Acupuncture combined with curcumin disrupts platelet-derived growth factor β receptor/extracellular signal-regulated kinase signalling and stimulates extracellular matrix degradation in carbon tetrachloride-induced hepatic fibrosis in rats

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Original paper

INTRODUCTION

Hepatic fibrosis occurs following injury to the liver by a variety of causes, including autoimmune conditions, alcohol or drug misuse, cholestatic and metabolic diseases, and viral hepatitis. Its pathogenesis is characterised by excessive deposition of extracellular matrix (ECM). Activation of hepatic stellate cells (HSCs) is the central event in liver fibrogenesis, leading to ECM overproduction.1 2 It has been established that platelet-derived growth factor (PDGF) is one of the most powerful profibrogenic cytokines to stimulate HSC activation and ECM synthesis.3 Clinical evidence has also shown that the serum PDGF level correlates with the degree of fibrosis in patients.4 PDGF transmits its signal through its receptor PDGF-βR and the downstream mitogen-activated protein kinase (MAPK) cascades including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 pathways.3 Emerging data also suggest that connective tissue growth factor (CTGF) is a master switch in liver tissue repair governing matrix production and could be a potentially valuable biomarker of active fibrogenesis.5 Furthermore, matrix metalloproteinases (MMPs) and their specific inhibitors (tissue inhibitors of metalloproteinases (TIMPs)) play an important role in fibrogenesis and fibrolysis. The highly controlled interplay between MMPs and TIMPs is responsible for a constant turnover of liver matrix and the maintenance of homeostasis.6

Currently, established therapeutic therapies for hepatic fibrosis are still lacking. Increasingly, acupuncture treatment has shown anti-fibrotic promise in animal studies and clinical trials. It is traditionally demonstrated that acupuncture at ST36 Zusanli, LR3 Taichong, LR14 Qimen and BL18 Ganshu may have important implications in liver disease treatment,7 but the mechanisms are poorly

Acupuncture treatment has been increasingly used to treat chronic liver diseases. We previously reported that acupuncture combined with curcumin, a natural anti-fibrotic compound, could remarkably attenuate liver fibrosis in chemically intoxicated rats, but the underlying molecular mechanisms are poorly understood. The present study was aimed at investigating the effects of acupuncture combined with curcumin on platelet-derived growth factor (PDGF) signalling and extracellular matrix (ECM) regulation in the fibrotic liver.

Methods

A total of 60 Sprague-Dawley male rats were randomly divided into control, model, sham, acupuncture, curcumin and combination treatment groups. During the establishment of fibrosis using carbon tetrachloride (CCl4), acupuncture at LR3, LR14, BL18 and ST36 and/or curcumin treatment by mouth were performed simultaneously. After treatment, serum PDGF levels were measured. Protein and mRNA expression of key effectors in PDGF pathway and fibrinolysis in the liver was determined.

Results

Acupuncture combined with curcumin potently reduced serum PDGF levels and selectively disrupted the PDGF-βR/extracellular signal-regulated kinase (ERK) cascade. Combination treatment also significantly repressed expression of connective tissue growth factor and upregulated expression of matrix metalloproteinase-9, promoting fibrinolysis in the fibrotic liver.

Conclusions

The beneficial effects of acupuncture and its combination with curcumin could be attributed to the disruption of PDGF-βR/ERK pathway and stimulated ECM degradation in the fibrotic liver. Acupuncture treatment significantly enhanced curcumin effects at the molecular level. These findings may provide molecular insights into the potential of acupuncture combined with curcumin for prevention of hepatic fibrosis.
understood. We previously reported that acupuncture at the aforementioned four points combined with curcumin, a natural polyphenolic compound with potent antifibrotic properties, reduced liver fibrosis through improving liver function and histology and inhibiting ECM synthesis in rats intoxicated by carbon tetrachloride (CCl₄), and that acupuncture enhanced the antifibrotic effects of curcumin. However, the molecular basis for acupuncture benefits and the coordinated effect was not elucidated. In addition, our previous studies revealed that the PDGF signalling pathway in activated HSCs could be disrupted by curcumin resulting in decreased collagen production in vitro, but the in vivo effects were still unknown. We hypothesised that acupuncture at LR3, LR14, BL18 and ST36 combined with curcumin could interfere with the profibrogenic PDGF signal transduction in the fibrotic liver and affect the ECM regulation system, leading to beneficial effects on liver fibrosis. To test the hypothesis, we conducted controlled experiments in rats with hepatic fibrosis induced by CCl₄, a classical model largely imitating hepatic fibrosis in humans.

**MATERIALS AND METHODS**

**Animals**

Male Sprague-Dawley rats (180–220 g body weight) used in this study were obtained from Shanghai Slaccas Laboratory Animals (Shanghai, China). All experimental procedures were approved by the institutional and local committee on the care and use of animals and all animals received humane care according to the National Institutes of Health (USA) Guidelines. All rats were maintained under a 12 h light/dark cycle at a controlled temperature (25°C) with free access to food and tap water until the day of the experiment.

**Experimental protocols**

A total of 60 healthy male Sprague-Dawley rats were randomly assigned into the following groups: control, model, sham, acupuncture, curcumin and combination use (n=10/group). The liver fibrosis model for rats was established through repeated intraperitoneal injections with a mixture of CCl₄ and olive oil (1:1 (v/v), 1 ml/kg body weight) over a total period of 6 weeks. Rats in all groups except the control group were injected with an olive oil solution of CCl₄ three times in the first week, and then two times every remaining week. Rats in the control group were injected with the same volume of saline. The treatments were performed simultaneously with the establishment of the fibrosis model. Rats in the acupuncture group received acupuncture at LR3, LR14, BL18 and ST36 three times per week during the course of the experiment. Rats in the sham group were given similar manipulations at four non-point locations, that is, 0.5 cm laterally to LR14 and BL18, and a similar distance laterally to LR3 and ST36. Rats in the curcumin group were given curcumin orally at 200 mg/kg every day (6 days/week) for 6 weeks. Rats in the combination group received acupuncture and curcumin administration simultaneously according to the described methods. Rats in the model group were given saline of the same volume as curcumin every day (6 days/week) for 6 weeks.

**Acupuncture protocols**

Rats were kept in specially designed holders with their forelimbs exposed. Pairs of stainless steel acupuncture needles (0.25 mm diameter; Jiangsu Provincial Hospital of Traditional Chinese Medicine, Nanjing, China) were inserted bilaterally into LR3, LR14 and BL18 to a depth of approximately 2 mm. The needles were rotated for 1 min in each acupuncture point and remained in situ for 15 min, as in a therapeutic procedure. Then, electroacupuncture at ST36 was performed bilaterally using a G-6850 electroacupuncture instrument (Shanghai Medical Electronic Instrument Factory, Shanghai, China) that produced constant current square-wave electrical stimulation to stimulate the acupuncture points for 10 min with parameters of 50 Hz, 4 volts and 1–3 mA (increased gradually). The electrical intensity was just strong enough to elicit slight twitches of the hind limbs.

**Detection of serum PDGF levels**

At 48 h after the last treatment in the experiment, all rats were weighed and killed after being anaesthetised by an intraperitoneal injection of pentobarbital at 50 mg/kg. Blood was obtained from each rat through the common carotid artery, and underwent centrifugation at 7500 g for 2 min to obtain plasma. Serum PDGF levels in rats were determined using an ELISA kit purchased from BD Biosciences (San Jose, California, USA) according to the protocol provided by the manufacturer.

**Western blot assay**

Liver extracts were prepared from pieces of liver tissue excised from every rat in the rat model using ice-cold radioimmunoprecipitation assay lysis buffer containing 150 mM NaCl, 50 mM Tris, 0.1% SDS, 1% Nonidet P-40 and 0.5% deoxycholate supplemented with protease inhibitors. Protein concentrations were determined using the BCA protein assay kit according to the protocol provided by the manufacturer (Pierce Chemical, Rockford, Illinois, USA). Samples of 40 μg of total protein were subjected to 10% SDS-PAGE, transferred to a polyvinylidene fluoride membrane (Millipore, Burlington, Massachusetts, USA), and blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween. Target proteins were respectively detected by primary antibodies against PDGF-βR, ERK, JNK, p38, CTGF; MMP-9 and TIMP-1, and horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, California, USA). β-Actin was probed as an internal control. Protein bands were visualised using chemiluminescence reagent (Amersham, Chalfont St Giles, Bucks, UK). The densities of bands were normalised with the internal invariable control β-actin. The level of target protein bands was densitometrically determined by using Quantity Ones 4.4.1 (Bio-Rad Laboratories, Hercules, California, USA). The variation in the density
was expressed as fold changes compared with the control in the blot.

**RNA isolation and quantitative real-time PCR**

Total RNA was extracted from frozen liver tissues using TRI reagent according to the protocol provided by the manufacturer (Sigma-Aldrich, St Louis, Missouri, USA). Total RNA (1 μg) was treated with DNase I to eliminate genomic DNA contamination, followed by synthesis of the first strand using the reverse transcription system (Promega, Madison, Wisconsin, USA). Reverse transcription was carried out as follows: 42°C for 5 min, 95°C for 5 min and 4°C for 5 min (one cycle). Real-time PCR was performed in 25 μl of reaction solution containing 12.5 μl 2×iQSYBR Green Supermix (Bio-Rad Laboratories, Hercules, California, USA), 300 nM primers and complementary DNA. The cycles for PCR were as follows: 95°C for 7 min, 40 cycles of 95°C for 20 s, 54°C for 50 s and 72°C for 30 s. Melting curves were determined by heat-denaturing PCR products over a 55°C temperature gradient at 0.2°C/s from 60 to 95°C. Fold changes in the messenger RNA (mRNA) levels of target genes related to the invariant control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were calculated as described. The following primers were used in real-time PCR: PDGF-βR: (forward (F)) 5'-CTGCCACAGCATGAGGATTGAT-3', (reverse (R)) 5'-GCCAGATGGCTGAGATCACCAC-3'; ERK: (F) 5'-GACACAGCACCTCAGCAA-3', (R) 5'-GGAGATGGCTGAGATCACCAC-3'; JNK: (F) 5'-AGTTGGCTCGCATCATAGTT-3', (R) 5'-GGGCCCTCTGGAAAGCTGT-3'; and GAPDH: (F) 5'-GGCCCTCTGGAAAGCTGT-3', (R) 5'-CCGGCTCTGTTCCACACCTTCT-3'. mRNA levels were expressed as fold changes after normalisation with GAPDH.

**Statistical analysis**

The data were analysed with SPSS V11.5 (SPSS, Chicago, Illinois, USA) and presented as mean with standard deviation of N detections, where N represents the number of animals in each group. Differences between means were evaluated using one-way analysis of variance (ANOVA) followed by Tukey multiple comparison test. A p value of <0.05 was considered to be significant.

**RESULTS**

**Acupuncture and/or curcumin decreased serum PDGF levels in rats with CCl₄-induced hepatic fibrosis**

We first examined the serum levels of PDGF, a powerful profibrogenic cytokine, in rats, using the ELISA method. The results showed that the serum PDGF levels in the model rats were significantly increased compared with the control group (p=0.005). Although no significant reduction in PDGF levels was observed in either the sham group or the acupuncture group, acupuncture manipulation seemed to be more potent. Curcumin showed significant reducing effects compared with the model group (p=0.05). Notably, combination treatment resulted in the most significant reduction in serum PDGF concentration compared with the acupuncture group (p=0.005) and curcumin group (p=0.04) (figure 1). Collectively, these data indicate that acupuncture might enhance curcumin inhibition of PDGF production in vivo in hepatic fibrosis.

**Acupuncture and/or curcumin disrupted PDGF-βR/ERK signalling in rats with CCl₄-induced hepatic fibrosis**

We next determined whether the treatments could affect PDGF signal transduction, that is, the PDGF-βR/MAPK cascades in the fibrotic liver. Our data showed that the protein abundance of PDGF-βR was significantly upregulated in the model group, but acupuncture, curcumin and their combination all decreased PDGF-βR protein expression (figure 2A). PCR assays demonstrated that curcumin and combination treatment led to a significant reduction in PDGF-βR mRNA expression (p=0.02 and 0.01, respectively), which was significantly upregulated in the model group, but acupuncture alone did not show significant effects. Additionally, combination use was more effective than acupuncture manipulation (p=0.04) but not curcumin treatment (figure 2B).

PDGF-βR can activate the downstream MAPK cascades, including ERK, JNK and p38 pathways. The present data demonstrated increased protein abundance of the three mediators, especially ERK, in the model group. Acupuncture or curcumin inhibited ERK protein expression to different degrees and their combination resulted in the most notable effects (figure 3A). Interestingly, the protein expression of JNK and p38 was not apparently affected, suggesting a selective inhibitory effect on MAPK pathways (figure 3A). To further confirm the critical role of ERK, we subsequently determined ERK mRNA expression, showing that it was significantly elevated in the model rats compared with the control...
group (p=0.02), but acupuncture and curcumin significantly reduced it compared with the model group (both p=0.03), and that their combination was more effective than acupuncture (p=0.02) or curcumin (p=0.03) alone (figure 3B). Taken together, these molecular data indicate that acupuncture and/or curcumin may selectively disrupt the PDGF-βR/ERK pathway in the fibrotic liver, contributing to attenuated fibrogenesis.
Acupuncture and/or curcumin reduced CTGF expression in rats with CCl4-induced hepatic fibrosis

CTGF is increasingly recognised as being an important player in liver fibrogenic pathways. It exhibits overexpression in fibrosing tissues and cytokine-stimulated expression in HSCs.5 Our present data demonstrated elevated CTGF protein abundance in the model group concomitant with a significant increase in CTGF mRNA expression compared with the control group (p=0.02). However, acupuncture or curcumin reduced CTGF protein abundance, and significantly downregulated CTGF gene expression compared with the model group (p=0.04 and 0.03, respectively). Combination treatment led to a more significant reduction in CTGF mRNA expression than that of acupuncture (p=0.03) or curcumin (p=0.03) (figure 4). Acupuncture at non-acupuncture points failed to reduce CTGF expression in the sham group. Collectively, these data suggest that acupuncture and/or curcumin could inhibit CTGF expression in the fibrotic liver at the protein and gene levels and that acupuncture could improve curcumin effects in the combined treatments.

Acupuncture and/or curcumin stimulated fibrolysis in rats with CCl4-induced hepatic fibrosis

ECM degradation is controlled by a fine balance between activities of proteinases and their inhibitors. The MMPs are a family of zinc metalloendopeptidases and responsible for the turnover of the ECM.12 We observed significantly decreased MMP-9 expression in the liver with established fibrosis, whereas acupuncture or curcumin upregulated MMP-9 expression and their combination produced the most remarkable reduction in MMP-9 protein abundance (figure 5), suggesting that fibrolysis may be stimulated. Interestingly, we found that although TIMP-1, which prevents ECM degradation, was upregulated in the fibrotic liver, its protein expression was not obviously affected by acupuncture and/or curcumin (figure 5). These findings indicate that the intrinsic inhibitory machinery of ECM degradation may not be involved in the antifibrotic effects of the treatments.

DISCUSSION

Hepatic fibrogenesis is a complex process involving a number of cytokines and signal transductions. These molecular recognitions may provide potential targets for intervention of this disease. Recently, acupuncture has increasingly shown benefits for prevention and treatment of chronic liver diseases including fibrosis.13,14 Our previous study also demonstrated that acupuncture combined with curcumin could potently attenuate liver fibrosis in rats.8 Our present investigation attempted to elucidate the underlying molecular mechanisms. We herein discovered that selective disruption of PDGF-βR/ERK pathway, and suppressed CTGF expression and upregulated MMP-9 expression in the fibrotic liver could contribute to the beneficial effects of acupuncture and/or curcumin treatments.

PDGF is one of the most potent proliferative factor in liver fibrosis. It promotes HSC proliferation and chemotaxis, and stimulates collagen production through autocrine and paracrine pathways.3 Therefore, reducing PDGF production is beneficial for attenuating HSC activation and fibrogenesis. Our present data showed that acupuncture could achieve this effect and significantly enhance curcumin reduction of PDGF levels in vivo. These effects may account for the beneficial effects on liver histology we previously observed. Furthermore, PDGF-βR expression is a major phenotypic change during HSC activation and mediates the fibrogenic PDGF signal transduction.5 Clinical evidence also shows that
PDGF-βR is highly expressed in myofibroblast-like cells in livers from patients with either cirrhosis or hepatitis. Our results from this study showed that acupuncture combined with curcumin markedly reduced PDGF-βR protein abundance and gene expression in the fibrotic liver, which may contribute to inhibition of HSC activation and recovery from fibrotic injury. Our subsequent detection of MAPK cascades demonstrated that the ERK pathway was selectively inhibited by acupuncture and/or curcumin at the protein and mRNA levels, suggesting a selective disruption of PDGF-βR/ERK signalling. These findings are consistent with the evidence that the ERK signalling is central to PDGF-stimulated proliferation and migration of HSCs during the progression of liver fibrosis. It is postulated that interruption of the PDGF-βR/ERK pathway may result in reduced profibrogenic behaviours of HSCs including cytokine release and ECM synthesis. Moreover, our results highlight the critical role of the PDGF-βR/ERK pathway as a target for treating liver fibrosis and indicate that acupuncture and/or curcumin may have specific target pathway effects in preventing HSC activation and fibrogenesis.

CTGF was originally discovered as a secreted ‘PDGF-related mitogen’ in human umbilical vein endothelial cells. Increasingly, CTGF has been found to contribute to the ECM accumulation in liver wound healing and fibrogenesis by promoting fibre–fibre, fibre–matrix and matrix–matrix interactions through direct molecular interactions with matrix components. CTGF reduction could markedly attenuate HSC activation and decrease production of type I and III collagens in liver fibrosis. In the present study, we showed that acupuncture and/or curcumin reduced CTGF expression at the protein and mRNA levels. These effects may contribute to the inhibited production of ECM components in rats with hepatic fibrosis received these treatments in our previous studies. Furthermore, CTGF can be stimulated by profibrogenic growth factors, for instance, it has been reported that PDGF could promote CTGF expression in activated HSCs. However, the precise signalling pathway that mediates PDGF-driven CTGF expression remains to be defined. Given our present finding that the disrupted PDGF-βR/ERK pathway mediated the antifibrotic effects of acupuncture and/or curcumin, it could be postulated that the ERK may be an upstream effector controlling CTGF gene expression in liver fibrotic cascades. This may also be partially supported by recent evidence that activation of ERK, but not JNK or p38, mediated CTGF secretion and proliferation and differentiation in human osteoblasts.

Compelling evidence has documented the association of MMPs with liver fibrosis. MMP-9 is a gelatinase with proteolytic activity and can degrade gelatins and ECM molecules including type I collagen. Many in vivo studies have shown that MMP-9 was sharply upregulated in the early period of liver fibrosis resulting in basement membrane-like ECM remodelling and HSC mobilisation; however, with fibrosis progression, MMP-9 expression was dramatically depleted favouring ECM accumulation. Our results partially confirmed this concept. MMP-9 protein abundance was maximally decreased in the established fibrosis as shown in figure 5, suggesting that MMP-9 was an early signal in hepatic wound healing. Acupuncture combined with curcumin remarkably increased MMP-9 expression, which may stimulate the process of fibrolysis in the fibrotic liver. This is consistent with the previous report that acupuncture could reduce collagen deposition in the fibrotic liver and ameliorate liver injury. However, we showed that TIMP-1 was upregulated in the established fibrosis, which is in line with the published data that TIMP-1 of high level deteriorated fibrogenesis due to its broad inhibitory effects on MMPs. However, acupuncture and/or curcumin did not apparently affect TIMP-1 expression, suggesting a selective intervention of ECM equilibrium. Together, impacts on MMPs/TIMPs system in liver fibrosis may be an important mechanism for the fibrolysis effects produced by acupuncture combined with curcumin. What role the PDGF-βR/ERK signalling plays in these effects and whether there are other MMPs or TIMPs involved merit further investigation.

We attempted to elucidate the molecular basis for acupuncture combined with curcumin in prevention of liver fibrosis. As shown in this study, although acupuncture sometimes did not exhibit significant molecular effects,
indeed significantly enhanced the curcumin effects at the molecular level. These findings may provide molecular evidence supporting the concept that acupuncture treatment can affect some intracellular events such as signal transduction and performance of functional proteins, which may, at least partially, underlie the antifibrotic potential of acupuncture. Undoubtedly, in-depth recognition of the pathophysiology of hepatic fibrosis will further help provide molecular insights into acupuncture treatment against chronic liver diseases.

CONCLUSIONS

In summary, we demonstrated that acupuncture combined with curcumin potently inhibited PDGF production and disrupted PDGF-βR/ERK cascade in rats with CCl₄-induced hepatic fibrosis. The treatments also reduced CTGF expression and increased MMP-9 expression. Acupuncture treatment markedly enhanced curcumin effects at the molecular level. These molecular insights may explain how acupuncture combined with curcumin inhibited HSC activation and stimulated ECM degradation implicated in the management of hepatic fibrosis.

Summary points

- The platelet-derived growth factor β receptor/extracellular signal-regulated kinase pathway was selectively disrupted.
- Combination treatment reduced connective tissue growth factor and stimulated fibrosis.
- Acupuncture enhanced the effects of curcumin at the molecular level.

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Contributors

X-RZ and FZ conducted the experiments and drafted the manuscript. Z-LZ, JM and D-SK participated in acupuncture manipulation and data analysis. A-YW and W-XC provided essential technical supports and helpful suggestions. G-XN and YL provided necessary financial support and were partly responsible for the project. S-ZZ was the principal designer of the study and responsible for all aspects of this work. S-ZZ also critically revised the manuscript.

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Ethics approval

This study was conducted with the approval of Nanjing University of Chinese Medicine, Nanjing, Jiangsu, China. All experimental procedures were approved by the institutional and local committee on the care and use of animals.

Provenance and peer review

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