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Original article

Tetramethylpyrazine attenuates carbon tetrachloride-caused liver injury and fibrogenesis and reduces hepatic angiogenesis in rats

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ABSTRACT

Liver fibrosis represents a frequent event following chronic insult to trigger wound healing reactions with abnormalities of angiogenesis in the liver. Capillarization of liver sinusoidal endothelial cell (LSEC) is the pivotal event during liver angiogenesis. In the current study, we sought to investigate the effect of tetramethylpyrazine (TMP) on carbon tetrachloride (CCl_4)-induced liver injury and fibrosis in rats, and to further examine the molecular mechanisms of TMP-induced anti-angiogenic effect. We found that TMP significantly ameliorated histopathological feature of liver fibrosis characterized by decreased collagen deposition, hepatocyte apoptosis, and expression of biochemical indicators, such as aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP). Moreover, TMP appeared to play an essential role in controlling pathological angiogenesis. In addition, TMP attenuated angiogenesis by downregulation of vascular endothelial growth factor-A (VEGF-A), vascular endothelial growth factor receptor 2 (VEGF-R2), platelet-derived growth factor-BB (PDGF-BB), and platelet-derived growth factor-β receptor (PDGF-βR), four important factors transmitting pro-angiogenic pathways. Besides, TMP inhibited LSEC capillarization in CCl_4-induced liver fibrotic model with the morphological features of increasing sinusoidal fenestrae. Importantly, we found that disruption of angiogenesis is required for TMP to inhibit hepatocyte apoptosis in rats. Treatment with TMP significantly inhibited the expression of Bax, and up-regulated Bcl-2 expression. Interestingly, treatment with angiogenesis-inducer AngII dramatically eliminated the effect of TMP on Bax/Bcl-2 axis. Overall, these results provide novel perspectives to reveal the protective effect of TMP on liver, opening up the possibility of using TMP based anti-angiogenic drugs for the liver diseases.

1. Introduction

Liver fibrosis occurs as compensatory responses to tissue repairing process in a wide range of chronic liver injures, and angiogenesis is a typical feature of liver fibrosis [1]. Recent studies established a pivotal role for liver sinusoidal endothelial cells (LSECs) in liver angiogenesis because they play an important role in the parenchymal distribution of nutrients and oxygen, modulation of the hepatic vascular tone, blood pathogens clearance, and intrahepatic cells communication [2]. Endothelial cells form the wall of hepatic sinusoids and comprise approximately 16% of the cells in liver under physiological conditions [3]. The LSEC has a unique phenotype among all mammalian endothelial cells, LSEC have open fenestrae grouped into sieve plates and lack an organized basement membrane. The capillarization of LSEC is a key step in the pathogenesis of hepatic angiogenesis, which is characterized by formation of an organized basement membrane and lack of fenestration [4].

LSEC capillarization is also accompanied by overexpression of Platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31), CD34 and von Willebrand factor (vWF), three markers of endothelial cells [5]. In addition, LSEC capillarization is also coupled with the sequential overexpression of platelet-derived growth factor (PDGF) [6], platelet-derived growth factor-β receptor (PDGF-βR) [7], vascular endothelial growth factor receptor 2 (VEGF-R2) [8], epidermal growth factor receptor (EGFR) [9] and hypoxia inducible factor 1α (HIF-1α) [10], which transmit

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the pro-angiogenesis VEGF, PDGF, and EGF pathways, respectively. Furthermore, chemical stimuli play a critical role in LSEC capillarization and angiogenesis [4]. Accumulating evidence demonstrated that capillarized LSECs not only preceded fibrosis, but also permitted for pathological sinusoidal angiogenesis, suggesting that inhibition of LSEC capillarization was a key step in anti-angiogenesis and restoration of liver functions [2].

Recently, new therapeutic agents and strategies are required for the management of anti-angiogenesis in liver fibrosis. The use of natural products as a realistic option for the treatment of liver fibrosis has broadly been accepted. Natural products are an important source of potential anti-angiogenic remedies. Chuanxiong has been widely used as one of a disease-preventing medicine throughout history. It has a variety of pharmacological activities including treatment of heart disease [11], fibrosis [12] as well as antibacterial [13]. Recent studies have shown that the extracts of Chuanxiong could protect the liver under a variety of pathological circumstances [14], but the evidence for hepatoprotection by a single Chuanxiong-derived component is rarely seen. We herein hypothesized that TMP contributed predominantly to the hepatoprotective effects by inhibiting the pathological angiogenesis. In this study, we sought to investigate the effect of TMP on carbon tetrachloride (CCl_4)-induced liver injury and fibrosis in rats, and to further examine the molecular mechanisms of TMP-induced anti-angiogenic effect.

2. Material and methods

2.1. Reagents and antibodies

TMP (purity >98%) and angiotensin-II (AngII) were purchased from Sigma (St Louis, MO, USA). Alzet osmotic pumps (models 2004 and 2ML4) were purchased from the Durect Corporation (Cupertino, CA, USA). Primary antibodies against α-SMA, α1(I) procollagen, and fibronectin were purchased from Epitomics (SanFrancisco, CA, USA). Primary antibodies against VEGF-A, VEGFR2, PDGF-BB, PDGF-Rβ, EGF, EGFR and HIF-1α were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibodies against MMP2, MMP9, TIMP-1, TIMP-2, Cleaved Caspase-9, Bax, Bcl2, EGFR and β-actin were purchased from Cell Signaling Technology (Danvers, MA, USA). The horseradish peroxidase-conjugated secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

2.2. Animals and experimental design

Male Sprague–Dawley rats (180–220 g body weight) were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All animals were maintained at approximately 22 ± 2 °C and 55 ± 5% relative humidity, with a 12-h light: 12-h dark cycle, and had free access to laboratory chow and tap water. The experimental was approved by the institutional and local committee on the care and use of animals of Nanjing University of Chinese Medicine (Nanjing, China). All animals were cared for in accordance with the National Institutes of Health (USA) guidelines. A mixture of CCl_4 (0.1 ml/100 g body weight) and olive oil [1:1 (w/v)] was used to induce liver fibrosis in rats. After adaptive feed for a week, sixty-four rats were randomly divided into eight groups (eight rats per group) (Fig. 1A). Group 1 was the vehicle control in which rats were not administrated CCl_4 or TMP but intraperitoneally (i.p.) injected with olive oil. Group 2 was the CCl_4 group in which rats were i.p. injected with CCl_4 without TMP treatment. Groups 3 were treatment groups in which rats were i.p. injected with CCl_4 and orally given TMP at 100 mg/kg. Group 4 was the positive control in which rats were i.p. injected with CCl_4 and treated with colchicine (Yifeng Pharmacy, Nanjing, China). Group 5 was CCl_4 + saline group in which rats were i.p. injected with CCl_4 and continuous infusion of saline with mini-Osmotic pumps. Group 6 was the CCl_4 + AngII group in which rats were i.p. injected with CCl_4 and continuous infusion of AngII (25 μg/kg/h) with mini-Osmotic pumps. Group 7 was CCl_4 + TMP + saline group in which rats were i.p. injected with CCl_4, orally given TMP at 100 mg/kg and continuous infusion of saline with mini-Osmotic pumps. Group 8 was CCl_4 + TMP + AngII group in which rats were i.p. injected with CCl_4, orally given TMP at 100 mg/kg and continuous infusion of AngII with mini-Osmotic pumps. TMP and Colchicine (0.1 mg/kg) were suspended in sterile phosphate buffered saline (PBS) and given once daily by gavage during weeks 2–5. At the end of experiment, rats were sacrificed after being anesthetized by i.p. injection pentobarbital (50 mg/kg). Blood was collected, and livers were isolated and weighted quickly and stored at −80 °C until analysis.

2.3. Liver histopathology

Hematoxylin-eosin (HE) staining, sirius red collagen staining and masson-trichrome staining were performed as we previously described [15]. Representative views of liver sections were shown. All histopathological analyses were undertaken by an experienced histopathologist in a blinded manner.

2.4. Measurement of hepatic enzyme levels

Levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) in serum samples were evaluated using enzyme-linked immunosorbent assay methods according to the kit protocols (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Experiments were performed in triplicate.

2.5. Determination of hepatic hydroxyproline

The hydroxyproline levels in the liver tissue and serum were determined using a kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the protocol. Briefly, three small pieces of liver tissues randomly excised from the liver of every rat were hydrolyzed in 6 N HCl at 110 °C for 24 h, and subsequently, they were neutralized with NaOH. Isopropanol in citrate acetate-buffered chloramine T was added to aliquots of the hydrolysate, followed by the addition of Ehrlich reagent. The chemical reaction occurred in the dark for 25 min at 60 °C. After centrifugation, absorbance of the supernatant of each sample was read at 558 nm using a 96-well plate spectrometer. *trans*-hydroxyproline was used as the standard for quantification. Values were normalized to the control. Experiments were performed in triplicate.

2.6. RNA isolation and real-time PCR

Total RNA was extracted from rat liver samples using Trizol reagent (Biouniquer Technology Co., Ltd, Nanjing, China) according to the manufacturer's protocol. Amplification kit was purchased from Bio-Rad Laboratories (Berkeley, CA, USA). Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as the invariant control. All primers were obtained from GenScript (Piscataway, NJ, USA) and are listed as follows: α-SMA: (forward) 5′-CCGACCGAATGCAGAAGGA-3′, (reverse) 5′-ACAGAGTATTTGCGCTCCGGA-3′; α(I)Procollagen: (forward) 5′-CCTCAAGGGCTCCAACGAG-3′, (reverse) 5′-TCAATCACTGTCTTGCCCCA-3′; Fibronectin: (forward) 5′-TGTCACCCACCACCTTGA-3′, (reverse) 5′-CTGATTGTTCTTCAGTGCGA-3′; GAPDH: (forward) 5′-GGCCCCTCTGGAAAGCTGTG-3′; (reverse) 5′-CCGCCTGCTTCACCACCTTCT-3′. Results were from triplicate experiments.