THE EFFECT OF CURCUMIN ON THE PROLIFERATION AND EXTRACELLULAR MATRIX PRODUCTION IN ETHANOL-INDUCED HEPATIC STELLATE CELLS

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ABSTRACT

Objectives: In various liver disease models, including those for alcoholic liver diseases, curcumin, a polyphenolic compound derived from *Curcuma longa*, is known to have an hepatoprotective effect. However, the mechanism of action underlying its effects on alcohol-induced hepatic fibrosis remains unknown. We aimed to investigate the mechanisms of action underlying the effects of curcumin, mainly involving the transforming growth factor (TGF)-β/Smad pathway.

Methods: Hepatic stellate cells (HSCs), LX2, were incubated with 50 mM ethanol with or without curcumin (1 and 10 µM). Viable HSCs were counted using a LUNA™ automated cell counter, whereas the expressions of TGF-β, Smad3, tissue inhibitor of metalloproteinases-1 (TIMP-1), and type 1 collagen mRNA were measured using quantitative reverse transcriptase polymerase chain reactions.

Results: Curcumin significantly suppressed ethanol-induced HSCs proliferation. The antiproliferative effect of curcumin appeared to be dose dependent. In addition, the mRNA expressions of TGF-β, Smad3, TIMP-1, and type 1 collagen decreased in the cells treated with curcumin.

Conclusion: Curcumin seems to attenuate ethanol-induced HSCs proliferation through the suppression of TGF-β and appears to reduce the production of extracellular matrix as shown by the decreased expression of type 1 collagen.

Keywords: Curcumin, Transforming growth factor-β, Smad, Tissue inhibitor of metalloproteinases-1, Type 1 collagen.

INTRODUCTION

Heavy alcohol consumption is a causal factor of multiorgan dysfunction, including alcoholic liver disease (ALD) [1]. The clinical manifestations of ALD may vary depending on the disease stage, which can range from steatosis (fatty liver) to alcoholic hepatitis (fatty liver with inflammation). The condition can continue to progress to liver fibrosis, cirrhosis, and hepatocellular carcinoma [2].

Liver fibrosis is an excessive accumulation of extracellular matrix (ECM) as a response to liver injuries. Hepatic stellate cells (HSCs) are known to play an important role in the development of liver fibrosis. HSCs remain inactive in the normal liver as retinoid storage cells and are activated during the processes leading to liver fibrosis. Many *in vitro* and *in vivo* studies have confirmed that chronic alcoholism induces HSC activation [3-5]. Activation of HSCs is characterized by increased cell proliferation, enhanced matrix production, and expression of a number of proliferative and profibrogenic cytokines and their receptors [5].

Transforming growth factor (TGF)-β is an important profibrogenic cytokine that plays a key role in HSC activation and proliferation. TGF-β inhibits ECM degradation by suppressing the matrix metalloproteinases (MMPs) and promoting its natural inhibitor, the tissue inhibitor of metalloproteinases (TIMPs) [6-8]. The most recognized pathway in myofibroblast formation and matrix production is the TGF-β/Smad pathway [7]. TIMP-1 is expressed by HSCs as a response to hepatic inflammation. The majority of these markers are involved in the TGF-β/Smad pathway through phosphorylation of Smad 2/3 and affect the transcription level in nucleus [9-11].

Curcumin is the major active substance of the plant *Curcuma longa*. Curcumin is a polyphenol, and it is well known for its anti-inflammatory activity in hepatocytes. Many studies show that curcumin exerts a hepatoprotective effect in various liver disease models, including ALD [12]. However, the underlying mechanism by which curcumin exerts its protective effects on alcohol-induced hepatic fibrosis remains unknown. In this study, we aimed to investigate the mechanism of action of curcumin on alcohol-induced hepatic fibrosis mainly through TGF-β/Smad pathway.

METHODS

Materials

Human immortalized HSCs (LX2) were obtained commercially from Merck Millipore (USA). Curcumin was purchased from Plamed, China. Absolute alcohol was purchased from Merck, USA. Dimethyl sulfoxide was purchased from Vivantis (Malaysia). Dulbecco's Modified Eagle's Medium (DMEM)-high glucose, fetal bovine serum (FBS), penicillin/streptomycin, and amphotericin B (Fungizone®) were purchased from Biowest (USA). MTS Assay Kit was purchased from Promega (USA). High Pure RNA Isolation Kit, Transcriptor First Strand cDNA Synthesis Kit, and FastStart DNA Master SYBR Green I kit were obtained from Roche (USA).

LX2 culture

Human HSCs, LX2, were cultured and maintained at 37°C in a 5% CO_2 air atmosphere in DMEM-high glucose supplemented with 10% heat-inactivated FBS, 10 U/L penicillin, 100 µg/mL streptomycin, and 2.5 µg/mL amphotericin B. Curcumin were given to the cells with or without alcohol.

Cells were seeded at 5000 cells per well in a 12-well plate and incubated for 72 h. Curcumin 1 and 10 µM were added to the cells with or without prior treatment with alcohol 50 µM for 24 h. The dose of curcumin was selected on the basis of our previous study by Lin *et al*. [13] in the cultured HSC from rats. After 24 h of curcumin treatment,

cells were harvested and counted for cell viability using Trypan Blue Exclusion Method. Harvested cells were isolated for total RNA and further synthesized to cDNA for real-time polymerase chain reaction (PCR) analysis. Experiments were done 3 times in duplicate.

Quantitative reverse transcription PCR analysis

Total cellular RNA was extracted from 1,000,000 cells following trypsinization, using High Pure RNA Isolation Kit (Roche, USA). Then, 1 μg of RNA was converted to cDNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche, USA). A quantitative real-time PCR was performed on 100 ng cDNA using FastStart DNA Master SYBR Green 1 kit (Roche, USA) according to the manufacturer's protocol with β-actin as housekeeping gene. Primer sequences for TGF-β-Fwd: 5'-TGAACCGGCCTTTCCTGCTTCTACATG-3'; TGF-β-Rev: 5'-GCGGAAGTCAATGTACAGCTGCCGC-3'; Smad3-Fwd: GTCTGCAAGAT CCCACCAG, Smad3-Rev: AGCCCTGGTTGACCGACT; collagen 1A1 (Col1A1)-Fwd: AGGTCCCCCTGGAAAGAA, Col1A1-Rev: AATCCTCG AGCACCCTGA; TIMP1-Fwd: GGCTGTGAGGAATGCACA; TIMP1-Rev: TGG AAGCCCTTTTCAGAGC; β-actin-Fwd: 5'GCTGGAAGGTGGACAGCGA-3'; β-actin-Rev: 5'-GGCATCGTGATGGACTCCG-3'.

Statistical analysis

Results were presented as mean±standard deviation. Statistical analysis was performed using one-way analysis of variance. Statistical significance was determined at the level of $p<0.05$.

RESULTS

Our results showed that alcohol increased the cell viability by approximately two-fold that of the control. Treatment with curcumin appeared to normalize cell viability; however, a higher dose of curcumin leads to a significant decrease of cell viability compared with the control (Fig. 1).

The increased cell viability was in accordance with the increase in TGF-β mRNA and Smad3 mRNA expressions. With curcumin treatment, the increases in TGF-β mRNA and Smad3 mRNA expressions approached normal values (Figs 2 and 3).

Treatment with alcohol resulted in an increase in the expression of Col1A1. Curcumin at a low dose did not reverse the increase in Col1A1 expression; however, a high dose of curcumin increased the expression of Col1A1 even more markedly than alcohol alone (Fig. 4).

The mRNA expression of TIMP increased with alcohol. Curcumin considerably decreased the mRNA expressions of TIMP-1, even to values below the normal (Fig. 5). However, no dose-dependent effect was observed.

DISCUSSION

It has been demonstrated in previous studies that curcumin had antifibrotic activity in many liver diseases, including ALD; however, the exact underlying mechanism remains unknown [14-17]. The TGF-β/Smad signaling pathway is a major pathway involved in fibrogenesis [7,8]; therefore, we have investigated whether curcumin exerts any effects on this pathway.

Many cytokines are involved in the development of fibrosis. TGF-β and platelet-derived growth factor (PDGF) are the two major cytokines involved in fibrogenesis. PDGF is a potent proliferative cytokine, and TGF-β is a potent fibrogenic factor that greatly influences the cellular processes such as differentiation, proliferation, apoptosis, and migration [7,18-20]. After alcohol consumption, the primary metabolite of ethanol and acetaldehyde induces hepatocyte destruction and the release of profibrogenic cytokines. Profibrogenic cytokines stimulate HSC activation through paracrine signaling. After HSC activation, these cells produce TGF-β and continued fibrogenesis through paracrine signaling [21,22]. Alcohol could directly activate HSC through the toll-like receptor-4 (TLR4) signaling pathway and thus increase

profibrogenic cytokine production [17].

Our study showed that there was a significant increase in proliferation of HSCs and the level of TGF-β mRNA expression under direct stimulation with alcohol. This result supports the mechanisms underlying the alcohol toxicity proposed in a previous study, in which the TLR4 signaling pathway led to the production of pro-inflammatory mediators and profibrogenic factor including TGF-β and PDGF. In the development of liver fibrosis, TLR4 is also expressed on recruited macrophages, hepatocytes, sinusoidal endothelial cells, and HSC [17]. Consequently, these cells can contribute to liver inflammation through TLR4 signaling, by releasing proinflammatory cytokines and chemokines. As a result, HSCs can also contribute to the development of alcoholic fibrosis by enhancing TGF-β signaling [17]. This mechanism explains the results of our study, in which the direct stimulation of HSCs with alcohol can
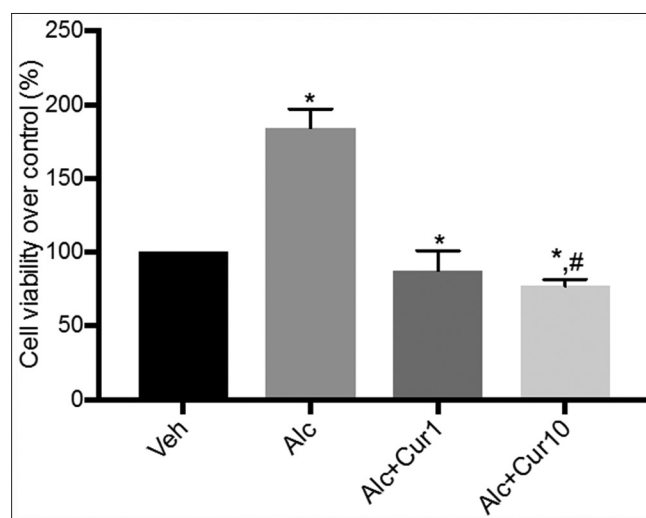

Fig. 1: Number of viable cells after treatment with vehicle only, alcohol, alcohol+curcumin 1 μM, and alcohol+curcumin 10 μM. Results are presented as mean±standard deviation, *p<0.05 versus vehicle, #p<0.05 versus alcohol. Veh: Vehicle; Alc: Alcohol; Alc+Cur1: Alcohol+curcumin 1 μM; Alc+Cur10: Alcohol+curcumin 10 μM
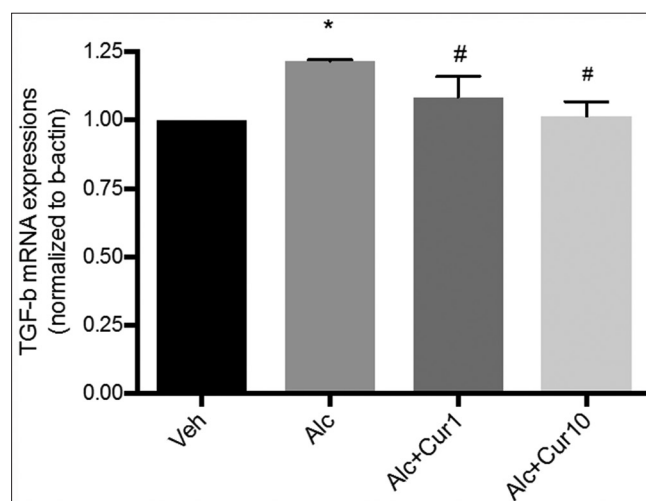

Fig. 2: Transforming growth factor-β mRNA expressions after LX-2 treatment with vehicle only, alcohol, alcohol+curcumin 1 μM, or alcohol+curcumin 10 μM. Results are presented as mean+standard deviation, *p<0.05 versus vehicle, #p<0.05 versus alcohol. Veh: Vehicle; Alc: Alcohol; Alc+Cur1: Alcohol+curcumin 1 μM; Alc+Cur10: Alcohol+curcumin 10 μM
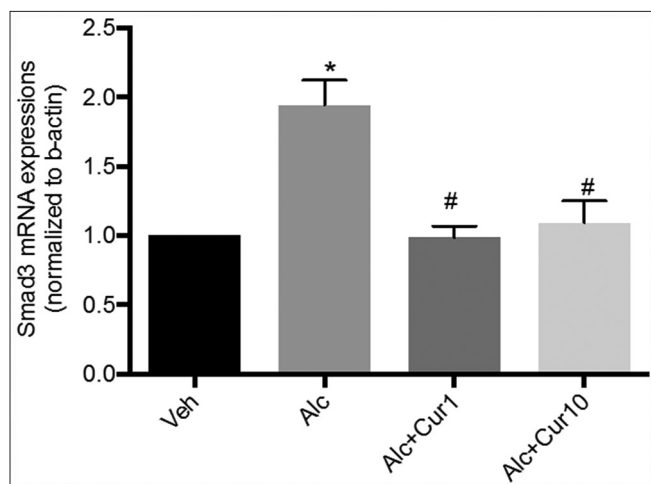
Fig. 3: Smad3 mRNA expressions after LX-2 treatment with vehicle only, alcohol, alcohol+curcumin 1 µM, or alcohol+curcumin 10 µM. Results are presented as mean+standard deviation, *: p<0.05 versus vehicle, #: p<0.05 versus alcohol. Veh: Vehicle; Alc: Alcohol; Alc+Cur1: Alcohol+curcumin 1 µM; Alc+Cur10: Alcohol+curcumin 10 µM
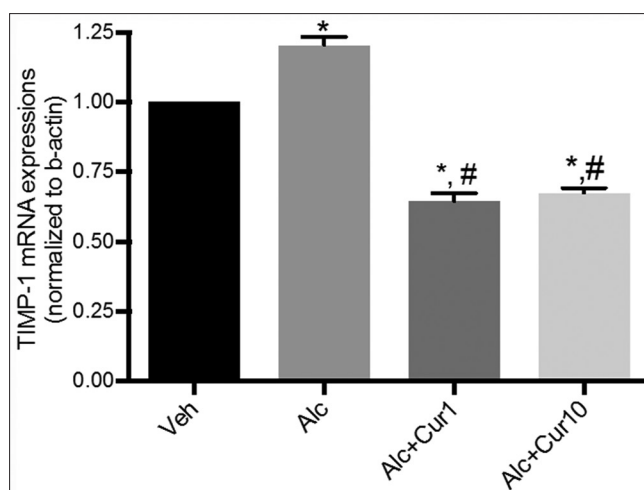

Fig. 5: Tissue inhibitor of metalloproteinases-1 mRNA expressions after LX-2 treatment with vehicle only, alcohol, alcohol+curcumin 1 µM, or alcohol+curcumin 10 µM. Results are presented as mean±standard deviation, *p<0.05 versus vehicle, #p<0.05 versus alcohol. Veh: Vehicle; Alc: Alcohol; Alc+Cur1: Alcohol+curcumin 1 µM; Alc+Cur10: Alcohol+curcumin 10µM
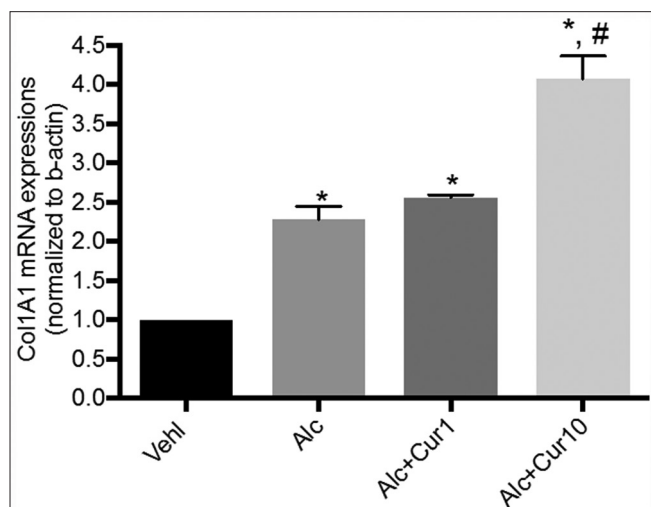

Fig. 4: Collagen 1A1 mRNA expressions after LX-2 treatment with vehicle only, alcohol, alcohol+curcumin 1 µM, or alcohol+curcumin 10 µM. Results are presented as mean±standard deviation, *p<0.05 versus vehicle, #p<0.05 versus alcohol. Veh: Vehicle; Alc: Alcohol; Alc+Cur1: Alcohol+curcumin 1 µM; Alc+Cur10: Alcohol+curcumin 10 µM

increase the expression of TGF-β, thus stimulating HSC proliferation.

The major pathway responsible for HSC activation is TGF-β/Smad pathway. There are several types of Smad proteins in the Smad family that is involved in the TGF-β/Smad signaling pathway. Yet Smad3, a regulatory Smad protein, seems to be the most important Smad in fibrogenesis. Phosphorylated Smad2/3, in collaboration with coSmad 4, is transported into the nucleus and acts as a transcription factor for the profibrogenic marker α-SMA and Col1A1 [7,23,24]. A previous study showed that the activation of HSC was correlated with increasing levels of Smad 3 mRNA expression [17]. In our study, we confirmed that induction by ethanol significantly increased the expression of Smad3 mRNA and profibrogenic marker Col1A1 expression. Activation of HSCs releases TGF-β and promotes fibrogenesis by decreasing MMPs and promoting its natural inhibitor, TIMP [7]. In the present study, we confirmed that induction of ethanol significantly increased TIMP-1 expressions. Thus,

we confirmed that, in our model, ethanol induction was successful. Further, HSCs were markedly activated, and all the profibrogenic markers of HSC activation were significantly increased. These results are in accordance with those of a previous study, suggesting that ethanol induction will result in a significant increase in Smad3, TIMP-1, and Col1A1 expression on LX2 cells induced by ethanol [25,26].

Our study showed that curcumin suppressed the proliferation of HSCs under stimulation with ethanol. Curcumin significantly decreased the mRNA TGF-β expression, and this effect appeared to be dose dependent. Our result is consistent with a previous study in which curcumin reduced TGF-β expressions in the livers of CCl_4-treated rats. Altogether, these findings suggest that the downregulation of TGF-β by curcumin may be the key underlying mechanisms of its antifibrogenic effects [26]. The examination of the signal molecules in the TGF-β/Smad pathway showed that curcumin decreased Smad3 mRNA expression during HSC activation [26]. Our study confirmed that curcumin decreased Smad3 expression. TGF-β also promotes fibrogenesis by inhibiting MMPs and promoting its natural inhibitor, TIMPs [27]. Our study showed that inhibition of TGF-β in activated HSC by curcumin was in line with decreasing TIMP-1 expressions. This result was in accordance with a previous study done by Liu *et al.* [26].

As described above, ethanol induction activated the HSCs through TLR signaling. TLR4, one receptor for lipopolysaccharide receptor, can trigger two different signaling pathways, in which one is a myeloid differentiation factor 88-dependent pathway and leads to the rapid activation of nuclear factor-κB (NF-κB) [26]. NF-κB is commonly associated with liver fibrosis, including ALD. Although NF-κB is not required for the activation of HSCs, an increase in the p65/p50 heterodimer, with a concomitant decrease in the NF-κB inhibitory protein, IκBα, promotes the survival of activated HSCs. Overexpression of NF-κB in activated HSCs, however, has been shown to inhibit α1(I) and α2(I) collagen mRNA expression in culture-activated HSCs [26,28]. Many studies showed that curcumin reduces the transcription activation of NF-kB [29-31]. These facts suggest our result that curcumin treatment did not affect Col1A1 expression.

CONCLUSION

Collectively, our results suggest that the antiproliferative effects of curcumin played by its ability to inhibit TGF-β in alcohol-induced HSC. Inhibition of the TGF-β/Smad pathway by curcumin decreases Smad3

and TIMP-1 expressions. Thus, curcumin will affect matrix degradation during fibrogenesis. Therefore, this study suggested the possibility of using curcumin as a potential candidate in the treatment of ALD.

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AUTHORS' CONTRIBUTIONS

RR and BW contributed to the literature search, experimental procedures, and writing of the manuscript. ML and VS contributed to the study design, statistical design, and writing of the manuscript.

CONFLICTS OF INTEREST STATEMENT

The authors state that there were no conflicts of interest to declare regarding the publication of this work.

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