Arch Pharm Res Vol 27, No 12, 1238-1244, 2004

Archives of Pharmacal Research
http://apr.psk.or.kr

Suppressive Effects of *Platycodon grandiflorum* on the Progress of Carbon Tetrachloride-Induced Hepatic Fibrosis

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(Received October 8, 2004)

The suppressive effects of Platycodi Radix (Changkil: CK), the root of *Platycodon grandiflorum* A. DC (Campanulaceae), on the progress of acute carbon tetrachloride (CCl_4)-induced hepatic fibrosis were investigated in the rat. CK significantly suppressed CCl_4-induced hepatic necrosis and inflammation, as determined by the serum enzymatic activities of alanine and aspartate aminotransferase and serum tumor necrosis factor-α levels, in dose-dependent manners. In addition, the increased hepatic fibrosis after acute CCl_4 treatment was suppressed by the administration of CK. CK also significantly prevented the elevation of hepatic α1(I) procollagen (type I collagen) mRNA and α-smooth muscle actin (α-SMA) expressions in the liver of CCl_4-intoxicated rats and also suppressed the induction of α-SMA and type I collagen in cultured hepatic stellate cells, in dose-dependent manners. These results suggest that the suppressive effects of CK against the progress of acute CCl_4-induced hepatic fibrosis possibly involve mechanisms related to its ability to block both hepatic inflammation and the activation of hepatic stellate cells.

Key words: Platycodi Radix, Carbon tetrachloride, Hepatic fibrosis, Hepatic stellate cells

INTRODUCTION

Hepatic fibrosis is a common pathological feature of progressive chronic liver diseases, and is characterized by increased synthesis and deposition of newly formed extracellular matrix components (Maher and McGuire, 1990; Milani *et al.*, 1990; Friedman, 1993; Friedman, 2000). The increased extracellular matrices mechanically interfere with blood flow and reduce liver functions. The most important cells directly related to hepatic fibrosis are hepatic stellate cells (HSCs, referred to as Ito cells or fat-storing cells) (Friedman, 1993; Friedman, 2000). HSCs are non-parenchymal liver cells, with a characteristic stellate morphology, which reside in the perisinusoidal space of Disse. When liver injury occurs, HSCs change their phenotypes from vitamin A-storing resting cells to myofibroblast-like cells expressing α-smooth muscle actin (α-SMA), which is considered the activated state (Ramadori *et al.*, 1990). These transformed (activated) HSCs enhance the production of extracellular matrix components, especially collagens, and promote hepatic fibrosis (Brenner *et al.*, 2000). Following chemically-induced injury, the HSCs are the main component of the fibrogenic cell population (Ramadori *et al.*, 1990; Pinzani and Gentilini, 1999). Carbon tetrachloride (CCl_4) is widely used as an experimental model for the study hepatic fibrosis (Davis and Madri, 1987; Nakatsukasa *et al.*, 1990; Iredale *et al.*, 1996), with the formation of the trichloromethyl free radicals ($CCl_3^{\bullet}$ and/or $CCl_3OO^{\bullet}$) believed to be the basis for the toxic effects of CCl_4 (Castro *et al.*, 1974; Brattin *et al.*, 1985).

Recently, herbs have begun to attract attention as health-beneficial foods (physiologically functional foods) and raw materials for the development of drugs. Herbal medicines derived from plants are being increasingly utilized to treat a wide variety of clinical diseases, even though relatively little is known about their modes of action. Platycodi Radix, the root of *Platycodon grandiflorum* A. DC (Campanulaceae), is used as a traditional oriental medicine, the biological significance of which has previously been reviewed (Lee, 1973). The roots of *P. grandiflorum*

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have been reported to have wide ranging health benefits. In Korea, the root of *P. grandiflorum* (four years old) is used both as a food and as a folk remedy for adult diseases, such as bronchitis, asthma and pulmonary tuberculosis, hyperlipidemia and inflammatory diseases, as well as being taken as a sedative (Lee, 1973). In our previous studies, it was observed that Changkil (CK), the root of *P. grandiflorum* cultivated for more than twenty years (Lee, 1991, Patent on the method of cultivating the perennial balloonflower, Patent No. 045971, Korea), prevented hypercholesterolemia and hyperlipidemia (Kim *et al.*, 1995) and enhanced the functions of macrophages (Choi *et al.*, 2001). Recently, CK was shown to exhibit protective effects against acetaminophen-, carbon tetrachloride- and *tert*-butyl hydroperoxide-induced hepatotoxicity in rats (Lee *et al.*, 2001; Lee and Jeong, 2002; Lee *et al.*, 2004), and antioxidant effects in $FeCl_2$-ascorbate induced lipid peroxidation and in relation to superoxide radical scavenging activity (Lee and Jeong, 2002).

Even though CK showed hepatoprotective activity in our previous examinations, the elucidation of its effect on hepatic fibrosis remains. Therefore, the present study was undertaken to evaluate the suppressive effects of CK on the early phase of acute CCl_4-induced progress of hepatic fibrosis and elucidate the mechanism(s) underlying these protective effects in the rat and the α-SMA and type I collagen expression of rat cultured HSCs. Our results indicate that CK significantly prevented CCl_4-induced progress of hepatic fibrosis.

MATERIALS AND METHODS

Materials

CK derived from the roots of *Platycodon grandiflorum* (twenty-two years old) was prepared as described previously (Lee and Jeong, 2002). The composition of CK has been previously published (Kim *et al.*, 1995), and consists of saponin (~2.5%), inulin (~60%) and oligosaccharide (~25%). The chemicals and cell culture materials were obtained from the following sources: CCl_4, olive oil, diagnostic kits for aspartate aminotransferase (AST) and alanine aminotransferase (ALT), metrizamide and antibodies to α-SMA from Sigma Co.; antibody to type I collagen from Calbiochem Inc.; TNF-α enzyme linked immunosorbent assay (ELISA) kit from the R&D Systems Inc.; Dulbecco's modified essential medium (DMEM), fetal bovine serum (FBS) and penicillin-streptomycin solution from Life Technologies, Inc.; Western blotting detection reagents (ECL) from Amersham Pharmacia Biotech.; all other chemicals were of the highest commercial grade available.

Animals and treatment

Male Sprague-Dawley rats (220-250 g) were purchased from Dae Han Laboratory Animal Research and Co. (Daejeon, Korea). The animals were allowed free access to Purina Rodent Chow and tap water, maintained in a controlled environment at 21±2°C and 50±5% relatively humidity with a 12 h dark/light cycle and acclimatized for at least 1 week before use. CK in saline was administered intragastrically at 10-100 mg/kg once daily. Three hours after the administration of CK, the rats were injected with CCl_4 (0.5 mL/kg body weight intraperitoneally, dissolved in olive oil). Groups of control animals were given the respective vehicles. Twelve, 24 and 72 h after the CCl_4 treatment, the rats were anesthetized with CO_2, blood removed by cardiac puncture, to determine the serum ALT and AST activities and serum TNF-α levels, and the animals decapitated. After bleeding, the livers were weighed and a thin slice preserved in a buffered formalin solution to obtain histological sections. The remaining livers were frozen quickly in dry ice/methanol and stored at -70°C for α1(I) procollagen (type I collagen) mRNA analysis.

Measurement of Serum ALT and AST Activities and Serum TNF-α Levels

Hepatic necrosis was assessed by quantifying the serum activities of ALT and AST using a spectrophotometric diagnostic kit obtained from the Sigma Chemical Co. Hepatic inflammation was measured by quantifying the serum TNF-α levels using an ELISA kit obtained from the R&D Systems Inc.

Histopathology and immunohistochemical examination

Fresh liver tissues, previously trimmed to a thickness of approximately 2 mm, were placed in plastic cassettes and immersed in neutral buffered formalin for 24 h. Fixed tissues were routinely processed, and then embedded in paraffin, sectioned, deparaffinized and rehydrated using standard techniques. The extents of CCl_4-induced necrosis and fibrosis were evaluated by assessing the morphological changes in liver sections stained with hematoxylin and eosin (H&E) and Masson's trichrome, respectively, using standard techniques. α-SMA for the detection of activated hepatic stellate cells was immunchistochemically assessed by the avidin-biotin-peroxidase complex method, as previously described (Sakaida *et al.*, 1998).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis for type I collagen mRNA

Total RNA was prepared from frozen livers using the acidic phenol extraction procedure of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). cDNA synthesis, semiquantitative RT-PCR for rat αl(I) collagen (sense; 5'-CTGACTGGAAGAGCGGAGAG-3', antisense; 5'-TGA-

GTTTGGGTTGTTGGTCT-3') and glycerol aldehyde 3-phosphate dehydrogenase (GAPDH, sense; 5'-GGC-AAGTTCAATGGCACAGT-3', antisense; 5'-AAGGTG-GAGGAATGGGAGTT-3') mRNAs, and the analyses of the results, were all performed as described previously (Brandsten *et al.*, 1999). PCR reactions fragments were subjected to electrophoresis through a 2.5% agarose gel and visualized by ethidium bromide staining and UV irradiation. Prior to analysis, the PCR product band intensities were checked to ensure their saturation intensity had not been reached.

Isolation and culture of hepatic stellate cells

Hepatic stellate cells (HSCs) were isolated from male Sprague-Dawley rats by in situ collagenase perfusion and differential centrifugation, using Metrizamide density gradients, as previously described (Kristensen *et al.*, 2000). Isolated cells were plated at a density of 5×10^5 cells/mL of culture medium on uncoated plastic culture dishes. The culture medium was changed every 2 days after plating. The hepatic stellate cells were initially cultured for 5 days in DMEM supplemented with 10% fetal bovine serum and antibiotics (100 U/mL of penicillin G and 100 μg/mL of streptomycin sulfate) in humidified air containing 5% CO_2, were then growth-arrested in DMEM containing serum-free medium for 24 h. The cells were incubated either in the presence or absence of CK for an additional 2 days in serum-free medium.

Western blot analysis for α-SMA and type I collagen in HSCs

Whole cell extracts were prepared by treating the cells with lysis buffer (150 mM NaCl, 100 mM Tris, pH 8.0, 1% Tween 20, 50 mM diethyldithiocarbamate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL trypsin inhibitor and 10 μg/mL leupeptin). The lysates were sonicated on ice for 20 s and then centrifuged at 10,000×g for 10 min to throw down the particulate material. The protein concentration of the supernatant was measured by the Bradford method (Bradford, 1976). SDS-PAGE was performed under reducing conditions on 10% polyacrylamide gels. The resolved proteins were transferred onto polyvinylidene difluoride membranes. After blocking, the membranes were incubated with monoclonal antibody to α-SMA or type I collagen. The secondary antibody to IgG conjugated to horseradish peroxidase was used. The blots were probed with an ECL Western blot detection system, according to the manufacturer's instructions.

Statistical analysis

All experiments were repeated at least three times and the results reported as means±SD. ANOVA was used to evaluate the difference between multiple groups. If significance was observed between groups, a Dunnet's 't' test was used to compare the means of the two specific groups in question, with $P < 0.05$ considered significant.

RESULTS AND DISCUSSION

Hepatic fibrosis represents the main complication of most chronic liver disorders, which regardless of its etiology, is characterized by excessive deposition of extracellular matrix components (Friedman, 2000). Hepatic fibrogenesis is accompanied by hepatocellular necrosis and inflammation. HSCs are regarded as the primary target cells for inflammatory stimuli in the injured liver (George *et al.*, 1999; Li and Friedman, 1999), and activated HSCs have been identified as the primary source of excess accumulation of extracellular matrix components in liver fibrosis (Friedman, 1993, 2000).

The CCl_4-treated rat is frequently used as an experimental model for the study of hepatic fibrosis (Davis and

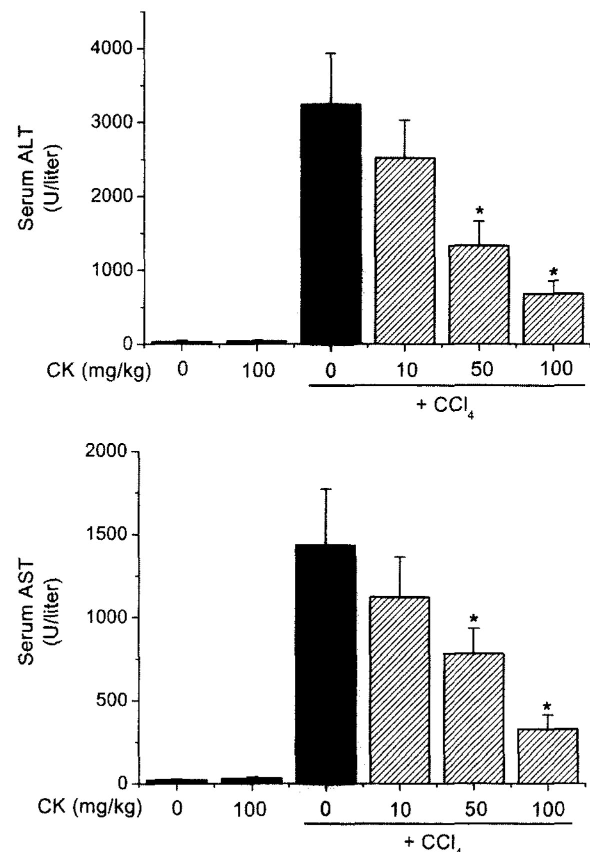

Fig. 1. Effects of CK on the CCl_4-induced serum ALT and AST activities. Rats were simultaneously treated with CCl_4 (0.5 mL/kg, i.p.) and/or CK (10-100 mg/kg, i.g.). Necrotic hepatotoxicity was determined 24 h later by quantifying the serum activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Each value represents the mean±SD of five mice. *$P < 0.05$, significantly different from the CCl_4.

Madri, 1987; Nakatsukasa *et al.*, 1990; Iredale *et al.*, 1996). The effects of CK on early phase liver necrosis and inflammation following acute CCl_4 intoxication were investigated to address if CK suppresses the early phase progress of acute CCl_4-induced hepatic fibrosis. A single dose of CCl_4 caused necrotic hepatotoxicity in rats, as indicated by the increases in ALT and AST serum levels 24 h after the administration of CCl_4. CK treatment prevented the CCl_4-induced elevation of the ALT and AST serum levels, in dose-dependent manners (Fig. 1). Treatment with CK alone resulted in no changes in the serum ALT and AST activities compared to the control. Histopathological studies showed that CCl_4, compared to the control, induced degeneration in hepatocytes and hepatic cords, and focal necrosis in the pericentral and mid-zonal areas, with infiltration of inflammatory cells. By microscopic examination, the severe hepatic lesions induced by CCl_4 were markedly reduced by CK, which was in good agreement with the results of the serum aminotransferases activities (data not shown).

The effects of CK on the changes in the inflammatory responses caused by CCl_4 were assessed by measuring the serum TNF-α levels. A single dose of CCl_4 treatment increased the serum TNF-α level at 12 h, as determined by ELISA (Fig. 2). CK significantly prevented CCl_4-induced increases in the serum TNF-α level. Taken together, these results suggest that CK reduces the necrosis and inflammatory responses following acute CCl_4 treatment. Studies have shown that expansion and activation of the HSCs population follows those of the monocyte/macrophage populations, and both cell types accumulate exclusively within areas of necrosis. It induces extracellular matrix deposition by simultaneously stimulating the synthesis of new matrix components, increasing the synthesis of enzymes that inhibit extracellular matrix degradation and decreasing the synthesis of matrix degrading proteases. Soluble factors, such as TNF-α and IL-1β, released by Kupffer cells lead to HSCs proliferation and promote the synthesis of collagen, proteoglycans and hyaluronate by these cells. Preventing the production of TNF-α and IL-1β can block the induction of collagen. Kupffer cells could be activated by toxic metabolite(s) from CCl_4, which might result in acute liver toxicity. Kupffer cell-derived stimulatory factors, such as TNF-α, are responsible for liver injury and subsequent fibrogenesis. TNF-α is the principal mediator of inflammatory responses, and is closely associated with the acute hepatotoxicity induced by immunological and chemical toxicants (Kunstle *et al.*, 1999). Lipopolysaccharide potently stimulates the release of cytokines, including TNF-α, from activated Kupffer cells (Beutler and Kruys 1995). In our previous studies, CK was observed to suppress TNF-α production by lipopolysaccharide in the Raw264.7 macrophage cell line (data not shown). In addition, in the current study, CK inhibited the increase in the serum TNF-α level following acute CCl_4 intoxication. These results raise the possibility of CK inhibiting the activation of mononuclear phagocytes (e.g. Kupffer cells) and hence the expression of TNF-α. Inhibition of TNF-α production by CK may contribute to its therapeutic efficacy against chemical hepatitis as well as hepatic fibrosis.

Hepatic fibrosis is a fundamental, clinically serious problem in various types of chronic liver diseases. To investi-

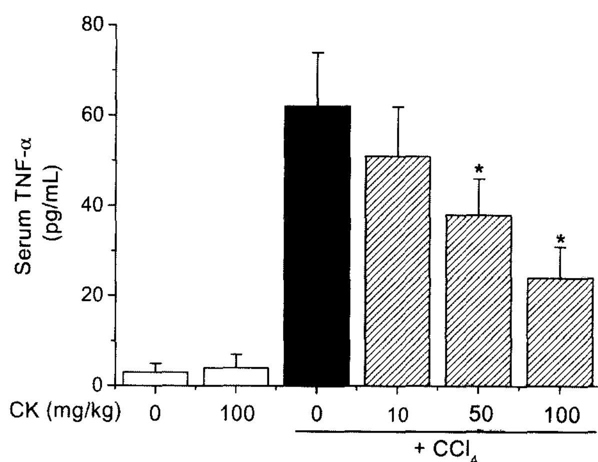

Fig. 2. Effects of CK on the CCl_4-induced serum TNF-α levels. Rats were simultaneously treated with CCl_4 (0.5 mL/kg, i.p.) and/or CK (10-100 mg/kg, i.g.). Serum TNF-α levels were determined 12 h later by ELISA. Each value represents the mean±SD of five mice. *$P<0.05$, significantly different from the CCl_4.

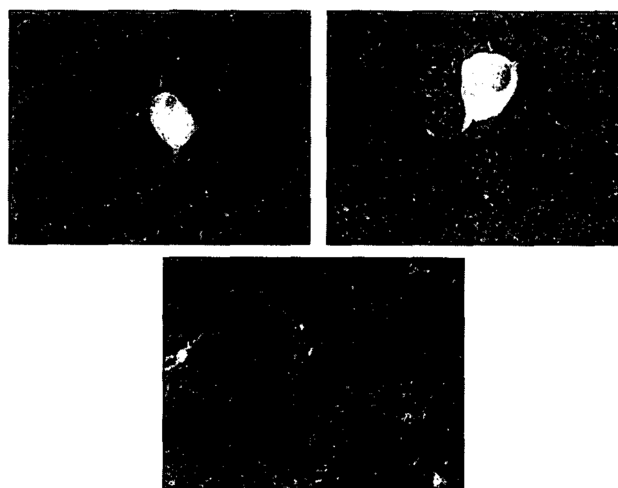

Fig. 3. Effects of CK on the CCl_4-induced collagen fiber accumulation in the liver; Masson's trichrome staining. Rats were simultaneously treated with CCl_4 (0.5 mL/kg, i.p.) and/or CK (100 mg/kg, i.g.). Subsequently, CK or vehicles alone, as controls, were treated once daily for 3 consecutive days, and liver samples obtained 24 h after the final treatment. The liver sections were stained with Masson's trichrome (200×). (A) liver from vehicles alone; (B) liver from treated with CCl_4 alone; (C) liver from mouse treated with CK plus CCl_4.

gate the suppressive effect of CK on the progress of acute CCl_4-induced hepatic fibrosis, rats were given a single treatment of CCl_4. The effects of CK on the extent of early phase hepatic fibrosis were evaluated by histopathological examination of liver sections by H&E staining following 3 days of CCl_4 treatment. In contrast to the control group (Fig. 3A), acute CCl_4 treatment caused mild pericentral fibrosis, extensive hemorrhagic necrosis and disruption of tissue architecture (Fig. 3B). Masson's trichrome staining, which was used to assess the extracellular matrix (collagen), revealed that rats treated with CCl_4 exhibited a progressive fibrogenic liver morphology. Treatment with CK significantly decreased the extent of these fibrotic changes induced by acute CCl_4 treatment (Fig. 3C). The CK-treated rat livers showed no histological alterations compared to the control rat livers (data not shown). Because activation of HSCs is a key event in the early phase of fibrogenesis in the liver, the expression of α-SMA, a typical marker of activation of HSCs, was detected by immunohistochemical staining in the acute CCl_4-treated livers after 3 days. Considerable α-SMA expression was detected in CCl_4-treated rats (Fig. 4B) compared with control rat livers (Fig. 4A). In contrast, treatment with CK markedly reduced the positive areas of α-SMA in the livers of rats treated with CCl_4 (Fig. 4C).

Histological examinations showed significant reduce in the profile of collagen fiber deposition and HSCs activation (α-SMA expression) in the extracellular matrix of the CCl_4-treated rat livers when also treated with CK (Figs. 3 and 4). Therefore, the effect of CK on type I collagen was determined by assessing the mRNA levels of αl(I) procollagen by RT-PCR. Treatment with CCl_4 increased the level of expression of αl(I) procollagen mRNA (Fig. 5), which was almost reversed when the rats were treated with CK. The amount of β-actin, which was present as an internal control, remained unchanged. These results suggest that CK suppresses type I collagen expression at the transcriptional level, and thus contributes to decreasing the early progress of fibrogenic responses in the liver following acute CCl_4 treatment.

Hepatic fibrogenesis is a fundamental, clinically serious problem in various types of chronic liver diseases. HSCs are believed to play a pivotal role in fibrogenesis in the liver (Friedman 1993; Friedman 2000). The inhibitory effects of CK on type I collagen and α-SMA (the phenotypic marker of activated HSCs) expressions in activated HSCs were investigated by measuring the protein levels using

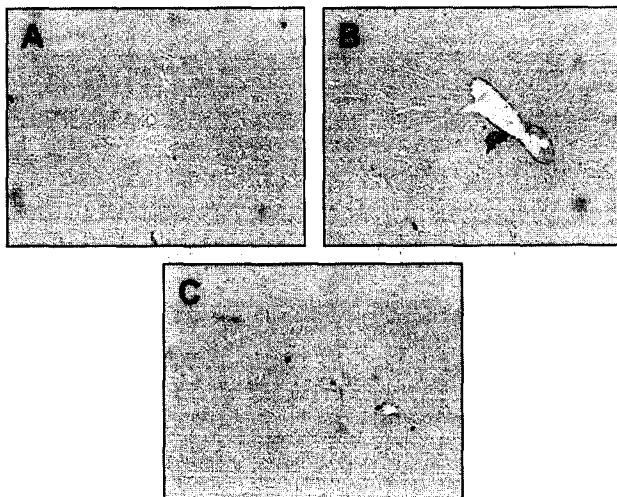

Fig. 4. Effects of CK on the CCl_4-induced α-SMA expression in the liver; Immunohistochemistry for α-SMA. Rats were simultaneously treated with CCl_4 (0.5 mL/kg, i.p.) and/or CK (100 mg/kg, i.g.). Subsequently, CK or vehicles alone, as controls, were treated once daily for 3 consecutive days, and liver samples obtained 24 h after the final treatment. The expression and localization of α-SMA in the liver was detected by immunohistochemical staining using a monoclonal antibody for α-SMA (200x). (A) liver from vehicles alone; (B) liver from treated with CCl_4 alone; (C) liver from mouse treated with CK plus CCl_4.

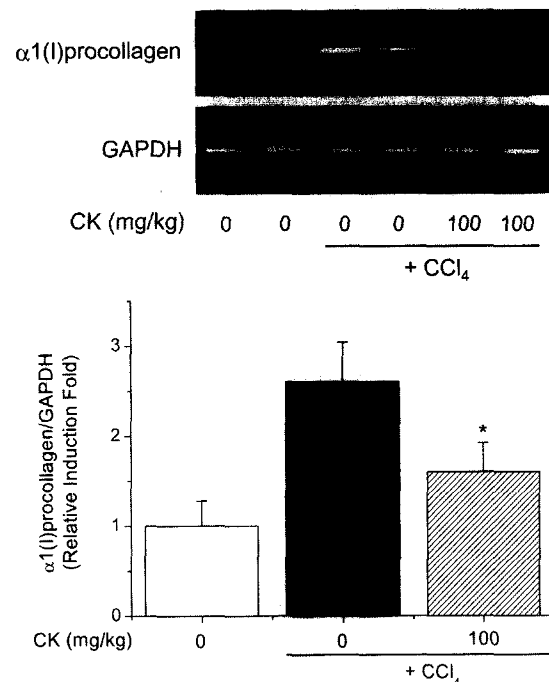

Fig. 5. Effect of CK on the CCl_4-induced expression of α1(I) procollagen mRNA in the liver. Rats were simultaneously treated with CCl_4 (0.5 mL/kg, i.p) and/or CK (100 mg/kg, i.g.). Subsequently, CK or vehicles alone, as controls, were treated once daily for 3 consecutive days, and liver samples obtained 24 h after the final treatment. Total RNA was prepared, and RT-PCR performed, as described in the Materials and Methods section. The PCR products were separated on an 1.2% agarose gel and stained with ethidium bromide. GAPDH, the housekeeping gene, was used as an internal control. One of three representative experiments is shown. The ratio of the RT-PCR products of α1(I) procollagen to GAPDH was calculated. The induction-fold represents the mean±SD of three separate experiments. $^*P < 0.05$, significantly different from the CCl_4.

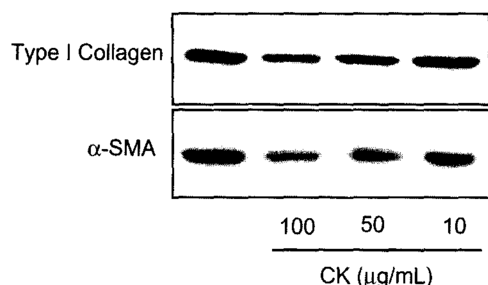

Fig. 6. Effects of CK on expressions of type I collagen and α-SMA in cultured hepatic stellate cells. Activated hepatic stellate cells were cultured with/without CK for 2 days, as described in the Materials and Methods section, and the cell lysates separated by SDS-PAGE, transferred to a nitrocellulose membrane and blotted with anti-type I collagen or α-SMA antibodies. These blots are each representative of three independent experiments.

Western blot analysis. The control HSCs showed marked expression of type I collagen in HSCs lysates (Fig. 6). In contrast, two days of CK treatment decreased the type I collagen compared with the activated HSCs. CK also suppressed the α-SMA expression, an established marker for myofibroblastic differentiation, in activated HSCs *in vitro* when evaluated by Western blot analysis (Fig. 6). This suppression of the α-SMA expression suggests CK may have blocked the synthesis of type 1 collagen, as discussed above, at least partially, by preventing the differentiation of HSCs into myofibroblasts in cultures of HSCs *in vitro*. To eliminate the possibility that CK might be toxic toward HSCs, the cell viability was assessed using the MTT assay, which showed CK did not alter the cell viability at the concentrations used in the experiment (data not shown).

In the present study, CK was demonstrated to have preventive effects against the necrosis and inflammatory responses, and subsequently suppressed the progress of early-phase acute CCl_4-induced fibrotic responses in the liver. CK significantly inhibited the increases in the serum transaminases and TNF-α levels following a single CCl_4 treatment (Fig. 1 and 2). It is therefore likely that CK inhibits the activation of Kupffer cells during CCl_4 intoxication, thereby decreasing the subsequent fibrogenic progress in the liver. In addition, CK was confirmed to suppress not only the induction of α-SMA, but also the expression of type I collagen in cultured activated HSCs (Fig. 6). During hepatic fibrosis, HSCs in the necrotic area are probably activated by locally excreted cytokines and/or conformational changes in Disse's cavity (Pinzani and Gentilini, 1999; Friedman, 2000). After activation, HSCs proliferate and transform into myofibroblasts expressing collagen and α-SMA, resulting in liver fibrosis (Knittel *et al.*, 1992; Friedman, 1993, 2000). Therefore, agents that inhibit HSCs activation, either *in vivo* or *in vitro,* might be candidates for the therapeutic prevention of chronic liver injury and/or liver fibrosis (Friedman, 1993, 2000). In the previously study, the saponins derived from CK were shown to exhibit protective effects against tert-butyl hydroperoxide-induced hepatotoxicity in rats, and to have potent antioxidant effects, such as a superoxide radical scavenging activity generated by the xanthine and xanthine oxidase system, and a reduction of the reactive oxygen species production by *t*-BHP in hepatocytes (Lee *et al.*, 2004). Since oxidative stress has recently been associated with HSCs activation (Svegliati Baroni *et al.*, 1998) and has been shown to modulate collagen gene expression (Casini *et al.*, 1991), the saponins derived from CK could prevent HSCs activation and inhibit the progress of CCl_4-induced hepatic fibrosis. However, further studies are needed in order to clarify the exact mechanism(s).

In conclusion, CK suppresses the progress of CCl_4-induced hepatic fibrosis *in vivo,* and these effects may be due to its ability to block the activation of Kupffer cells and HSCs. Recently, much attention has focused on the protective biochemical function of naturally occurring antioxidants in biological systems, and on their mechanism of action. This study; therefore, provides biological evidence supporting the use of CK for the prevention and/or treatment of liver disorders, such as hepatic fibrosis, and suggests that CK could function as a chemopreventive agent in living systems.

ACKNOWLEDGEMENTS

This work was supported by grants from the BioGreen 21 Program of Rural Development Administration, funded by Ministry of Science and Technology of the Korean government, the Plant Diversity Research Center of 21st Century Frontier Research Program (PF0320505-00), and the Research Center for Proteineous Materials by KOSEF. The authors are grateful to Jangsaeng Doraji Co., Ltd., Chinju, South Korea for providing the root of *P. grandiflorum*.

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