Alteration of AP-endonuclease1 expression in curcumin-treated fibrotic rats

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ABSTRACT

Background. Apurinic/apyrimidinic endonuclease1/ redox factor-1 (APE1/Ref-1) is a multifunctional protein involved in DNA base excision repair and redox regulation of many transcription factors. It is an important pro-survival protein activated in response to oxidative stress. Increased level of this essential redox sensitive protein correlates closely with cellular survival against oxidative insults. Curcumin (diferuloylmethane) a naturally occurring compound derived from turmeric has attracted interest because of its anti-inflammatory, anti-oxidative, and chemopreventive activities. Material and methods. The current study evaluates the in vivo role of curcumin in protecting and treating liver injury and fibrogenesis caused by carbon tetrachloride (CCl4) in rats. It also addresses the possible involvement of the multifunctional protein APE1 in hepatoprotection. Analysis of APE1 expression was performed at mRNA and protein levels by reverse transcriptase (RT)-PCR and western blotting respectively. Profile of HSCs-activation related genes were assayed by RT-PCR and pro-inflammatory cytokines levels were determined by enzyme-linked immune assays. Results. Here we show that oral administration of curcumin was accompanied by a robust increase in APE1 protein and mRNA levels, and improved the histological architecture of rat liver. In addition, curcumin attenuated oxidative stress by increasing the content of hepatic glutathione within normal values, leading to the reduction in the level of lipid hydroperoxide. Curcumin remarkably suppressed inflammation by reducing levels of inflammatory cytokines, including tumor necrosis factor-α (TNF-α), nuclear factor-kappa B (NF-κB) and interleukin-6 (IL-6). It also inhibited hepatic stellate cells (HSCs) activation by elevating the level of PPARγ and reducing the abundance of transforming growth factor-β (TGF-β). We found that oral administration of curcumin at 200 mg/kg dose not only protected against CCl4-induced hepatic injury, but also resulted in more than two-fold induction of APE1 protein expression in CCl4-induced rat group. Conclusions. It can be concluded that curcumin reduced markers of liver damage in rats treated with CCl4, with concomitant elevation in APE1 protein level indicating a possible protective effect with unknown mechanism. The induction of DNA repair enzymes may be an important and novel strategy for hepatic protection against oxidative injury.


INTRODUCTION

The pathogenesis of liver fibrosis is not yet completely understood. Hepatic fibrosis is due to an imbalance in extracellular matrix (ECM) breakdown and deposition.1-3 Research over the past two decades has established that hepatic stellate cells (HSCs) are the primary ECM-producing cell type during hepatic fibrogenesis.1,2 HSCs activation, characterized by enhanced cell proliferation, overproduction of ECM and de novo synthesis of α-smooth muscle actin, is triggered by the release of mitogenic platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) from activated HSCs and fibrogenic transforming growth factor (TGF-β1), mostly from Kupffer cells1,2 as well as the dramatic down-expression of peroxisome proliferator-activated receptor-γ (PPARγ).4-6 The alteration in PPARγ activity plays an important role in HSCs activation.7,8

Oxidative stress has been recently recognized as a fundamental factor in the pathogenic changes observed in liver fibrosis. It plays an important role in pathophysiological changes that progress to liver cirrhosis.
Cellular response to oxidative stress is a highly regulated and complex biological process. APE1 is a ubiquitous and remarkably multifunctional protein. It plays a central role in the base excision repair (BER) pathway for damaged bases and DNA single-strand breaks induced by reactive oxygen species (ROS) and alkylating agents, and hence is an attractive target for possible hepatoprotection, as it is a critical component of the oxidative stress up-regulated proteins. To prevent ROS accumulation, cells have several antioxidant systems including enzymes and redox-sensitive molecules (Trx, APE1/Ref-1) that protect the cells from oxidative stress. Activation of APE1/Ref-1 increases the binding of oxidative stress regulating transcription factors (Hif-1, p53, Ap-1). In aged rats, APE1/Ref-1 is decreased and responses to stress in the brain are defective. There are many studies on the relationship between p53 and APE1/Ref-1.

Discoveries during the past 2 decades have begun to clarify genetic regulation of fibrogenesis and have thereby indicated areas of potential therapeutic intervention. Therefore, the development of interventions designed to impede or reverse hepatic fibrosis is of high priority and is urgently needed. Most evolving antifibrogenic therapies will be aimed at inhibiting HSCs activation. The antioxidant curcumin, a polyphenolic antioxidant purified from turmeric, is an important and widely used spice of the diet in several countries. It has received attention as a promising dietary supplement for the treatment of hepatic fibrogenesis.

In vitro, curcumin induces apoptosis and inhibits activation and proliferation of HSCs. In addition, it prevents formation and development of the extracellular matrix by inhibiting collagen α1(I), fibronectin and α-smooth muscle actin gene expression, by enhancing matrix metalloproteinase-2 and -9 expression and suppressing CTGF expression. In addition to several intracellular signaling pathways are modulated by curcumin in HSCs.

The current study was designed to investigate the prophylactic effects and the mechanisms of curcumin on liver fibrosis in rats, and the concomitant modular expression of APE1 and thus to explore the possible relationship between them.

MATERIALS AND METHODS

Animals

Seventy male Sprague-Dawley rats, weighing 90-120 g were used in this study. The animals were maintained under controlled temp (25 ± 2 °C) and constant periodic conditions (12 h light, 12 h dark). The dams had free access to water and to standard food (20% protein, 54% carbohydrates, 4% lipid, 7% ash, 10% moisture). The principles of laboratory animal’s care were followed in all experimental protocols and were approved by ethics committees for animal’s research at Faculty of Medicine, Alexandria University.

Induction of hepatic injury and fibrogenesis using CCl₄

Rats were divided into three groups: control group (group 1) received subcutaneous injection of empty vehicle, CCl₄-induced group (group 2) received subcutaneous injection of CCl₄ (0.3 mL/kg body weight) once per week for 4 weeks followed by twice injections weekly for 12 weeks) and pre-cur & CCl₄-induced (group 3) where rats were administered curcumin (200 mg/kg) by gavage tube twice weekly for 4 weeks before CCl₄ administration as in group 2.

At the end of fibrosis-induction period, 10 rats from groups 2 and 3 were treated by curcumin administration (200 mg/kg; suspended in sterile PBS) for 8 weeks.

The study aimed to investigate the molecular changes caused by CCl₄-induced fibrosis and curcumin treatment in a dose- and time-dependent manner; therefore three rats from each group (groups 1, 2 and 3) were sacrificed by cervical dislocation at time intervals of 6, 10 and 16 weeks following CCl₄ injection and at weeks 20 and 24 following curcumin treatment (i.e. after 4 and 8 weeks of curcumin treatment respectively). Blood sample from each sacrificed rat was collected by heart puncture. Sera were collected and stored at -80 °C for liver function biochemical analyses (ALT, AST, albumin, total protein, and bilirubin).

Liver tissues were removed from each sacrificed rat; small portion was fixed in 10% neutral-buffered formalin (NBF) for histological analysis and the remaining tissue was immediately frozen in liquid nitrogen then stored at -80 °C.

Histopathological analysis

The fixed liver tissues in formalin were dehydrated in ascending grades of alcohol, and then cleaned by immersing the tissues in xylene for 1 h (three times), followed by impregnation in melted paraffin, in wax, then dried at oven for 1 h. Then the specimens were embedded in paraffin and were left to so-
lidify at room temperature. Using a rotatory micro-
tome, multiple sections of 5 µm thick were cut
and were mounted on clean glass slides. All sections
were stained with hematoxylin and eosin (H&E)
and examined for any histopathological changes. Pathological diagnosis of each liver specimen was
assessed and graded from 0 to VI by two patholo-
gists in a blinded manner according to the criteria
described by Wang, et al.23

Analyses of hepatocytic
death and hepatic injury parameters

Blood was collected from each rat by heart punc-
ture when sacrificed. After coagulation, sera were
collected and stored at -20 °C for further analyses.
Activities of serum aspartate aminotransferase
(AST), alanine aminotransferase (ALT), total pro-
tein and albumin levels in sera were analyzed using
routine laboratory assay kits.

Determination of lipid peroxidation

Part liver tissue (10% w/v) was washed with sali-
ne solution, minced and homogenized in ice cooled
buffer 50 mm potassium phosphate pH 7.5, the ho-
mogenate was centrifuged at 10,000 rpm for 20 min
at 4 °C, and the supernatant was used for the as-
say. Level of lipid per oxidation was determined ba-
sed on Ohkawa, et al.,24 colorimetric method.
Thiobarbiutic acid (TAB) reacts with malondialde-
hyde (MDA) in acidic medium at temp 95 °C for 30
min to form thiobarbiutic acid reactive product the
absorbance of the resultant pink product can be
measured at 534 nm and is expressed in nmol/
g.tissue.

Determination of reduced glutathione (GSH) and
 glutathione peroxidase activity (GPX)

Part liver tissue (10% w/v) was washed with sali-
ne solution, minced and homogenized in ice cooled
buffer1.15% kcl, 0.01 M sodium phosphate buffer pH
7.4 the homogenate was centrifuged at 10,000 rpm for 20 min
at 4 °C , and the supernatant was used for the as-
say. The concentration of GSH was determined as described by Eliman;22 Eliman’s reagent (5,5’, dithiobis (2-
nitrobenzoic acid), DTNB) was reacted with GSH
giving 2-nitro-5-thiobenzoic acid , a yellow colored
product with a maximum absorbance at 412 nm, GSH level is expressed as mg/g liver.

Total glutathione peroxidase (Gpx) was determi-
ined spectrophotometrically using cumene hydroper-
oxide as substrate. The excess amount of reduced
 glutathione was determined using Ellman method.
Gpx activity were calculated as (µg/min/mL), the
consumed GSH (µg/mL)/time of incubation (min).

Total RNA isolation and reverse transcriptase
polymerase reaction (RT-PCR)

Total RNA was extracted from frozen liver tissues
according to Chomczynski and Sacchi procedure26
using GStractTM RNA Isolation Kit II Guanidinium
Thiocyanate method. The extracted dried pellets
were resuspended in 50-100 µl RNAase free H2O and
stored at -80 °C. Alterations in the steady–state
mRNA levels of genes relevant to hepatic stellate ce-
lls (HSCs) activation and fibrosis development in
CCl4 induced rats is determined using reverse-trans-
criptase PCR analysis. Using one-step RT-PCR (RT/
PCR Master Mix. Gold Beads, BIORON) reaction,
the cDNA was synthesized and used for amplifica-
tion of target gene(s) using specific primer sets.
Briefly, total RNA (1-3 µg) and random primer (3
µM) mixture were denatured at 70 °C for 5 min and
placed on ice. The incubated mixture was added to
the RT/PCR Gold mix that contains all the compo-
nents necessary for cDNA synthesis and amplifica-
tion in one tube. The cDNA synthesis reaction was
performed at 42 °C for 60 min then 5 min at 94 °C
for RTase inactivation. The primers then subjected
to PCR cycles. Annealing temperature and time need
to be optimized for each primer/template combina-
tion. We investigated the expression of pro-fibrotic
genes that is induced by activated hepatic stellate ce-
lls (HSCs) (TGF-β, CTGF, TIMP-1, STAP and α-
SMA) vs. anti-fibrotic genes (MMP-2 and PPARγ) as
well as the expression of rat albumin, APE1 and
p53.

The sequences of the primers set for

- TTMP: F-GCCCCAACCCACCCACAGA- R-TTT-
  GCAAGGGATGGCTGAACAG (405 bp).
- MMP-2: F-GAGAAAAAGGCACGGGTGAGTACG-
R- TTTCCCCGCAAGGCCAATG (145 bp).
- CTGF: F-ATCCCTGCGACCCACACAG- R-
CCTCATCGCCTATGCCGTAAG (450 bp).
- TGF-β-1: F-CGGAAGCAGTGCCAGAA- R-
CAACTGCTTTGGAAGGTCG (470 bp).
- APE-1: F-CTGCGGACTTCTCTCATCAATAC-
R-CTCTATCGCCTATGCCGAACG (200 bp).
- p53: F-CACACGGATATGACAGCAGCATC- R-GTCG-
TCCAGAATGCATCC (600 bp).
- α-SMA: F-ACTGGGACGACATGGAAA AG- R- CATCTCCAGAGTCACAGACA (280 bp).
- STAP: F-ATGGAGAAAGTGCCGGGGAC- R- TGGCCCTGAAAGGCACTGT (340 bp).
- Rat-albumin: F-GCTCTGAGACTGCCTGTGT- R-ACAAGGTTGGCCCTCAAG (400 bp).
- GAPDH: F-TGCTGGTGCTGACCTGTGT- R-ACAAGGTTGGCCCTCAAG (400 bp).

Nuclear proteins extraction

Nuclear extract was prepared as described by Schreiber et al.27 500 mg of frozen tissue was homogenized in 5 mL of ice-cold extraction buffer A (0.6% NP-40, 150 mM NaCl, 10 mM HEPES pH 6.9, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride). The homogenate was centrifuged at 2,000 rpm for 30 s at 0 °C. After incubation for 5 min on ice, the supernatant was centrifuged at 5,000 rpm for 5 min at 0 °C. The supernatant is the cytoplasmic fraction was collected and stored at -80 °C. The precipitate was resuspended in extraction buffer B (25% glycerol, 420 mM NaCl, and 20 mM HEPES pH 7.9, 1.2 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 5 mg/mL each of aprotinin, leupeptin, and pepstatin). After being kept on ice for 20 min, it was transferred to a centrifuge tube and centrifuged at 12,000 rpm for 25 min at 0 °C. The supernatant was transferred to a new tube and stored at -80 °C.

Cytokines determination

IL-6, TNF-α, and TGF-β levels were assayed in total cell extracts prepared from liver tissues and

Figure 1. Representative microscopic photograph of liver of CCl₄-induced or curcumin-treated rats by hematoxylin-eosin (H&E) staining. A-0 to A-16 shows the histological findings of the experimental rats of group 2 that was fibrosis-induced with CCl₄ injection for 16 weeks. The results are represented at time intervals of 0, 6, 10, and 16 of CCl₄ injection and at week 24 which represents the result after 8 weeks of curcumin treatment of fibrotic liver. (0) Represents rat’s liver section of normal control. A-24 shows fibrosis-induced liver that has been treated with curcumin for 8 weeks. B-0 to B-16 shows results of group 3 that was pre-cur & CCl₄-induced for 16 weeks. Treatment with curcumin before CCl₄ injection has protective effect on rat liver as represented in figure B during the follow up weeks. B-24: Sections of curcumin pre-treated, fibrosis-induced then curcumin treated rats for 8 weeks (week 24) showing thick wall of portal track collapsed with congested hepatocytes cell forming pseudo nodules, rest of hepatocytes was vacuolated.
sera samples. Cytokines levels were determined using commercially available kits. The analyses were performed according to the manufacturers’ guidelines. IL-6 level assayed using BioLegend ELISA MAX™ Set Standard Rat IL-6 Kit (Cat.430502). TNF-α was determined using Rat TNF-α Frprotech kit (cat# 900-K25) and TGF-β using Quantikine kit (Cat# MB100B). Nuclear factor-κB p50/65 DNA-binding activity in nuclear extracts of hepatic tissue samples was evaluated. Analysis was performed according to manufacturer’s protocol guidelines using commercial Kit (NF-κB p50/65 Transcription Factor Assay Colorimetric. Cat. No. SGT510 Themicon International Inc. Temecula, CA, USA).

**Western blotting**

According to the method described by Burnette, APE1 and p53 immunoblots were performed on prepared cytosolic and total cell extracts respectively. Briefly, 50 µg protein lysates from each sample were separated on 12% SDS-PAGE then transferred to a nitrocellulose membrane. Primary antibody to APE1 (rabbit Polyclonal anti-APE1) and p53 (sc-DO-1, Santa Cruz Biotechnology) was used. Then incubated with HRP-conjugated secondary antibodies (Santa Cruz; diluted).

**Hydroxyproline (hyp) assay in rat liver**

A colorimetric assay was performed as described by Nakamura, et al. Briefly, lyophilized liver sections (0.5 g) were hydrolyzed for 20 h in 6 mol/L HCl at 100 °C, redissolved in water, and centrifuged to remove any impurities. Samples were incubated for 10 min in 0.05 mol/L chloramine-T (Fisher, Fair Lawn, NJ, USA) at room temperature, followed by 15-min incubation in Ehrlich’s-perchloric acid solution at 65 °C. Sample absorbencies were assessed at 560 nm and resulting values compared to a hyp standard curve. Each sample was assayed in duplicate. The hyp content was expressed as micrograms per gram of wet liver.

**Statistical analysis**

All experiment was repeated two or more times independently and typically graph are represented. In some cases data are presented as mean ± SD. Data were analyzed by student T-test and different considered significant at p < 0.05 or at p < 0.01.

**RESULTS**

**Pre-treatment with curcumin ameliorated CCl₄-induced liver injury through apoptosis**

Representative microscopic photographs of liver sections stained with Hematoxylin-eosin (H&E) staining of CCl₄-induced and pre-cur & CCl₄-induced groups then treated are shown in figure 1. We assessed the fibrosis degree (score) according to the pathological index described by Wang, et al. Stained and examined liver sections of CCl₄-induced rats were graded from score 0 (at week zero, control), II (at week 6), III (at week 10) and from IV to V, (at week 16). At score IV-V the CCl₄-injection was stopped and curcumin treatment was started.

Pre-treatment with curcumin for 4 weeks has protective effect on rat liver as represented in the stained sections in figure 1-B where the fibrosis grade assessment was I at week 6, II at week 10, III to IV at week 16 of CCl₄-injection compared to advanced progression that has been observed in CCl₄-induced rats of group 2. Appearance of area of apoptotic cells in different stages of pre-cur & CCl₄-induced induced group was observed as well. Sections of fibrotic rats (group 2) that has been treated with curcumin for 8 weeks indicated portal track collapsed with congested hepatocytes cell forming pseudo nodules, rest of hepatocytes was vacuolated.

The general conditions of rats improved after curcumin treatment within 14 days as assessed by change in the body weight and hyp content. During fibrogenesis induction, the body weight of group 2 rats dramatically reduced within the first 6 weeks (the mean loss body mass was 35.40 ± 8.34 g) and then slowly increased within 6-16 week (increased by 19.44 ± 6.78 g). From week 20, the body weight of rats slightly increased in treatment group with no obvious changes in control group, showing that rats become adapted to toxicity. In addition, hyp content significantly (p< 0.05) attenuated in wet liver of pre-cur & CCl₄-induced group to 396.8 ± 116.3 mg/g liver compared to 566.0 ± 237.4 mg/g liver in CCl₄-induced.

**Curcumin reduced CCl₄-induced hepatocytes toxicity and injury**

Curcumin pre-treatment ameliorated the significant elevation in sera ALT and AST activities compared to CCl₄ injected rats without curcumin
pre-treatment (Figure 2A and 2B). At weeks 20 and 24 (curcumin treatment for 4 and 8 weeks) the results indicated significant (p < 0.05) reduction in the ALT and AST serum activities in both groups. Also pre-treatment with curcumin induces less reduction in total serum protein and albumin levels compared to CCl₄-induced rats (Figure 2C and 2D). Alterations in albumin mRNA level as a marker of hepatocytes synthetic function was found to be highly reduced in liver tissues of CCl₄-induced rats compared to pre-cur & CCl₄-induced rats (Figure 7). Curcumin treatment for 4 and 8 weeks of fibrotic liver, restored hepatocytes function as indicated by significant elevation (p < 0.05) in sera total proteins and albumin levels as well.

Figure 2. Changes in liver function indices. The activities of serum AST and ALT as well as the total protein and albumin levels were analyzed during CCl₄-induced fibrosis and curcumin. A significant elevation in AST and ALT activities were observed in all groups. In pre-cur & CCl₄-induced rat curcumin protected rat liver from CCl₄-induced in serum elevated ALT and AST activities as well as reduction in total protein and albumin levels compared to the CCl₄-induced group without curcumin pre-treatment (group 2; CCl₄-induced). Curcumin administration for 4 and 8 weeks (at weeks 20 and 24), restored hepatocytes function as indicated by significant elevation (p < 0.05) in sera total proteins and albumin levels as well.
Curcumin treatment reduces oxidative stress in the liver of CCl₄-induced rats

Curcumin treatment reduced lipid hydroperoxide (calculated as MDA level) production in the liver of CCl₄-induced rats. A significant increase in MDA level during CCl₄-injection vs. the pre-cur & CCl₄-induced experimental rats was observed. Treatment of fibrotic rats with curcumin was shown to significantly reduce the lipid peroxidation level (p< 0.05) in both groups at weeks 20 and 24 of the follow up period. Figure 3A)

GSH level and GPX activity were reduced significantly in CCl₄-treated rats, while pretreatment with curcumin (pre-cur & CCl₄-induced) reduced the CCl₄-mediated oxidative stress. Treatment with curcumin for 4 and 8 weeks significantly elevated (p <0.05) the GSH level and GPX activities in both groups.

Modulation of sera and liver inflammatory cytokines levels during the course of fibrosis induction and curcumin treatment

Hepatic fibrogenesis is commonly associated with inflammation. Levels of inflammatory cytokines; TNF-α, TGF-β, and IL-6 were determined in serum
and liver homogenates. TNF-α, TGF-β, and IL-6 levels were elevated significantly (p < 0.05) during the 16 weeks of fibrosis induction compared to the control group (Figures 4 and 5). Pre-treatment with curcumin reduced the CCl₄-induced HSCs activation and inflammation as indicated by marked reduction in TNF-α, TGF-β, and IL-6 levels during the course of fibrosis induction compared to CCl₄-induced rats. Curcumin treatment of both groups (group 2 and 3) induced-hepatocytes regeneration and inhibition of HSCs activation and inflammation as indicated by significant reduction in sera and total cell extracts TNF-α, TGF-β, and IL-6 levels (p < 0.05) at weeks 20 and 24. In addition, NF-κB level in nuclear extracts of CCl₄-induced and curcumin pre- and post-fibrosis treated rats showed a significant

Figure 4. Time course study of the change in TNF-α and TGF-β levels in sera and total cell extracts of CCl₄-induced and curcumin-treated groups. Data indicate significant elevation (p < 0.05) in both TNF-α (A) TGF-β (B) levels sera samples and cell extracts during the follow up weeks fibrotic rat liver versus control group. Pre-cur administration ameliorated the CCl₄-induced hepato-toxicity as indicated in group 3 compared to group 2. A significant reduction (p < 0.05) in both cytokines levels was observed at weeks 20 and 24.
elevation (p < 0.05) during fibrosis development in the rat liver vs. control group. Treatment by curcumin reduced significantly (p < 0.05) NF-κB level at weeks 20 and 24 (Figure 5).

Curcumin modulates the expression of fibrotic vs. anti-fibrotic genes that are related to HSCs activation

We investigated the changes in the mRNA levels of pro-fibrotic genes (TGF-β, CTGF, TIMP-1, α-SMA, and STAP) and anti-fibrotic genes (MMP-2 and PPARγ) by reverse transcriptase-PCR. Previous reports showed that curcumin inhibited in vitro HSCs activation by suppressing expression of genes relevant to cell growth and fibrogenesis.19,30 Curcumin pre- and post-fibrosis induction treatment suppressed pro-fibrotic genes expression including TGF-β, CTGF, TIMP-1, α-SMA, and STAP and induced MMP-2. A marked elevation of TGF-β, CTGF, MMP-2, α-SMA, and STAP mRNA levels were observed as well (Figure 6). Pre-cur treatment ameliorated the CCl₄-induced HSCs activation and pro-fibrotic genes expression compared to rats in group 2 (Figure 6); 8 weeks of curcumin treatment for groups 2 and 3 reduced pro-fibrotic genes expres-

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**Figure 5.** Time course study of IL-6 (A) and NF-κB (B) in the CCl₄-induced liver fibrosis and treated rats. Data indicate significant elevation (p < 0.05) in IL-6 during the follow up weeks 6, 10 and 16 in the total cell extracts vs. control. The pre-treatment with curcumin before CCl₄ injection protected the hepatocytes as indicated by change in IL-6 level when compared to samples of group 2. Curcumin injection attenuated hepatic stellate cells activation and reduced IL-6 significantly (p < 0.05) in total cell extracts and sera samples at weeks 20 and 24. Also a significant elevation (p < 0.05) in NF-κB during the follow up period of fibrosis development in the rat liver versus control was observed. Pre-treatment and treatment by curcumin showed significant reduction (p < 0.05) in NF-κB at weeks 20 and 24.

525

Fibrosis-induction weeks

Curcumin treatment

Time (week)

0 6 16 24

TGF-β
CTGF
TIMP-1
MMP-2
α-SMA
STAP
GAPDH

Reduction in p53 mRNA and protein levels were observed during the course of fibrosis induction associated with elevated TGF-β mRNA and protein expression (Figure 7). Curcumin pre-treatment has protected rat liver during the first 6 weeks possibly by inducing apoptosis as confirmed by the presence of apoptotic bodies in histological examination and by detection of p53 expression at both mRNA and protein levels.

**DISCUSSION**

CCl₄ is one of the most widely used hepatic toxins for experimental induction of hepatic fibrosis and cirrhosis in laboratory animals. CCl₄-induced fibrosis or cirrhosis in experimental animals resembled human cirrhosis in some aspects of morphology and pathophysiology. Thus, CCl₄-induced hepatic fibrosis due to stimulation of lipid peroxidation and the production of free radicals has also been used to assess the efficacy of anti-fibrotic reagents and

**Curcumin anti-fibrotic effect may be correlated with alteration in APE1 mRNA and protein levels**

In the present study we report for the first time that APE1 protein and mRNA levels are reduced significantly (p < 0.01) in APE1 both protein and mRNA levels from weeks 6 to 16 in CCl₄-induced (20 to 6% and 44 to 20% respectively) and pre-cur & CCl₄-induced groups (70 to 28% and 95 to 50%, respectively) was observed. Upon curcumin treatment a significant (p< 0.01) re-elevation in APE1 mRNA and protein levels was observed (30 and 95% in CCl₄-induced and 66 and 70% in pre-cur & CCl₄-induced for mRNA and protein levels respectively).

**Figure 6.** Reverse transcriptase (RT)-PCR analyses of fibrosis vs. anti-fibrotic genes profile. The alterations in the mRNA levels were analyzed by RT-PCR. Curcumin modulates the expression of genes related to HSCs growth and fibrogenesis. mRNA levels of genes related to fibrogenic transformation, including, TGF-β, CTGF, TIMP-1, α-SMA, and STAP were significantly elevated during the CCl₄-injection weeks. On the contrary, marked reduction in MMP-2 was observed. Curcumin treatment for 8 weeks reduced pro-fibrotic genes expression (TGF-β, CTGF, TIMp-1, α-SMA, and STAP) in both groups and induced increase in MMP-2 level. GAPDH was used as an invariant internal control.
Figure 7. The change in p53 and APE1 mRNA and protein levels in CCl₄-induced fibrogenesis. A and B. Show the results of group 2 (CCl₄-induced) and group 3 (pre-cur treated & CCl₄-induced) of mRNA and protein levels respectively. Elevation in p53 mRNA level at weeks 6 to 16 of Cur- pre-treated & CCl₄-induced was detected. In addition, APE1 level is elevated at week 6 due to CCl₄-induced oxidative stress, its level is reduced significantly at week 16 and upon curcumin treatment for 8 weeks (week 24) a significantly induced APE1 mRNA and protein level (A and B) was observed. Moreover PPARγ, which plays crucial role during in vivo liver injury mRNA is reduced markedly during CCl₄-induced fibrogenesis in group 2 compared to less reduction in pre-cur treated & CCl₄-induced rats. Curcumin treatment attenuated the HSCs and possibly mediated an increase in APE1 and PPARγ mRNA levels at week 24. C and D. Represent the fold change in APE1 mRNA and protein levels in relative to GAPDH and β-actin levels respectively. Curcumin treatment may correlates with the significant (p< 0.05) expression of APE1 mRNA and proteins levels in cur- pre-treated & CCl₄-induced vs. CCl₄-fibrosis group. Representatives of three independent experiments are shown.

to verify correlation between pathophysiologic feature of the liver and serum marker of fibrosis.³³

In the present work, we have performed a study on a chronic model of liver fibrosis obtained by CCl₄ treatment combined with alcohol in drinking water during the last 4 weeks. We investigated therapeutic effects of curcumin on hepatic fibrosis and the variation of correlated cytokines. On this basis, oral administration of curcumin at 200 mg/kg dose significantly reduced elevated indices of liver, serum transaminases, and hyp contents in liver tissue. Histological examination showed curcumin evidently alleviated the progression of hepatic fibrosis. Therefore, intra-gavage administration of curcumin had apparently inhibitory effect on hepatic fibrosis induced by CCl₄ in rats.
Herein we present the relationship between the oxidative stress induced HSCs activation, hepatic injury and fibrosis development and APE1/Ref-1. In addition, we examined p53 activity during CCl4-induced liver fibrosis. We predicted APE1/Ref-1 would have an anti-fibrotic effect via effects on fundamental cellular antioxidant system. Our results indicated a significant elevation in the total antioxidant defense system activity with concomitant elevation in APE1 mRNA and protein levels at very early follow up time of fibrosis induction (weeks 2-4; data not shown). A significant reduction in APE1 mRNA and protein levels was observed during weeks 6, 10 and 16 of follow up period. The degree of reduction was milder in the group of rat pre-treated with curcumin before fibrosis induction (group 3).

Due to chronic liver injury and chronic HSCs activation, p53 level was elevated only in group 3, rats pre-treated with curcumin followed by CCl4 injection, consistently with appearance of apoptotic bodies in the microscopic examination.

Regulation of critical DNA repair enzymes such as APE1 in hepatic fibrosis presents a logical approach to therapeutic intervention against injury and fibrogenesis caused by carbon tetrachloride (CCl4); however, the functional significance of the DNA repair pathways in CCl4-induced hepatic fibrosis has not been directly tested. Although the mechanism was not completely clear, the present study suggests the unique finding that curcumin may act via induction of APE1 expression by unknown mechanism to afford protection to hepatic fibrosis.

APE1 is a critical cellular protein that performs multiple functions. In addition to its repair activity, APE1 acts as a transcriptional cofactor, as well as a suppressor of reactive oxygen species via a redox site. Many of these functions could lead to hepatoprotection by curcumin.

Results from histopathological analyses of rats pre-treated with curcumin before CCl4 injection (group 3) demonstrated area of apoptotic cells. In addition, results from determination of the level of hepatic injury-related biochemical markers indicated that the CCl4-elevated partially the levels of hepatocytes biochemical markers. Curcumin treatment of experimental rats of groups 2 and 3 for 8 weeks induced hepatocytes regeneration and improved histological and biochemical results significantly.

Lipid peroxidation and necrosis are significantly suppressed in the liver of animals supplemented with antioxidants. It was further demonstrated that de novo synthesis of GSH was a prerequisite for curcumin to inhibit HSC activation. In this study, we hypothesized that curcumin might protect the liver against CCl4-induced injury and fibrosis by attenuating...
ting oxidative stress. Curcumin attenuated oxidative stress as demonstrated by the reduction in the levels of lipid hydroperoxide in the CCl₄ rat model. These observations are supported by other studies. Our results in this study indicated that oral administration of curcumin not only increased the level of total hepatic GSH but also significantly improve activities of GST and catalase in the liver.

Pro-inflammatory TNF-α, IFN-γ, and IL-6 are major players in hepatic inflammation. TNF-α stimulates the development of hepatic fibrosis. IL-6 produced by activated HSCs facilitates the production of ECM, including type I collagen, leading to hepatic fibrosis. We reported in the present study that curcumin significantly reduced the levels of TNF-α, TGF-β and IL-6 in sera samples and the total cell extracts prepared form liver homogenates. As represented, sera and total cell extracts levels of TNF-α, TGF-β and IL-6 were significantly increased in the rats injected with CCl₄, and to a milder extent in the group of rats pre-treated with curcumin before CCl₄ injection vs. mock-treated group. Curcumin treatment for 8 weeks at the end of time course of fibrosis-induction, inhibited hepatic stellate cells activation and reduced the levels of TNF-α, TGF-β and IL-6 in sera samples and total cell extracts significantly (p < 0.05).

Further experiments revealed that curcumin enhanced the activity of antioxidant enzymes and modulated cytokines level by inducing expression of the genes. Activation of HSCs is triggered by various cytokines and chemokines, including the potent mitogens PDGF and EGF released from Kupffer cells and activated HSCs and oxidative stress. Results in this report further confirmed the in vivo effect of curcumin on the suppression of expression of these genes in the CCl₄ rat model demonstrated by semi-quantitative RT-PCR. In addition, HSCs activation coincides with a dramatic reduction in the abundance of PPARγ, which might play an important role in hepatic fibrogenesis.

It has been previously demonstrated that curcumin dramatically induced gene expression of PPARγ in activated HSCs. 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. It was of interest to verify that the treatment with curcumin diminished the role of CCl₄ in the reduction of the number of PPARγ-positive HSC in the rat model. Our results demonstrated that curcumin protected the rat liver from CCl₄-caused injury and fibrogenesis in vivo by suppressing hepatic inflammation, attenuating hepatic oxidative stress and inhibiting HSCs activation. The mRNA level of PPARγ was significantly reduced in the experimental rats of group 2 during the CCl₄-induced time course. Pre-treatment with curcumin prior to CCl₄ injection did not inhibit completely the expression of PPARγ compared to group 2. In addition, treatment with curcumin after fibrosis induction in both groups, significantly elevated PPARγ mRNA level. 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.

In agreement with our work, Chen, et al. confirmed the previous work suggesting that curcumin activation of PPARγ results in the inhibition of NF-κB activation in HSCs. In addition, they report that both NF-κB and the ERK MAP kinase pathway are required for the expression of CTGF, a key fibrogenic growth factor produced by HSCs. Furthermore, our results suggest and agree with this study that curcumin may suppress CTGF expression in HSCs by inhibiting the activation of NF-κB.

Collectively, our findings have important implications for understanding the pathophysiology of liver fibrosis and suggest that combined targeting of portal myofibroblasts activation could represent a central strategy to treat or delay the progression of chronic liver fibrosis. Curcumin can heal rat hepatic fibrosis. Effects of reducing the expression of correlated cytokines may be mechanisms of therapeutic effects of curcumin on hepatic fibrosis. This is in full agreement with recent studies.

Our results show that curcumin may have multiple targets in liver, including inhibition of HSCs activation and inhibition of fibrotic signaling in portal myofibroblasts, thereby modulating several central cellular events in a rat model of liver disease. Further studies will be devoted to understanding the causal role of APE1 activation in curcumin hepatoprotective function.

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