mechanisms by which ginsenoside-Rg1 inhibited the expression of PDGFRβ in HSCs, we evaluated the effect of ginsenoside-Rg1 on the gene promoter activity. Our results revealed that ginsenoside-Rg1 suppressed the promoter activity of PDGFRβ by reducing NF-κB DNA activity. NF-κB has been shown to play a critical role in regulating the promoter activity of PDGFRβ in HSCs (Chen and Zhang, 2003). Thus, these results suggest that the effect of ginsenoside-Rg1 on the expression of PDGFR-β is mainly exerted at the level of transcription.

A previous study has demonstrated that PDGF-BB activates NAD(P)H oxidases in HSCs, resulting in the formation of ROS (Adachi et al., 2005). ROS are believed to be involved in proliferation of HSCs (Galli et al., 2005; Adachi et al., 2005). Thus, ROS play a critical role in liver fibrosis (Parola and Robino, 2001). Indeed, antioxidants seem to be promising for the inhibition of liver fibrosis. Antioxidants, such as vitamin E (Wong et al., 1994; Houglum et al., 1997), flavonoids (Kawada et al., 1998), N-acetyl-L-cysteine (Kawada et al., 1998) and (−)-epigallocatechin-3-gallate (Chen and Zhang, 2003), can effectively inhibit the proliferation of HSCs. For instance, (−)-epigallocatechin-3-gallate can block the activation of two transcription factors, AP-1 and NF-κB, resulting in inhibition of PDGFRβ expression (Chen and Zhang, 2003). Ginsenoside-Rg1 is an antioxidant (Liu et al., 2002). We speculate that the following two mechanisms might be involved in inhibition of ROS by ginsenoside-Rg1: (I) ginsenoside-Rg1 directly scavenges ROS formation induced by PDGF-BB; (II) ginsenoside-Rg1 inhibits PDGF-BB-mediated ROS formation by down-regulating the expression of PDGFRβ.

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