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Green tea polyphenol epigallocatechin-3-gallate inhibits oxidative damage and preventive effects on carbon tetrachloride–induced hepatic fibrosis

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Abstract

The aim of the study was to examine the effects of epigallocatechin-3-gallate (EGCG) on hepatic fibrogenesis and on cultured hepatic stellate cells (HSCs). The rat model of carbon tetrachloride (CCl_4)-induced hepatic fibrosis was used to assess the effect of daily intraperitoneal injections of EGCG on the indexes of fibrosis. Histological and hepatic hydroxyproline examination revealed that EGCG significantly arrested progression of hepatic fibrosis. EGCG caused significant amelioration of liver injury (reduced activities of serum alanine aminotransferase and aspartate aminotransferase). The development of CCl_4-induced hepatic fibrosis altered the redox state with a decreased hepatic glutathione and increased the formation of lipid peroxidative products, which were partially normalized by treatment with EGCG, respectively. Moreover, EGCG markedly attenuated HSC activation as well as matrix metalloproteinase (MMP)-2 activity. In cultured stellate cell, the expression of MMP-2 mRNA and protein were substantially reduced by EGCG treatment. Concanavalin A-induced activation of secreted MMP-2 was inhibited by EGCG through the influence of membrane type 1-MMP activity. These results demonstrate that administration of EGCG may be useful in the treatment and prevention of hepatic fibrosis.

Keywords: Green tea; Matrix metalloproteinase; Oxidative stress; Hepatic stellate cell; Fibrosis

1. Introduction

Hepatic fibrosis is a dynamic and sophisticatedly regulated wound healing response to chronic hepatocellular injury. Hepatic stellate cells (HSCs) are recognized as the primary cellular source of matrix components in chronic liver disease and, therefore, play a critical role in the development and maintenance of liver fibrosis [1].

Experimental and clinical results indicate that oxidative stress could represent a common link between the different types of chronic liver injury and hepatic fibrosis [2]. Carbon tetrachloride (CCl_4) is a xenobiotic used extensively to induce oxidative stress. Chronic CCl_4 treatment is frequently used in rats to produce an experimental model to study hepatic fibrosis [3,4]. Liver fibrosis induced by CCl_4 is associated with the exacerbation of lipid peroxidation and the depletion of antioxidant status [5,6]. In this regard, reduction of oxidative stress may be a potential and effective therapeutic strategy for prevention and treatment of hepatic fibrosis.

Many studies have demonstrated that in hepatic fibrosis progression the expression of matrix metalloproteinases (MMPs) involved in fibrillar collagen degradation (e.g., MMP-1 in humans and MMP-13 in rats) is very limited, whereas the expression of MMP-2 is markedly increased. Evidence from experimental and clinical studies indicates that MMP-2 expression and increased activity is one of the major causes of liver fibrosis [7–9]. MMP-2 is constitutively expressed and secreted as a latent zymogen (pro-MMP-2), which is activated by a membrane-linked process mediated by membrane type 1 (MT1)-MMP and tissue inhibitor of metalloproteinase-2 (TIMP-2) [10,11]. Once activated, MMP-2 is able to degrade the normal subendothelial matrix, hastening its replacement by fibrillar collagen. The presence

Abbreviations: HSC, hepatic stellate cell(s); EGCG, Epigallocatechin-3-gallate; MMP, matrix metalloproteinase; SMA, smooth muscle actin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GSH, glutathione; TBARS, thiobarbituric acid-reactive substances; ConA, Concanavalin A.

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of this newly formed collagen lattice can further promote HSC activation [8].

Green tea is a widely consumed beverage in many countries. It has been shown that an aqueous extract of polyphenols from green tea *Camellia sinensis* reduces liver fibrosis in rats induced by bile duct ligation. Epigallocatechin-3-gallate (EGCG), a major polyphenol, which accounts for 10%–15% of the total green tea [12], was implicated as the main active ingredient. EGCG is a potent antioxidant that has attracted considerable attention for its role in preventing oxidative stress-related diseases including cancers, cardiovascular diseases and fibrosis [13–15]. Recently, Chen et al. [16] have reported that in the mice model, the nonfibrotic hepatic damage induced by a single dose of CCl_4 was reduced following EGCG pretreatment. In vitro studies have shown that EGCG exerts antifibrogenic effects by decreasing the synthesis of type I collagen, by reducing cell proliferation and by inducing apoptosis on cultured HSCs [17–19]. These findings led us to propose that EGCG administration could become a promising treatment for hepatic fibrosis.

Therefore, the aim of this study was to investigate the protective effects of EGCG on CCl_4-induced hepatic fibrogenesis and to investigate some of the mechanisms involved.

2. Materials and methods

2.1. Materials

EGCG (purity >95%) was purchased from Sigma-Aldrich Chemical (St Louis, MO, USA). Antibodies against MMP-2 and TIMP-2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and antibodies against MT1-MMP were obtained from Chemicon (Temecula, CA, USA).

2.2. Animals and treatment

Six-week-old male Sprague–Dawley rats weighing 180–200 g were housed in conventional cages with free access to water and rodent chow at 20–22°C with a 12-h light–dark cycle. All procedures involving laboratory animal use were in accordance with the guidelines of the instituted animal care and use committee of Sun Yat-Sen University for the care and use of laboratory animals. Rats were randomly divided into four groups: (A) normal control group ($n=8$) — rats received an intraperitoneal injection of olive oil at 1 ml/kg body weight per rat twice a week for 7 weeks. (B) EGCG control group ($n=8$) — rats received an intraperitoneal injection of olive oil at 1 ml/kg body weight per rat twice a week for 7 weeks. At the same time, the rats were intraperitoneally administered EGCG (25 mg/kg daily for 7 weeks). (C) Fibrosis group ($n=12$) — rats received intraperitoneal injection of CCl_4 (1 ml/kg body weight, 1:1 in olive oil, twice a week) for 7 weeks. (D) EGCG-treated fibrosis group (EGCG-treated group, $n=14$) — rats received intraperitoneal injection with CCl_4 (1 ml/kg body weight, 1:1 in olive oil, twice a week) for 7 weeks. At the same time, the rats were intraperitoneally administered EGCG (25 mg/kg daily for 7 weeks). At 24 h after the final injection of CCl_4, a laparotomy was performed and blood was drawn from the abdominal aorta under ether anesthesia. The serum was stored at −80°C after separation until assayed as described below. Liver samples were collected and frozen in liquid nitrogen and stored at −80°C.

2.3. Measurement of serum aminotransferase levels

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were determined using commercial kits produced by Institute of Shanghai Biological Products affiliated with the Ministry of Health.

2.4. Histological and immunohistochemical stains

Liver samples from all animals were processed for light microscopy. Tissue sections were fixed in 10% neutral buffered formalin and embedded in paraffin. Paraffin sections were stained with hematoxylin–eosin (H&E) and Masson's trichrome stain. The degree of liver damage was examined blindly by a liver pathologist under a light Olympus microscope, scored with the Knodell index as follows [20]: 0, absence of fibrosis; 1, portal fibrosis; 2, fibrous portal expansion; 3, bridging fibrosis (portal–portal or portal–central linkage); and 4, cirrhosis. Histopathological evaluation was performed twice in four sections per slide for all animals in each group. The fibrotic area per liver section was quantified by morphometric analysis of the sections using the Image J software package (NIH, Bethesda, MD, USA) and expressed as percentage of the total area. The immunohistochemical stains for α-smooth muscle actin (SMA) proteins were carried out using anti-α-SMA antibodies (Santa Cruz, CA, USA).

2.5. Hepatic glutathione and thiobarbituric acid-reactive substances measurements

The glutathione (GSH) concentrations in the liver homogenate were determined with a GSH-400 colorimetric assay kit (Calbiochem, San Diego, CA, USA) according to the manufacturer's instructions. Liver tissue was homogenized with 2 ml of 10% (w/v) metaphosphoric acid solution at 4°C. Each sample was then centrifuged at $3000\times g$ for 10 min at 4°C. After vortexing, a 200-ml aliquot of the centrifuged supernatants was read at 412 nm in a spectrophotometer. The values of unknown samples were drawn from a standard curve plotted by assaying different known concentrations of GSH. The liver homogenate for lipid peroxidation was prepared with 2 ml of 50 mM potassium phosphate buffer, pH 7.4, and thiobarbituratic acid-reactive substances (TBARS) were determined [21]. The fluorescence of the samples was detected at an excitation wavelength of 515 nm and an emission wavelength of 555 nm in a F4500 fluorescence spectrophotometer (Hitachi, Japan), and 1,1,3,3-tetramethoxypropane was used as the TBARS standard.

2.6. Hepatic hydroxyproline concentration

Liver collagen concentration was determined by measuring hydroxyproline content in liver samples and was performed as described previously [22]. Briefly, liver samples were homogenized and hydrolyzed in 6 N HCl at 110°C for 18 h. After filtration of the hydrolysate through a 0.45-mm Millipore filter, chloramine T was added to a final concentration of 2.5 mM. The mixture was then treated with 410 mM paradimethyl-amino-benzaldehyde and incubated at 60°C for 30 min. The concentration of hydroxyproline in each sample was determined spectrophotometry at 560 nm using a standard curve generated from known quantities of hydroxyproline. Each liver sample was measured in triplicate, and the mean value of hydroxyproline used for analysis. Results were expressed as micrograms per gram of wet tissue.

2.7. MMP-2 activity assay

The liver was homogenized in lysis buffer containing a protease inhibitor cocktail (Boehringer Mannhein, Lewes, UK). MMP-2 activity of tissue homogenates was examined by gelatine zymography. Fifty micrograms of protein was subjected to substrate-gel electrophoresis using sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (7.5%) containing 0.1% gelatin (Sigma-Aldrich). After electrophoresis, SDS was removed by soaking the gels three times for 30 min at room temperature in buffer (50 mM Tris–HCl, pH 8.0, 5 mM $CaCl_2$, 0.02% NaN_3 and 2.5% Triton X-100) and incubated for 24 h at 37°C with the same buffer lacking Triton X-100. Gels were then stained with 0.1% Coomassie brilliant blue R-250 and destained until clear bands became evident. Quantitative results of the assays were obtained by densitometry.

2.8. Hepatic stellate cell culture study

Rat HSCs were kindly donated by Professor Shi-gang Xiong (Department of Pathology, Keck School of Medicine, University of Southern California, USA). HSCs were isolated from normal Wistar rats by in situ digestion of the liver and arabinogalactan gradient ultracentrifugation as described previously [23]. They were cultured in Dulbecco's minimal essential medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Bio-Whittaker, Walkersville, MD, USA), 100 IU/ml penicillin and 100 μg/ml streptomycin. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2, and the medium was changed twice a week. Cell viability was tested by trypan blue exclusion. All experiments were performed with HSCs from Passages 6–15, which were exposed to serum-free culture medium containing 0.1% bovine serum albumin for 24 h.

2.9. Gelatin zymography

HSCs (5.5×10^4/well) in 6-well plastic dishes were cultured with concanavalin A (ConA) (20 μg/ml) in the presence or absence of EGCG at the indicated concentration for 24 h. MMP-2 activity in the conditioned medium of cultured HSCs was analyzed by zymography.

2.10. Reverse transcriptase–polymerase chain reaction

HSCs (5.5×10^4/well) in six-well plastic dishes were cultured with ConA (20 μg/ml) in the presence or absence of EGCG at the indicated concentration for 24 h. Total RNA was extracted by the use of TRIzol reagent (Sigma-Aldrich) according to the manufacturer's instructions. Reverse transcription with oligo (dT) priming was used to generate cDNA from total RNA (2-μg) extracts. The synthesized cDNA for MMP-2, TIMP-2, MT1-MMP and β-actin were amplified using specific sets of primers. Rat MMP-2 forward primer was 5′ GCT GAT ACT GAC ACT GGT ACT G 3′ and reverse primer was 5′ CAA TCT TTT CTG GGA GCT C 3′ [24]. Rat MT1-MMP (MMP-14) forward primer was 5′ GTA CTA CCG CTT CAA TGA GG 3′ and reverse primer was 5′ CAC TGC CAG TAC CAG GAG 3′ [25]. Rat TIMP-2 forward primer was 5′ ATT TAT CTA CAC GGC CCC 3′ and reverse primer was 5′ CAA GAA CCA TCA CTT CTC TTG 3′ [26]. β-Actin primer was designed based on published cDNA sequences. The forward primer was 5′ TGG GAC GAT ATG GAG AAG AT 3′ and the reverse primer was 5′ ATT GCC GAT AGT GAT GAC CT 3′ . Each polymerase chain reaction (PCR) mixture contained the appropriate set of forward and reverse primers (0.2 μM), each dNTP (deoxyribonucleoside triphosphate) at 0.25 mM, 1.25 U Taq polymerase and 2.5 mM $MgCl_2$ in a PCR buffer. The PCR procedure consisted of 28 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1min, with initial denaturation of sample cDNA at 95°C for 3 min and an additional extension period of 10 min after the last cycle. The PCR products were subjected to 1.5% agarose gel electrophoresis, staining with ethidium bromide and quantitation by densitometry using the Image Master VDS system and associated software (Pfizer, NY).

2.11. Western blotting

Cells were washed with cold PBS and lysed by the lysis buffer containing protease inhibitor cocktail. Cell extracts (50 μg per lane) were separated via 10% gel electrophoresis and electroblotted onto PVDF (polyvinylidene fluoride) membranes. Nonspecific binding sites were blocked by incubating PVDF membranes for 1 h in phosphate-buffered saline containing 5% low-fat dry milk. Membranes were probed with primary antibodies overnight at 4°C, followed by application of a secondary horseradish peroxidase-conjugated second antibody. Blots were developed using an enhanced chemiluminescence detection system (ECL, Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to the manufacturer's instructions.

2.12. Cellular MT1-MMP activity assay

HSCs (1×10^4/well) in 24-well plastic dishes were cultured with ConA (20 μg/ml) in the presence or absence

Table 1
Effect of EGCG on serum ALT and AST activities in hepatic fibrotic rats induced by CCl_4

	Normal control	EGCG control	Fibrosis	EGCG-treated
AST (IU/L)	65.3±28.5	66.6±30.6	234.6±63.2*	138.4±45.8†
ALT (IU/L)	36.2±6.1	35.7±7.8	186.2±36.6*	96.4±20.5†

* $P<.05$ compared with normal control group.
† $P<.05$ compared with fibrosis group.

of EGCG at the indicated concentration for 24 h. MT1-MMP activity was determined with the use of a commercial kit (Amersham) according to the manufacturer's instructions. Briefly, MT1-MMP was extracted from cultured HSCs by extraction buffer and its activity detected through activation of the modified prodetection enzyme and the subsequent cleavage of its chromogenic peptide substrate. The resultant color was read at 405 nm in a microplate spectrophotometer. The concentration of active MT1-MMP in a sample was determined by interpolation from a standard curve.

2.13. Statistics

Results are expressed as the mean±S.E.M. of at least three separate experiments. Results were analyzed by one-way analysis of variance followed by the Student–Newman–Keuls test. Differences with P values of $<.05$ were considered significant.

3. Results

3.1. Effect of EGCG on liver function tests

AST and ALT in serum were used for the biochemical markers to evaluate the hepatic injury. The serum AST and ALT levels were significantly increased in the fibrosis group compared to that in the normal group, but were significantly decreased in the EGCG-treated group (Table 1).

3.2. EGCG decreased liver fibrosis and HSC activation in CCl4-treated rats

Liver fibrosis of the rats was evaluated by two histological methods, H&E staining and Masson's trichrome staining, and both methods showed the same pattern. The histological analysis of the livers from normal and EGCG control rats indicated normal architecture (Figs. 1A, B and 2A, B). A experimental design involving CCl_4 administration was used to produce general liver morphological changes and fibrosis evidenced by both qualitative and quantitative histopathological examinations. Representative photographs of liver morphology are shown in Figs. 1C and 2A-c: in contrast to normal rat liver morphology (Figs. 1A, B and 2A, B), CCl_4-induced fibrosis was evidenced by disruption of tissue architecture, extension of fibers, large fibrous septa formation, pseudolobe separation, and collagen accumulation. These alterations were remarkably reduced in the liver sections of the rats that received both

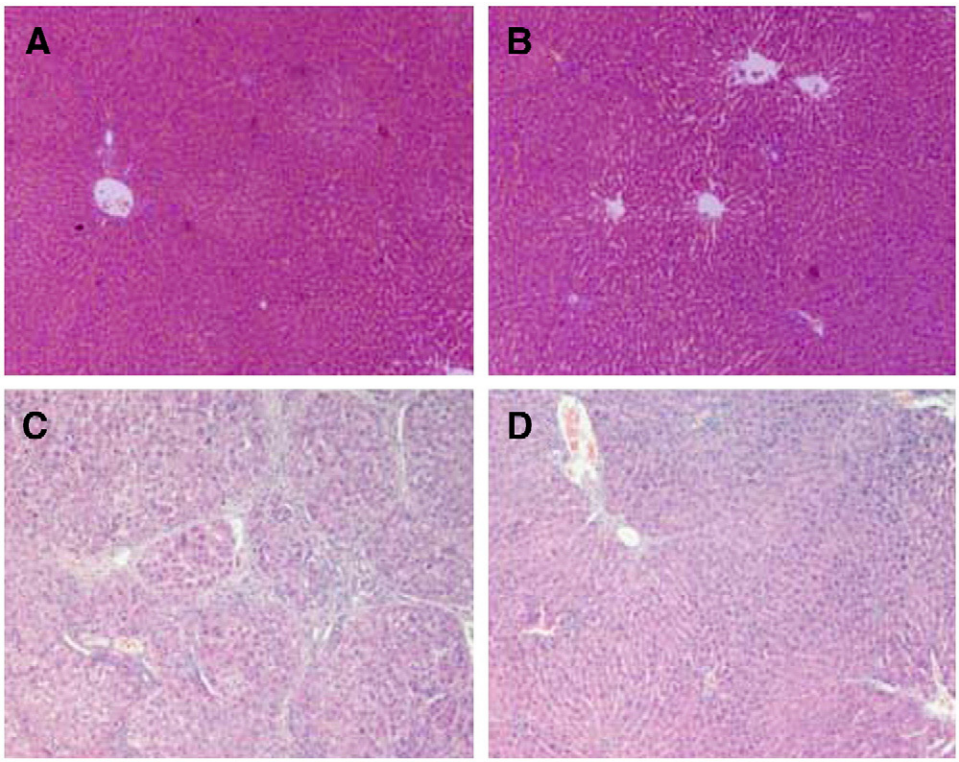

Fig. 1. Hepatic histopathology during development of CCl_4-induced cirrhosis with or without concurrent EGCG treatment. Liver sections were stained with H&E (original magnification ×100). (A) Normal control. (B) EGCG control group. H&E staining showed a normal architecture. (C) fibrosis group. Light microscopy examination showing large fibrous septa formation. (D) EGCG-treated group. H&E staining showing that collagen fiber bundles were markedly reduced (original magnification ×100).

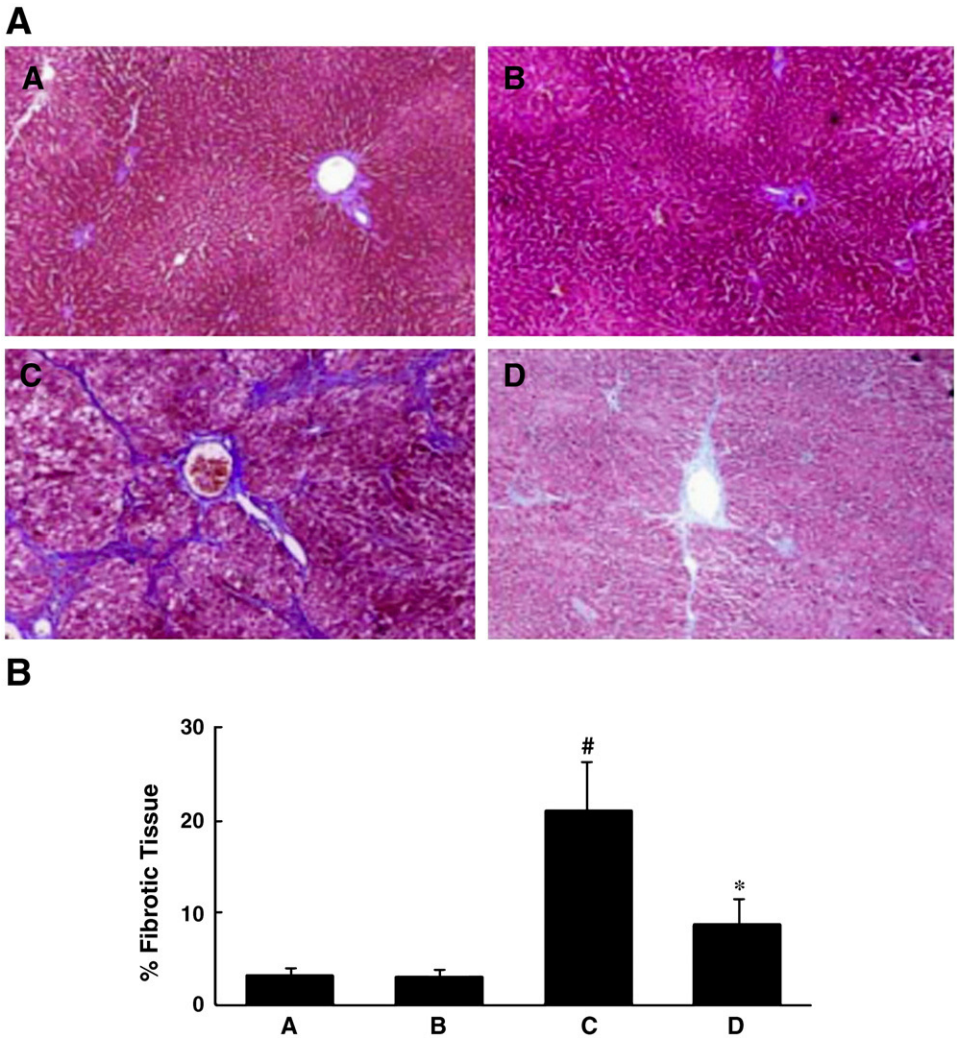

Fig. 2. Liver sections stained with Masson's trichrome (original magnification ×100). (A) Normal control. (B) EGCG control group. This section also showed a normal architecture. (C) Liver fibrosis group. Light microscopy examination, and also H&E staining, showing large fibrous septa formation. (D) EGCG-treated group. Masson's trichrome staining also showed that collagen fiber bundles were markedly reduced. $^{\#}P<.05$ vs. normal control groups; $^{*}P<.05$ vs. fibrosis group.

EGCG and CCl_4 treatment for 7 weeks (Figs. 1D and 2A-d). By Knodell index analysis of liver fibrosis, it was verified that the scoring of the EGCG-treated group had obviously improved compared with the fibrosis group (Table 2); The fibrotic area in the EGCG-treated group was significantly decreased to 8.8±2.6%, compared with 21.1±5.2% in the fibrosis group ($P<.05$, Fig. 2B). There were no significant differences between the normal control and EGCG control groups in histological observation.

Table 2
Effect of EGCG on the pathological grading of hepatic fibrotic rats induced by CCl_4

Groups	*n*	Stage 0	Stage 1	Stage 2	Stage 3	Stage 4	Mean±S.E.
Normal control	8	8	0	0	0	0	0.00±0.00
EGCG control	8	8	0	0	0	0	0.00±0.00
Fibrosis	12	0	0	1	3	8	3.58±0.19*
EGCG-treated	14	0	8	5	1	0	1.50±0.17†

* $P<.05$ compared with normal control group.
† $P<.05$ compared with fibrosis group.

In parallel to the observed improvement of liver histology, fibrosis was also quantified by measurement of hepatic hydroxyproline levels (Fig. 3). There was a significant increase in hepatic hydroxyproline levels in rats with CCl_4-induced cirrhosis (fibrosis group) compared to normal controls (576±29 vs. 162±17μg/g wet liver, $P<.05$, respectively), and administration of EGCG prevented the increase in hepatic hydroxyproline content in CCl_4-treated rats (326±21 vs. 576±29 μg/g in EGCG-treated group and fibrosis group, respectively).

Activated HSCs are the major source of matrix proteins in diseased liver [1]. Accordingly, we evaluated α-SMA, an indicator of HSC activation, by immunohistochemical staining in liver samples. In normal control livers, α-SMA-positive cells were present in portal veins and hepatic arteries (Fig. 4A). After 7 weeks of CCl_4 treatment, α-SMA-positive

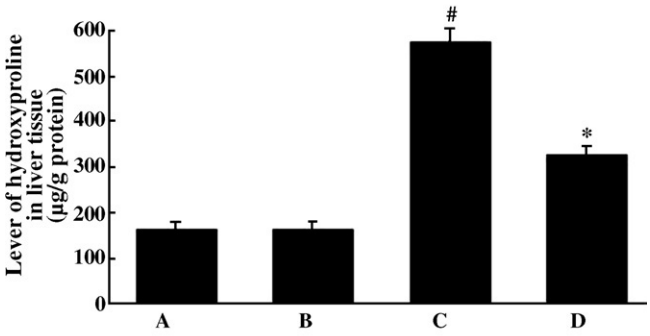

Fig. 3. Effect of EGCG on the hydroxyproline content of liver in fibrotic rats induced by CCl_4. Data represent mean±S.E.M. from at least eight rats. #$P<.05$ vs. normal control groups; *$P<.05$ vs. fibrosis group. (A) normal control group; (B) EGCG control group; (C) fibrosis group; (D) EGCG-treated group.

cells occupied most of the fibrous tissue (Fig. 4C). Concurrent EGCG treatment for 7 weeks significantly reduced the number of α-SMA-positive cells in the fibrous areas (Fig. 4D), indicating inhibition of HSC activation.

3.3. Effect of EGCG treatment on the hepatic redox state

To determine whether EGCG therapy had any effect on the redox state of liver tissue, the hepatic concentrations of GSH and TBARS were measured in the hepatic homogenate of rats. GSH constitutes the first line of defense against free radicals. As shown in Fig. 5A, hepatic GSH levels were significantly decreased in the fibrosis group (3.5±0.1 μmol/g) compared to normal control (5.9±0.2 μmol/g), but were increased significantly following treatment with EGCG to 5.3±0.2 μmol/g ($P<.05$). TBARS increased about 3.2-fold in the fibrosis group compared with that of the normal control group (Fig. 5B), and chronic EGCG administration decreased the hepatic level of TBARS (0.78±0.15 μmol/g in EGCG-treated group, $P<.05$, Fig. 5B).

3.4. The effect of EGCG on activity of hepatic MMP-2

Increased MMP-2 activity is believed to be associated with the development of fibrosis. To study the effect of EGCG on the fibrosis induced by CCl_4, we tested the activation of MMP-2 in different treatment groups. MMP-2 activity in liver homogenates was measured by gelatine zymography. As shown in Fig. 6A, two prominent bands appear after zymography and correspond to the proenzyme (72 kDa, pro-MMP-2) and the active form (66 kDa) of MMP-2. The intensity of both bands increased in fibrosis group and were decreased in the EGCG-treated group (Fig. 6A). Quantitative analysis by densitometry showed, compared with normal control group, CCl_4 treatment

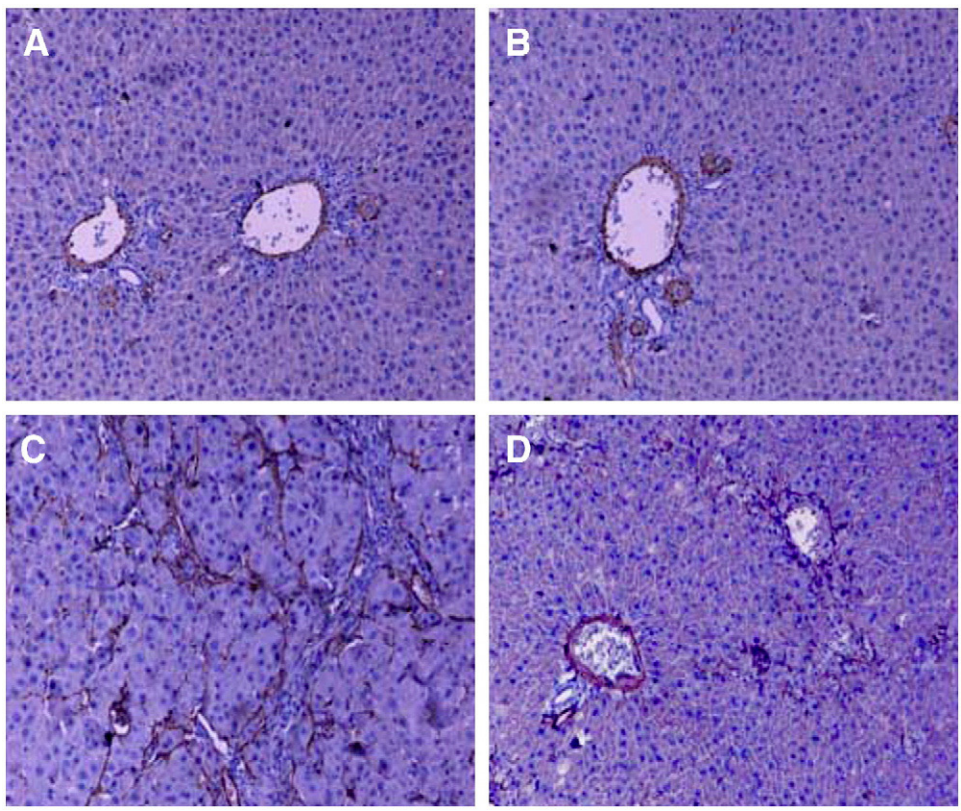

Fig. 4. Hepatic immunohistochemical stain for α-SMA. (A) Normal control group. α-SMA-positive cells are restricted to the portal vein and hepatic artery walls only. (B) EGCG control group. EGCG alone group showed normal hepatic morphology. (C) Liver fibrosis group. Abundant α-SMA staining in the fibrous areas indicates the presence of activated HSCs. (D) EGCG-treated group. The number of α-SMA-positive cells in the fibrous areas is reduced significantly (original magnification ×200).

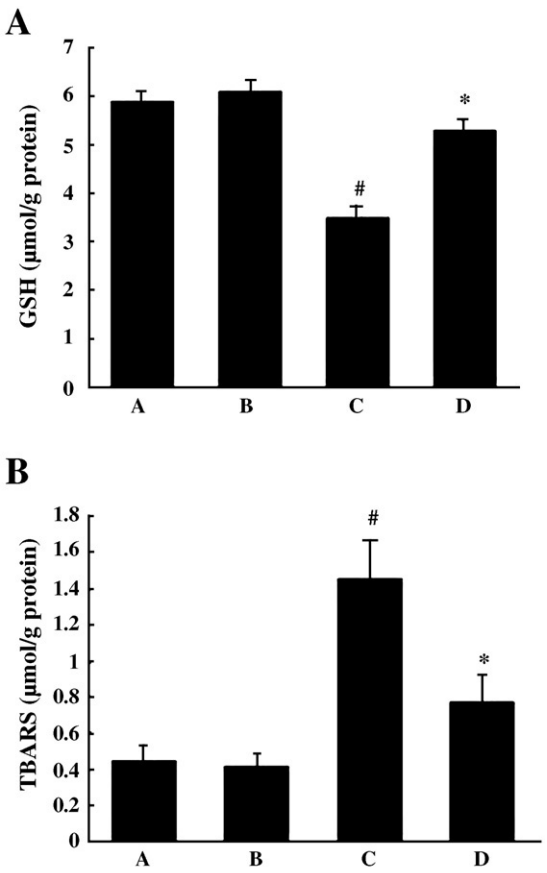

Fig. 5. Effect of EGCG on hepatic homogenate GSH (A) and TBARS (B) levels in fibrotic rats induced by CCl_4. Data represent mean±S.E.M. from at least eight rats. $^{\#}P<.05$ vs. normal control groups; $^{*}P<.05$ vs. fibrosis group. (A) normal control group; (B) EGCG control group; (C) fibrosis group; (D) EGCG-treated group.

markedly stimulated the hepatic MMP-2 tissue activity by 98%. EGCG significantly reduced the hepatic MMP-2 tissue activity by 26±3.4%, suggesting that EGCG decreases the level of activity of MMP-2 in the liver (Fig. 6B, $P<.05$).

3.5. EGCG influence on ConA-induced activation of MMP-2

Because activated stellate cells are the predominant source of MMP-2 in liver injury, we examined whether EGCG suppresses MMP-2 expression and activation in cultured rat HSCs. ConA stimulation of cells can induce a drastic increase in the secretion and proteolytic activation of MMP-2; hence, it is widely used for the study of MMP-2 expression and activation. To investigate the inhibitory effects of EGCG on MMP-2 activation, the release of MMP-2 in the medium was assessed by gelatin zymography. MMP-2 is secreted as inactive zymogen pro-MMP-2 by HSCs and ConA induces pro-MMP-2 activation [27,28]. Zymography of culture media of unstimulated HSCs revealed a gelatinolytic band that represented pro-MMP-2 (72 kDa) (Fig. 7A). After addition of ConA to HSCs and culture for 24 h, pro-MMP-2 levels were increased. In addition, less intense bands of activated form of MMP-2 (66 kDa) were present in zymograms. EGCG at 5, 10, 25 or 50 μM significantly reduced the ConA-induced activation of MMP-2 by 23.5±6.1%, 59.4±8.7%, 74.7±13.5% and 84.5±10.6%, respectively (Fig. 7B), suggesting that EGCG inhibited pro-MMP-2 activation in a dose-dependent manner.

3.6. EGCG inhibition of MT1-MMP activity

To examine the influence of EGCG on the expression of components of the MMP-2 activation complex, we assessed MT1-MMP and TIMP-2 expression in ConA-stimulated HSCs treated with or without the indicated concentrations of EGCG. The results showed that the expression of TIMP-2 mRNA and protein were not affected by ConA (Fig. 8A). EGCG had no influence on the level of TIMP-2, as compared with the HSCs cultured without EGCG. Previous studies have suggested that the ConA-induced activation of pro-MMP-2 on the cell surface is mediated by MT1-MMP [27]. Therefore, the possibility that EGCG could prevent ConA-induced MMP-2 activation by inhibiting MT1-MMP expression in HSCs was further investigated. Expression of MT1-MMP mRNA and protein was increased by ConA-stimulation, and the presence of the EGCG had no influence on the level of MT1-MMP (Fig. 8A, B). However, the cell-associated MT1-MMP activity was strongly inhibited by treatment of HSCs with EGCG in a dose-dependent manner (Fig. 9). These results indicated that EGCG reduced ConA-

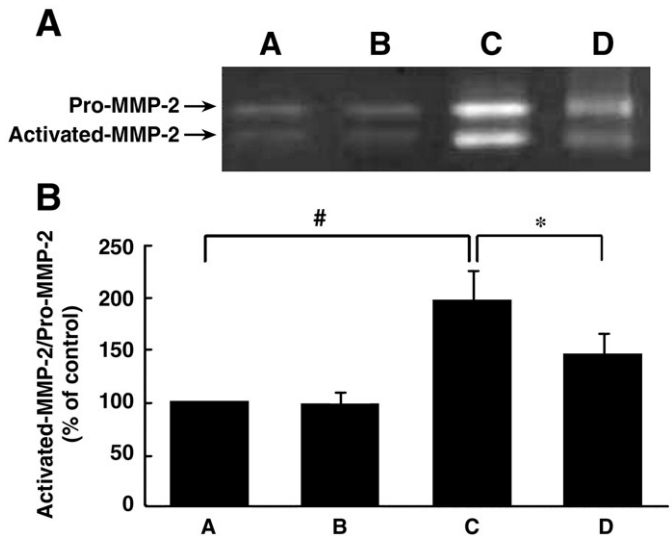

Fig. 6. Effect of EGCG treatment on liver tissue activity of MMP-2 collagenase measured by gelatin zymography. (A) Fifty micrograms of each liver protein sample was analyzed by gelatin zymography. (B) Quantitative analysis by densitometry showed a significant reduction in MMP-2 tissue activity after 7 weeks of EGG treatment. The values are expressed as mean±S.E.M. for three different experiments, and the relative percentage of activated MMP-2 to pro-MMP-2, compared with that of normal control groups. $^{\#}P<.05$ vs. normal control groups; $^{*}P<.05$ vs. fibrosis group. (A) normal control group; (B) EGCG control group; (C) fibrosis group; (D) EGCG-treated group.

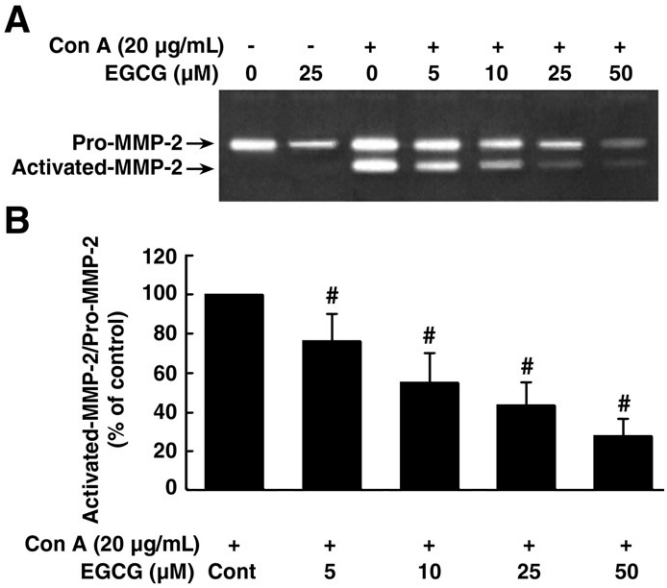

Fig. 7. EGCG inhibits ConA-induced activation of MMP-2 in a concentration-dependent manner in HSCs. (A) HSCs were cultured with ConA (20 μg/ml) in the presence or absence of EGCG for 24 h. The panel displays a representative gelatin zymogram of the conditioned medium from HSCs cultured with ConA in the presence or absence of the indicated concentrations of EGCG. (B) Quantitative results of the gelatin zymogram of conditioned media from HSCs cultured with ConA in the presence or absence of indicated concentrations of EGCG. The values are expressed as mean±S.E.M. for three different experiments, and the relative percentage of activated MMP-2 to pro-MMP-2, compared with that of the conditioned medium from HSCs cultured with ConA in the absence of EGCG. $^{\#}P<0.05$ vs. control.

induced MMP-2 activation through direct inhibition of MT1-MMP activity.

3.7. EGCG inhibition of pro-MMP-2 expression in cultured rat HSCs

The possibility of EGCG affecting the amount of pro-MMP-2 expression in HSCs was assessed by reverse transcriptase (RT)–PCR and Western blot analyses. Serum-starved HSCs were cultured with or without ConA (20 μg/ml) in the presence or absence of EGCG for 24 h. The expression of MMP-2 mRNA and protein was up-regulated by ConA stimulated HSCs. EGCG alone or in the presence of ConA markedly reduced MMP-2 mRNA after 24 h of incubation period (Fig. 10A, $P<.05$). Subsequently, as shown by Western blotting, the quantity of pro-MMP-2 protein could also be significantly reduced by EGCG treatment (Fig. 10B, $P<.05$).

4. Discussion

The present study demonstrates that EGCG, a major polyphenol of green tea, prevents the development of

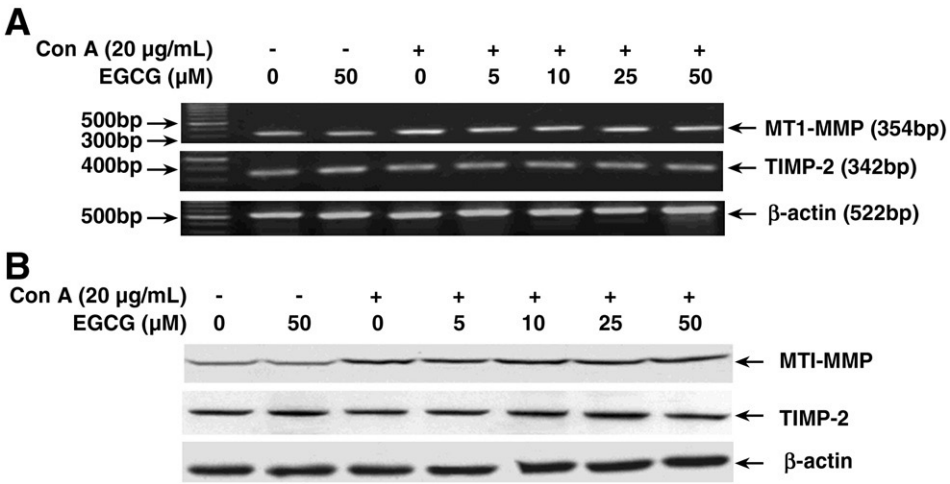

Fig. 8. EGCG does not affect MT1-MMP and TIMP-2 expressions in HSCs. HSCs were cultured with ConA in the presence or absence of EGCG for 24 h. Thereafter, the expression of pro-MT1-MMP and TIMP-2 mRNA was assessed by RT-PCR (A). Protein in cell lysates was assessed by Western blot analysis (B). Results from three independent experiments were similar.

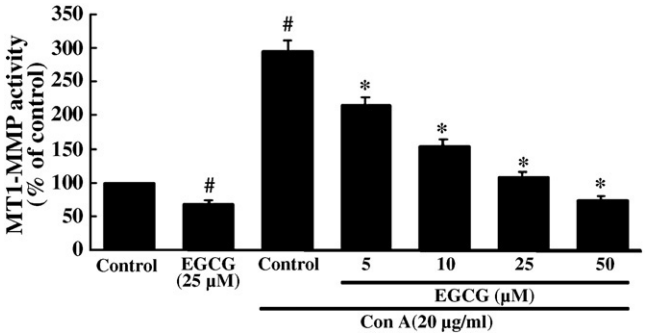

Fig. 9. EGCG inhibit MT1-MMP activity in HSCs. HSCs were cultured with or without ConA in the presence or absence of indicated concentrations of EGCG for 24 h. Thereafter, MT1-MMP was extracted from cells and its activity was determined using an enzymatic assay detailed in Materials and methods. Data represent mean±S.E.M. of three independent experiments. $^{\#}P<.05$ vs. control; $^{*}P<.05$ vs. ConA treatment.

hepatic fibrosis in a rat model of CCl_4-induced liver fibrosis. This result was confirmed both by liver histology as well as quantitative measurement of the hepatic hydroxyproline content, a marker of collagen deposition in liver.

Oxidative stress has been thought to be a major cause of CCl_4-induced liver injury, in which CCl_4 is metabolized by cytochrome P450 in liver cells to yield the trichloromethyl free radical. These radicals cause lipid peroxidation, which produces hepatocellular damage and enhanced production of fibrotic tissue [5]. The results of the present study demonstrate that treatment of rats with EGCG had a markedly protective effect against CCl_4-induced hepatotoxicity in rats, as evidenced by decreased serum AST and ALT activities. The hepatic concentration of TBARS, an index of lipid peroxidation, in CCl_4-administred rats increased significantly as reported previously [6]. In contrast, EGCG treatment significantly suppressed the increase of TBARS formation induced by CCl_4 in rats. GSH acts as an antioxidant both intracellularly and extracellularly in conjunction with various enzymatic processes, multiple intracellular functions including detoxification of reactive oxygen intermediates, and reduction of low-molecular-weight thiols and sulfides and mixed disulfides of proteins [29]. It has been suggested that the lipid peroxidates generated after intoxification are eliminated by GSH peroxidase in the presence of GSH [30]. In the current study, we found that there is a significant decrease in hepatic GSH levels in CCl_4-administrated rats, whereas EGCG treatment significantly increased hepatic GSH levels. Consistent with our findings, EGCG attenuates oxidative stress by increasing the level of GSH in cultured HSCs [31]. In vitro studies have also demonstrated that the hepatocyte protective action of EGCG is mainly due to its antioxidative effects [32–34]. Recently, Chen et al. [16] have shown that a single-dose EGCG injection improved hepatic injury in rats induced by CCl_4 administration through the inhibition of lipid peroxidation and reduction of proinflammatory nitric oxide-generated mediators. Taken together, these findings indicate that EGCG exerts a therapeutic effect on CCl_4-induced liver fibrosis in rats, possibly through its antioxidant action.

In the present study, we found that EGCG inhibited rat liver fibrosis development along with suppression of

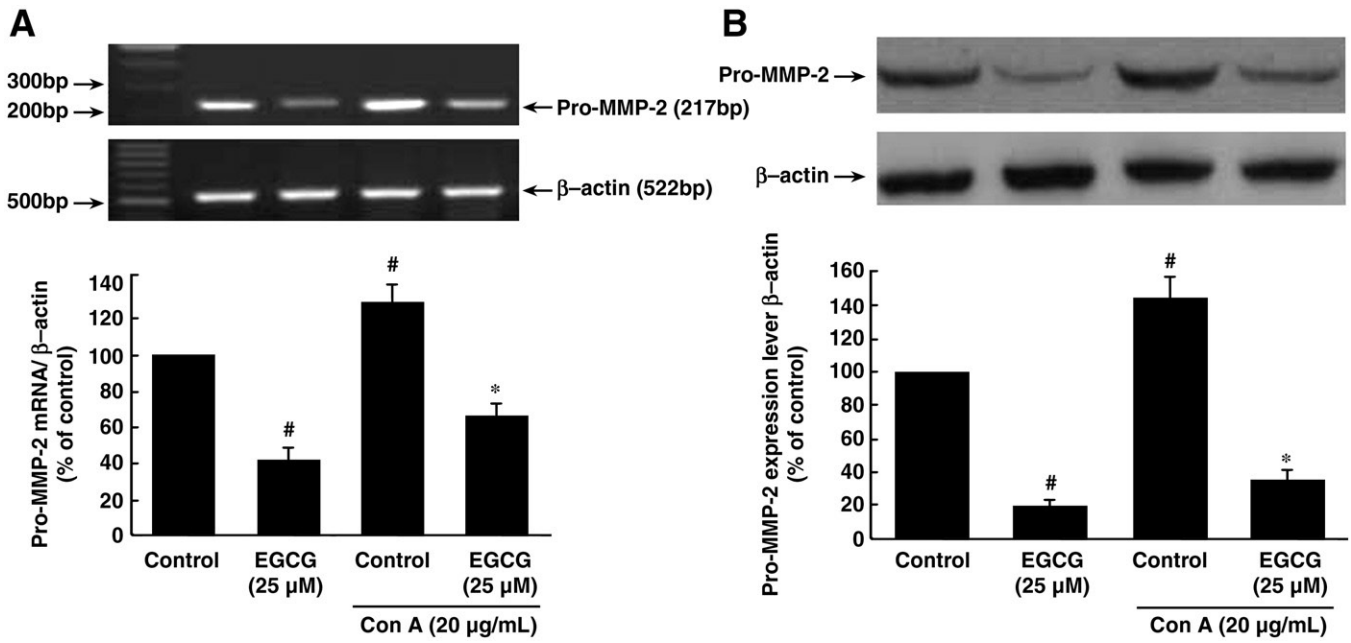

Fig. 10. EGCG inhibits the expression of pro-MMP-2 expression in HSCs. The expression of pro-MMP-2 mRNA was assessed by RT-PCR analysis (A). Expression of pro-MMP-2 protein in cell lysates assessed by Western blot analysis (B). (A) Representative RT-PCR showing pro-MMP-2 mRNA (top), β-actin (center) and cumulative data (bottom). (B) Representative Western blots showing pro-MMP-2 protein levels in cell lysates (top), β-actin (center) and cumulative data (bottom). Results depict the mean±S.E.M. of three independent experiments. $^{\#}P<.05$ vs. respective control; $^{*}P<.05$ vs. ConA treatment.

α-SMA-positive cells. α-SMA is one of the well-known markers of activated HSCs, suggesting that EGCG might attenuate HSC activation. Previous studies have shown that oxidative stress is associated with liver fibrosis and activation of HSCs either directly or through paracrin stimulation by injured hepatocytes [35,36]. Therefore, EGCG may prevent fibrosis through inhibiting oxidant-dependent activation and proliferation of HSCs.

MMP-2 expression and increased activity is one of the major causes of hepatic fibrosis. Increased MMP-2 activity is believed to be associated with an increase in destruction of the normal liver architecture, promoting its replacement by interstitial collagen [7–9]. In the present study, we revealed that the hepatic levels of MMP-2 activity were significantly decreased following EGCG treatment of CCl_4-administred rats. Since the cellular source of MMP-2 in the liver is thought to be the HSCs [37], therefore, we investigated the effects of EGCG on MMP-2 expression and activation in cultured rat HSCs. Our results showed that EGCG suppressed the expression of endogenous MMP-2 protein and mRNA expression, and, more importantly, the activation of the secreted MMP-2.

MMP-2 is secreted as an inactive proenzyme. Several reports have demonstrated that activation of pro-MMP-2 at the cell surface through a trimolecular complex that includes MT1-MMP and TIMP-2. MT1-MMP complexed with TIMP-2 serves as a cell surface receptor for pro-MMP-2 by promoting its pericellular proteolysis and consequent activation [10,38]. However, excess TIMP-2 was observed to specifically inhibit both MMP-2 gelatinolytic activity and pro-MMP-2 activation by MT1-MMP [39]. Our results suggest that the prevention of MMP-2 activation by EGCG is likely to be mediated by the direct inhibition of MT1-MMP activity. A similar result in human vascular smooth muscle cells was reported [13], but the mechanism remains unknown. Previous studies have demonstrated that MMP-2 expression requires the activation of nuclear transcription factor-κB (NF-κB) [40]. Since EGCG prevents the activation of NF-κB [41], its effect on MMP-2 expression is quite likely achieved through this inhibition.

MMP-2 activation is a critical pathophysiological mechanism relating to survival of HSCs mediated during the response to oxidative stress [42,43]. Previous studies have shown that GSH markedly inhibits MMP-2 activity in transformed fibroblast cells [44] and GSH appears to be an effective inhibitor of MMP in cold preserved liver allografts [45]. Therefore, the partial suppression of MMP-2 activity following EGCG treatment of CCl_4-administred rats may be explained by attenuation of oxidative stress induced activation of HSCs after liver injury.

Oxidative stress may be a common factor in chronic liver diseases of different etiologies [2]. Many agents have been proposed for the prevention and treatment of fibrosis. However, there is no established therapy for the resolution of hepatic fibrosis. New clinical approaches need to be developed to improve the efficiency of current treatments. The antioxidant potential of EGCG is far grater than that of vitamin E and/or C [46], which might allow it to succeed where other antioxidants have failed in preventing hepatic fibrosis. In vitro studies have shown that EGCG exerts antifibrogenic effects by decreasing the synthesis of type I collagen, by reducing cell proliferation and by inducing apoptosis on cultured HSCs [17–19]. Taken together, these findings support the suggestion that EGCG is a promising agent for the treatment of hepatic fibrosis.

In summary, our results demonstrate that treatment with EGCG markedly decreased the development of hepatic fibrosis, and this was associated with changes in the redox state and MMP-2 activity of the liver tissue. We also demonstrate that EGCG strongly inhibited the pro-MMP-2 expression as well as the conversion of pro-MMP-2 into its activated form, through the direct inhibition of MT1-MMP activity in cultured HSCs. Therefore, the administration of EGCG may be an optional therapeutic and preventive measure against oxidative stress-induced liver injury and hepatic fibrosis.

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