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Curcumin Protects the Rat Liver from CCl_4-Caused Injury and Fibrogenesis by Attenuating Oxidative Stress and Suppressing Inflammation

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ABSTRACT

We previously demonstrated that curcumin, a polyphenolic antioxidant purified from turmeric, up-regulated peroxisome proliferator-activated receptor (PPAR)-γ gene expression and stimulated its signaling, leading to the inhibition of activation of hepatic stellate cells (HSC) in vitro. The current study evaluates the in vivo role of curcumin in protecting the liver against injury and fibrogenesis caused by carbon tetrachloride (CCl_4) in rats and further explores the underlying mechanisms. We hypothesize that curcumin might protect the liver from CCl_4-caused injury and fibrogenesis by attenuating oxidative stress, suppressing inflammation, and inhibiting activation of HSC. This report demonstrates that curcumin significantly protects the liver from injury by reducing the activities of serum aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase, and by improving the histological architecture of the liver. In addition, curcumin attenuates oxidative stress by increasing the content of hepatic glutathione, leading to the reduction in the level of lipid hydroperoxide. Curcumin dramatically suppresses inflammation by reducing levels of inflammatory cytokines, including interferon-γ, tumor necrosis factor-α, and interleukin-6. Furthermore, curcumin inhibits HSC activation by elevating the level of PPARγ and reducing the abundance of platelet-derived growth factor, transforming growth factor-β, their receptors, and type I collagen. This study demonstrates that curcumin protects the rat liver from CCl_4-caused injury and fibrogenesis by suppressing hepatic inflammation, attenuating hepatic oxidative stress and inhibiting HSC activation. These results confirm and extend our prior in vitro observations and provide novel insights into the mechanisms of curcumin in the protection of the liver. Our results suggest that curcumin might be a therapeutic antifibrotic agent for the treatment of hepatic fibrosis.

Hepatic fibrosis is the wound response to chronic hepatic injury, including alcohol abuse, viral infection, and cholestasis. It is characterized by excessive production and deposition of extracellular matrix (ECM) molecules (Friedman, 2003; Bataller and Brenner, 2005). Research over the past two decades has established that hepatic stellate cells (HSC) are the primary ECM-producing cell type during hepatic fibrogenesis (Friedman, 2003; Bataller and Brenner, 2005). HSC activation, characterized by enhanced cell growth and overproduction of ECM, is triggered by the release of mitogenic platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) from activated HSC and fibrogenic transforming growth factor (TGF)-β1, mostly from Kupffer cells (Friedman, 2003; Bataller and Brenner, 2005). This process is coupled with the sequential up-expression of PDGF-β receptor (PDGF-βR) (Wong et al., 1994), type I and II receptors for TGF-β (Tβ-RI and Tβ-RII) (Friedman et al., 1994), and EGF receptor (EGFR) (Kömüves et al., 2000). In addition, HSC activation coincides with a dramatic reduction in the peroxisome proliferator-activated receptor (PPAR)-γ (Galli et al.,

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ABBREVIATIONS: ECM, extracellular matrix; HSC, hepatic stellate cell(s); PDGF, platelet-derived growth factor; TGF, transforming growth factor; PDGFR, platelet-derived growth factor receptor; Tβ-RI, type I receptor for transforming growth factor-β; Tβ-RII, type II receptor for transforming growth factor-β; EGFR, epidermal growth factor receptor; PPAR, peroxisome proliferator-activated receptor; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, and alkaline phosphatase; ELISA, enzyme-linked immunosorbent assay; IFN, interferon; TNF, tumor necrosis factor; IL, interleukin; RT, room temperature; GSH, glutathione; GSSG, oxidized glutathione; MES, 2-(*N*-morpholino)ethanesulfonic acid; LPO, lipid peroxidation; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SMA, smooth muscle actin; GCL, glutamate-cysteine ligase; cGCL, heavy catalytic subunit of glutamate-cysteine ligase (73 kDa); mGCL, light regulatory subunit of glutamate-cysteine ligase (31 kDa); IHC, immunohistochemical/immunohistochemistry.

2000; Marra et al., 2000; Miyahara et al., 2000). Inflammation is a key event in the stimulation of HSC activation and hepatic fibrosis, which are induced by oxidative stress (Lee et al., 1995; Tsukamoto et al., 1995; Greenwel et al., 2000).

We previously reported that the polyphenolic antioxidant curcumin, the yellow pigment in curry from turmeric, inhibited activation of HSC in vitro by reducing cell proliferation, inducing apoptosis and suppressing ECM gene expression (Xu et al., 2003). Further studies demonstrated that curcumin induced gene expression of PPARγ in activated HSC in vitro, leading to the activation of PPARγ signaling. The latter is a prerequisite for curcumin to inhibit HSC activation (Zheng and Chen, 2004, 2006; Zhou et al., 2007). The current study evaluates the in vivo role of curcumin in the protection of the liver from injury and fibrogenesis caused by carbon tetrachloride (CCl_4) in a rat model and further explores the underlying mechanisms. We hypothesize that curcumin might protect the liver from CCl_4-induced injury and fibrogenesis by attenuating oxidative stress, suppressing inflammation, and inhibiting activation of HSC. Results in this study support our hypothesis, extend our prior in vitro observations, and provide novel insight into the mechanisms of curcumin in the protection of the liver.

Materials and Methods

Establishment of a Rat Model with Hepatic Injury and Fibrogenesis Caused by CCl_4. The rat model was established using the method originally described by Proctor and Chatamra (1982) and since used by many others (Pérez Tamayo, 1983; Kobayashi et al., 2000; Rivera et al., 2001), with minor modifications. Thirty male Sprague-Dawley rats (250–300 g) were randomly divided into five groups (six rats/group). Group 1 was the vehicle control in which rats were not administrated CCl_4 or curcumin, but they were intraperitoneally (i.p.) injected with the vehicle olive oil. Group 2 was the curcumin control in which rats were injected with olive oil as described for group 1, without CCl_4, but they were orally given curcumin. Group 3 was the CCl_4 group in which rats were i.p. injected with CCl_4, without curcumin treatment. Group 4 was a treatment group in which rats were injected with CCl_4 and treated with curcumin at 200 mg/kg. Group 5 was another treatment group in which rats were injected with CCl_4 and treated with curcumin at 400 mg/kg. All rats were fed with chow diet and kept at 21–25°C under a 12-h dark/light cycle. Rats in groups 3, 4, and 5 were i.p. injected with a mixture of CCl_4 (0.1 ml/100 g body weight) and olive oil [1:1 (v/v)] every other day for 8 weeks. Curcumin (200 mg/kg; 400 mg/kg body weight) was suspended in sterile PBS and given once daily by gavage. The control animals in groups 1 and 2 were similarly handled, including i.p. injection with the same volume of olive oil and oral administration of the same volume of PBS with or without curcumin. Forty-eight hours after the last CCl_4 injection, rats were sacrificed after being anesthetized by i.p. pentobarbital (50 mg/kg). A small portion of the liver was removed for immunohistochemical studies by fixation with 10% formalin and subsequent embedment with paraffin. The remaining liver was cut in pieces and rapidly frozen with liquid nitrogen for extraction of total RNA, hepatic proteins.

Analyses of the Pathological Indexes for Hepatocytic Death and Hepatic Injury. Blood was collected from each rat by heart puncture when sacrificed. After coagulation, sera were collected and stored at −20°C for further analyses. Activities of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) in fresh sera (1 ml/sample) were analyzed by the Chemical Lab in the Department of Pathology, Louisiana State University Health Science Center in Shreveport.

Enzyme-Linked Immunosorbent Assays. Levels of hepatic or serum interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-6, PDGF, and TGF-β in rats were determined by using a corresponding ELISA kit purchased from BD Biosciences (San Jose, CA) according to the protocol provided by the manufacturer. In brief, microplates were coated with 100 μl/well of capture antibody, and then they were incubated overnight at 4°C. After washes, the plates were blocked with Assay Diluent (BD Biosciences) at room temperature (RT) for 1 h. One hundred microliters of a serum sample, or a liver extract in PBS supplemented with protease inhibitors, was added to each well of the plate, followed by incubation for 2 h at RT. Working Detector (100 μl; BD Biosciences) was loaded into each well, and the plate was incubated for an additional 1 h at RT before the addition of Substrate Solution (100 μl; BD Biosciences). The reaction was stopped by adding Stop Solution (50 μl; BD Biosciences). The absorbance was read at 450 nm, with reference wavelength at 570 nm using a 96-well plate spectrometer (SpectraMax 190; Molecular Devices, Sunnyvale, CA). Calculation of the concentrations of the cytokines was performed in a log-log linear regression according to the instructions in the protocol.

Determination of the Content of Hepatic Hydroxyproline. This experiment was performed using a colorimetric method described by Bergman and Loxley (1970), with modifications. In brief, three small pieces of liver tissues randomly excised from the liver of every rat in the rat model were hydrolyzed in 6 N HCl at 110°C for 24 h, and subsequently they were neutralized with NaOH. Isopropanol in citrate acetate-buffered chloramine T (Sigma-Aldrich St. Louis, MO) was added to aliquots of the hydrolysate, followed by the addition of Ehrlich reagent (Sigma). The chemical reaction occurred in dark for 25 min at 60°C. After centrifugation, absorbance of the supernatant of each sample was read at 558 nm using a 96-well plate spectrometer (SpectraMax 190). *Trans*-hydroxyproline was used as the standard for quantification. Results were expressed as micromoles of hydroxyproline per gram of hepatic tissue.

GSH Assays. Levels of hepatic glutathione (γ-glutamyl-cysteinyl-glycine; GSH) and oxidized GSH (GSSG) were determined using the enzyme immune assay kit GSH-400 (Cayman Chemical, Ann Arbor, MI), following the protocol provided by the manufacturer. In brief, small pieces of the liver tissue collected from the rat model were homogenized and sonicated in 0.5 ml of ice-cold buffer (50 mM MES, pH 7.0, containing 1 mM EDTA) and centrifuged at 10,000*g* for 15 min at 4°C. The supernatant was collected and deproteinated using the reagent in the kit. A sample (50 μl/well) and the Assay Cocktail (150 μl/well; Cayman Chemical) were mixed and incubated in dark on a plate shaker. The absorbance was measured at 404 nm after 25-min incubation. A GSSG standard was generated according the instructions. The concentration of total GSH was calculated according to the equation in the protocol.

Analyses of Lipid Peroxidation. The lipid peroxidation (LPO) assays were carried out using a Lipid Hydroperoxide Assay kit purchased from Cayman Chemical. A piece of liver tissue was collected from each rat of the rat model. Immediately after sonication in PBS, lipid hydroperoxides were extracted from the samples into chloroform using the extraction buffer provided by the manufacturer. Chromogenic reaction was carried out at RT for 5 min. The absorbance was read at 500 nm using a 96-well plate spectrometer (SpectraMax 190). 13-Hydroperoxy-octadecadienoic acid was used as the standard. Cellular lipid hydroperoxide was calculated and expressed as nanomoles per milligram of total protein.

RNA Isolation and Real-Time PCR. Total RNA was extracted from frozen liver tissues using TRI Reagent according to the protocol provided by the manufacturer (Sigma-Aldrich). Real-time PCR was performed as we described previously (Chen et al., 2002). Total RNA (1 μg) was treated with DNase I to eliminate genomic DNA contamination, followed by synthesis of the first strand using reverse transcription system (Promega, Madison, WI). Reverse transcription was carried out as follows: 42°C for 30 min, 95°C for 5 min, and 4°C for 5 min (one cycle). Real-time PCR was performed in a 25 μl of reaction

solution containing 12.5 μl of 2X iQSYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA), 300 nM primers, and cDNAs. The cycles for PCR were as follows: 95°C for 7 min, 40 cycles of 95°C for 20 s, 54°C for 30 s, and 72°C for 30 s. Melting curves were determined by heat-denaturing PCR products over a 35°C temperature gradient at 0.2°C/s from 60 to 95°C. mRNA -fold change relative to GAPDH was calculated as suggested by Schmittgen et al. (2000). GAPDH was used as an internal control. The primers were as follows: PDGF-βR, (forward) 5′-CTG CCA CAG CAT GAT GAG GAT TGA T-3′ and (reverse) 5′-GCC AGG ATG GCT GAG ATC ACC AC-3′; EGFR, (F) 5′-TGC ACC ATC GAC GTC TAC AT-3′ and (R) 5′-AAC TTT GGG CGG CTA TCA G-3′; Tβ-RI, (F) 5′-ATC CAT GAA GAC TAT CAG TTG CCT-3′ and (R) 5′-CAT TTT GAT GCC TTC CTG TTG GCT-3′; Tβ-RII, (F) 5′-TGT GCT CCT GTA ACA CAG AG-3′ and (R) 5′-GAT CTT GAC AGC CAC GGT CT-3′; CTGF, (F) 5′-TGT GTG ATG AGC CCA AGG AC-3′ and (R) 5′-AGT TGG CTC GCA TCA TAG TTG-3′; αI(I)-collagen, (F) 5′-CCT CAA GGG CTC CAA CGA G-3′ and (R) 5′-TCA ATC ACT GTC TTG CCC CA-3; fibronectin, (F) 5′-TGT CAC CCA CCA CCT TGA-3′ and (R) 5′-CTG ATT GTT CTT CAG TGC GA-3′; α-SMA, (F) 5′-CCG ACC GAA TGC AGA AGG A-3′ and (R) 5′-ACA GAG TAT TTG CGC TCC GGA-3′; PPAR-γ, 5′-ATT CTG GCC CAC CAA CTT CGG-3′ and (R) 5′-TGG AAG CCT GAT GCT TTA TCC CCA-3′; glutamate-cysteine ligase (GCL)c, (F) 5′-GTC TTC AGG TGA CAT TCC AAG C-3′ and (R) 5′-TGT TCT TCA GGG GCT CCA GTC-3′; GCLm, (F) 5′-CTG CTA AAC TGT TCA TTG TAG G-3′ and (R) 5′-CTA TGG GTT TTA CCT GTG-3′; and GAPDH, (F) 5′-GGC AAA TTC AAC GGC ACA GT-3′ and (R) 5′-AGA TGG TGA TGG GCT TCC C-3′. mRNA levels were expressed as -fold changes after normalization with GAPDH.

Western Blotting Analyses. Liver extracts were prepared from pieces of liver tissues excised from every rat in the rat model using ice-cold radioimmunoprecipitation assay lysis buffer containing 150 mM NaCl, 50 mM Tris, 0.1% SDS, 1% Nonidet P-40, and 0.5% deoxycholate supplemented with protease inhibitors. Protein concentrations were determined using the BCA Protein Assay kit according to the protocol provided by the manufacturer (Pierce Chemical, Rockford, IL). Forty micrograms of total proteins was subjected to 10% SDS-polyacrylamide gel electrophoresis. GCLc or GCLm proteins were, respectively, detected by using primary antibodies against GCLc or GCLm and secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). β-Actin was probed as an internal control. Protein bands were visualized by using chemiluminescence reagent (Amersham, Chalfont St. Giles, Buckinghamshire, UK).

Sirius Red Collagen Staining. Rat liver tissues were fixed with 10% buffered neutral formalin, and then they were embedded in paraffin. Thin sections (5 μm) were deparaffinized and stained with picro-Sirius red for 1 h at RT. After washes, sections on the slides were dehydrated in 100% ethanol and in xylene, and then they were mounted in Permount (Thermo Fisher Scientific, Waltham, MA). Representative views of liver sections are shown.

Immunofluorescence Staining. After deparaffin, thin sections (5 μm) of the liver tissues were blocked with 1% bovine serum albumin, and then they were incubated with the primary antibodies overnight at 4°C. The primary antibodies used in this study were rabbit antibodies against PDGF-βR (1:200), EGFR (1:200), Tβ-RII (1:200), Tβ-RI (1:200) (all Santa Cruz Biotechnology, Inc.), or PPAR-γ (1:50; Abcam Inc., Cambridge, MA), and mouse monoclonal antibodies against α-SMA (1:100; Sigma-Aldrich). After three washes with PBS, sections on slides were incubated with goat anti-rabbit rhodamine-conjugated secondary antibodies and donkey anti-mouse Alexa Fluor 488-conjugated secondary antibodies (Invitrogen, Carlsbad, CA) at RT for 1 h. Sections incubated with secondary antibodies alone were used as negative controls. Sections were viewed in a single plane under an MRC 1024 laser confocal microscope (Bio-Rad Laboratories). Images were captured using LaserSharp software (Bio-Rad Laboratories). Representative views of liver sections are shown.

Statistical Analyses. Differences between means were evaluated using an unpaired two-sided Student's t test ($p < 0.05$ considered as significant). Where appropriate, comparisons of multiple treatment conditions with control were analyzed by analysis of variance with the Dunnett's test for post hoc analysis.

Results

Curcumin Protected the Liver against CCl_4-Induced Injury and Suppressed Hepatic Fibrogenesis in the Rat Model. CCl_4 causes hepatic injury, including hepatocytic necrosis, steatosis, and inflammation (Pérez Tamayo, 1983). Low-dose and long-term administration of CCl_4 induces hepatic fibrogenesis, which largely imitates hepatic fibrosis in human diseases (Pérez Tamayo, 1983). A CCl_4 rat model was established as described under *Materials and Methods* to evaluate the effect of curcumin on protecting the liver from injury and fibrogenesis. The process lasted for 8 weeks. The liver rapidly recovers from CCl_4-caused injury (Johnson et al., 1992; Shi et al., 1998). Therefore, rats were scarified within 48 h after the last CCl_4 injection, which provided a better chance to evaluate the CCl_4-caused hepatic injury and to assess the effect of curcumin on protection of the liver (Johnson et al., 1992; Shi et al., 1998). The effects of curcumin on the protection of the liver from injury and fibrogenesis were initially evaluated by histological analyses. Representative views of liver sections are shown in Fig. 1. As shown in tissue sections stained with hematoxylin and eosin, compared with sections from livers in the controls (groups 1 and 2) (Fig. 1A), CCl_4 caused prominent hepatic steatosis, necrosis, and formation of regenerative nodules and fibrotic septa between the nodules (group 3). Oral administration of curcumin daily for 8 weeks significantly improved the state of steatosis with a significant reduction in the number of macro- and microvesicular steatosis, and it apparently sup-

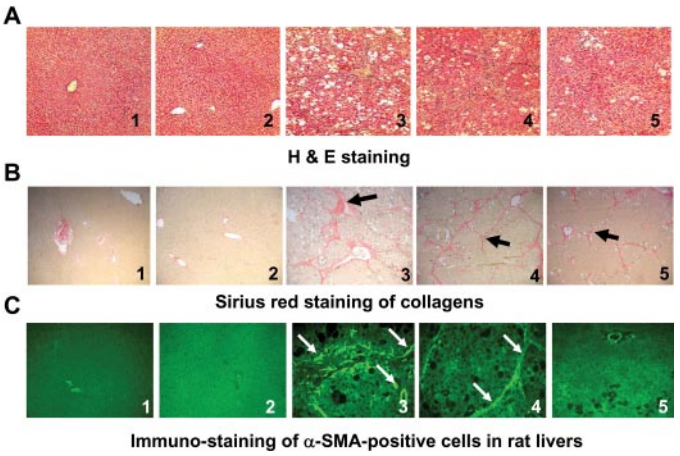

Fig. 1. Curcumin protects the liver from CCl_4-caused injury and fibrogenesis in the CCl_4 rat model. Rats were grouped: group 1, vehicle control (no CCl_4, no curcumin); group 2, curcumin control (no CCl_4); group 3, CCl_4 control (with CCl_4, no curcumin); group 4, curcumin-treated group (200 mg/kg + CCl_4); and group 5, curcumin-treated group (400 mg/kg + CCl_4). A piece of the liver tissue from each rat in the CCl_4 rat model was fixed with formalin, and then it was embedded in paraffin. Thin sections were cut and stained with hematoxylin and eosin (H&E) (A), Sirius red (B), and immunofluorescence using antibodies against α-SMA (C). Representative views from each group (n = 6/group) are presented (original magnification, 25×). The numbers in the views represent the groups of rats in the experiments. Arrows indicated collagen deposition stained by Sirius red (B) or an area positively labeled with α-SMA (C).

pressed hepatic fibrogenesis by reducing the thickness of bridging fibrotic septa (group 4 and 5, respectively).

To assess the impact of curcumin on hepatic fibrogenesis caused by CCl_4, liver sections were stained with picro-Sirius red for detecting the deposition of collagens. Compared with sections from the control groups (Fig. 1B, 1 and 2), liver sections from rats injected with CCl_4 showed prominent red staining in the fibrotic septa between nodules (Fig. 1B, 3), suggesting a high level of collagen deposition. Curcumin treatment remarkably reduced the size stained with Sirius red in the liver (Fig. 1B, 4 and 5).

α-SMA is the unique marker for activated HSC. To evaluate the effect of curcumin on HSC activation in vivo, α-SMA-positive cells were examined in liver tissues from the CCl_4 rat model using immunofluorescence staining. Compared with that in the control groups (Fig. 1C, 1 and 2), the number of the α-SMA-positive cells around the fibrotic septa was significantly increased by CCl_4 (Fig. 1C, 3), confirming that CCl_4 stimulated the activation of HSC in the rat model. The number of the α-SMA-positive cells was apparently reduced by oral administration of curcumin at both doses (200 and 400 mg/kg b.wt.) (Fig. 1C, 4 and 5), suggesting that curcumin inhibited HSC activation in the rat model.

Hydroxyproline is an amino acid found almost exclusively in collagens. Determination of the content of hydroxyproline in the liver tissue is regarded as a good method to quantify fibrosis and to evaluate the effectiveness of new potentially antifibrotic agents (Kusunose et al., 2002; Yoshiji et al., 2002). The efficacy of the treatment with curcumin on the protection of the liver from fibrogenesis was further evaluated by using a quantitative method to determine the content of hepatic hydroxyproline in the rat model. As shown in Table 1, compared with the vehicle control (group 1), the content of hepatic hydroxyproline was significantly higher in rats injected with CCl_4 (group 3). The level of hepatic hydroxyproline was significantly reduced in the rats treated with curcumin at 200 and 400 mg/kg b.wt. (group 4 and 5, respectively). However, there was no dose-dependently significant difference between the two groups (groups 4 and 5), suggesting that overdose of curcumin might be used for the treatment. These results confirmed that curcumin suppressed hepatic fibrogenesis in the rat model.

Additional biochemical analyses of serum enzymes were performed to verify the role of curcumin in the protection of the liver from injury. As shown in Table 2, compared with those in the controls (groups 1 and 2), the activities of serum alkaline phosphatase, ALT, and AST were significantly higher in rats injected with CCl_4 (group 3). The levels of serum alkaline phosphatase, ALT, and AST were significantly reduced by administration of curcumin at 200 and 400 mg/kg (group 4 and 5, respectively). However, no dose-dependent difference was observed between these two groups. Taken together, these results demonstrated that curcumin not only protected the liver against CCl_4-induced injury but also inhibited HSC activation, and it reduced hepatic fibrogenesis caused by chronic CCl_4 intoxication.

Curcumin Suppressed Inflammation in the CCl_4 Rat Model. Inflammation is commonly associated with hepatic fibrogenesis during chronic liver diseases (Marra, 2002). To begin to explore the mechanisms underlying the protective effects of curcumin, we proposed that curcumin might protect the liver against CCl_4-induced injury by suppressing inflammation in the liver. A piece of liver tissue and a blood sample were collected from every rat in each group. The levels of the proinflammatory cytokines TNF-α, IFN-γ, and IL-6 were determined in the liver and in serum. As shown in Fig. 2, compared with those in the control groups 1 and 2, the levels of the three proinflammatory cytokines in group 3 were significantly elevated by CCl_4 in both the liver and serum. Curcumin treatment significantly reduced the levels of TNF-α, IFN-γ, and IL-6 in groups 4 and 5. However, there was no dose-dependently significant difference between these two groups. These results indicated that curcumin suppressed inflammation caused by CCl_4, which might lead to the protection of the liver from injury.

Curcumin Reduced the Levels of PDGF and TGF-β in the CCl_4 Rat Model. PDGF is the most potent growth factor in stimulating HSC proliferation (Bachem et al., 1989a; Pinzani et al., 1989), whereas TGF-β is the major profibrogenic factor during hepatic fibrogenesis (Gressner et al., 2002). To elucidate the underlying mechanisms of curcumin in the suppression of hepatic fibrogenesis in the rat model, it was plausible to examine the effect of curcumin on the production of PDGF and TGF-β in the rat model. A piece of liver tissue and a blood sample were collected from every rat in each group, and the levels of PDGF and TGF-β in the liver and in serum were analyzed by ELISA. As shown in Fig. 3, compared with those in the vehicle control (group 1), the levels of PDGF and TGF-β in the liver (Fig. 3A) and in the serum (Fig. 3B) were dramatically increased in group 3 injected with CCl_4. The levels of PDGF and TGF-β in the liver (Fig. 3A) and in the serum (Fig. 3B) were significantly reduced by curcumin treatment at both 200 and 400 mg/kg (groups 4 and 5, respectively). Collectively, these data indicated that curcumin significantly reduced the levels of PDGF and TGF-β in the rat model, which might result in the inhibition of HSC activation stimulated by CCl_4.

Curcumin Abrogated the Effect of CCl_4 on Altering the Steady-State mRNA Levels of Genes Relevant to HSC Activation in the Liver in the CCl_4 Rat Model. As indicated, HSC activation is coupled with the sequential up-expression of PDGF-βR, EGFR, Tβ-RI, and Tβ-RII. In addition, HSC activation coincides with a dramatic reduction in PPARγ. We previously reported that curcumin inhibited HSC activation in vitro demonstrated by suppressing expression of genes relevant to cell growth and fibrogenesis (Xu et al., 2003; Zheng and Chen, 2004). In addition, we observed that curcumin induced expression of endogenous PPARγ

TABLE 1
Determination of the content of hepatic hydroxyproline in the rat model
Values are expressed as mean ± S.D. (n = 6).

	Group 1	Group 2	Group 3	Group 4	Group 5
Hydroxyproline (μmol/g)	3.51 ± 0.80	3.11 ± 0.41	5.91 ± 1.21*	4.12 ± 0.64‡	3.98 ± 0.93‡

* $p > 0.05$ vs. vehicle group 1.
‡ $p > 0.05$ vs. rat group 3.

gene and stimulated PPARγ activity (Xu et al., 2003), which was required for curcumin to inhibited HSC activation in vitro (Xu et al., 2003; Zheng and Chen, 2004). To verify these effects of curcumin on the regulation of expression of genes relevant to HSC activation in the rat model, total RNA was extracted from the liver of each rat in the rat model. The steady-state levels of mRNA of genes relevant to HSC activation in the liver were evaluated using real-time PCR. As shown in Fig. 4A, compared with that in the vehicle control (group 1), the mRNA level of PPARγ was markedly reduced by 68% in the rats administered with CCl_4 (group 3). The level of PPARγ mRNA was significantly elevated by simultaneous treatment with curcumin by approximately 480% in group 4. There was no significant difference between groups treated with curcumin at 200 and 400 mg/kg (group 4 and 5, respectively). It was of interest to observe that curcumin alone increased the mRNA level of PPARγ as well (group 2). In great contrast, the mRNA levels of α-SMA, the marker of activated HSC, and αI(I) collagen were significantly increased by approximately 3.4- and 5.3-fold, respectively, in the CCl_4 group (Fig. 4B, 3). Curcumin treatment apparently abrogated the stimulatory effect of CCl_4 and reduced the abundance of mRNA of α-SMA and αI(I) collagen by approximately 48 and 47%, respectively (Fig. 4B, 4). These results suggested that curcumin might inhibit HSC activation and suppress fibrogenesis in the rat model. Additional experi-

TABLE 2

Quantitation of ALP, ALT, and AST in the sera from rats in the model with CCl_4-caused hepatic injury

Blood was collected from rats in each group in the rat model through heart puncture when sacrificed. After coagulation, the activities of serum AST, ALT, and ALP were analyzed. Values are expressed as mean ± S.D. (n = 6).

Group	ALP	ALT	AST
		U/l	
1. Vehicle control	108.33 ± 37.59	45 ± 7.54	137.67 ± 50.9
2. Curcumin control	82.83 ± 41.85	35.67 ± 11.04	96.5 ± 30.41
3. CCl_4 control	370.17 ± 11.71*	802.17 ± 13.21*	1771.13 ± 96.08*
4. Curcumin 200 mg/kg	263.5 ± 15.11‡	511.83 ± 18.63‡	1046 ± 20.53‡
5. Curcumin 400 mg/kg	249.33 ± 14.85‡	482.17 ± 48.42‡	1097.83 ± 94.62‡

* $p < 0.05$ vs. the level in group 1 or 2.
‡ $p < 0.05$ vs. the level in group 3.

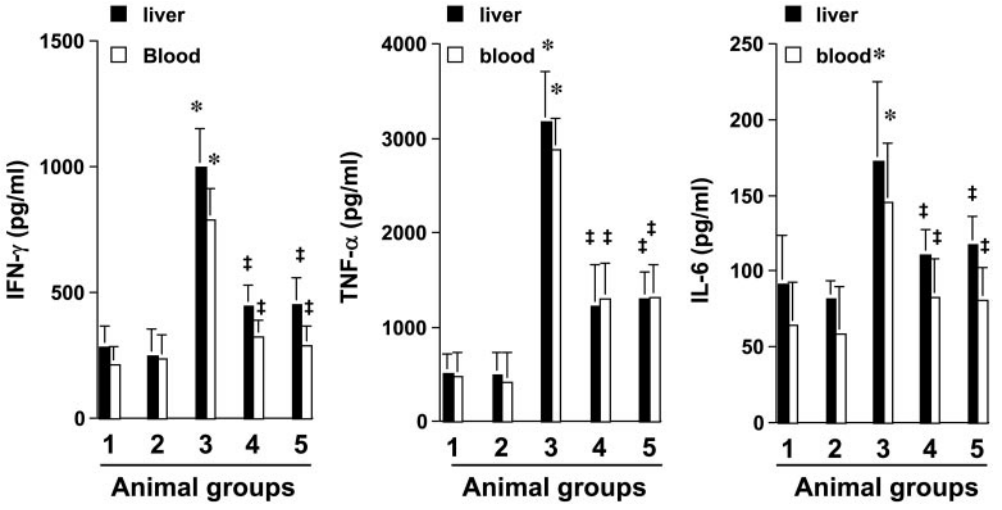

Fig. 2. Curcumin suppresses inflammation in the CCl_4 rat model. Rats were grouped: group 1, vehicle control (no CCl_4, no curcumin); group 2, curcumin control (no CCl_4); group 3, CCl_4 control (with CCl_4, no curcumin); group 4, curcumin-treated group (200 mg/kg + CCl_4); and group 5, curcumin-treated group (400 mg/kg + CCl_4). A piece of liver tissue or blood was collected from each rat in the rat model. Levels of IFN-γ (A), TNF-α (B), and IL-6 (C) in the liver and in serum were determined by ELISA as described under *Materials and Methods*. Values are expressed as mean ± S.D. (n = 6/group). *, $p < 0.05$ versus the level in group 1 or 2 (first or second column on the left side); ‡, $p < 0.05$ versus the level in group 3 (third column).

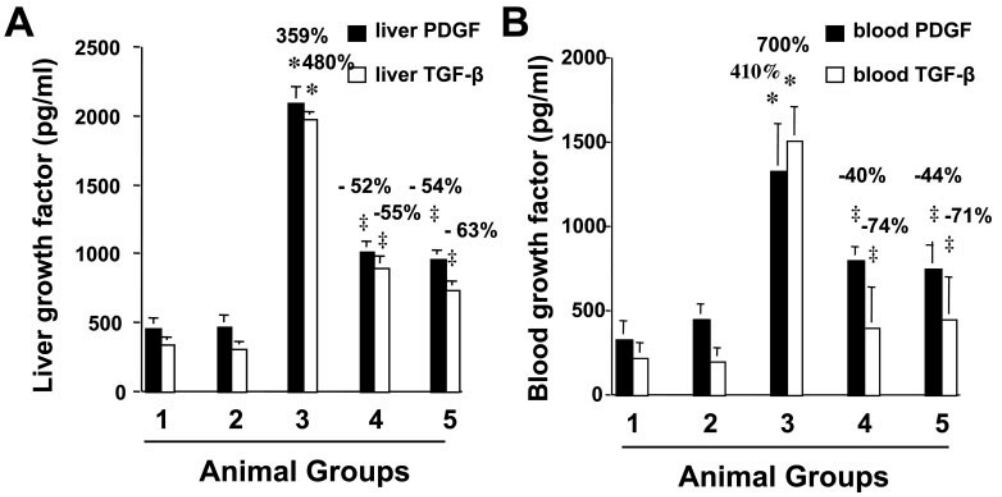

Fig. 3. Curcumin reduces the levels of PDGF and TGF-β in the CCl_4 rat model. Rats were grouped: group 1, vehicle control (no CCl_4, no curcumin); group 2, curcumin control (no CCl_4); group 3, CCl_4 control (with CCl_4, no curcumin); group 4, curcumin-treated group (200 mg/kg + CCl_4); and group 5, curcumin-treated group (400 mg/kg + CCl_4). Levels of PDGF-BB and TGF-β in the liver (A) or in serum (B) were determined by ELISA as described under *Materials and Methods*. Values are expressed as mean ± S.D. (n = 6/group). *, $p < 0.05$ versus the level in group 1 or 2 (first or second column on the left side); ‡, $p < 0.05$ versus the level in group 3 (third column).

ments demonstrated that, compared with the controls (group 1 and 2), CCl_4 significantly induced expression of genes relevant to cell proliferation, including PDGF-βR and EGFR (Fig. 4A, 3), and genes relevant to fibrogenesis, including Tβ-RI and Tβ-RII and fibronectin (Fig. 4B, 3). Curcumin markedly eliminated the role of CCl_4 in the rat model by reducing the mRNA levels of these genes (Fig. 4, A and B, 4 and 5). Curcumin showed no dose-dependent effect on regulation of expression of the genes, suggesting that over-dose of curcumin might be used for the treatment. Taken together, these results indicated that curcumin abrogated the effect of CCl_4 on altering the steady-state levels of mRNA of genes relevant to HSC activation in the liver in the CCl_4 rat model.

Curcumin Eliminated the Impact of CCl_4 on Regulating the Abundance of Proteins Relevant to HSC Activation in the Liver in the CCl_4 Rat Model. Immunohistochemical (IHC) experiments were performed to further evaluate the impact of curcumin on regulating the expression of genes relevant to HSC growth. Liver sections from each group were doubly immunolabeled with antibodies against PDGF-βR and against α-SMA, the marker of activated HSC. As shown in Fig. 5A, as expected, few cells in the liver sections from the normal control groups were recognized by antibodies against PDGF-βR or against α-SMA (Fig. 5A, 1 and 2), suggesting few activated HSC in the normal livers in the control rats. Administration of CCl_4 caused a significant increase in the number of cells recognized by the antibodies against PDGF-βR (top view with red fluorescence in Fig. 5A, 3) or α-SMA (middle view with green fluorescence in Fig. 5A, 3). Merging the images demonstrated that these labeled cells by the two different antibodies were the same cells (bottom view in Fig. 5A, 3), suggesting the colocalization of PDGF-βR and α-SMA in these cells (i.e., activated HSC). Curcumin treatment significantly reduced the number of cells labeled with PDGF-βR and α-SMA antibodies (Fig. 5A, 4 and 5), suggesting that curcumin might suppress HSC activation by inhibiting cell growth of HSC in the rat model. The similar pattern was observed when the liver sections from the rat model were doubly labeled with antibodies against EGFR, another important mitogenic protein relevant to cell proliferation, and with antibodies against α-SMA (Fig. 5B).

Additional IHC was performed to verify the effect of curcumin on regulating expression of genes relevant to fibrogenesis, including Tβ-RI and Tβ-RII. As shown in group 1 and 2 in Fig. 6, the expression of Tβ-RI and Tβ-RII is hardly detectable in the normal rat liver. Curcumin has no apparent impact on the abundance of Tβ-RI and Tβ-RII (Fig. 6, 2). It is noteworthy that administration of CCl_4 significantly increased the number of cells recognized by the antibodies against Tβ-RI or Tβ-RII (the top views with red fluorescence in Fig. 6A, 3 or in Fig. 6B, 3, respectively) or α-SMA (middle views with green fluorescence in Fig. 6, A and B, 3). Curcumin significantly reduced the number of the positively labeled cells (Fig. 6, 4 and 5). These results collectively confirmed the prior observations in Fig. 4, and they indicated that curcumin suppressed expression of genes relevant to fibrogenesis, leading to the inhibition of HSC activation in the rat model.

Prior studies have demonstrated that the elevation in the expression of α-SMA, the marker of activated HSC, is coupled with the reduction in the expression of PPARγ in activated HSC (Galli et al., 2000; Marra et al., 2000; Miyahara et al., 2000; Xu et al., 2003). To verify our prior in vitro observation that curcumin induced gene expression of PPARγ in HSC, IHC of the liver sections was conducted using antibodies against PPARγ and α-SMA. As shown in Fig. 7, there were numbers of PPARγ positively stained cells, which were negatively labeled with α-SMA antibodies, in liver sections from groups 1 and 2. Along with the increase in the number of α-SMA-positively labeled cells in the liver from rats injected with CCl_4 (group 3), the number of cells labeled with PPARγ was remarkably reduced. Simultaneous treatment with curcumin apparently eliminated these roles of CCl_4 in the alteration of the number of PPARγ-positive cells and α-SMA-positive cells in the liver (groups 4 and 5). Taken together, these results demonstrated that curcumin inhibited

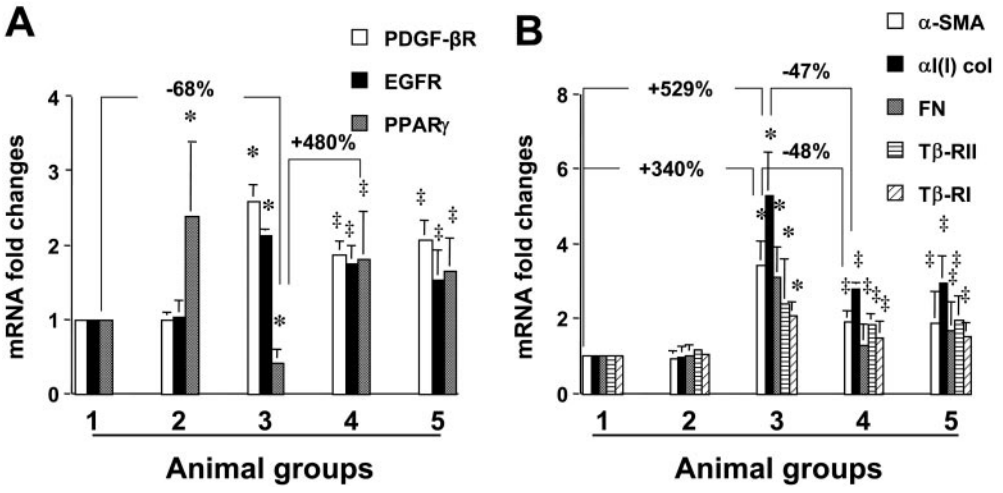

Fig. 4. Curcumin modulates the expression of genes related to HSC growth and fibrogenesis. Total RNA was prepared from liver tissues from each group of rats: group 1, vehicle control (no CCl_4, no curcumin); group 2, curcumin control (no CCl_4); group 3, CCl_4 control (with CCl_4, no curcumin); group 4, curcumin-treated group (200 mg/kg + CCl_4); and group 5, curcumin-treated group (400 mg/kg + CCl_4). The steady-state levels of mRNA in the liver were analyzed by real-time PCR assays. A, mRNA levels of genes relevant to cell growth, including PDGF-βR, EGFR, and PPARγ. B, mRNA levels of genes relevant to fibrogenesis, including α-SMA, αI(I) collagen, fibronectin (FN), Tβ-RII, and Tβ-RI. Values are presented as means $\pm$ S.D. ($n = 6$). GAPDH was used as an invariant internal control for calculating mRNA -fold changes. *, $p < 0.05$ versus the level in group 1 or 2 (first or second columns on the left side). ‡, $p < 0.05$ versus the level in group 3 (third columns).

expression of genes relevant to HSC activation and diminished the role of CCl_4 in the suppression of expression of PPARγ gene in HSC, which verified our prior in vitro observations.

Curcumin Significantly Attenuated Oxidative Stress in the Liver Caused by CCl_4. Oxidative stress resulting from the metabolism of CCl_4 in the liver plays a critical role in damaging the liver and promoting hepatic fibrogenesis. GSH is the predominant low-molecular-weight thiol and the most important nonenzyme antioxidant in mammalian cells (Wu et al., 2004). GSH effectively protects cells against oxidative stress-caused damage by scavenging free radicals, removing hydrogen peroxide (H_2O_2), and suppressing lipid peroxidation (Blair, 2006). We recently demonstrated that curcumin inhibited HSC activation in vitro through attenuating oxidative stress via de novo synthesis of glutathione (Zheng and Chen, 2007). To evaluate the impact of curcumin on attenuating oxidative stress in vivo, the level of hepatic GSH and the ratio of reduced GSH (i.e., GSH) versus GSSG were determined. The GSH/GSSG ratio is often regarded as a sensitive indicator of oxidant stress (Fridovich, 1978; Jones, 2002). As shown in Fig. 8, A and B, compared with that in the vehicle control (group 1), the level of total GSH and the ratio of GSH/GSSG were significantly reduced by CCl_4 by 59 and 42%, respectively (group 3). Oral administration of curcumin apparently eliminated the inhibitory effect of CCl_4 by increasing the level of total hepatic GSH by 88 and 75%, respectively (Fig. 8A, 4 and 5) and by elevating the ratio of hepatic GSH/GSSG by 52 and 61%, respectively (Fig. 8B, 4 and 5). Furthermore, we conducted additional experiments to verify the effect of curcumin on the attenuation of oxidative stress in the liver of the rat model. As shown in Fig. 8C, CCl_4 increased the level of lipid hydroperoxide, a major product of LPO, by 62% (Fig. 8C, 3) compared with that in the normal control (group 1). Oral administration of curcumin significantly diminished the CCl_4-elevated level of lipid hydroperoxide in the rat model by 40 and 42%, respectively (Fig. 8C, 4 and 5). These data collectively suggested that curcumin might protect the liver against CCl_4-caused injury by attenuating oxidative stress.

Curcumin Apparently Eliminated the Inhibitory Effect of CCl_4 on the GCL Activity by Inducing Gene Expression of GCL in the Rat Model. Additional experiments were conducted to elucidate the underlying mechanisms of curcumin in the elevation of the level of hepatic GSH

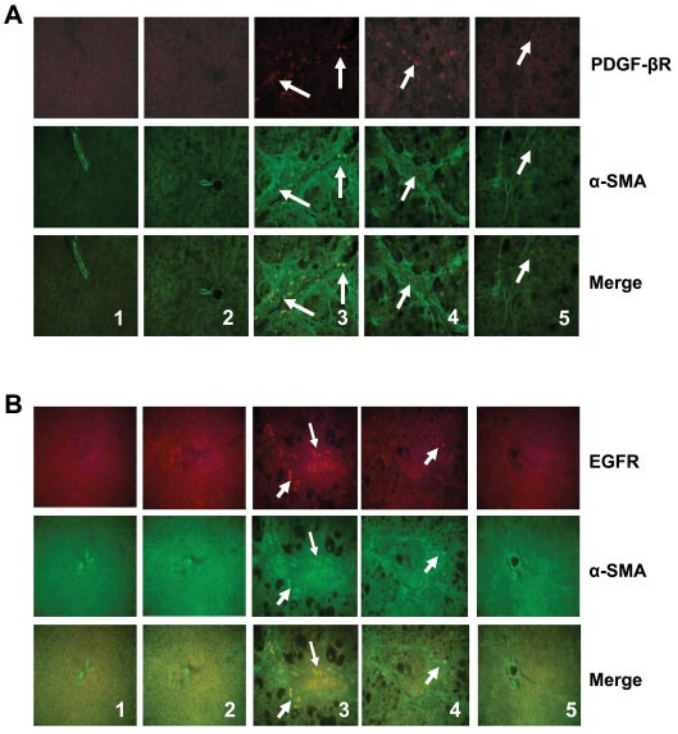

Fig. 5. Curcumin reduces the number of activated HSC doubly labeled with antibodies against PDGF-βR, or EGFR, and α-SMA in the CCl_4 rat model. Rats were grouped: group 1, vehicle control (no CCl_4, no curcumin); group 2, curcumin control (no CCl_4); group 3, CCl_4 control (with CCl_4, no curcumin); group 4, curcumin-treated group (200 mg/kg + CCl_4); and group 5, curcumin-treated group (400 mg/kg + CCl_4). A piece of the liver tissue from each rat in the CCl_4 rat model was fixed with formalin and embedded in paraffin. Thin sections were doubly stained with primary antibodies against PDGF-βR (A) or EGFR (B), plus antibodies against α-SMA and subsequently with goat anti-rabbit rhodamine-conjugated secondary antibodies and donkey anti-mouse Alexa Fluor 488-conjugated secondary antibodies. Sections were viewed under an MRC 1024 laser confocal microscope to visualize PDGF-βR-positive cells (top views with red fluorescence in A), EGFR-positive cells (top views with red fluorescence in B), and α-SMA-positive cells (middle views with green fluorescence in A and B). The merged images are presented in the bottom views in A and B. Representative fluorescent images from each group (n = 6) of the rat model are presented. Arrows indicated examples of positively stained cells (original magnification, 25×).

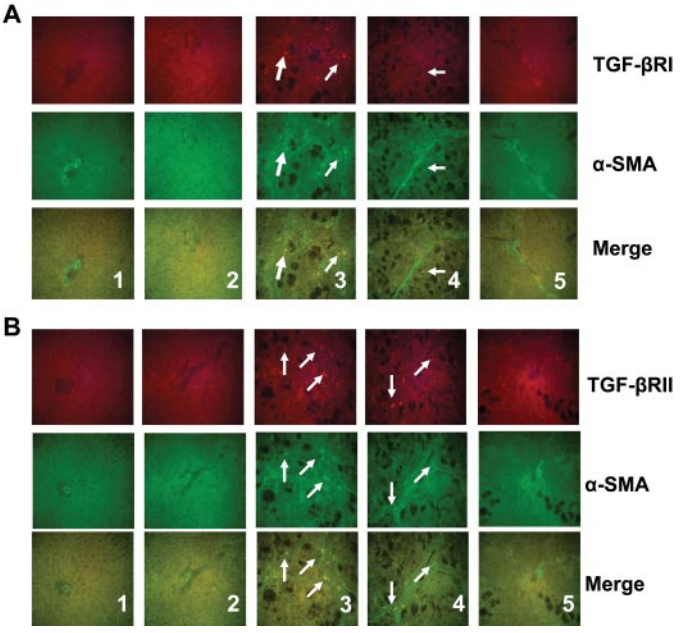

Fig. 6. Curcumin reduces the number of activated HSC doubly labeled with antibodies against Tβ-RI or Tβ-RII and α-SMA in the CCl_4 rat model. Rats were grouped: group 1, vehicle control (no CCl_4, no curcumin); group 2, curcumin control (no CCl_4); group 3, CCl_4 control (with CCl_4, no curcumin); group 4, the curcumin-treated group (200 mg/kg + CCl_4); and group 5, curcumin-treated group (400 mg/kg + CCl_4). A piece of the liver from each group in the CCl_4 rat model was fixed with formalin, and then it was embedded in paraffin. Thin sections were doubly stained with primary antibodies against Tβ-RI (A) or Tβ-RII (B) plus antibodies against α-SMA and subsequently with goat anti-rabbit rhodamine-conjugated secondary antibodies and donkey anti-mouse Alexa Fluor 488-conjugated secondary antibodies. Sections were viewed under an MRC 1024 laser confocal microscope to visualize Tβ-RI-positive cells (top views with red fluorescence in A), Tβ-RII-positive cells (top views with red fluorescence in B), and α-SMA-positive cells (middle views with green fluorescence in A and B). The merged images are presented in the bottom views in A and B. Representative fluorescent images from each group (n = 6) of the rat model are presented. Arrows indicated examples of positively stained cells (original magnification, 25×).

and the attenuation of oxidative stress. GSH is sequentially synthesized from glutamate, cysteine, and glycine, which is mainly controlled by the rate-limiting enzyme GCL. GCL is composed of two subunits, the heavy catalytic subunit GCLc (73 kDa) and the light regulatory subunit GCLm (31 kDa). We postulated that oral administration of curcumin might eliminate the inhibitory effect of CCl_4 on the GCL activity in the rat model by inducing expression of GCL subunit genes, leading to the elevation of the GSH content and the attenuation of oxidative stress. To test the postulation, the GCL activity was analyzed in liver tissues collected from rats in each group. As shown in Fig. 9A, CCl_4 significantly reduced the GCL activity in the rat model (group 3). Oral administration of curcumin apparently eliminated the inhibitory effect of curcumin on the activity of GCL (Fig. 9A, 4 and 5). Further experiments revealed that curcumin significantly enhanced the levels of the transcript and protein of GCL subunits GCLc and GCLm in the rat model, demonstrated by real-time PCR (Fig. 9B, 4 and 5) and Western blotting analyses (Fig. 9C, 4 and 5). These results collectively demonstrated that curcumin apparently eliminated the inhibitory effect of CCl_4 on the GCL activity by inducing gene expression of GCL in the rat model.

Discussion

Hepatic fibrosis caused by CCl_4 has been extensively used in experimental models in rats. Hepatic responses in rats to chronic CCl_4 stimulation are shown to be superficially similar to human cirrhosis (Pérez Tamayo, 1983). CCl_4 metabolism in the liver results in the stimulation of lipid peroxidation and the production of free radicals (Basu, 2003), which causes necrosis of hepatocytes, induces inflammation, and further promotes progression of hepatic fibrogenesis. The number of six rats in each group was chosen as the sample size in this report. A difference within two groups with a p value of less than 5% was considered to be significant (positive). This probability was computed under the assumption that the treatment difference or strength of association equaled the minimal detectable difference. In the present study, we demonstrated that curcumin significantly reduced the pathological indexes for hepatocytic death and hepatic injury caused by CCl_4 in the rat model. In addition, curcumin attenuated hepatic oxidative stress and dramatically suppressed inflammation initiated by CCl_4. Furthermore, curcumin apparently reduced the deposition of collagens and altered the expression pattern of genes relevant to HSC activation in this rat model. Taken together, our results demonstrated that curcumin protected the rat liver from CCl_4-caused injury and fibrogenesis by suppressing hepatic inflammation, attenuating hepatic oxidative stress, and inhibiting HSC activation. However, it is noteworthy that,

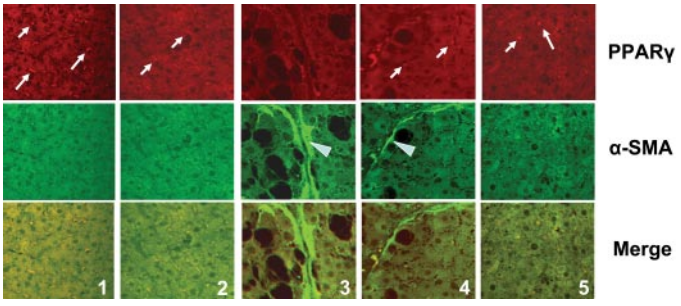

Fig. 7. Curcumin eliminates the role of CCl_4 in the reduction of the number of PPARγ-positive cells in the CCl_4 rat model. Rats were grouped: group 1, vehicle control (no CCl_4, no curcumin); group 2, curcumin control (no CCl_4); group 3, CCl_4 control (with CCl_4, no curcumin); group 4, curcumin-treated group (200 mg/kg + CCl_4); and group 5, curcumin-treated group (400 mg/kg + CCl_4). A piece of the liver from each rat in the CCl_4 rat model was fixed with formalin, and then it was embedded in paraffin. Thin sections were doubly stained with primary antibodies against PPARγ and antibodies against α-SMA and then subsequently stained with goat anti-rabbit rhodamine-conjugated secondary antibodies and donkey anti-mouse Alexa Fluor 488-conjugated secondary antibodies. Sections were viewed under an MRC 1024 laser confocal microscope to visualize PPARγ-positive cells (top views with red fluorescence) and α-SMA-positive cells (middle views with green fluorescence) in the liver tissues. The merged images are presented in the bottom views. Representative fluorescent images from each group (n = 6) of the rat model are presented. Arrows indicated examples of positively stained cells (original magnification, 60×).

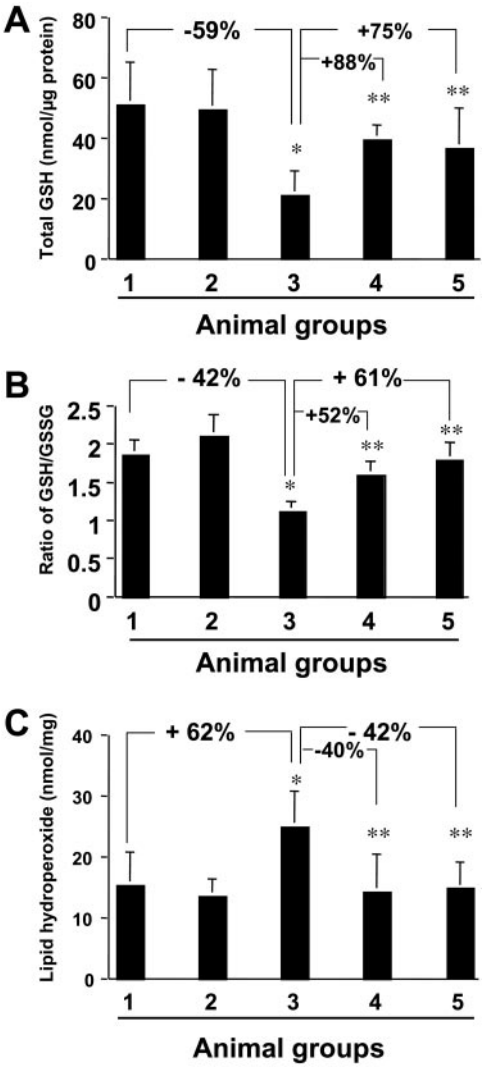

Fig. 8. Curcumin increases the levels of hepatic GSH and reduces the production of hepatic lipid hydroperoxide in the CCl_4 rat model. A piece of the liver tissue was collected from each rat in all of the groups: group 1, vehicle control (no CCl_4, no curcumin); group 2, curcumin control (no CCl_4); group 3, CCl_4 control (with CCl_4, no curcumin); group 4, curcumin-treated group (200 mg/kg + CCl_4); and group 5, curcumin-treated group (400 mg/kg + CCl_4). Levels of hepatic GSH (A), the ratio of GSH/GSSG (B), and lipid hydroperoxide (C) were determined as described under *Materials and Methods*. Values are expressed as mean ± S.D. (n = 6). *, $p < 0.05$ versus the level in group 1 (first column on the left side); **, $p < 0.05$ versus the level in group 3 (third column).

except for some of our histological experiments, most of our biochemical and molecular assays indicated no significant difference between administration of curcumin at 200 and 400 mg/kg in the protection of the liver from injury, the attenuation of oxidative stress, the suppression of inflammation, and the regulation of the gene expression. We assumed that overdose of curcumin might be used in our experiments compared with curcumin at 100 mg/kg in some other reports (Sreepriya and Bali, 2006). An even lower dose of curcumin at 40 mg/kg seemed to be effective in the treatment of mice with cystic fibrosis (Egan et al., 2004). Additional experiments are ongoing using lower doses of curcumin in the rat model.

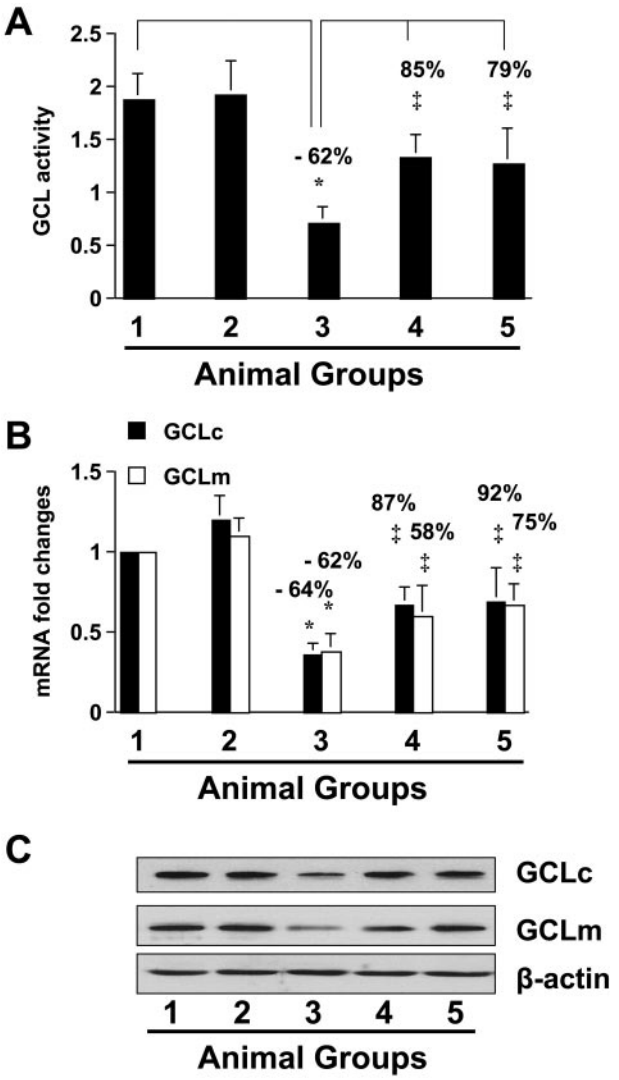

Fig. 9. Curcumin eliminates the inhibitory effect of CCl_4 on the GCL activity by inducing gene expression of GCL in the rat model. A piece of the liver tissue was collected from each rat in all of the groups: group 1, vehicle control (no CCl_4, no curcumin); group 2, curcumin control (no CCl_4); group 3, CCl_4 control (with CCl_4, no curcumin); group 4, curcumin-treated group (200 mg/kg + CCl_4); and group 5, curcumin-treated group (400 mg/kg + CCl_4). A, determination of hepatic GCL activity. Values are presented as means ± S.D. ($n = 6$). *, $p < 0.05$ versus the activity in group 1. ‡, $p < 0.05$ versus the activity in group 3. B, real-time PCR analyses of the mRNA levels of GCLc and GCLm. Values are presented as means ± S.D. ($n = 6$). GAPDH was used as an invariant internal control for calculating mRNA -fold changes. *, $p < 0.05$ versus the level in group 1; ‡, $p < 0.05$ versus the level in group 3. C, Western blotting analyses of the protein abundance of GCLc and GCLm. β-Actin was used as an invariant control for equal loading. Representatives of three independent experiments are shown.

In this report, traditional methods of assessing hepatic fibrosis, such as histopathological analysis and hydroxyproline measurements, were performed to determine the effectiveness of curcumin on the protection of the liver from CCl_4-induced fibrogenesis. Results from histopathological analyses demonstrated that curcumin significantly reduced the size stained with Sirius red in the liver. In addition, results from determination of the level of hepatic hydroxyproline indicated that the CCl_4-elevated levels of hepatic hydroxyproline were significantly reduced by oral administration of curcumin, which confirmed the histological observations in Fig. 1. Both results suggested that curcumin protected the liver from fibrogenesis caused by CCl_4 in the rat model. However, it is noteworthy that the haphazard distribution of fibrous tissue within the liver, such as in septa, bands, or expanded portal spaces, could interfere with measurement of the level of hepatic hydroxyproline and mislead the final conclusion. A recent study indicated that hydroxyproline ratio in a normal liver kept a fair degree of reproducibility (Gomes et al., 2006). However, in livers with fibrosis, the levels of hydroxyproline varied significantly among samples excised from the same liver, suggesting that the level of hydroxyproline in the liver might have limited value in quantifying the extent of fibrosis (Gomes et al., 2006).

Inflammation is commonly associated with hepatic fibrogenesis during chronic liver diseases (Marra, 2002). CCl_4 is metabolized in the liver by cytochrome P450 into the free radical CCl_3 (Burk et al., 1984). The free radical attacks hepatocytes and causes necrosis of parenchymal cells, which promotes inflammatory responses in the liver (Pérez Tamayo, 1983). Proinflammatory TNF-α, IFN-γ, and IL-6 are major players in hepatic inflammation. Concentrations of serum TNF-α and IL-6 are remarkably increased in patients with alcoholic cirrhosis (Byl et al., 1993; Diez Ruiz et al., 1993). TNF-α stimulates the development of hepatic fibrosis (Yin et al., 1999). IL-6 induces hepatic inflammation and collagen synthesis in vivo (Choi et al., 1994). IL-6 produced by activated HSC facilitates the production of ECM, including type I collagen, leading to hepatic fibrosis (Natsume et al., 1999). We reported in the present study that curcumin significantly reduced the levels of TNF-α and IL-6 in the liver and in serum in the CCl_4 rat model. Our observations are consistent with and supported by prior observations. Fibrotic changes were less evident in IL-6-deficient mice injected with CCl_4 (Natsume et al., 1999). Curcumin ameliorated the alcoholic and nonalcoholic experimental pancreatitis and reduced mRNA levels of IL-6, TNF-α, and inducible NO synthase (Gukovsky et al., 2003). IFN-γ plays a crucial role in modulating immune responses. Prior studies reported the inhibitory role of IFN-γ in HSC proliferation in vitro (Rockey et al., 1992; Shen et al., 2002). However, our study showed that the level of IFN-γ was positively correlated with serologic markers of hepatic injury and fibrogenesis. Prior clinical reports also indicated that IFN-γ was positively associated with elevated levels of ALT in chronic hepatitis B patients (Lee et al., 1999; Hyodo et al., 2003). The role of the elevated level of IFN-γ in the development of hepatic injury and fibrogenesis remains unclear. It was postulated that the elevation in the level of IFN-γ might be advantageous in the

control of hepatic viral infection (Wang et al., 2007). Therefore, it is plausible to assume that in addition to its role in the induction of inflammation, the elevation of the level of IFN-γ in the rat model might be an advantageous response for the animals to protect the liver from CCl_4-caused injury and to suppress HSC proliferation. Additional experiments are necessary to determine the role and mechanisms of INF-γ in hepatic fibrogenesis.

Oxidative stress is closely associated with hepatic fibrosis (Poli and Parola, 1997). Lipid peroxidation and necrosis are significantly suppressed in the liver of animals supplemented with antioxidants such as flavonoid, silymarin, or vitamin E (Parola and Robino, 2001). We recently reported that curcumin elevated the level of cellular GSH and induced de novo synthesis of GSH in HSC by stimulating the activity and gene expression of GCL, a key rate-limiting enzyme in GSH synthesis (Zheng and Chen, 2007). It was further demonstrated that de novo synthesis of GSH was a prerequisite for curcumin to inhibit HSC activation (Zheng and Chen, 2007). In this study, we hypothesized that curcumin might protect the liver against CCl_4-induced injury and fibrosis by attenuating oxidative stress. Results in this report indicated that oral administration of curcumin not only increased the level of total hepatic GSH but also significantly improve the ratio of GSH/GSSG in the liver. Further experiments revealed that curcumin enhanced the activity of GCL by inducing expression of the genes. Curcumin attenuated oxidative stress demonstrated by the reduction in the levels of lipid hydroperoxide in the CCl_4 rat model. These observations are supported by other studies. Treatment with 100 mg/kg curcumin prevented the drop in the content of hepatic GSH, diminished lipid peroxidation, and minimized hepatocarcinogenesis in rats (Sreepriya and Bali, 2006). It bears emphasis that our results do not exclude any other mechanisms involved in the antioxidant capacity of curcumin and in the curcumin elevation of the level of hepatic GSH. The level of cellular GSH is mainly determined by GSH synthesis (GSH supply) and GSH consumption (GSH demand). This current report focused on the effect of curcumin on GSH synthesis. Additional experiments are ongoing to evaluate the role of curcumin in regulating gene expression and activity of enzymes involved in consuming GSH, including GSH transferase and GSH peroxidase. In addition, we could not exclude the roles of any mechanisms and enzymes in the removal of lipid peroxidation products, which requires additional studies

Activation of HSC is triggered by various cytokines and chemokines, including the potent mitogens PDGF and EGF (Bachem et al., 1989b; Pinzani et al., 1989), and the fibrogenic factor TGF-β (Gressner et al., 2002), released from Kupffer cells and activated HSC (Friedman, 1999). The process of HSC activation is associated with sequential up-regulation of corresponding receptors, including PDGF-βR (Pinzani et al., 1996), EGFR (Wong et al., 1994; Kömüves et al., 2000), and type I and II TGF-β receptors (Friedman et al., 1994). We previously demonstrated that curcumin significantly suppressed gene expression of PDGF-βR, EGFR, and type I and II TGF-β receptors in HSC in vitro (Zheng and Chen, 2004, 2006; Zhou et al., 2007). Results in this report further confirmed the in vivo effect of curcumin on the suppression of expression of these genes in the CCl_4 rat model demonstrated by real-time PCR analyses and immunohistochemical assays. In addition, HSC activation coincides with a dramatic reduction in the abundance of PPARγ (Marra et al., 2000; Miyahara et al., 2000), which might play an important role in hepatic fibrogenesis. We previously demonstrated that curcumin dramatically induced gene expression of PPARγ in activated HSC, which facilitated its *trans*-activation activity, leading to the inhibition of HSC proliferation, the induction of apoptosis, and the suppression of ECM production (Xu et al., 2003; Zheng and Chen, 2004). It was further reported that the interruption of the signaling pathways for TGF-β, PDGF, and EGF by curcumin was required for the curcumin induction of gene expression of PPARγ in vitro (Zheng and Chen, 2006; Zhou et al., 2007). It was of interest to verify that the treatment with curcumin diminished the role of CCl_4 in the reduction of the number of PPARγ-positive HSC in the rat model.

In summary, the results from this study supported our initial hypothesis and demonstrated that curcumin protected the rat liver from CCl_4-caused injury and fibrogenesis in vivo by suppressing hepatic inflammation, attenuating hepatic oxidative stress and inhibiting HSC activation. These results confirm and extend our prior in vitro observations, and they provide novel insights into the mechanisms of curcumin in the protection of the liver. Our results suggest that curcumin might be a therapeutic anti-fibrogenic candidate for the treatment of hepatic fibrosis.

References

Bachem MG, Melchior R, and Gressner AM (1989a) The role of thrombocytes in liver fibrogenesis: effects of platelet lysate and thrombocyte-derived growth factors on the mitogenic activity and glycosaminoglycan synthesis of cultured rat liver fat storing cells. *J Clin Chem Clin Biochem* **27:**555–565.

Bachem MG, Riess U, and Gressner AM (1989b) Liver fat storing cell proliferation is stimulated by epidermal growth factor/transforming growth factor alpha and inhibited by transforming growth factor beta. *Biochem Biophys Res Commun* **162:**708–714.

Basu S (2003) Carbon tetrachloride-induced lipid peroxidation: eicosanoid formation and their regulation by antioxidant nutrients. *Toxicology* **189:**113–127.

Bataller R and Brenner DA (2005) Liver fibrosis. *J Clin Invest* **115:**209–218.

Bergman I and Loxley R (1970) The determination of hydroxyproline in urine hydrolysates. *Clin Chim Acta* **27:**347–349.

Blair IA (2006) Endogenous glutathione adducts. *Curr Drug Metab* **7:**853–872.

Burk RF, Lane JM, and Patel K (1984) Relationship of oxygen and glutathione in protection against carbon tetrachloride-induced hepatic microsomal lipid peroxidation and covalent binding in the rat. Rationale for the use of hyperbaric oxygen to treat carbon tetrachloride ingestion. *J Clin Invest* **74:**1996–2001.

Byl B, Roucloux I, Crusiaux A, Dupont E, and Deviere J (1993) Tumor necrosis factor alpha and interleukin 6 plasma levels in infected cirrhotic patients. *Gastroenterology* **104:**1492–1497.

Chen A, Zhang L, Xu J, and Tang J (2002) The antioxidant (−)-epigallocatechin-3-gallate inhibits activated hepatic stellate cell growth and suppresses acetaldehyde-induced gene expression. *Biochem J* **368:**695–704.

Choi I, Kang HS, Yang Y, and Pyun KH (1994) IL-6 induces hepatic inflammation and collagen synthesis in vivo. *Clin Exp Immunol* **95:**530–535.

Diez Ruiz A, Santos Perez JL, Lopez Martinez G, Gonzalez Calvin J, Gil Extremera B, and Gutierrez Gea F (1993) Tumour necrosis factor, interleukin-1 and interleukin-6 in alcoholic cirrhosis. *Alcohol Alcohol* **28:**319–323.

Egan ME, Pearson M, Weiner SA, Rajendran V, Rubin D, Glockner-Pagel J, Canny S, Du K, Lukacs GL, and Caplan MJ (2004) Curcumin, a major constituent of turmeric, corrects cystic fibrosis defects. *Science* **304:**600–602.

Fridovich I (1978) The biology of oxygen radicals. *Science* **201:**875–880.

Friedman SL (1999) Cytokines and fibrogenesis. *Semin Liver Dis* **19:**129–140.

Friedman SL (2003) Liver fibrosis—from bench to bedside. *J Hepatol* **38 (Suppl 1):**S38–S53.

Friedman SL, Yamasaki G, and Wong L (1994) Modulation of transforming growth factor beta receptors of rat lipocytes during the hepatic wound healing response. Enhanced binding and reduced gene expression accompany cellular activation in culture and in vivo. *J Biol Chem* **269:**10551–10558.

Galli A, Crabb D, Price D, Ceni E, Salzano R, Surrenti C, and Casini A (2000) Peroxisome proliferator-activated receptor gamma transcriptional regulation is involved in platelet-derived growth factor-induced proliferation of human hepatic stellate cells. *Hepatology* **31:**101–108.

Gomes AT, Bastos CG, Afonso CL, Medrado BF, and Andrade ZA (2006) How variable are hydroxyproline determinations made in different samples of the same liver? *Clin Biochem* **39:**1160–1163.

Greenwel P, Dominguez-Rosales JA, Mavi G, Rivas-Estilla AM, and Rojkind M (2000) Hydrogen peroxide: a link between acetaldehyde-elicited alpha1(I) collagen

gene up-regulation and oxidative stress in mouse hepatic stellate cells. *Hepatology* **31:**109–116.
Gressner AM, Weiskirchen R, Breitkopf K, and Dooley S (2002) Roles of TGF-beta in hepatic fibrosis. *Front Biosci* 7:d793–d807
Gukovsky I, Reyes CN, Vaquero EC, Gukovskaya AS, and Pandol SJ (2003) Curcumin ameliorates ethanol and nonethanol experimental pancreatitis. *Am J Physiol Gastrointest Liver Physiol* **284:**G85–G95.
Hyodo N, Tajimi M, Ugajin T, Nakamura I, and Imawari M (2003) Frequencies of interferon-gamma and interleukin-10 secreting cells in peripheral blood mononuclear cells and liver infiltrating lymphocytes in chronic hepatitis B virus infection. *Hepatol Res* **27:**109–116.
Johnson SJ, Hines JE, and Burt AD (1992) Immunolocalization of proliferating perisinusoidal cells in rat liver. *Histochem J* **24:**67–72.
Jones DP (2002) Redox potential of GSH/GSSG couple: assay and biological significance. *Methods Enzymol* **348:**93–112.
Kobayashi N, Ito M, Nakamura J, Cai J, Gao C, Hammel JM, and Fox IJ (2000) Hepatocyte transplantation in rats with decompensated cirrhosis. *Hepatology* **31:**851–857.
Kömüves LG, Feren A, Jones AL, and Fodor E (2000) Expression of epidermal growth factor and its receptor in cirrhotic liver disease. *J Histochem Cytochem* **48:**821–830.
Kusunose M, Qiu B, Cui T, Hamada A, Yoshioka S, Ono M, Miyamura M, Kyotani S, and Nishioka Y (2002) Effect of Sho-saiko-to extract on hepatic inflammation and fibrosis in dimethylnitrosamine induced liver injury rats. *Biol Pharm Bull* **25:** 1417–1421.
Lee KS, Buck M, Houglum K, and Chojkier M (1995) Activation of hepatic stellate cells by TGF alpha and collagen type I is mediated by oxidative stress through c-myb expression. *J Clin Invest* **96:**2461–2468.
Lee M, Lee M, Lee SK, Son M, Cho SW, Park S, and Kim HI (1999) Expression of Th1 and Th2 type cytokines responding to HBsAg and HBxAg in chronic hepatitis B patients. *J Korean Med Sci* **14:**175–181.
Marra F (2002) Chemokines in liver inflammation and fibrosis. *Front Biosci* **7:**d1899–d1914
Marra F, Efsen E, Romanelli RG, Caligiuri A, Pastacaldi S, Batignani G, Bonacchi A, Caporale R, Laffi G, Pinzani M, et al. (2000) Ligands of peroxisome proliferator-activated receptor gamma modulate profibrogenic and proinflammatory actions in hepatic stellate cells. *Gastroenterology* **119:**466–478.
Miyahara T, Schrum L, Rippe R, Xiong S, Yee HF, Jr., Motomura K, Anania FA, Willson TM, and Tsukamoto H (2000) Peroxisome proliferator-activated receptors and hepatic stellate cell activation. *J Biol Chem* **275:**35715–35722.
Natsume M, Tsuji H, Harada A, Akiyama M, Yano T, Ishikura H, Nakanishi I, Matsushima K, Kaneko S, and Mukaida N (1999) Attenuated liver fibrosis and depressed serum albumin levels in carbon tetrachloride-treated IL-6-deficient mice. *J Leukoc Biol* **66:**601–608.
Parola M and Robino G (2001) Oxidative stress-related molecules and liver fibrosis. *J Hepatol* **35:**297–306.
Pérez Tamayo R (1983) Is cirrhosis of the liver experimentally produced by CCl4 and adequate model of human cirrhosis? *Hepatology* **3:**112–120.
Pinzani M, Gesualdo L, Sabbah GM, and Abboud HE (1989) Effects of platelet-derived growth factor and other polypeptide mitogens on DNA synthesis and growth of cultured rat liver fat-storing cells. *J Clin Invest* **84:**1786–1793.
Pinzani M, Milani S, Herbst H, DeFranco R, Grappone C, Gentilini A, Caligiuri A, Pellegrini G, Ngo DV, Romanelli RG, et al. (1996) Expression of platelet-derived growth factor and its receptors in normal human liver and during active hepatic fibrogenesis. *Am J Pathol* **148:**785–800.
Poli G and Parola M (1997) Oxidative damage and fibrogenesis. *Free Radic Biol Med* **22:**287–305.
Proctor E and Chatamra K (1982) High yield micronodular cirrhosis in the rat. *Gastroenterology* **83:**1183–1190.
Rivera CA, Bradford BU, Hunt KJ, Adachi Y, Schrum LW, Koop DR, Burchardt ER, Rippe RA, and Thurman RG (2001) Attenuation of CCl_4-induced hepatic fibrosis by $GdCl_3$ treatment or dietary glycine. *Am J Physiol Gastrointest Liver Physiol* **281:**G200–G207.
Rockey DC, Maher JJ, Jarnagin WR, Gabbiani G, and Friedman SL (1992) Inhibition of rat hepatic lipocyte activation in culture by interferon-gamma. *Hepatology* **16:**776–784.
Schmittgen TD, Zakrajsek BA, Mills AG, Gorn V, Singer MJ, and Reed MW (2000) Quantitative reverse transcription-polymerase chain reaction to study mRNA decay: comparison of endpoint and real-time methods. *Anal Biochem* **285:**194–204.
Shen H, Zhang M, Minuk GY, and Gong Y (2002) Different effects of rat interferon alpha, beta and gamma on rat hepatic stellate cell proliferation and activation. *BMC Cell Biol* **3:**9.
Shi J, Aisaki K, Ikawa Y, and Wake K (1998) Evidence of hepatocyte apoptosis in rat liver after the administration of carbon tetrachloride. *Am J Pathol* **153:**515–525.
Sreepriya M and Bali G (2006) Effects of administration of Embelin and Curcumin on lipid peroxidation, hepatic glutathione antioxidant defense and hematopoietic system during *N*-nitrosodiethylamine/phenobarbital-induced hepatocarcinogenesis in Wistar rats. *Mol Cell Biochem* **284:**49–55.
Tsukamoto H, Rippe R, Niemela O, and Lin M (1995) Roles of oxidative stress in activation of Kupffer and Ito cells in liver fibrogenesis. *J Gastroenterol Hepatol* **10 (Suppl 1):**S50–S53.
Wang J, Gujar SA, Cova L, and Michalak TI (2007) Bicistronic woodchuck hepatitis virus core and gamma interferon DNA vaccine can protect from hepatitis but does not elicit sterilizing antiviral immunity. *J Virol* **81:**903–916.
Wong L, Yamasaki G, Johnson RJ, and Friedman SL (1994) Induction of beta-platelet-derived growth factor receptor in rat hepatic lipocytes during cellular activation in vivo and in culture. *J Clin Invest* **94:**1563–1569.
Wu G, Fang YZ, Yang S, Lupton JR, and Turner ND (2004) Glutathione metabolism and its implications for health. *J Nutr* **134:**489–492.
Xu J, Fu Y, and Chen A (2003) Activation of peroxisome proliferator-activated receptor-gamma contributes to the inhibitory effects of curcumin on rat hepatic stellate cell growth. *Am J Physiol Gastrointest Liver Physiol* **285:**G20–G30.
Yin M, Wheeler MD, Kono H, Bradford BU, Gallucci RM, Luster MI, and Thurman RG (1999) Essential role of tumor necrosis factor alpha in alcohol-induced liver injury in mice. *Gastroenterology* **117:**942–952.
Yoshiji H, Kuriyama S, Yoshii J, Ikenaka Y, Noguchi R, Nakatani T, Tsujinoue H, Yanase K, Namisaki T, Imazu H, et al. (2002) Tissue inhibitor of metalloproteinases-1 attenuates spontaneous liver fibrosis resolution in the transgenic mouse. *Hepatology* **36:**850–860.
Zheng S and Chen A (2004) Activation of PPARgamma is required for curcumin to induce apoptosis and to inhibit the expression of extracellular matrix genes in hepatic stellate cells in vitro. *Biochem J* **384:**149–157.
Zheng S and Chen A (2006) Curcumin suppresses the expression of extracellular matrix genes in activated hepatic stellate cells by inhibiting gene expression of connective tissue growth factor. *Am J Physiol Gastrointest Liver Physiol* **290:** G883–G893.
Zheng S and Chen A (2007) de novo synthesis of glutathione is a prerequisite for curcumin to inhibit HSC activation. *Free Radic Biol Med* **43:**444–453.
Zhou Y, Zheng S, Lin J, Zhang QJ, and Chen A (2007) The interruption of the PDGF and EGF signaling pathways by curcumin stimulates gene expression of PPAR-gamma in rat activated hepatic stellate cell in vitro. *Lab Invest* **87:**488–498.

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