Saikosaponin-d attenuates the development of liver fibrosis by preventing hepatocyte injury[1]

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Abstract: Treatment of liver fibrosis and cirrhosis remains a challenging field. Hepatocyte injury and the activation of hepatic stellate cells are the 2 major events in the development of liver fibrosis and cirrhosis. It is known that several Chinese herbs have significant beneficial effects on the liver; therefore, the purpose of the present study was to investigate the therapeutic effect of saikosaponin-d (SSd) on liver fibrosis and cirrhosis. A rat model of liver fibrosis was established using the dimethylnitrosamine method. Liver tissue and serum were used to examine the effect of SSd on liver fibrosis. A hepatocyte culture was also used to investigate how SSd can protect hepatocytes from oxidative injury induced by carbon tetrachloride. The results showed that SSd significantly reduced collagen I deposition in the liver and alanine aminotransferase level in the serum. Moreover, SSd decreased the content of TGF-β1 in the liver, which was significantly elevated after dimethylnitrosamine induced liver fibrosis. Furthermore, SSd was able to alleviate hepatocyte injury from oxidative stress. In conclusion, SSd could postpone the development of liver fibrosis by attenuating hepatocyte injury.

Key words: saikosaponin-d, liver fibrosis, oxidative stress, cytotoxicity.

Résumé : Le traitement de la fibrose du foie et de la cirrhose reste un défi. Le dommage aux hépatocytes et l'activation des cellules hépatiques étoilées constituent deux événements majeurs du développement de la fibrose du foie et de la cirrhose. Il est connu que les herbes chinoises ont des effets bénéfiques significatifs sur le foie; cependant, l'étude présente a pour but d'examiner l'activité thérapeutique de la saikosaponine-d (SSd) sur la fibrose du foie et la cirrhose. Un modèle de fibrose du foie a été chez établi chez le rat à l'aide de diméthylnitrosamine. Le tissu hépatique et le sérum ont été utilisés pour examiner l'effet de la SSd sur la fibrose du foie. La culture d'hépatocyte a aussi été employée pour examiner l'effet protecteur exercé par la SSd contre un dommage oxydatif causé par le tétrachlorure de carbone. Les résultats montrent que la SSd a réduit de façon significative le dépôt de collagène I dans le foie et le niveau d'alanine aminotransférase sérique. De plus, la SSd a diminué le contenu en TGF-β1 du foie, lequel était significativement élevé suite à la fibrose du foie induite par le diméthylnitrosamine. Qui plus est, la SSd était capable d'alléger le dommage hépatique causé par le stress oxydatif. En conclusion, la SSd pourrait retarder le développement de la fibrose du foie en protégeant l'hépatocyte de certains dommages.

Mots-clés : saikosaponine-d, fibrose du foie, stress oxydatif, cytotoxicité.

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Introduction

Liver fibrosis and cirrhosis are common pathways of different liver diseases (Bataller and Brenner 2005) and are characterized by the excessive deposition of extracellular matrix (ECM) (Safadi and Friedman 2002). Over the past several decades, it has been known that hepatic stellate cells (HSCs) play an important role during hepatic fibrogenesis (Safadi and Friedman 2002). Hepatic stellate cells are non-parenchymal liver cells, which comprise about 15% of the total number of resident cells within the liver. In normal liver, they are the principal storage sites for retinoids (Kmiec 2001). Following liver damage or inflammation, HSCs undergo a process known as activation, which is a

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transdifferentiation of quiescent and retinoid-storing cells into proliferative and secretive myofibroblast-like HSCs (Hazra et al. 2004). The activation of HSCs could be initiated by the stimulation of cytokines, oxidative stress, and the deposition of degraded ECM (Pinzani and Marra 2001). The activated HSCs then increase the production of ECM and different cytokines, such as TGF-β1, which further stimulates the production of ECM (Kershenobich Stalnikowitz and Weissbrod 2003).

Cellular oxidative stress is one of the factors that cause liver disease. Several primary anti-oxidant defence systems, such as superoxide dismutase and glutathione peroxidase, are known to exist in cells. These systems can scavenge reactive oxygen species, such as superoxide. It is known that several chemicals or drugs can cause oxidative stress in the liver, such as dimethylnitrosamine (DMN) and carbon tetrachloride (CCl_4). These chemicals are typically used to induce liver diseases, such as fibrosis, in rats (Pritchard et al. 1987).

Saikosaponin-d (SSd) belongs to the saponin family and has a chemical structure similar to that of steroid hormones. SSd is also the major medicinal component of the Chinese herb chaihu. Chaihu, or bupleurum, is the most important herb in the treatment of liver disease, according to traditional Chinese medicine. It has been demonstrated that SSd has an anti-inflammatory effect (Bermejo Benito et al. 1998; Bu et al. 2000; Liang and Cui 1998). It can increase serum concentrations of adrenocorticotropic hormone and corticosterone (Iwama et al. 1986; Nose et al. 1989), and it can also elevate corticotropin-releasing factor mRNA levels in the hypothalamus (Dobashi et al. 1995); moreover, it can activate the phagocytosis of macrophages (Ushio and Abe 1991*a*; Ushio et al. 1991*b*), modulate T lymphocyte function (Kato et al. 1994), and upregulate interleukin (IL)-2/IL-4 production in thymocytes (Kato et al. 1995; Kato et al. 1994). However, the molecular mechanisms involved in therapeutic effects of SSd on liver fibrosis and cirrhosis remain to be determined. The objective of the current study was to investigate the mechanism of SSd in the alleviation of liver fibrosis and cirrhosis.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM), pyruvate, penicillin, streptomycin, and fetal bovine serum were purchased from GIBCO/BRL (Life Technology). A rat hepatoma cell line (CRL-1548) was purchased from the American Type Cell Culture Collection. All chemical reagents were purchased from Sigma-Aldrich Canada, Ltd.

Animal model

Eighteen male Sprague–Dawley rats, 4 weeks old and weighing 80–90 g, were divided into 3 groups, with 6 rats per group. Our experiments were approved by the Ethics Committee of our institution, and were carried out in accordance with the *Guide to the Care and Use of Experimental Animals*. Rats in the liver fibrosis model group were given an i.p. injection of DMN (10 mg/kg body mass) 3 times per week (3 consecutive daily injections and 4 days off per week) for 4 weeks, as previously reported (George et al. 2001). Rats in the SSd-treated group received DMN

injections, as described above, and 1.8 mg/kg body weight of SSd (i.p.) daily for 4 weeks. Rats in the normal group received PBS. After 4 weeks (28 days), the rats were sacrificed and sera were obtained for alanine aminotransferase and type-IV collagen C terminal assays. The livers were removed and fixed with 10% neutral buffered formalin for histological and immunohistological examinations.

Biochemical analyses of serum

Serum alanine aminotransferase was determined by routine clinical biochemistry methods. A type-IV collagen C terminal assay was performed using an immunoassay kit from Shanghai Seng Xiong Technology Enterprise Company (Shanghai, China).

Histological and immunohistochemical stainings

The livers were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections (4 μm thick) were cut, mounted on glass slides, and deparaffinized. Sections were subjected to either hematoxylin–eosin staining or Sirius red staining for histological analysis. The grade of fibrosis in the livers of DMN-treated rats was evaluated with ImagePro Plus version 5 (Aperio Technologies), as described previously (Scheuer 1991). For immunohistochemical staining, sections were incubated with proteinase K (20 μg/mL) for 5 min and then blocked with peroxidase blocking reagent (0.03% hydrogen peroxide and 0.2% NaN_3) for 30 min. Sections were incubated with rabbit polyclonal antibody against alpha smooth-muscle actin (α-SMA) (1:200 dilution) at 4 °C overnight. Goat anti-rabbit IgG conjugated with peroxidase-labelled polymer was then incubated with the sections for 30 min at room temperature. Reaction products were visualized by incubating with 3,3-diaminobenzidine chromogen in imidazole buffer (containing hydrogen peroxide and NaN_3 at pH 7.5).

Cell culture

A rat hepatoma cell line (CRL-1548) was cultured and grown in DMEM – Ham's F12 medium supplemented with 100 U of penicillin/mL, 100 μg of streptomycin/mL, 10% fetal bovine serum, 1% fungizone, and 0.011% sodium pyruvate in a humidified, 37 °C incubator in an atmosphere of 95% air – 5% CO_2.

Measurement of lactate dehydrogenase release

The cells (4×10^4) were seeded into 96-well plates and incubated with SSd (2 μg/mL) for 48 h in DMEM – Ham's F12 medium with 10% FBS. At the end of pretreatment with SSd, the cells were incubated with CCl_4 (at final concentration of 2.5 mmol/L) for 1 and 3 h. At the end of the experiment, cell culture media were collected and lactate dehydrogenase (LDH) was measured using a routine laboratory method (Castilla et al. 2004). Briefly, the culture media were incubated with 0.2 mmol/L β-NADH and 0.4 mmol/L pyruvic acid in PBS (pH 7.4). The LDH concentration in the samples was proportional to the linear decrease in the absorbance at 340 nm.

Determination of lipid peroxidation

CRL-1548 cells were cultured in DMEM – Ham's F12 medium. The cells (4×10^5) were seeded into 6-well plates and treated with SSd (2 μg/mL) for 48 h. At the end of the

Fig. 1. Histological and immunohistological examination of rat liver in different experimental groups. The livers from the rats of 3 different groups were collected and fixed in a 10% paraformaldehyde solution and stained with either hematoxylin–eosin or Sirius red for liver histology or collagen I, respectively. The tissue sections were also subjected to immunohistological analysis with an antibody against α-SMA. With hematoxylin–eosin staining, the liver sections from PBS-treated rats showed normal hepatic lobule structure, with a central vein in the centre. Liver sections from dimethylnitrosamine (DMN)-treated rats displayed irregular hepatic nodules with an excessive deposition of extracellular matrix, whereas liver sections from DMN+saikosaponin-d (SSd)-treated rats showed relatively normal hepatic lobules with some deposition of extracellular matrix. After staining with Sirius red, there was collagen I staining around the central vein in the PBS-treated control group. There was less collagen I staining in the liver sections of DMN+SSd-treated rats than in the liver sections of rats treated with DMN alone. A similar pattern of α-SMA immuno-staining was observed in each of the 3 groups.
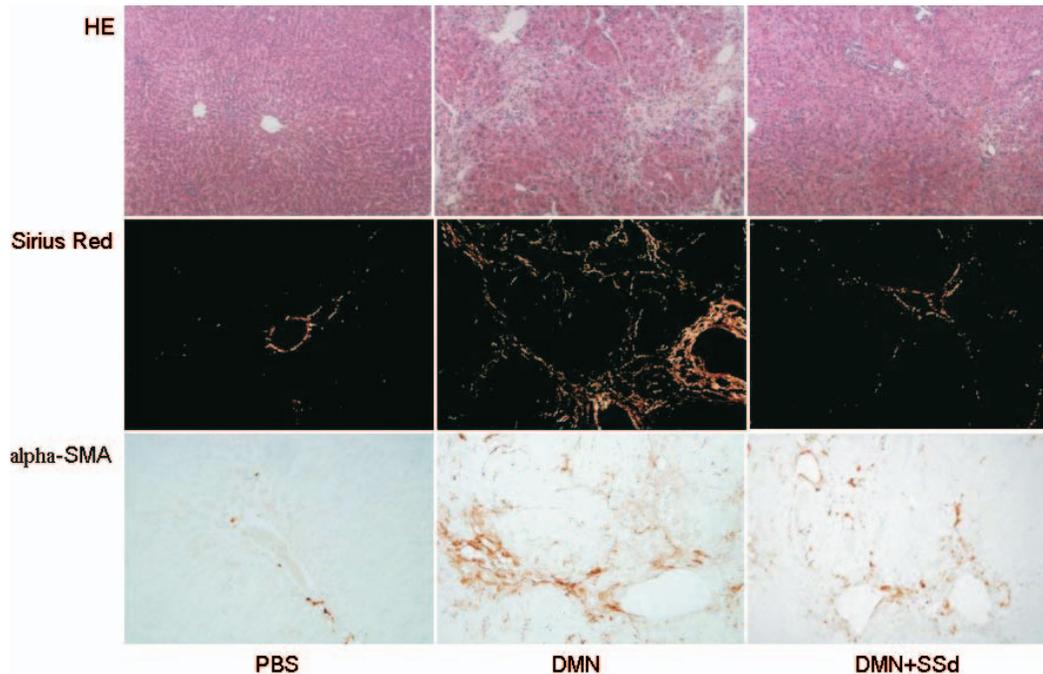

pretreatment with SSd, cells were then incubated with CCl_4 (at a final concentration of 2.5 mmol/L) or a combination of SSd and CCl_4 at the same concentrations for 1 and 3 h. At the end of the experiment, cells were harvested and cellular malondialdehyde (MDA) was determined by a procedure described previously (Wasowicz et al. 1993). Briefly, CRL-1548 cells were collected and lysed. The samples (50 μL) were treated with trichloroacetic acid (10%) and centrifuged at 16 800g at 4 °C for 5 min. EDTA (1.34 mmol/L) and GSH (0.65 mmol/L) were added to the supernatant to prevent further lipid peroxidation during the assay. The samples were mixed with 0.25 mL HCl (25%) and 0.25 mL of thiobarbituric acid (1% diluted in 50 mmol/L NaOH). The mixture was heated at 100 °C for 1 h, and then extracted with 600 μL butanol – pyridine (15:1). After centrifugation at 16 800g for 10 min, the solvent layer containing thiobarbituric-acid-reactive substances was evaluated by measuring the absorbance of the samples at 532 nm. A standard curve was generated using 1,1,3,3-tetraethoxypropane diluted in ethanol as the source of MDA.

Statistics analysis

A statistical significance of difference was performed by employing a 1-way ANOVA and Fisher's PLSD test as a posthoc test with StatView software (version 5.0, SAS Institute Inc.). All measurements were expressed as the means ± SE. Differences were considered to be significant at $p < 0.05$.

Fig. 2. Analysis of the collagen area in liver sections. Sirius red staining sections were evaluated with ImagePro Plus. Five fields were taken from each tissue section, with a final magnification of 200 ×. The percentage of collagen relative to the total tissue area was calculated. There was a significant increase in the percentage of collagen versus the total tissue area. Treatment with saikosaponin-d significantly decreased the percentage of collagen relative to the total tissue area. Data represent the means ± SE from 6 liver sections. *, $p < 0.05$. DMN, dimethylnitrosamine; SSd, saikosaponin-d.
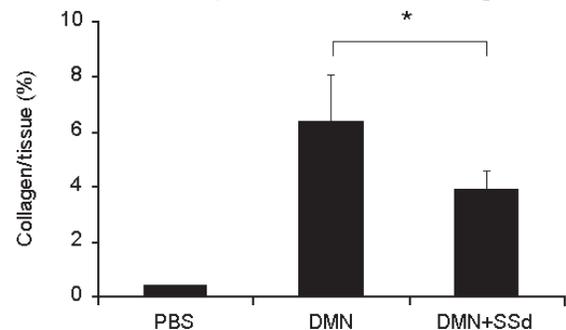
Results

Saikosaponin-d attenuated liver fibrosis

A liver fibrosis model in rat was established and treatment with SSd attenuated the injury to the liver induced by DMN (Fig. 1). The staining of collagen I with Sirius red

Table 1. The grade of liver fibrosis in liver sections from 3 groups.

Group	Grade				
	0	I	II	III	IV
PBS	6	0	0	0	0
DMN	0	0	1	4	1
DMN+SSd	0	1	4	1	0

Note: The liver sections were stained with Sirius red and the grade of liver fibrosis was evaluated using 2 individual pathologists at the China–Japan Friendship Hospital. The grade of fibrosis was based on Scheuer's method, as follows: grade 0, normal hepatic nodules with no excess collagen deposition; grade I, a small deposition of collagen around the central vein and portal triad; grade II, a significant deposition of collagen around the central vein; grade III, the presence of collagen connecting the central vein to itself or to the portal triad; grade IV, a significant collagen deposition, forming a bridge in the liver, and the presence of separated regenerated hepatocyte nodules with the collagen deposition. DMN, dimethylnitrosamine; SSd, saikosaponin-d.

demonstrated that there was a significantly increased deposition of collagen I in the liver of DMN-treated rats, whereas a lesser deposition of collagen I was observed in the liver of DMN+SSd-treated rats. A quantitative assessment of collagen I levels in the liver indicated that the total collagen area increased about 10-fold after DMN administration, whereas SSd treatment significantly reduced total collagen area compared the result obtained when no SSd treatment was given (Fig. 2). Moreover, α-SMA immunostaining was examined in the liver. As shown in Fig. 1, there was an increased staining of α-SMA in the liver of DMN-treated rats, and α-SMA staining was significantly reduced in the liver of DMN+SSd treated rats.

DMN-induced liver fibrosis cannot be completely resolved by SSd treatment, as indicated in Table 1. Moreover, we demonstrated that SSd was able to reduce the DMN-induced elevation of the level of serum alanine aminotransferase, as well as that of serum-soluble collagen type IV (Fig. 3).

Saikosaponin-d reduced lipid peroxidation in the liver

To investigate the mechanism of SSd involved in the reduction of liver fibrosis, we used a rat hepatoma cell line (CRL-1548) (Benedict et al. 1973), which has the characteristics of rat hepatocytes. After pretreatment with 2 μg/mL SSd for 48 h, the cells were challenged with 2.5 mmol/L CCl_4 for 1 and 3 h. As shown in Fig. 4, SSd alone did not induce cytotoxicity to CRL-1548 cells but SSd did attenuate CCl_4-induced cytotoxicity to these cells, as indicated by the LDH assay. Moreover, we demonstrated that protective effect of SSd on hepatocytes was due to the reduction in lipid peroxidation in the cells, which was determined using an MDA assay, as shown in Fig. 5.
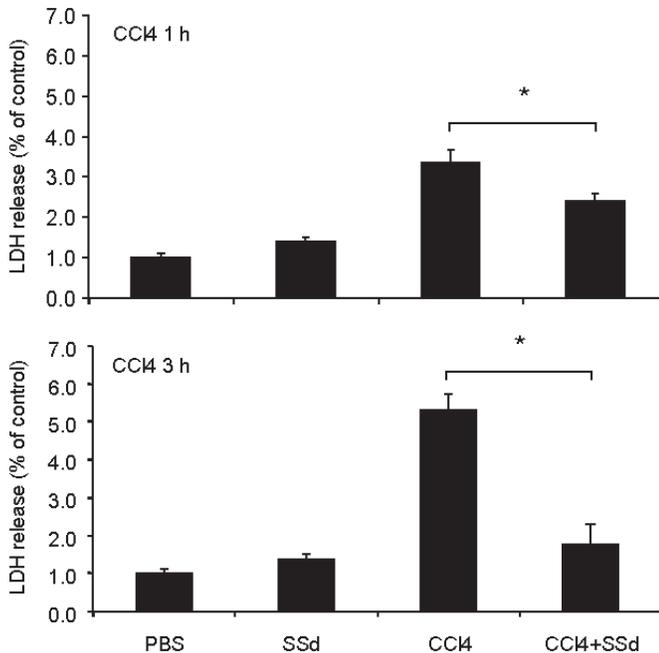
Discussion

Liver fibrosis and cirrhosis is one of the end-stages of liver diseases and the treatment of this condition is still a challenge in the field of liver disease. Although it is known that HSCs play an important role in the development of liver

Fig. 3. Serum alanine aminotransferase and type-IV collagen (IV-C) peptide levels in 3 different groups of rats. Sera were collected from the rats at the end of the experiment. Serum alanine aminotransferase and collagen IV-C peptide were determined according to the procedures described in Materials and methods. There was a significant increase in both the alanine aminotransferase and collagen IV-C levels in the DMN (dimethylnitrosamine)-treated rats, whereas treatment with SSd (saikosaponin-d) decreased alanine aminotransferase and collagen IV-C levels back to those close to normal. Data represent the means ± SE from 6 rats. *, $p < 0.05$.
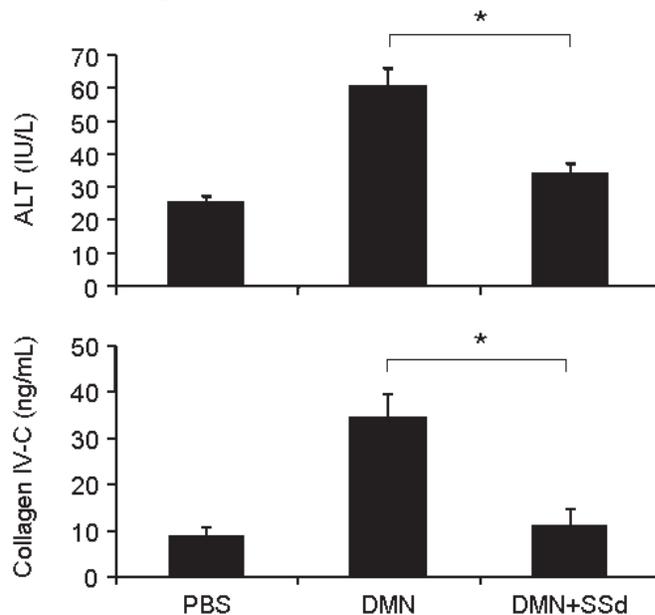
fibrosis, a therapeutic treatment that targets HSCs has yet to be found and is an important research topic (Beljaars et al. 2002; Elsharkawy et al. 2005). Another major area of research in the prevention and treatment of liver fibrosis and cirrhosis is the protection of hepatocytes from injury. Since the liver is a major organ, mediating the metabolism of a variety of drugs and chemicals in the body, hepatocytes can be easily damaged by drug or chemical metabolites, which induce oxidative stress in liver cells (Beddowes et al. 2003; Minamide et al. 1998). The protection of hepatocytes from injury has been investigated extensively (Garcia-Rubio et al. 2005; Lee et al. 2000, 2004; Nakahira et al. 2003; Senthilkumar et al. 2004) and Chinese herbal combinations have been documented to be effective in the protection of hepatocytes or HSCs from injury (Chiu et al. 1997; Kim et al. 2005; Lee et al. 2004; Park et al. 2002; Shimizu 2000). One of these Chinese herbs, chaihu, and its extract, SSd, are extensively used in the treatment of liver disease in traditional Chinese medicine and SSd has been shown to confer protection from liver injury in a rat model of CCl_4-induced liver disease (Abe et al. 1982). In the present study, we demonstrated that SSd can reduce liver injury and fibrosis induced by another chemical, DMN. Moreover, we found that the attenuation of liver injury and fibrosis using SSd was due to the reduction of lipid peroxidation and the protection hepatocytes from injury, as demonstrated by the production of MDA in the cells and LDH in the culture medium, respectively. One previous study demonstrated that SSd prevented an enhanced CCl_4 hepatotoxicity induced by by phenobarbitone (Abe et al.

Fig. 4. Saikosaponin-d (SSd) attenuated CCl4-induced lactate dehydrogenase (LDH) release in hepatocytes. CRL-1548 cells were cultured as described in Materials and methods. After cells were incubated with or without 2 μg/mL SSd for 48 h, the cells were incubated with or without CCl4 for 1 and 3 h. Conditioned media were then collected for the analysis of LDH release. SSd treatment alone did not increase LDH release compared with the control (PBS-treated) group, as indicated in the SSd group. CCl4 treatment alone significantly induced LDH release, as indicated in the CCl4 group, whereas pretreatment with SSd for 48 h attenuated the release of LDH (as indicated in the CCl4+SSd group). Data represent the means ± SE from 6 independent experiments. *, $p < 0.05$.

Fig. 5. Saikosaponin-d (SSd) attenuated CCL4-induced lipid peroxidation in hepatocytes. CRL-1548 cells were cultured as described in Materials and methods. After cells were incubated with or without 2 μg/mL SSd for 48 h, the cells were incubated with or without CCl4 for 1 and 3 h. Cells were harvested and cytoplasmic proteins were prepared for the analysis of malondialdehyde (MDA) production in the cells. SSd treatment alone did not increase MDA production compared with the control (PBS-treated) group, as indicated in the SSd group. CCl4 treatment alone significantly stimulated MDA production, as indicated in the CCl4 group, whereas pretreatment with SSd for 48 h prevented the cells from producing MDA (as indicated in the CCl4+SSd group). Data represent the means ± SE from 6 independent experiments. *, $p < 0.05$.
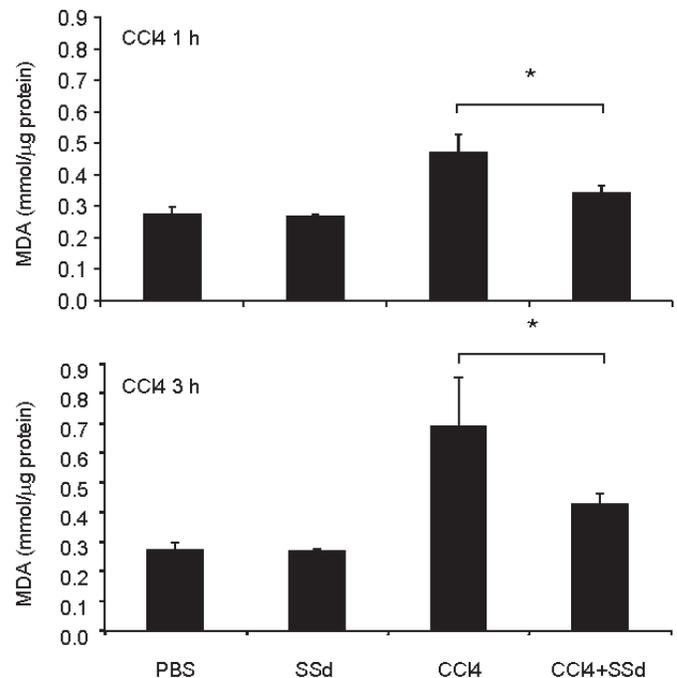
1985). However, the detailed mechanism of SSd regulation of lipid peroxidation remains to be further investigated.

Another possible mechanism of SSd in attenuation of DMN-induced liver fibrosis and cirrhosis is SSd regulation of TGF-β1 gene expression. TGF-β1 is a critical cytokine, not only liver fibrosis, but also in the process of fibrosis in other organs (Bataller and Brenner 2005; Kotecha 1996; Palomar et al. 2005). In liver fibrosis and cirrhosis, it has been demonstrated that TGF-β1 could not only serve as a marker for liver fibrosis and cirrhosis (Chen et al. 2005; Yasuda et al. 1996), but could also stimulate HSCs to produce ECM (Gressner 1996). SSd downregulation of TGF-β1 was found in a rat model of glomerulosclerosis (Li et al. 2005). This finding indicates that some extracts of Chinese herbs could have a potentially significant effect on the regulation of growth factors and may be involved in the regulation of signal transduction and transcription of growth factors.

The development of liver fibrosis and cirrhosis is a complex process, which includes hepatocyte injury and HSC activation. Activated HSCs are responsible for the degradation of normal ECM in the liver, such as type-IV collagen, and the secretion of abnormal ECM in the liver, such as type-I collagen. Thus, we employed type-IV collagen C terminus in the serum as a marker of liver fibrosis, and type-I collagen in the liver as an indicator of liver fibrosis. In the cur-

rent study, we demonstrated that SSd reduced collagen type I deposition in the liver and type-IV collagen C terminus in the serum. However, SSd was unable to reverse the collagen I content back to its normal level, although the type-IV collagen C terminus in the serum was close to normal. This might be due to the concentration of SSd and the severity of liver injury, because we observed that lower concentrations of SSd (<1 μg/mL) could not protect hepatocytes from injury induced by CCl4 in vitro (data not shown). Therefore, i.p. injection with SSd may not be the optimal route for SSd delivery in the treatment of liver fibrosis, and low concentrations of SSd in the liver might explain the results obtained in current study. Further studies should focus on how to specifically deliver SSd into the liver. It is known that mannose-6-phosphate-modified albumin can specifically target the liver (Gonzalo et al. 2006; Greupink et al. 2006), and it has been successfully delivered a Chinese herbal extract, glycyrrhetin, to the liver and significantly reduced ECM deposition in the liver (Zhang et al. 2002). Whether SSd can be linked to mannose-6-phosphate-modified albumin remains to be further investigated.

In conclusion, we have successfully established a rat model of liver fibrosis and demonstrated that SSd can reduce collagen I deposition and liver injury. Moreover, we

demonstrated that the effect of SSd on liver fibrosis may be related to its ability to reduce lipid peroxidation in hepatocytes and protect hepatocyte from CCl$_4$-induced injury.

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