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Cortex Dictamni extract induces apoptosis of activated hepatic stellate cells via STAT1 and attenuates liver fibrosis in mice

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ABSTRACT

Ethnopharmacological relevance: In traditional Chinese medicines, Cortex Dictamni is prescribed for the treatment of a variety of inflammatory diseases such as acute rheumatoid arthritis, skin inflammation and jaundice.
Aim of the study: This study was designed to investigate the effect of ethanol extract of Cortex Dictamni on treatment of hepatic fibrosis and its possible mechanisms.
Materials and methods: The in vivo effect of Cortex Dictamni extract (CDE) was evaluated by measuring histological changes and collagen content in CCl_4-indcued hepatic fibrosis mice. Viability, apoptosis and protein expression of hepatic stellate cells (HSC) were analyzed by MTT, Annexin V staining and Western blot respectively.
Results: CDE alleviated CCl_4-induced hepatic fibrosis in mice and showed a much stronger inhibition of cell viability in activated HSC cell line HSC-T6 than that in normal hepatocyte L02 cells. Furthermore, CDE induced apoptosis of HSC-T6 cells associated with increased expressions of cleaved PARP and cleaved caspase-3. Interestingly, CDE activated STAT1 in HSC-T6 cells and the effect of CDE on apoptosis of HSC-T6 cells could be neutralized using JAK/STAT1 signaling inhibitor AG490.
Conclusions: These findings suggest that CDE possesses anti-fibrosis activity with selectively induction of activated HSC apoptosis via activating STAT1, which might be a novel strategy for hepatic fibrosis therapy.

1. Introduction

Liver fibrosis is characterized by an excessive deposition of extracellular matrix (ECM) proteins, of which type I collagen predominates (Friedman, 2000). As the main source of accumulated ECM including type I collagen, activated hepatic stellate cells (HSCs), which are proliferative and fibrogenic, have been evidenced to play a key role in the development of liver fibrosis (Bataller and Brenner, 2001; Lotersztajn et al., 2005). Thus, eliminating the effects of HSCs such as inducing their apoptosis has become an important strategy for the treatment of liver fibrosis (Bataller and Brenner, 2001; Oakley et al., 2005). However, in physiological conditions, quiescent HSCs play critical roles in the regulation of retinoid homeostasis and ECM remodeling by producing ECM components as well as metalloproteases and their inhibitors (Sarem et al., 2006). And hepatocytes play a major role in liver regeneration (Michalopoulos and DeFrances, 1997). Thus, induction of the quiescent HSC apoptosis and inhibition of the hepatocyte viability may cause side effects and impede recovery from liver fibrosis.

In recent years considerable effort has been devoted to targeting HSCs for the treatment of liver fibrosis. Astragaloside IV displays antifibrotic effects and inhibition of the proliferation of HSCs (Liu et al., 2009). Tetrandrine inhibits activation of rat hepatic stellate cells (Chen et al., 2005). And there have also been a number of studies recently looking at the effects of various components of traditional Chinese medicine on HSC apoptosis (Yao et al., 2002; Deng et al., 2010). However, Strategies for selective targeting activated HSCs have not yet been achieved. For these reasons, the proper approach to inducing HSC apoptosis for the resolution of liver fibrosis is still facing enormous challenges.

Signal transducers and activators of transcription (STAT) proteins can be mainly activated by cytokines and play important roles in antiviral defense, acute phase response, hepatic injury, repair, inflammation, transformation, and hepatitis (Gao, 2005). Among the STAT family members, STAT1 has been reported to attenuate liver fibrosis through inhibition of HSC proliferation, activation and function (Jeong et al., 2006), and promotes apoptosis by inducing pro-apoptotic or inhibiting anti-apoptotic genes in different

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tissues (Stephanou and Latchman, 2003). These findings suggest that induction of apoptosis in HSCs via STAT1 signaling may become an optimal strategy for the treatment of liver fibrosis. However, no agent capable of targeting activated HSC apoptosis via STAT1 has been reported so far.

Cortex Dictamni has been widely used for a variety of inflammatory diseases, such as acute rheumatoid arthritis, skin inflammation and jaundice in China. A variety of components such as dictamine, fraxinellone, obacunone and lignin were found in this medicinal plant (Jiang et al., 2006). Fraxinellone attenuates acute liver injury in mice (Ran et al., 2007). Dictamine and lignin have anti-fibrosis effects respectively on interstitial fibrosis (Anjaneyulu and Chopra, 2004). Nevertheless, the scientific evidence for the effects of the herbal drug Cortex Dictamni on liver fibrosis is still lack. Therefore, the present study was designed to investigate the anti-fibrosis effect of the ethanol extract from Cortex Dictamni (CDE) and found that the extract protected mice from hepatic fibrosis and stimulated apoptosis in activated HSCs via STAT1 with less influence on normal hepatocytes.

2. Materials and methods

2.1. Plant material and chemicals

The Chinese herbal drug Bai-Xian-Pi (Cortex Dictamni) was purchased from Nanjing Medicinal Material Co. (Nanjing, China) and identified Dr. Boyang Yu (Department of Complex Prescription of TCM, China Pharmaceutical University) as Dictamnus dasycarpus Turcz. A specimen was deposited at State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University with voucher number N050118. The material herb was used to make the ethanol extract as previously reported (Sun et al., 2009).

2.2. HPLC analysis

HPLC analysis was applied on a Waters 600 series HPLC system consisting of a Waters 600 pump, a 2487 UV detector, an online degasser and a LC Work Station equipped with Empower™ software. CDE was applied to YMC-pack Pro C18 column (5 μm, 250 × 4.6 mm, YMC Co., Ltd, Japan) and detected at 236 nm. Column temperature was set up at 25 °C and the flow rate was 1 ml/min. The mobile phase was methanol:water (60:40, vol/vol) (Fig. 1A).

2.3. Cells and reagents

Activated hepatic stellate cell line HSC-T6 and normal hepatocyte cell line L02 cells were purchased from Chinese Academy of Medical Sciences and cultured in RPMI 1640 medium (Gibco, NY). Colchicine was purchased from Shanghai Kefeng Co., Ltd. (Shanghai, China). Anti-cleaved-PARP, anti-cleaved-caspase 3, anti-Bad, anti-pY-STAT1, anti-mouse IgG and anti-rabbit IgG were bought from Cell Signaling Technology (Cell Signaling Technology, MA, USA). Anti-Actin, anti-α-SMA, and anti-Tubulin were bought from Santa Cruz (Santa Cruz Biotechnology, CA, USA). AG490 was purchased from Sigma (Sigma-Aldrich, USA).

2.4. Animal treatment

Male ICR mice (body weight 18–22 g) were supplied by the Experimental Animal Center of Nanjing Medical University (Nanjing, China). Animal care and research protocols were based on the principles and guidelines adopted by the Guide for the Care and Use of Laboratory Animals (NIH publication No: 85-23, revised in 1985). The mice were maintained at 24 ± 1 °C and 40–60% relative humidity and were given 30, 60 and 120 mg/kg of CDE or 2.5 mg/kg of colchicine intragastrically daily for 8 weeks, respectively. Those in normal and CCl_4 groups were given equal volume of vehicle solution. After one-week administration, all mice except those in normal group were intraperitoneally injected with CCl_4 (0.3% CCl_4/olive oil 10 ml/kg body weight twice a week) for seven weeks. Then, the mice were sacrificed 24 h after the last injection by bleeding. Liver and spleen were taken away, and weighted. Then livers were divided into two portions: (1) preserved in 10% formalin for histological examination, (2) frozen for hydroxyproline assay at −70 °C.

2.5. Determination of morphometric collagen

The liver sections imbedded in paraffin were cut (5 μm) and stained with hematoxylin–eosin (H&E) and Masson's trichrome to determine the collagen distribution (Malkusch et al., 1995).

2.6. Determination of hydroxyproline content

Hydroxyproline content in the liver was determined by the spectrophotometric method as the instruction manual for hydroxyproline assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) (Lange and Malyusz, 1994). The data was expressed as hydroxyproline (mg)/wet liver weight (g).

2.7. Assessment of cell viability

Cells were cultured in 96-well plate at a density of 5×10^4 cells/ml in the media (0.2 ml) for indicated time in the presence or absence of CDE. Cell proliferation was measured at 540 nm by MTT uptake.

2.8. Assessment of cell apoptosis

Cells were stained with Annexin V-FITC (Jingmei, Beijing, China). Then the cells were measured by flow cytometry. Samples were analyzed by FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA). Annexin V^+ cells were considered as apoptotic cells.

2.9. Western blot analysis

HSC-T6 cells were seeded into 60 mm dishes at 1×10^6 cells/dish. In the next day, cells were treated with CDE (50 μg/ml) for the indicated time periods. Then they were harvested and extracted in the lysis buffer (Biyuntian, Hangzhou, China). The extracted proteins were separated by polyacrylamide/SDS gel and electrophoretically transferred onto polyvinylidene fluoride membranes (Roche, IN). The membranes were probed with antibodies overnight at 4 °C, and then incubated with a HRP coupled secondary antibody. Detection was performed using a LumiGLO chemiluminescent substrate system (KPL, Guildford, UK).

2.10. Statistical analysis

Results were expressed as mean ± SEM. Statistically evaluated by Student's *t* test when only two value sets were compared, and one-way ANOVA followed by Dunnett's test when the data involved three or more groups. $P < 0.05$ was considered to be significant.

3. Results

3.1. CDE attenuates CCl_4-induced liver fibrosis in mice

The contents of main components in CDE were determined by HPLC as 0.868% (w/w) of fraxinellone, 7.374% of obacunone

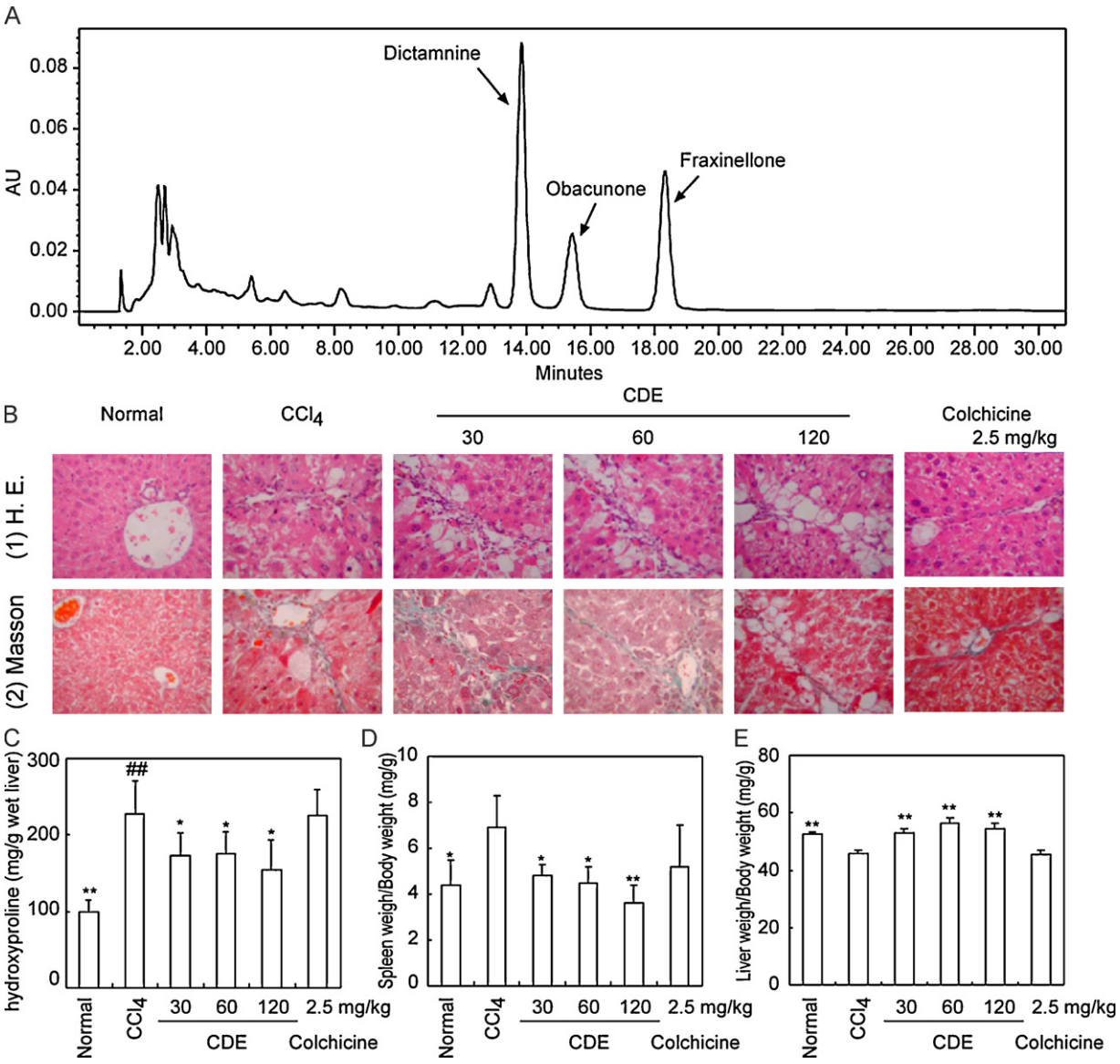

Fig. 1. Effect of CDE on CCl_4-induced liver fibrosis. (A) CDE was analyzed by HPLC. (B) The sections of mice liver were stained with (1) hematoxylin–eosin or (2) Masson and examined by a blinded histologist (Original magnification ×200). (C) Liver hydroxyproline content. (D) Spleen index. (E) Liver index. Data are expressed as the mean ± SEM. The number of mice in each column is 8–11. $^{*}P<0.05$, $^{**}P<0.01$ compared with CCl_4-treated group.

and 0.514% of dictamnine (Fig. 1A). As shown in Fig. 1B (1), adipose degeneration of hepatocytes, mild wall thickening of central venous, fibrous hyperplasia, and inflammatory cell infiltration were observed in the liver sections of CCl_4-treated mice. Against the pathological changes, the treatment with 120 mg/kg CDE showed a significant improvement. Colchicine relieved the fibrous hyperplasia but had no effect on inflammatory cell infiltration (Table 1). As stained by Masson, the collagen fibers in CCl_4-treated mice were

Table 1
Histopathological grading of hepatic fibrosis in mice. The liver tissue sections were stained with hematoxylin–eosin or Masson. The histological changes from hematoxylin–eosin staining were read on a scale of 0–3 (0, no change; 1, mild; 2, moderate; and 3, severe) and the fibrosis changes were analyzed by the Knodell scoring system (Knodell et al., 1981), score 0, absence of fibrosis; 1, fibrous portal expansion; 3, bridging fibrosis (portal-portal or portal-central linkage); 4, crrihosis. Data were expressed as the median and range. Each figure indicates median (range) of 8–11 animals. The Kruskal–Wallis test revealed a significant effect at $^{*}P<0.05$, $^{**}P<0.01$ *vs.* CCl_4-treated group.

Group	Hepatocyte regeneration	Central vein	Perisinuso	Portal area		Fibrosis
				Inflammation	Connective tissue proliferation	
Normal	0 (0–0)*	0 (0–0)*	0 (0–0)**	0 (0–0)*	0 (0–0)**	0**
CCl_4	0.5 (0–1)	0.5 (0–1)	1 (0.5–2)	0.5 (0–1)	1 (0–1)	2.4 (1–3)
CDE (mg/kg)						
30	1 (0–1)	0.5 (0–0.5)	1 (0.5–1)	1 (0–1)	1 (0–1)	1.9 (1–2.5)
60	1 (0.5–1)	0 (0–0.5)*	1 (0.5–1)	1 (0.5–2)	1 (0.5–2)	1.6 (1–2)*
120	1 (0.5–2)	0 (0–0)**	0.5 (0.5–1)*	0 (0–1)**	0.5 (0–1)*	0.6 (0–1.5)**
Colchicine	0.5 (0–1)	0 (0–0)**	0.5 (0–1)**	0.5 (0–1)	0 (0–1)**	0.8 (0–2)**

obviously more than those in naive mice. The collagen deposition in liver was decreased by 60 mg/kg of CDE, and the collagen fibers nearly disappeared by 120 mg/kg of CDE (Fig. 1B (2)).

Collagen content was also determined by measurement of hydroxyproline in the livers. The hydroxyproline level increased significantly after CCl_4 treatment ($P < 0.01$). CDE at 120 mg/kg significantly reduced the content of liver hydroxyproline. Colchicine only had the tendency to decrease the content (Fig. 1C). Meantime, a significant recovery of the spleen and liver weights was found to the normal levels in the groups with CDE treatment, while such effect was not found in the colchicine group (Fig. 1D and E).

3.2. CDE selectively reduces the cell viability of activated hepatic stellate cells

The effects of CDE on cell viability of different kinds of hepatic cells were compared using activated HSCs (HSC-T6), and normal hepatocytes (L02). Treatment with CDE reduced cell viability of HSC-T6 cells in a time- and dose-dependent manner (Fig. 2A and B). The extracts from the dose of 20, 40, 60 and 80 μg/ml did not display significant inhibition on cell viability of L02 cells (Fig. 2C). α-SMA, a marker of HSC activation (Rockey et al., 1992), was abundant in HSC-T6 cells but not in L02 cells (Fig. 2D).

3.3. CDE induces the apoptosis of HSC-T6 cells

HSC-T6 cells were treated with 25, 50 and 100 μg/ml of CDE for 6 h. As shown in Fig. 3A, CDE dose-dependently induced apoptosis of HSC-T6 cells. As revealed by Western blot analysis, pretreatment with 50 μg/ml CDE for 6 h remarkably enhanced the expression of pro-apoptosis protein Bad (Fig. 3B). The expressions of cleaved Caspase-3 and cleaved PARP were elevated by 50 μg/ml CDE treatment in HSC-T6 cells (Fig. 3C).

3.4. Apoptosis of HSC-T6 cells induced by CDE depends on the activation of STAT1

The expression of pY-STAT1 was elevated by 50 μg/ml CDE treatment in a time-dependent manner in HSC-T6 cells (Fig. 4A). The expressions of pY-STAT1 and Bad and the apoptosis of HSC-T6 cells induced by 50 μg/ml CDE were inhibited by 20 μM AG490 pretreatment (Fig. 4B and C).

4. Discussion

Chronic exposure to the hepatotoxin CCl_4 is a classical method of inducing liver fibrosis in mice (Rudolph et al., 2000; Inagaki et al., 2003; Kamada et al., 2003). The present study has found the therapeutic effect of CDE in CCl_4-induced hepatic fibrosis in mice. The hydroxyproline content and the collagen deposition in liver tissue were reduced with a significant improvement of spleen and liver weights in CDE treatment mice in comparison with those in the control mice receiving saline (Fig. 1). However, colchicine improved the fibrosis without having any significant effect on the weight of spleen and liver (Fig. 1D and E). In addition, CDE showed more hepatocyte regeneration than colchicine (Table 1). These findings suggest that CDE may have less unwanted effects on hepatocytes and immune cells in anti-liver fibrosis than colchicine. Activated HSCs play a critical role in the pathogenesis of hepatic fibrosis. However, selectively targeting activated HSCs without influencing normal cells remains an ideal strategy for therapy of hepatic fibrosis.

In vivo, CDE alleviated CCl_4-induced hepatic fibrosis in mice. Since HSCs play a critical role in the pathogenesis of liver fibrosis, we focused on the effect of CDE on HSC-T6 cells, which represented activated HSCs (Vogel et al., 2000). In vitro, CDE showed a much

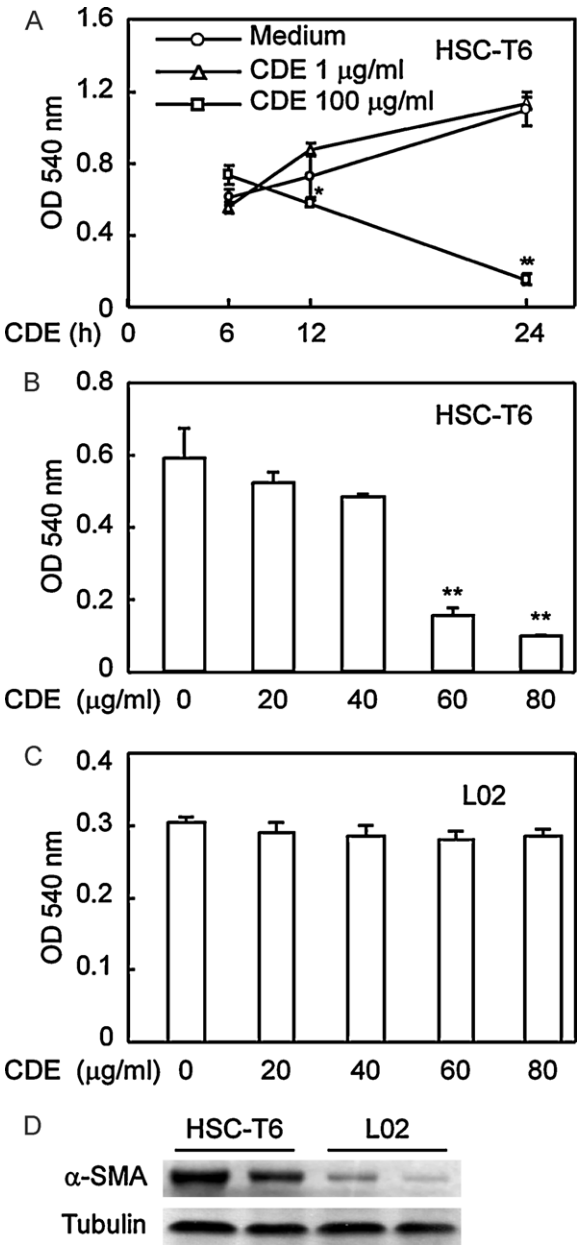

Fig. 2. Effect of CDE on cell viability of HSC-T6, and L02 cells. (A) HSC-T6 cells were treated with CDE (1 and 100 μg/ml) for 6, 12 and 24 h. (B) HSC-T6 cells were treated with CDE (20, 40, 60, and 80 μg/ml) for 24 h. (C) Inhibitory effect of CDE on cell growth. L02 cells were treated with CDE (20, 40, 60, and 80 μg/ml) for 24 h. MTT assays were performed to assess cell viability. (D) HSC-T6 and L02 cell lysates were tested for α-SMA and Tubulin by Western blot. Data are given in the form of mean ± SEM of three experiments and each experiment includes triplicate wells. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* medium treatment group.

stronger inhibition of cell viability in activated HSC cell line HSC-T6 than that in normal hepatocyte L02 cells (Fig. 2), which suggest CDE may be low toxic in the treatment of liver fibrosis. Furthermore, CDE induced apoptosis of HSC-T6 cells associated with increased expressions of cleaved PARP and cleaved caspase-3 (Fig. 3). On the other hand, STAT1 signaling pathway is known to be involved in growth inhibition, induction of apoptosis and inhibition of HSC activation (Thomas et al., 2004; Jeong et al., 2006). Clinical data have shown that IFN-γ, the STAT1 signaling stimulator, improves liver fibrosis (Weng et al., 2005). However, IFN-γ is a proinflammatory cytokine, which may promote the process of liver fibrosis via inflammation. Since STAT1 has higher selective function in

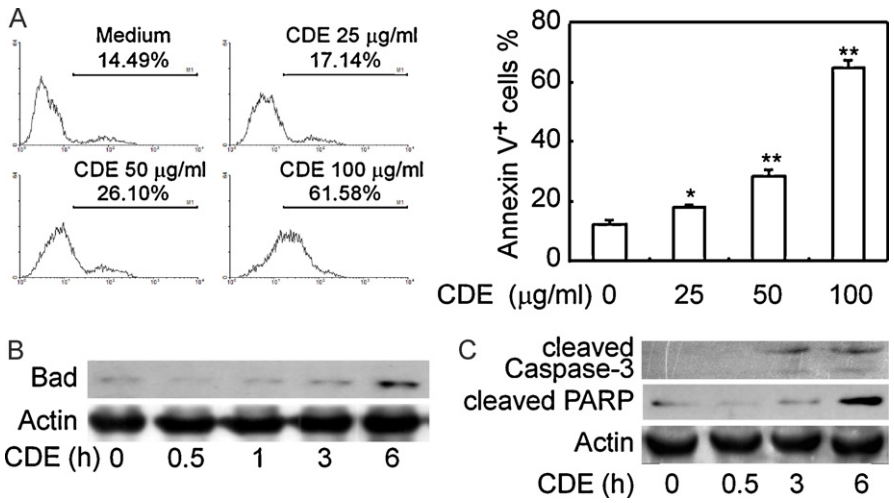

Fig. 3. Effect of CDE on apoptosis in HSC-T6 cells. (A) After treatment with different concentrations of CDE for 6 h, HSC-T6 cells were stained with Annexin V to determine apoptosis by flow cytometry. (B and C) After treatment of HSC-T6 cells with 50 μg/ml of CDE for indicated times, the total cell lysates were tested for Bad (B), cleaved Caspase-3 and cleaved PARP (C) by Western blot. Data are given in the form of mean ± SEM of three experiments and each experiment includes triplicate wells. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* medium treatment group. Representative data from three individual experiments are depicted here.

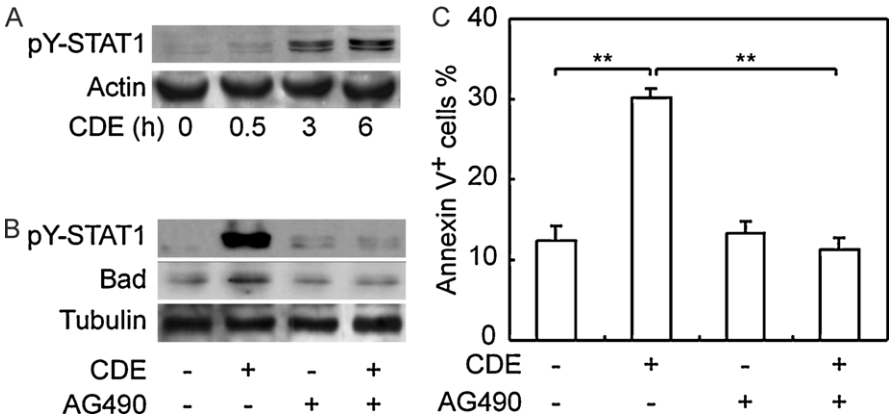

Fig. 4. Effect of CDE on the expression of pY-STAT1 in HSCs. (A) After the treatment of HSC-T6 cells with 50 μg/ml of CDE for 0.5, 1, 3, and 6 h, total cell lysates were tested for indicated proteins by Western blot. (B and C) HSC-T6 cells pretreated with or without 10 μM AG490 for 2 h were treated with or without 50 μg/ml of CDE for 6 hours. Then, total protein was isolated and assayed for Western blot (B), or cells were stained with Annexin V to determine apoptosis by flow cytometry (C). Data are given in the form of mean ± SEM of three experiments and each experiment includes triplicate wells. $^{**}P < 0.01$, compared as indicated. Representative data from three individual experiments are depicted here.

regulation of fibrosis than IFN-γ, induction of STAT1 signaling may be a useful strategy for regulating the development of liver fibrosis. For this purpose, we next found that the activation of STAT1 was elevated by 50 μg/ml of CDE treatment in HSC-T6 cells (Fig. 4A). And the activation of STAT1 contributed to the apoptosis of HSC-T6 cells induced by CDE (Fig. 4B and C). These results suggest provide a possibility to activate STAT1 signaling by the CDE instead of IFN-γ. Interestingly, CDE activated STAT1 in HSC-T6 cells and the effect of CDE on apoptosis of HSC-T6 cells could be neutralized using JAK/STAT1 signaling inhibitor AG490. Our findings suggest that CDE possesses antifibrotic activity with unique property to selectively target activated HSCs. This characteristic may be distinct from current drugs and beneficial to the long term treatment of liver fibrosis with few side effects. Furthermore, we examined the effects of CDE on the expression of α-SMA and the procollagen I. The extract also inhibited the protein expression of α-SMA and the mRNA expression of procollagen I in HSC-T6 cells (data not shown), which was in concordance with the result in inducing STAT1 activation. These effects of CDE contributed to the improvement of liver fibrosis. Induction of STAT1 activation by CDE may be a new character for liver fibrosis resolution.

5. Conclusion

In conclusion, we demonstrated that CDE attenuated CCl_4-induced liver fibrosis, and its anti-fibrosis effects may be related to the induction of apoptosis in activated hepatic stellate cells through activating STAT1 without damaging normal cells. A possible clinical application due to this unique character could be expected to have benefits for chronic liver disorders accompanied by liver fibrosis.

Conflict of interest

There is no conflict interest to disclose for all authors.

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Glossary

α-*SMA:* alpha-smooth muscle actin
CDE: Cortex Dictamni extract
ECM: extracellular matrix
HSC: hepatic stellate cells
HPLC: high performance liquid chromatography
H & E: hematoxylin–eosin
IFN-γ: interferon gamma
STAT: signal transducers and activators of transcription
PARP: poly ADP-ribose polymerase