Liver fibrosis is a scarring process that represents the liver's response to injury. Over time, this process can result in cirrhosis of the liver in which the architectural organization of the functional units of the liver becomes so disrupted that blood flow through the liver and liver function become disrupted. Once cirrhosis has developed, the serious complications of liver disease may occur including portal hypertension, liver failure and liver cancer. The risk of liver cancer is greatly increased once cirrhosis develops and cirrhosis should be considered to be a pre-malignant condition. Cirrhosis and liver cancer are now among the top ten causes of death worldwide, and in many developed countries liver disease is now one of the top five causes of death in middle-age (Griffiths et al., 2005; Bosetti et al., 2007). Therefore, prevention of the fibrosis is very important in order to improve these conditions.

HSCs activation leads to retinoid acid storage, remodeling of ECM and production of growth factors and cytokines (Tsukada et al., 2006). Suppression of HSC activation has been proposed as a therapeutic target against liver fibrosis (Bataller and Brenner, 2005; Lee et al., 2008). Many studies confirm that the strategy against TGF-β and its intracellular signaling pathway are of primary interest applying ligand scavengers, cytokine antagonist and a variety of large or small molecule synthetic inhibitors of Ser/Thr-kinase of Alk-5 TGF-β receptors to reverse liver fibrosis.
fibrosis (Gressner and Gressner, 2008; Gressner et al., 2002; Liu et al., 2006). However, since TGF-β has important anti-proliferative and anti-inflammatory effects in its normal expression, inhibition of TGF-β may be a double-edged sword. Therefore, alternative targets for therapeutic intervention are needed to treat this complication of liver fibrosis. Recent evidence supports that connective tissue growth factor (CTGF), a fibrogenic master switch in fibrotic liver diseases (Li et al., 2006; Gressner and Gressner, 2008), plays a key role in the pathogenesis of liver fibrosis (Paradis et al., 2002).

Protocatechuic aldehyde (PA), a naturally occurring compound, is extracted from the degradation of phenolic acids. Many bioactivities of PA have been reported previously. For example, antitumor and antioxidant activity (Kim et al., 2007), protection of vascular endothelial cells from ox-LDL induced injury (Han et al., 2007), suppression of TNF-α-induced ICAM-1 and VCAM-1 expression (Zhou et al., 2005). Although previous studies display that PA inhibits TGF-β1 in human lens epithelial cells cultured under diabetic conditions (Kim et al., 2007), inhibits hepatitis B virus replication (Zhou et al., 2007) and may inhibit HSC growth (Lv & Yao, 2006), there are no reports about PA on TGF-β1 and CTGF expression in TNF-α stimulated HSCs or liver fibrosis animal model. The aim of this study was to investigate the antifibrogenic effect and the possible mechanisms of PA in TNF-α stimulated HSCs and the animal model of carbon tetrachloride (CCL4) induced liver fibrosis, to clarify how to protect liver fibrosis in rats.

Materials and methods

Chemicals

Protocatechuic aldehyde (PA, Formula: C7H6O3, molecular weight: 138.12, CAS number: 139-85-5) was obtained from State Key Laboratory of Long-acting and Targeting Drug Delivery Technologies (Yantai, P.R. China). The purity of PA was 99.3% by HPLC. Dulbecco’s modified Eagle’s medium (DMEM, Grand Island, NY, US) and fetal bovine serum (FBS) were purchased from Gibco BRL (Shanghai, P.R. China). The ELISA kits of hyaluronic acid (HA), laminin (LN), collagen types I (PC I) and collagen types III (PC III) were purchased from Beixi Science (Shanghai, P.R. China). The ELISA kits of hyaluronic acid (HA), laminin (LN), collagen types I (PC I) and collagen types III (PC III) were purchased from Beixi Science (Shanghai, P.R. China). The kits of hydroxyproline were purchased from State Key Laboratory of Long-acting and Targeting Drug Delivery Technologies (Yantai, P.R. China). SP kits were purchased from Maixin Biotechnology Company (Fuzhou, P.R. China). HSC-T6 was purchased from Shandong Luye Pharmaceutical Company (Yantai, P.R. China). All other chemicals were analytical grade and purchased from commercial suppliers.

Animals

Adult male Sprague-Dawley rats were obtained from Shandong Luye Pharmaceutical Company (Yantai, P.R. China). All animals were housed individually under temperature (20±2°C) and humidity (50±10%) with a 12 h light/12 h dark cycle and had free access to food and water. The experimental procedures were approved by the institutional Animal Ethics Committee of Yantai University.

HSCs proliferation assay

Hepatic stellate cells T6 (HSCs) were cultured according to previous methods (Mi et al., 2008). On a 24-well plate, HSCs 5×10⁴ cells/mL were seeded in DMEM medium containing 20% FBS and incubated at 37°C in a humidified 5% CO₂ atmosphere. The control group and the control +PA 27 μM group (no TNF-α 20 ng/mL stimulation) were incubated at 37°C in a humidified 5% CO₂ atmosphere. After HSCs incubated with TNF-α 20 ng/mL, the cultures were replaced and incubated with PA 1, 3, 9, 27 and 81 μM for 24 h and 48 h, then measured by a SRB assay.

ELISA analysis

HSCs were treated with 20 ng/mL of TNF-α with or without 1, 3, 9, 27, 81 μM of PA in 0.5% FBS medium for 48 h. Cells were washed with PBS, then collected by scraping and centrifuged at 10000 g for 5 min. The collected cells were lysed with 2 mL of ice-cold lysis buffer (50 mM HEPES, 5 mM EDTA, 100 mM NaCl, 1% Triton X-100, pH 4) for 30 min, then centrifuged at 10000 g for 5 min. The supernatant was collated and the levels of TGF-β1, CTGF, PCI and PCIII were determined by ELISA kits.

CCL4-induced liver fibrosis model and experimental design

Fifty adult male Sprague-Dawley rats, aged 6 weeks and weighing 160-200 g. Except for the control rats (n = 10), all rats were subcutaneously injected with 400 g/L CCl4 (CCl4:olive oil=2:3) once every 3 days for continuous 6 weeks. The rats were randomly divided into two subgroups (n = 10) according to body weight and serum alkaline phosphatase (AKP): (I) model group, (II) PA 10 mg/kg group. All animals were treated daily via intragastric (i.g.) of corresponding drug for a continuous 4 weeks. After 4 weeks, all rats were anesthetized with 200 g/L urethane (5 mL/kg, i.p.). Blood samples were collected. The serum was separated by centrifugation at 4°C and kept at −20°C for assay. Both, their body weight and liver weight were obtained. Liver tissue was stored in cold saline for pathological diagnosis.

Biochemical determination

Serum levels of albumin (ALB), globulin (GLB), alanine aminotransferase (ALT) and glutamate-pyruvate transaminase (AST) were measured by routine laboratory methods using AutoLAB Analyzer Medical System (Italy). Serum levels of HA, LN and PCIII were measured by ELISA kits according to the manufacturer’s instructions. The contents of hepatic hydroxyproline (HYP) were measured by the kits according to the manufacturer’s instructions.

Fibrosis grade and histopathological changes

Liver condition was classified according to the standard formulated by China Medical Association (1995), and
fibrosis was graded from 0 to 4 (0: no fibrosis; 1: portal area fibrosis; 2: fibrotic septa between portal tracts; 3: fibrosis septa and structure disturbance of hepatic lobule and 4: cirrhosis). After grading, liver tissue was fixed in a 40 g/L solution of formaldehyde containing 0.1 μM PBS (pH = 7.4), then embedded in paraffin. Five micron thick sections were prepared. All the sections were stained with haematoxylin and mounted. As a negative control, primary antibody was replaced with PBS.

**Immunohistochemical detection for liver TGF-β1 and CTGF**

Immunohistochemical staining of CTGF and TGF-β1 were performed according to the instructions of the SP kit. The primary antibodies were polyclonal rabbit anti-human TGF-β1 (1:100, Santa Cruz company, US) and goat anti-human CTGF C-terminal peptide antibody (1:100, R&D company, US). The end compounds reacted with AEC reagent. The slides were counterstained with haematoxylin and mounted. As a negative control, primary antibody was replaced with PBS.

**Western blot analysis for liver TGF-β1 and CTGF**

The liver samples were homogenized, and lysed in lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 100 µg/mL phenylmethylsulfonyl fluoride, 1 µg/mL aprotinin, and 1% Triton X-100. After centrifugation at 1600 g, protein samples (20 µg) were resolved on 10% SDS-PAGE gels and transferred onto a polyvinylidene difluoride (PVDF) membrane in semi-dry system (Bio-Rad company, Hercules, CA). The membranes were incubated with goat anti-human CTGF C-terminal peptide antibody (1:1000, R&D company, US), polyclonal rabbit anti-human TGF-β1 (1: 200, Santa Cruz company, US), and β-actin (1:500, Shanghai Xiangsheng company, P. R. China). β-Actin was used as the loading control. Optical densities of the bands were scanned and quantified with the Gel Doc 2000 (Bio-Rad company, US). Data were normalized against those of the corresponding β-actin. Results were expressed as fold increase over the control.

**Statistical analysis**

The difference of fibrosis grades between groups were evaluated by Nonparametric Tests. Quantitative data from experiments were expressed as mean ± SD, significance was determined by one-way analysis of AVONA followed by Tukey’s test. *p<0.05 was considered statistically significant.

**Results and discussion**

PA inhibited the HSCs proliferation in a concentration-dependent manner at the concentration of 3–81 μM, as shown in Figure 1. As time increases, HSCs proliferation was attenuated, with IC_{50} values of 24.5 μM at 24 h and 12.9 μM at 48 h. Those data indicated that PA plays sustained and stable inhibition of the HSCs proliferation in a time- and concentration-dependent manner.

We evaluated the effect of PA on the release of TGF-β1, CTGF, PCI and PCIII in TNF-α-stimulated HSCs, as shown in Table 1. The results indicated that TNF-α-stimulated HSCs cultured for 48 h obviously increased the levels of CTGF, PCI and PCIII. However, treatment of PA from 3 to 81 μM, the levels of CTGF, PCI and PCIII were markedly decreased in a concentration-dependent manner. Among them, TGF-β1 was decreased only from 27 to 81 μM.

Histopathologic changes of liver sections were observed by HE staining and Masson's trichrome staining, as shown in Figure 2A and 2B. Liver tissue samples from the control rats showed normal lobular architecture with central veins, radiating hepatic cords and little collagen deposition (Figure 2A1 and 2B1). Liver tissue samples from the model group showed more fibrous tissues and extended into the hepatic lobules to separate them completely, a large number of inflammatory cells infiltrated in the intralobular and interlobular regions, the liver structure was disordered and there were more necrotic, fatty degenerated liver cells and more collagen deposition compared with the controls (Figure 2A2 and 2B2). Compared with the model group, liver fibrosis grade was reduced. Hepatocyte degeneration, necrosis and infiltration of inflammatory cells were all apparently ameliorated. In addition, collagen deposition was also markedly reduced (Figure 2C and 2D) in the PA 10 mg/kg group.

Serum levels of HA, LN and PCIII were all significantly increased in the animals of liver fibrosis. After administration of PA 10 mg/kg for 4 weeks, serum levels of HA, LN and PCIII were all significantly decreased, while ALB/GLB was increased, as shown in Table 2.

The liver factor, the contents of hepatic HYP, serum levels of ALT and AST were all significantly increased in the animals of liver fibrosis. As shown in Table 3, after administration of PA 10 mg/kg for 4 weeks, the liver factor, the contents of hepatic HYP, serum levels of ALT and AST were all significantly decreased.
Table 1. Effect of PA on TGF-β1, CTGF, PCI and PCIII expression in TNF-α stimulated HSCs.

<table>
<thead>
<tr>
<th>Group</th>
<th>Concentration (μM)</th>
<th>TGF-β1 (ng/mL)</th>
<th>CTGF (ng/mL)</th>
<th>PCI (μg/mL)</th>
<th>PCIII (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.59 ± 0.73</td>
<td>0.10 ± 0.02</td>
<td>25.17 ± 6.01</td>
<td>2.67 ± 0.45</td>
<td></td>
</tr>
<tr>
<td>Control + PA</td>
<td>2.50 ± 0.65</td>
<td>0.09 ± 0.02</td>
<td>23.98 ± 6.45</td>
<td>2.62 ± 0.49</td>
<td></td>
</tr>
<tr>
<td>TNF-α-treated</td>
<td>5.59 ± 1.06</td>
<td>0.39 ± 0.06</td>
<td>105.23 ± 14.01</td>
<td>7.72 ± 1.03</td>
<td></td>
</tr>
<tr>
<td>PA + TNF-α 1</td>
<td>5.13 ± 0.58</td>
<td>0.34 ± 0.04</td>
<td>97.53 ± 12.73</td>
<td>7.15 ± 0.98</td>
<td></td>
</tr>
<tr>
<td>PA + TNF-α 3</td>
<td>4.88 ± 0.81</td>
<td>0.30 ± 0.05a</td>
<td>83.97 ± 14.42a</td>
<td>6.16 ± 1.06a</td>
<td></td>
</tr>
<tr>
<td>PA + TNF-α 9</td>
<td>4.50 ± 0.71</td>
<td>0.27 ± 0.06b</td>
<td>69.80 ± 9.98b</td>
<td>5.12 ± 0.73b</td>
<td></td>
</tr>
<tr>
<td>PA + TNF-α 27</td>
<td>4.09 ± 0.69a</td>
<td>0.24 ± 0.02b</td>
<td>59.03 ± 7.80b</td>
<td>4.33 ± 0.57b</td>
<td></td>
</tr>
<tr>
<td>PA + TNF-α 81</td>
<td>4.08 ± 0.91a</td>
<td>0.19 ± 0.03b</td>
<td>43.74 ± 11.19b</td>
<td>3.87 ± 0.82b</td>
<td></td>
</tr>
</tbody>
</table>

Data represent the means ± SD (n = 6). HSCs treated with or without PA (1, 3, 9, 27, 81 μM) were stimulated or unstimulated with TNF-α (20 ng/mL) for 48 h. TGF-β1, CTGF, PCI and PCIII were determined by ELISA. PA indicated protocatechuic aldehyde. *p < 0.05, **p < 0.01 vs. TNF-α-treated group. Significance was determined by one-way analysis of ANOVA followed by Tukey’s test.

Figure 2. Effect of PA on pathological progress of liver morphology. (A1–A3): Representative light microscopic appearance of rat liver (hematoxylin staining; original magnification ×200) for control (A1), model (A2) and PA 10 mg/kg (A3). (B1–B3): Representative light microscopic appearance of liver (masson trichrome staining; original magnification ×200) for control (B1), model (B2) and PA 10 mg/kg (B3). (C): Effect of PA on pathological scores in liver; (D): Effect of PA on collagen expression in liver, results of collagen expression were expressed as fold increase over control group. #p < 0.01 vs the control group; **p < 0.01 vs. the model group. Significance of masson trichrome staining was determined by one-way analysis of ANOVA followed by Tukey’s test. Fibrosis scores of differences between groups were evaluated by Nonparametric Tests.
Immunostaining and western blot analysis of CTGF and TGF-β1 expression were low in the control animals, while the CTGF and TGF-β1 expression were higher in the animals of liver fibrosis, as shown in Figures 3 and 4. After administration of PA 10 mg/kg for 4 weeks, the CTGF and TGF-β1 expression were significantly lower compared with the model animals, especially the levels of CTGF expression.

Our present study showed that administration of PA 10 mg/kg for 4 weeks increases ALB/GLB in hepatic injury rats caused by CCl₄. Histological examination demonstrated that PA ameliorates liver histological change, attenuates the extent of necrosis. Moreover, PA decreased fibrosis grade. It suggested that PA significantly protects liver against fibrosis. In addition, administration of PA 10 mg/kg for 4 weeks decreased the serum levels of Table 2. Effect of PA on fibrosis grade, serum HA, LN, PCIII and ALB/GLB.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>HA (ng/mL)</th>
<th>LN (ng/mL)</th>
<th>PCIII ng/mL</th>
<th>ALB/GLB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>saline</td>
<td>15.8±3.5</td>
<td>9.2±2.1</td>
<td>15.2±4.2</td>
<td>1.58±0.13</td>
</tr>
<tr>
<td>Model</td>
<td>saline</td>
<td>41.5±6.3</td>
<td>27.8±6.2</td>
<td>40.9±7.9</td>
<td>0.93±0.12</td>
</tr>
<tr>
<td>PA 10</td>
<td>31.6±7.8b</td>
<td>19.4±3.8b</td>
<td>31.9±4.4b</td>
<td>1.15±0.16b</td>
<td></td>
</tr>
</tbody>
</table>

Data represent the means ± SD (n=10). PA indicated protocatechuic aldehyde. *p<0.05, †p<0.01, vs. the model group. Significance of other data was determined by one-way analysis of AVONA followed by Tukey’s test.

Table 3. Effect of PA in body weight, liver factor and content of HYP in liver tissue.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>Body weight (g)</th>
<th>Liver factor</th>
<th>HYP (mg/g)</th>
<th>ALT (U/L)</th>
<th>AST (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>saline</td>
<td>437±54</td>
<td>3.01±0.29</td>
<td>0.35±0.13</td>
<td>25±4</td>
<td>68±9</td>
</tr>
<tr>
<td>Model</td>
<td>saline</td>
<td>287±19</td>
<td>3.98±0.72</td>
<td>1.72±0.50</td>
<td>62±16</td>
<td>160±41</td>
</tr>
<tr>
<td>PA 10</td>
<td>304±21</td>
<td>3.31±0.32a</td>
<td>1.20±0.27b</td>
<td>45±8a</td>
<td>118±24a</td>
<td></td>
</tr>
</tbody>
</table>

Data represent the means ± SD (n=10). PA indicated protocatechuic aldehyde. *p<0.05, †p<0.01 vs. the model group. Significance was determined by one-way analysis of AVONA followed by Tukey’s test.

![Figure 3. Effect of PA on liver CTGF and TGF-β1 expression by immunostaining analysis. (A1–A3): Liver immunostaining for TGF-β1 (×200) and (B1–B3): Liver immunostaining for CTGF (×200) for control (A1, B1), model (A2, B2), PA 10 mg/kg (A3, B3). (C): Effect of PA on immunostaining for CTGF and TGF-β1 expression in liver, results were expressed as fold increase over control group. PA indicated protocatechuic aldehyde. #p<0.01 vs. the control group; **p<0.01 vs. the model group. Significance was determined by one-way analysis of ANOVA followed by Tukey’s test.](image-url)
III collagens, laminin and the serum level of type III

tion and ECM accumulation, attenuate Type I and type

through siRNA has been shown to suppress HSC activa-

ECM-degradation. CTGF knockdown by gene silencing

direction of anti-fibrosis, e.g., inhibit ECM synthe-

ment of cirrhosis.

However, systemic application of inhibitors of

fibrotic rats.

Serum levels of HA, LN and PCIII are the important

biomarkers reflecting the degree of hepatic fibrosis,

included hepatic HYP (Hayasaka & Saisho, 1998; Kornet

et al., 1996; Murawaki et al., 1996). After administration

of PA 10 mg/kg for 4 weeks, the serum levels of HA, LN

and PCIII, including the content of hepatic HYP, were

reduced. It suggested that PA prevents hepatic fibrosis

due to chronic liver injury, thus delaying the develop-

ment of cirrhosis.

Suppression of TGF-β1 expression attenuates liver

fibrosis. However, systemic application of inhibitors of

TGF-β has potentially safety hazards, so it is not suit-

able for human therapy (Gressner & Weiskirchen, 2003;

Gressner & Gressner, 2008). For the reason described

above, CTGF is a more interesting target for future

anti-fibrotic therapies (Blom et al., 2002). According

to present knowledge, a down-modulation of CTGF

activity would shift the TGF-β1/BMP-7 balance in the

direction of anti-fibrosis, e.g., inhibit ECM synthesis-

attenuate EMT and HSC-activation, and increase

ECM-degradation. CTGF knockdown by gene silencing

through siRNA has been shown to suppress HSC activa-

tion and ECM accumulation, attenuate Type I and type

III collagens, laminin and the serum level of type III

procollagen, and alleviate liver fibrosis in two toxic

models of rat (Li et al., 2006; George and Tsutsu, 2007;

Uchio et al., 2004). In addition, inhibiting CTGF expres-

sion alleviates the severity of liver fibrosis (Li et al., 2010;

Gressner & Gressner, 2008). Those studies suggest that

inhibiting CTGF and TGF-β1 might be much better in

liver fibrosis therapy. Based on these observations, we

evaluated the effect of PA on the expression of TGF-β1,

CTGF, PC I and PC III in TNF-α stimulated HSCs

in vitro, immunohistochemical staining and western

blot analysis of hepatic TGF-β1 and CTGF in vivo. Our

study clearly demonstrated that PA markedly inhibits

CTGF expression, but suitably inhibits TGF-β1. The

analogic results were also confirmed in vitro study.

In addition, PA inhibited the expression of PC I and PC

III in TNF-α stimulated HSCs.

Conclusion

In summary, PA inhibited HSCs proliferation, and reduced

TGF-β1, CTGF, PCI and PC III expression in stimulated

HSCs. PA increased serum ALB/GLB, decreased serum

levels of HA, LN and PCIII, reduced content of HYP in

fibrotic liver, and decreased hepatic fibrosis grade. In

addition, PA ameliorated liver histological morphology,

reduced TGF-β1 and CTGF expression of fibrotic liver.

In conclusion, PA potentially has antifibrogenic effects,

the mechanism might be due to the inhibition of CTGF

expression and HSCs activation. These findings suggest

PA can be a promising drug candidate for the amelio-

rating liver fibrosis.

Declaration of interest

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Laboratory of Long-acting and Targeting Drug Delivery

Technologies (Luye Pharma Group Ltd.).

References


209–218.


connective tissue growth factor: new targets for anti-fibrotic

therapy, Matrix Biol, 21, 473–482.

Bosetti C, Levi F, Lucchini F, Zatonski WA, Negri E, La Vecchia C.


J Hepatol, 46, 827–839.


of grading and staging of viral hepatitis. Zhonghua Chuanranbing


George J, Tsutsu M. (2007). siRNA-mediated knockdown of connect-

teive tissue growth factor prevents N-nitrosodimethylamine-


Gressner AM, Weiskirchen R, Breitkopf K, Dooley S. (2002). Roles of

TGF-beta in hepatic fibrosis. Front Biosci, 7, 793–807.


suppression of active transforming growth factor-β. High enough
to fall deeply? J Hepatol, 39, 856–859.

Gressner OA, Gressner AM. (2008). Connective tissue growth factor:

A fibrogenic master switch in fibrotic liver diseases. Liver Int, 28,

1065–1079.

Gressner OA, Lahme B, Rehbein K, Siliuschek M, Weiskirchen R,


inhibits TGF-beta-stimulated connective tissue growth factor

expression in hepatocytes via PPARgamma and SMAD2/3-


England and Wales–how should we group causes? Health Stat Q,

28, 6–17.


