• BASIC RESEARCH •

Effects of transmitters and interleukin-10 on rat hepatic fibrosis induced by CCl$_4$

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Abstract

AIM: To study the effects of transmitters ET, AgII, PGI$_2$, CGRP and GG on experimental rat hepatic fibrosis and the antifibrogenic effects of IL-10.

METHODS: One hundred SD rats were randomly divided into 3 groups: control group (N): intraperitoneal injection with saline 2 ml· kg^{-1} twice a week; the fibrogenesis group (C): intraperitoneal injection with 50 % CCl$_4$ 2 ml· kg^{-1} twice a week; IL-10 treated group (E): besides same dosage of CCl$_4$ given, intraperitoneal injection with IL-10 4 ug· kg^{-1} from the third week. In the fifth, the seventh and the ninth week, rats in three groups were selected randomly to collect plasma and liver tissues. The levels of ET, AgII, PGI$_2$, CGRP and GG were assayed by radioimmunoassay (RIA). The liver fibrosis was observed with silver staining.

RESULTS: The hepatic fibrosis was developed with the increase of the injection frequency of CCl$_4$. The ET, AgII, PGI$_2$, CGRP and GG levels in serum of group N were 71.84±60.2 ng· L^{-1}, 76.21±33.3 ng· L^{-1}, 313.03±101.71 ng· L^{-1}, 61.97±21.4 ng· L^{-1} and 33.62±14.37 ng· L^{-1}, respectively; the levels of them in serum of group C were 523.30±129.3 ng· L^{-1}, 127.24±50.0 ng· L^{-1}, 648.91±357.29 ng· L^{-1}, 127.15±62.0 ng· L^{-1} and 85.26±51.83 ng· L^{-1}, respectively; the levels of them in serum of group E were 452.52±99.5 ng· L^{-1}, 90.60±44.7 ng· L^{-1}, 475.57±179.70 ng· L^{-1}, 102.2±29.7 ng· L^{-1} and 38.05±19.94 ng· L^{-1}, respectively. The histological examination showed that the degrees of the rats liver fibrosis in group E were lower than those in group C.

CONCLUSION: The transmitters ET, AgII, PGI$_2$, CGRP and GG play a significant role in the rat hepatic fibrosis induced by CCl$_4$. IL-10 has the antagonistic action on these transmitters and can relieve the degree of the liver fibrosis.

INTRODUCTION

Hepatic fibrosis is a disease which is characterized by an increase of type I and type III collagens, proteoglycans fibroneetin and hyaluronic acid in extracellular matrix (ECM) deposition[1-9]. It is an inevitable phase during the formation of liver cirrhosis, which is an irreversible stage of several liver pathological changes[10-12]. So it is important for how to prevent and cure hepatic fibrosis, i.e. antifibrogenetic treatment. Transmitters play an important role in the portal hypertention which is associated with the fibrosis[13,14]. In our study, the transmitters endothelin (ET), angiotensin II(Ag II), prostacyclin (PGI$_2$), calcitonin-gene related peptide (CGRP) and glucagon (GG) were selected to explore their effects on hepatic fibrosis induced by CCl$_4$ and the antifibrogenesis effect of interleukin-10 (IL-10) was explored as well.

MATERIALS AND METHODS

Animals

One hundred clean SD rats weighing 140-180 g were randomly divided into 3 groups. The control group (group N) included 24 rats; the fibrogenesis group (group C) included 40 rats and the IL-10 treated group (group E) included 36 rats, respectively. All the rats were breeding in the routine condition (room temperature 22±2 ℃, humidity 55±5 %, lighting 12hrs per day, to drink tap water and eat in any time when they needed, animal food was provided by BK company in shanghai.).

Establishment of the fibrosis model

Rats in group N were injected intraperitoneally with saline 2 ml· kg^{-1} twice a week. Rats in group C and group E were injected intraperitoneally with 50 % CCl$_4$ 2 ml· kg^{-1} twice a week[15]. From the third week, rats in group E were injected intraperitoneally with IL-10 4 ug· kg^{-1} (dissolved in saline)[16] 20 minutes before they were injected with CCl$_4$. All injections were performed in Monday and Thursday, rats' body weight was recorded before the injection. In the fifth week, 3 rats in group C and 2 rats in group E died, in the seventh week, total 8 rats in group C and 4 rats in group E died, in the ninth week, total 10 rats in group C, 6 rats in group E and 3 rats in group N died. In 5,7,9 weeks, 10 rats of group C and E and 7 rats in the control group were selected randomly to collect their plasma and liver tissue samples.

Assessment of samples

The blood samples were added into the tubes with 30 μl 10 % EDTA and 40 μl trasylol in ice bath, the tubes were centrifuged at 3 000 rpm for 10 minutes at 4 ℃, then the plasma was frozen for the assessment. The plasma levels of ET, Ag II, PGI$_2$, CGRP and GG were assayed by radioimmunoassay (RIA, kits provided by EastAsia Immune-technology Institute, Beijing). Each plasma sample was taken 100 μl into the tube, then 200 μl buffer and 100 μl antiserum were added into each sample, they were agitated and incubated for 24 hour at 4 ℃; then 100 μl ^{125}I-marked serum was added, agitated and incubated for 24 hour at 4 ℃; also 500 μl precipitation was added, after incubation for 20 min at room temperature, the tubes were centrifuged at 3 500 rpm for 25 min at 4 ℃, the upper layer was carefully removed, the cpm account was measuredusing γ

radioimmunocounter. The blank control and the standard control was measured respectively at the same time. The liver tissue was made of paraffin section with silver staining.

Statistical analysis

All data were expressed as $\bar{x} \pm s$, t test was used for comparison between groups.

RESULTS

Plasma levels of ET, AgII, 6-K-PGF$_{1a}$, CGRP and GG

The plasma levels of ET, AgII, 6-K-PGF$_{1\alpha}$, CGRP and GG in group C were higher than those in the control ($P<0.05$). After the intervention of IL-10, the levels of them were decreased, and had no difference with group N ($P>0.05$). Furthermore, their levels were increased with the development of hepatic fibrosis.

Table 1 Plasma levels of ET, AgII, 6-K-PGF$_{1\alpha}$, CGRP and GG in fibrosis and normal rats (ng· L^{-1})

	n	ET	AgII	6-K-PGF$_{1\alpha}$	CGRP	GG
N	21	71.84±60.2	76.21±33.3	313.03±101.71	61.97±21.4	33.62±14.37
C[a]	30	523.30±129.3	127.24±50.0	648.91±357.29	127.15±62.0	85.26±51.83
E[b]	30	452.52±99.5	90.60±44.7	475.57±179.70	102.2±29.7	38.05±19.94

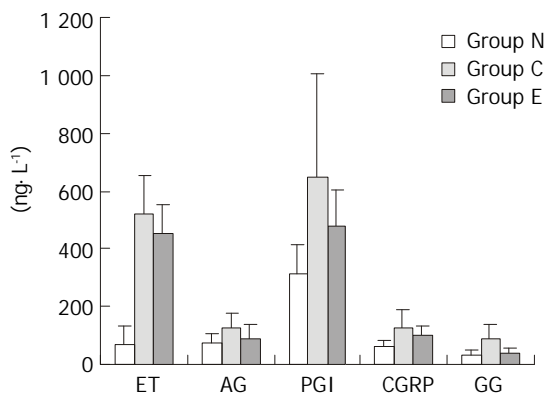[a]$P<0.05$ *vs* group N, [b]$P<0.05$ *vs* group N.

Figure 1 Plasma levels of ET, AgII, 6-K-PGF$_{1\alpha}$, CGRP and GG in fibrosis and normal rats.

Table 1 and Figure 1 showed that after the treatment of CCl$_4$, the plasma levels of ET, AgII, 6-K-PGF$_{1\alpha}$, CGRP and GG were increased, their levels were significantly higher than those in the normal controls ($P<0.05$). After treated with IL-10, their levels were obviously decreased, and there was no significant difference with those in the normal controls. It was showed that when the effective treatment was applied in the fibrosis rats, the levels of these transmitters showed the descending trend. It suggested that the levels of those transmitters were increased in liver fibrosis and they might play important pathogenic roles during the development of liver fibrosis.

Table 2 Plasma levels of ET, AgII, 6-K-PGF$_{1\alpha}$, CGRP and GG in fibrosis rats (ng· L^{-1})

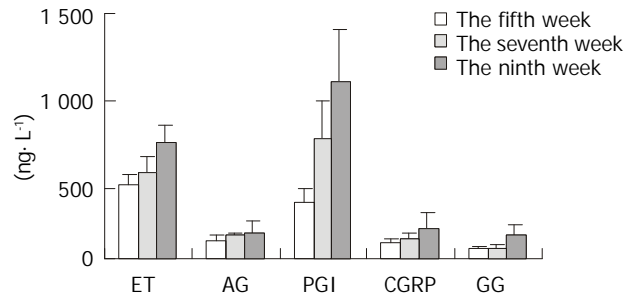| Week	n	ET	AgII	6-K-PGF1$_\alpha$	CGRP	GG
No.5	10	421.48±52.3	105.73±36.3	323.15±76.2	88.68±23.2	54.48±18.9
No.7	10	489.80±87.7	131.42±18.9	684.98±214.0	118.14±24.3	55.77±19.2
No.9	10	658.61±102.3	144.58±72.2	1081.61±294.3	174.65±87.7	141.66±50.8

Figure 2 Plasma levels of ET, AgII, 6-K-PGF$_{1\alpha}$, CGRP and GG in fibrosis rats.

Table 2 and Figure 2 showed that the levels of ET, AgII, 6-K-PGF$_{1\alpha}$, CGRP and GG were gradually increased and associated with the increase of CCl$_4$-treated frequency, especially in the ninth week ($P<0.05$). It suggested that there was close relation between the levels of the transmitters and the degrees of liver fibrosis.

Pathological assay

The histological feature showed that liver of control rats had no appreciable alterations (Figure 3). The degree of liver fibrosis in group C was up-going with the increas of the treatment frequency of CCl$_4$. In the fifth week, few reticular fiber deposited in the periportal tissue space. In the seventh week, the reticular fiber extended with hepatic plate but the full delimitation was not formed, while in the ninth week the integrity fibrous septum was developed in the interlobular septum, sometimes psedulobular could be seen (Figure 4,5,6). The degrees of inflammation of hepatocytes were decreased evidently in the seventh week after the treatment of IL-10, in the ninth week, the reticular fiber in the interlobular septum was limited remarkably, no psedulobular could be seen (Figure 7).
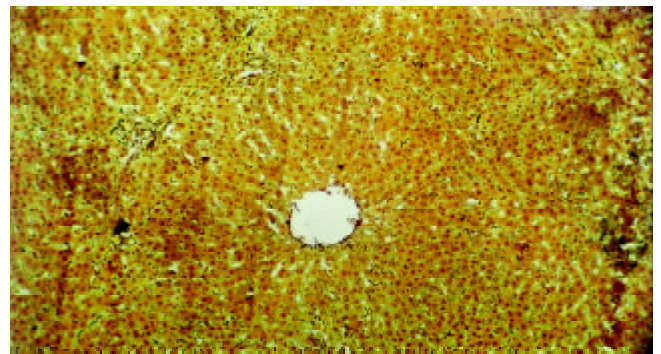

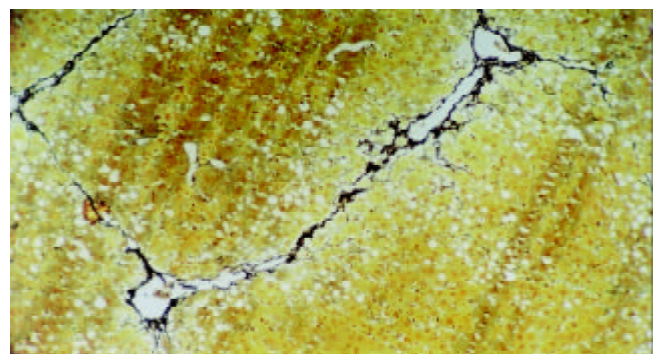**Figure 3** The liver of normal rat (silver staining, ×100).

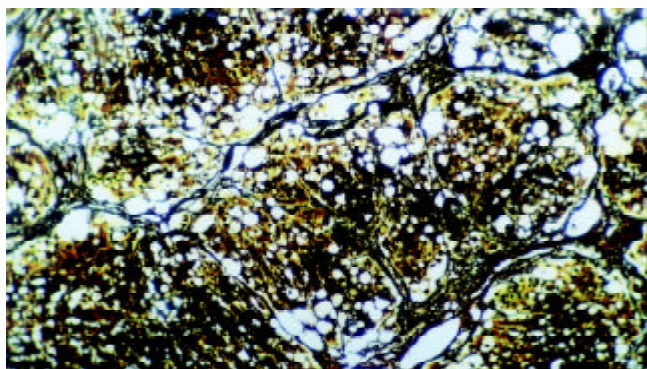**Figure 4** The liver of the rat in group C (the fifth week, silver staining, ×100).

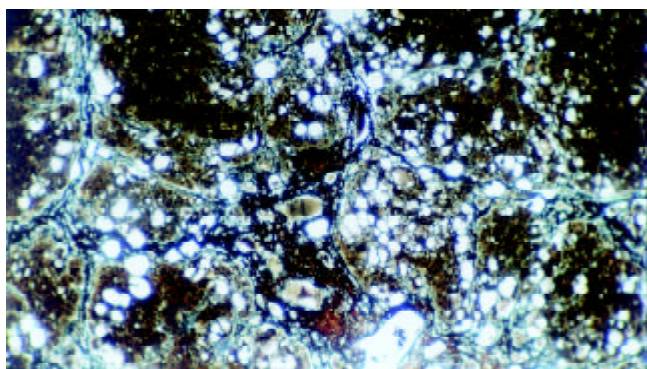**Figure 5** The liver of the rat in group C (the seventh week, silver staining, ×100).

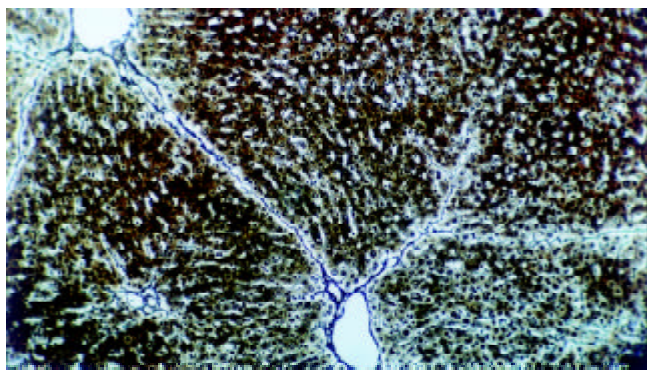**Figure 6** The liver of the rat in group C (the ninth week, silver staining, ×100).

Figure 7 The liver of the rat in group E (the ninth week, silver staining, ×100).

DISCUSSION

Endothelins are a family of polypeptides consisting of 21-amino acids[17-19]. ET-1 is initially noted for its powerful vasoconstrictor properties[20-24]. It is markedly overexpressed in different cellular elements in cirrhotic liver tissue, and particularly in sinusoidal endothelial cells and hepatic stellate cells (HSCs) in their activated phenotype located in the sinusoids of the regenerating nodules and at the edges of fibrous septa[25]. It plays an important role in the regulation of hepatic vascular tone. They elicit biological responses via the ET_A and ET_B receptors. ET-1 induces contraction, proliferation, and collagen synthesis of HSCs *in vitro*, which may be mediated *via* the ET_A receptors[26]. ET-1 is able to increase $[Ca^{2+}]_i$ in a dose-dependent fashion in HSCs, which results from both intracellular release of Ca^{2+} and extracellular Ca^{2+} influx via a dihydropyridine-insensitive pathway. ET-1-induced contractility of HSCs is maintained through all stages of

activation and is independent of the absolute number of ET_A-binding sites if a threshold level of expression is maintained. It has been shown that ET-1 could act as a cell growth promoter *via* the ET_A receptor to promote the proliferation of smooth muscle cell. Also, ET-1 is able to elicit MAPK (mitogen-activated protein kinase) activity in human HSCs with time-course and dose-response kinetics similar to those reported in mesangial cells through the ET_A receptor. Recent studies have shown that the ETR antagonist modifies the development of portal hypertension in carbon tertrachloride treated rats[27,28]. Some studies suggest that ET has two effects on HSC[29]. ET can inhibit the contraction and collagen synthesis in cells that have more ET_B receptors than ET_A receptors; it indicates that ET could restrict the development of liver fibrosis. The difference is linked to the active, contractile HSC phenotype. The cellular sites of action of AgII within the hepatic vasculature are incompletely defined; recent studies have shown that HSCs may be a potential cell target for the AgII actions in the hepatic vasculature[30]. Two different types of AgII receptors have been described. The AT1 receptors are present in most mesenchymal cells and mediate most of the biological effects of AgII. The AT2 receptors are mainly found in fetal cells, but their physiological role is not completely understood. AgII receptors (AT1 subtype) exist in many cells, including the human HSC[31], the activated HSCs may be an important target of the AgII in the hepatic vasculature[32]. The binding of AgII to AT1 receptor induces contraction and proliferation[33,34]. AgII causes a marked increase in $[Ca^{2+}]_i$ and cell contraction, which largely depends on the entrance of Ca^{2+} through L-type Ca^{2+} channels. In recent years, much attention has been focused on the growth-promoting effects of AgII and it has been found that AgII is also a mitogenic factor for activated HSCs through an MAPK- dependent pathway. So we could hypothesis that AgII plays a role in the proliferation of HSCs and in the progression of liver fibrosis. The inflammation may be the initial fibrogenic event. PGI_2, a potent vasodilator produced by the splanchnic endothelium, would account for much of the observed hyperemia[35]. Cyclooxygenase blockade reverses the splanchnic hyperemia[36]. The mechanism for the increase of portal PGI_2 remains unknown. Some have suggested of increase that blood pressure alone will increase the production of PGI_2. Theoretically, damage to any type of liver cell membrane can serve as a source of AA metabolites that initiate fibrosis. In the intact liver, the most probable target cells are the nonparenchymal cells such as endothelial cells. The inflammation may be the initial fibrogenic event. The inflammation involving the release of arachidonic acid (AA) from phospholipids by activation of phospholipae A_2 in damaged cell membranes and formation of bioactive AA metabolites (prostaglandins, thromboxane A_2 and leukotrienes) by way of 5' lipoxygenase pathway is one of the earliest biochemical events in hepatic fibrosis. The concentration of 6-keto-PGF1α, the stable metabolite of PGI_2, represents the plasma level of PGI_2. The enhanced production of 6-keto-$PGF_{1α}$ increases the TGF-$_{β1}$ gene expression by way of enhancing degranulation of platelets and inflammatory cells which are rich source of the fibrotic cytokine TGF-$_{β1}$[37]. As we all know, TGF-$_{β1}$ can promote the synthesis and deposition of ECM and inhibit the degradation of ECM[38,39]. CGRP is a highly potent vasodilator and is widely distributed in nerve fibers with relation to vascular structures[40]. The circulating CGRP is elevated in liver cirrhosis[41,42], but little information is known about CGRP in these patients[43]. Some authors have reported that CGRP could inhibit the lipid peroxidation on the liver, which antagonists the effects of ET[44]. So it is a protector in the liver fibrosis. Whether the CGRP has effects on the activation of HSC and the synthesis of collagen is not clarified. GG is a stress hormone whose release is stimulated by

catecholamines, cortisol, and growth hormone[45]. GG plays an important role in the formation of portal hypertension[46]. The present studies show that plasma GG levels are elevated in cirrhotic patients with portal hypertension. It is also clearly demonstrated that plasma GG levels is increased with the progression of cirrhosis. In addition, positive correlations has been found between plasma GG levels and Pugh's score or liver functions. In our study the increase of GG was associated with the failure of GG's degradation in liver and the hyperexcretion of pancreas. IL-10 is a potent anti-inflammatory cytokine that inhibits the synthesis of pro-inflammatory cytokines by T helper type 1 cells. It is produced locally in the liver and acts in an autocrine or paracrine way. IL-10 can inhibit a range of macrophage effecter functions, including nitric oxide and reactive oxygen intermediate production, MHC class II antigen expression, and eicosanoid synthesis. IL-10 can down-regulate expression of adhesion molecules, ICAM-1 and B7, on human monocytes, and also the nuclear transcription factor, nuclear factor κB. It is able to inhibit chemokine synthesis in T cells, neutrophils, and fibroblasts. Moreover, proinflammatory cytokines synthesis by a wide range of cells, particularly monocytes and macrophages, is profoundly inhibited by IL-10[47]. Previous reports indicated that IL-10 had a role in the remodeling of the extracellular matrix[48]. *In vitro*, IL-10 down regulates collagen type I while up regulates metalloproteinase gene expression. It also has antifibrogenic properties by down regulating profibrogenic cytokines, like TGF-β_1 and TNF-α[47,49]. Nelson *et al* had treated 24 patients with chronic hepatitis C with IL-10, they found that IL-10 normalized serum ALT levels, decreased hepatic inflammation, reduced liver fibrosis and was well tolerated in patients[16].

After that treatment of IL-10, all of the transmitters decreased. Therefore, transmitters play important roles in rat hepatic fibrosis induced by CCl$_4$. IL-10 decreases the levels of these transmitters so it has antifibrogenesis effect.

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