The regulatory role of AT 1 receptor on activated HSCs in hepatic fibrogenesis: effects of RAS inhibitors on hepatic fibrosis induced by CCl₄

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Abstract

AIM To assess the effect of ACE inhibitor and AngII type 1 (AT1) receptor antagonist in preventing hepatic fibrosis caused by CCl₄ administration in rats; to investigate whether or not there are expression of AT 1 receptors on hepatic stellate cells; and to observe the effect of AngII on proliferation and ECM synthesis of cultured HSCs.

METHODS Studies were conducted in male Sprague-Dawley rats. Except for the hepatofibrotic model group and the control group, in three treated groups, either enalapril (5mg/kg), or losartan (10mg/kg), or enalapril + losartan were given to the fibrotic rats by daily gavage, and saline vehicle was given to model and normal control rats. After 6 weeks, liver fibrosis was assessed directly by hepatic morphometric analysis, which has been considered the gold standard for the quantification of fibrosis. The expressions of AT 1 receptors and (α-smooth muscle actin, α-SMA) in liver tissue or isolated hepatic stellate cells (HSCs) were detected by immunohistochemical techniques. The effect of AngII on HSC proliferation was determined by MTT method. Effect of AngII on collagen synthesis of HSCs was determined by 3H-proline incorporation.

RESULTS Contrasted to the fibrosis in rats of the model group, groups of rats treated with either enalapril or losartan, or a combination of two drugs showed a limited expansion of the interstitium (4.23±3.70 vs 11.22±4.79, P<0.05), but no difference was observed among three treated groups (5.38±3.43, 4.96±2.96, 4.23±2.70, P>0.05). Expression of AT 1 receptors was found in fibrotic interstitium of fibrotic rats, whereas in normal control rats they were limited to vasculature only to a very slight degree. AT 1 receptors were also expressed on activated HSCs in the culture. At concentrations from 10⁻⁹ to 10⁻² mol/L, AngII stimulated HSC proliferation in culture in a dose-dependent manner. Increasing AngII concentrations produced corresponding increases in 3H-proline incorporation. Differences among groups were significant.

CONCLUSION Angiotensin-converting enzyme inhibitors and AT 1 blocker may slow the progression of hepatic fibrosis; activated HSCs express AT 1 receptors, and AngII can stimulate the proliferation and collagen synthesis of HSCs in a dose-dependent manner; and activation of RAS may be related to hepatic fibrogenesis induced by CCl₄.

INTRODUCTION

Liver fibrosis is a consequence of chronic liver injury from different causes, including alcohol, toxins, chronic viral infections, metabolic disease, etc. In liver fibrogenesis there is an increased deposition of extracellular matrix (ECM) in the perisinusoidal and periportal spaces. The activated hepatic stellate cells (HSCs), have now been identified as the primary source of ECM synthesis liver fibrogenesis[1-3]. The pathogenetic significane of HSC relies on their ability to be activated into myofibroblast-like cells with enhanced production of ECM[4,5]. Proliferative cytokine like platelet-derived growth factor and fibrogenetic cytokines like transforming growth factor-β (TGF-β) are major cytokines involved in the activation process, causing enhanced proliferation of HSC and matrix synthesis[6,7].
Researches increasingly show that locally synthesized angiotensin II (Ang II), as a fibrogenetic factor, is involved in cardiac fibrosis\cite{8,9}, renal interstitial fibrosis\cite{10,11}, and pulmonary fibrosis\cite{12,13}. Thus, treatment with angiotensin-converting enzyme (ACE) inhibitor or angiotensin II type 1 (AT1) receptor antagonist may attenuate the cardiac fibrosis that occurs in experimental myocardial infarction\cite{8}, and may also retard the progression of renal glomerulosclerosis and interstitial fibrosis\cite{14,15} and pulmonary fibrosis\cite{16}.

It is believed that Ang II has a direct fibrogenetic effect, independent of its systemic hemodynamic effect. The evidence supporting this notion comes from in vitro studies showing that the AT1 receptor is present on interstitial fibroblasts, such as cardiac fibroblasts\cite{17}, renal interstitial fibroblasts\cite{18}, and that Ang II directly increases ECM synthesis in cultured fibroblasts.

We hypothesized that the activation of local RAS might also be related to hepatic fibrogenesis. Thus, it is important to ascertain the existence of expression AT1 receptor on HSCs in the process of hepatic fibrosis. If this hypothesis is true, new preventive and therapeutic approaches may be provided to hepatic fibrosis. This study was designed: to assess effects of ACE inhibitor, enalapril, and AT1 receptor antagonist, losartan, on hepatic fibrosis induced by CCl4; and to investigate whether or not there is expression of AT1 receptors on HSCs.

METHODS

Animals and therapeutic regimen
Adult male Sprague-Dawley rats (Animal Centre of Shanghai Medical University, China) weighing 180g-200g were housed in temperature and light-controlled room. The rats were divided into five groups (n=10 in each group): the control group, model group and three treated groups. Except for rats of the control group, all rats were given subcutaneous injection of 40% CCl4 (CCl4:olive oil 2:3), 0.3mL/100g, every 3 days for 6 weeks). Simultaneous treatments with enalapril (Changzhou Pharmacy, China) (5mg·kg\(^{-1}\)·d\(^{-1}\)), losartan (Merk Co, England) (10mg·kg\(^{-1}\)·d\(^{-1}\)), or enalapril + losartan administration (once a day by gavage for 6 weeks) were withdrawn a day before the rats were killed for study.

Serum analysis
After sacrificing the rats, blood samples were immediately taken and centrifuged at 4°C, and plasma were kept at -20°C for assays. The aspartate transaminase (AST) and alanine transaminase (ALT) activities were determined by the standard enzymatic methods. The serum levels of angiotensin II and renin activity were determined by radioimmunoassays (kit purchased from Northern Biot Co, China).

Image analysis of liver fibrosis
Liver fibrosis was assessed directly by hepatic morphometric analysis, which has been considered to be the gold standard for the quantification of fibrosis. For image analysis, three liver fragments (> 10mm\(^2\)) were randomly taken in the right, median, and left lobes of each rat liver. The liver sections were fixed in a 10% solution of formaldehyde in 0.1mol/L phosphate-buffered saline (pH 7.4), and embedded in paraffin. Five-micrometer slides were prepared. Collagen expression was detected with standard van Gieson staining. The histomorphometric analysis was performed on a KS400 image analysis system (German). The liver slides were placed on the X-Y motorized stage of microscope after equalization of light intensity. The percentage of fibrosis or area of fibrosis could be obtained in microscopic fields. Total liver area of fibrosis was expressed as the mean of the percentage of fibrosis in the three liver fragments.

Expression of α-SMA and AT1 receptor in liver tissue
Immunohistochemical methods were used to detect the expressions of α-SMA and AT1 (anti-rat rabbit polyclonal AT1 antibody was the product of Santa Cruz Biot Co, USA) receptors in liver tissue.

Cells isolation and culture
HSCs were isolated from normal male Sprague-Dawley rat (500g-600g body weight) liver by a combination of pronase-collagenase perfusion and density gradient centrifugation, as previously described\cite{19}, and identified by positive ultraviolet autofluorescence. Primary HSCs were cultured in 24-well plastic culture plate at a density of 5×10\(^5\) cells/L, and maintained in Dulbecco’s modified Eagle medium (DMEM) with 15% fetal calf serum (FCS) (Sigma) and penicillin/ streptomycin. After seven days, the cells were wa shed with PBS, and the expression of α-SMA (Monoclonal antibody purchased from Maixin Biot Co, China) was determined by immunohistochemical method. The expression of AT1 receptor was detected by immunofluorescence staining with anti-rat rabbit polyclonal antibody (Santa Cruz Biot) and labeled with fluorescein-isothiocyanate (FITC, Sigma).

Effect of AngII on HSCs proliferation
The effect of AngII on HSC proliferation was determined by MTT method. After being cultured
for 4 days, HSCs were digested with 0.25% trypsin and then cultured in serum-free medium in 96-well culture plate (200µL per well), to which was added AngII (to make a series of final concentrations: 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} mol/L). Each concentration included six wells, while the serum-free medium served as a control. After being cultured for 48 hours, HSCs were supplemented with 20µL MTT (5g/L) (Fluka Co. Product) and incubated for another 6 hours. Then the supernatant was discarded by aspiration and the HSC preparation was shaken with 200µL DMSO for 10 minutes, before the OD value was measured at 490nm.

**Effect of AngII on collagen synthesis of HSCs**

Collagen synthesis was determined by ^3^H-proline incorporation. After cultured for 48 hours, HSCs were further cultured for additional 48 hours in serum -free medium containing 2µL ^3^H-proline in various concentrations of Ang II (the final concentrations being 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} mol/L). Subsequently, HSCs were digested with 0.25% trypsin and collected on glass fiber membrane, then washed with PBS. After being dried, HSCs were suspended in 10mL scintillation solution for 12 hours. The radioactivity was determined by liquid scintillation and expressed as cpm /2.5×10^5 cells.

**Statistical analysis**

Data were presented as mean ± SEM. Comparisons among three or more groups were made by one-way ANOVA followed by Dunnett’s t test. A value of P<0.05 was considered to be statistically significant.

**RESULTS**

**Effects of RAS inhibitors on rat serum levels of ALT and AST**

Contrasted with rats of the control group, as shown in Table 1, rats receiving CCl_4 had increased serum ALT and AST significantly. However, chronic administration of enalapril and losartan prevented the increase in ALT and AST (P<0.01, Table 1).

**Serum renin activity and Ang II levels**

Serum renin activity (µg·L^{-1}/h) of rats in the model group was three times higher than that of the control group (P<0.01). Serum Ang II (ng/L) levels of model group also increased two times than that of control group (P<0.01). Treatment with RAS inhibitors significantly decreased the serum renin activity and serum levels of Ang II (P<0.05, Table 2).

**Fibrosis quantification**

As expected, there was an increase in the area of fibrosis in rats of the model group compared with that of the control group. There was a significant decrease of fibrosis in all three treated groups (P<0.05), but no significantly difference was observed between the enalapril and losartan treated groups (P>0.05, Table 3).

**Table 1 Effects of RAS inhibitors on rat serum levels of ALT and AST**

|            | ALT(U/L)         | AST(U/L)        |
|------------|------------------|-----------------|
| Control    | 10               | 244.50±46.52    |
| Model      | 7                | 2680.86±1039.12 |
| Enalapril  | 7                | 785.29±262.40   |
| Losartan   | 8                | 777.50±270.32   |
| Enalapril+losartan | 8    | 449.60±130.36   |

*P<0.01 vs control; *P<0.05 vs Model; *P<0.05 vs Model.

**Table 2 Serum renin activity and Ang II levels**

|            | Renin(mg·L^{-1}/h) | Ang II(ng/L) |
|------------|--------------------|--------------|
| Control    | 10                 | 495.78±248.96|
| Model      | 7                  | 1505.46±49.72|
| Enalapril  | 7                  | 951.78±451.67|
| Losartan   | 8                  | 777.50±270.32|
| Enalapril+losartan | 8    | 449.60±130.36   |

*P<0.01 vs Control; *P<0.05 vs Model.

**Immunohistochemistry**

Expressions of AT1 receptors and α-SMA in liver tissue were detected by immunohistochamical methods. AT1 receptors mainly localized in the vasculature of liver tissue in the normal control rats. In rats of the model group, the expression of AT1 receptors mainly localized in the fibrotic areas, correlated with the expression of α-SMA. Enalapril, losartan, and combined treated groups showed a reduced AT1 receptor staining.

After culturing HSCs for seven days, the expression of AT1 receptors in HSC could be detected by FITC-immunofluorence, together with the expression of α-SMA (Figure 1A-1D).

**Effect of AngII on HSCs proliferation and collagen synthesis**

Over a wide range of concentrations from 10^{-9} to 10^{-5}mol/L, AngII stimulated cultured HSCs proliferation in a dose-dependent manner (Figure 2). Increasing AngII concentration produced an increase in ^3^H-proline incorporation (Figure 3). Differences among groups were significant (P<0.05).

**Table 3 Fibrosis measurements**

|                | Control          | Model            | Enalapril        | Losartan         | Enalapril+losartan |
|----------------|------------------|------------------|------------------|------------------|-------------------|
| Area of fibrosis | 0.94±0.33        | 11.22±4.79*      | 5.38±3.43*       | 4.96±2.96*       | 4.23±3.70*        |

*P<0.01 vs Control; *P<0.05 vs Model.
Figure 1A-1D A: Under the 328nm ultraviolet, HSCs had an autofluoresence. (×400 times) B: After cultured five days, HSCs transformed into myofibroblast-like cells. (×400 times) and expressed α-SMA (C, staining with DAB), labeled with FITC, expression of AT1 receptor was observed (D).

Figure 2 Effect of Ang II on HSC proliferation.

DISCUSSION

The present study demonstrates for the first time the expression of AT1 receptor subtype in fibrotic liver tissue and in primary cultured rat HSC.

In recent years, a series of investigations demonstrated that fibrogenesis was related to the activation of local RAS[20-24]. Expression of AT1 receptors in several types of interstitial cells, such as cardiac fibroblasts[17], renal interstitial fibroblasts[18] and lung fibroblasts[25]. In vivo study suggested that Ang II induced a net stimulation of collagen synthesis and expression of TGF-β via AT1 receptors in a dose-dependent manner[26-30]. Blockade of AT1 receptors has been shown to inhibit DNA and collagen syntheses in interstitial cells after myocardial infarction[31,32], renal and pulmonary injury[15,33], and retarded the progression of cardiac, renal and pulmonary interstitial fibrosis[34-41].

It is believed that the activation and proliferation of HSCs are major processes in hepatic fibrosis, although its pathogenesis has not been fully clarified[42-45]. The expression of AT1 receptors in HSCs and fibrotic liver tissue supports the notion that activation of local RAS might be related to hepatic fibrogenesis induced by CCl₄. Our results showed that in rat livers, the expression of AT1 receptors in fibrotic areas correlated with the expression of α-SMA. In primary cultured HSCs, the expression of AT1 receptors also correlated with α-SMA. These findings indicated that, as a growth factor of interstitial cells, Ang II might prompt activation of HSCs in the process of hepatic fibrosis. Although both ACE inhibitor and AT1 receptor antagonist could alleviate hepatic fibrosis, as our results showed, a combination of these two gave no added effects of slowing the progression of hepatic fibrosis.

Another interesting finding in our study was that ACE inhibitor and AT1 receptor antagonist could attenuate the hepatocytic injury induced by CCl₄, suggesting their cytoprotective effects by virtue of antifibrogenesis.

As the principal effector molecule of RAS, the production of a profibrogenic cytokine, TGF-β was
enhanced by Ang II in progressive fibrosis of heart and kidney. Recently, Powell et al. demonstrated that a statistically significant relationship was observed between inheritance of high TGF-α and angiotensinogen-producing genotype and the development of progressive hepatic fibrosis. Patients with chronic hepatitis C virus infection who inherited neither of the profibrogenic genotype had no or only minimal fibrosis. The documentation of a significant relationship between angiotensinogen genotype and fibrosis also suggested that Ang II might be another mediator of ECM production in the liver.

In conclusion, our investigation demonstrated that: 1) ACE inhibitor and AT1 receptor antagonist might slow the progression of hepatic fibrosis induced by CCl4, but a combination of the two gave no additive effect; 2) activated HSCs expressed AT1 receptors, and Ang II could stimulate the proliferation and collagen synthesis of HSCs in a dose-dependent manner; and 3) local activation of RAS in liver tissue might be related to hepatic fibrogenesis induced by CCl4.

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