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# III. 研究成果の刊行物・別刷

ELSEVIER

Hepatology Research 21 (2001) 147–158

Hepatology Research

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# Anti-fibrogenic effect of an angiotensin converting enzyme inhibitor on chronic carbon tetrachloride-induced hepatic fibrosis in rats

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Received 11 January 2001; received in revised form 5 March 2001; accepted 9 March 2001

**Abstract**

The tissue renin–angiotensin system has recently been demonstrated to reduce fibrogenesis in various organs. However, little has been clarified regarding its role in hepatic fibrosis. The purpose of this study was to investigate the effect of angiotensin-converting enzyme inhibitors on liver fibrogenesis induced in rats by low-dose chronic carbon tetrachloride administration. We used lisinopril that is absorbed in its active form and not metabolized in the liver to avoid any influence by the administration of the chemical. Carbon tetrachloride was administered twice a week i.p. Twelve and 24 weeks after the start of treatment, expanded periportal fibrosis or portal–portal bridgings and severe fat deposition were observed in the rats treated with carbon tetrachloride alone, and these findings were significantly reduced with the simultaneous treatment with lisinopril. The hydroxyproline content of the liver was significantly lower in the lisinopril-treated group. Angiotensin II up-regulated mRNA of pro α (I) collagen and transforming growth factor-β in isolated hepatic stellate cells. These results suggest that the local tissue renin–angiotensin system plays a role in rat hepatic fibrogenesis induced by chronic carbon tetrachloride administration and that hepatic fibrogenesis is significantly reduced by ACE inhibitors. 

*Keywords:* Rat hepatic stellate cells; Angiotensin II; Angiotensin-converting enzyme inhibitor; Carbon tetrachloride; Fibrosis

## 1. Introduction

The renin–angiotensin-aldosterone system plays an important role in the regulation of the systemic blood pressure, body fluid and elec-

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1386-6346/01/$ - see front matter 
PII: S1386-6346(01)00102-4

trolyte balance. Moreover, angiotensin-converting enzyme (ACE) inhibitors have been developed to block the renin–angiotensin system and reduce systemic blood pressure. On the other hand, the existence of a local tissue renin–angiotensin system has been recently postulated [1], and its existence is actually supported by the fact that the expressions of the mRNA set of angiotensinogen, renin, and ACE has been detected in various local tissues and/or organs [2]. Recently, a variety of physiological roles of angiotensin II (Ang II) have been clarified not only in the pathogenesis and maintenance of high blood pressure [3] but also in the stimulation of fibroblast proliferation and collagen synthesis by non-parenchymal cells [4–6]. Decrease in the levels of serum fibrosis markers and the amounts of collagen in cardiac muscle [7], inhibition of pulmonary fibrogenesis in irradiated rats [8] and improvement of proteinuria and glomerular lesions [9] have been achieved by the administration of ACE inhibitors. These inhibitory effects on fibrogenesis are tissue-specific and localized, indicating that Ang II functions independently in every tissue or organ in a paracrine or autocrine manner.

Hepatic fibrosis is a common pathological feature of progressive chronic liver diseases, and is characterized by an abnormal increase in extracellular matrices. The increased extracellular matrices mechanically interfere with blood flow and reduce liver functions. The most important cells directly related to hepatic fibrosis are hepatic stellate cells (HSCs, Ito cells) distributed in Disse's cavity [10–12]. When hepatic parenchymal cells are disturbed, HSCs enlarge, proliferate, and transform into myofibroblasts. These transformed HSCs locally synthesize and excrete a variety of extracellular matrices and promote hepatic fibrosis [13]. Non-parenchymal cell transformation into myofibroblasts has been demonstrated in every organ of the body, e.g., renal mesangial cells [14], and interstitial cells in the myocardium [15] in which Ang II stimulates fibrosis in kidneys and the heart, respectively. This pivotal role of the tissue renin–angiotensin system has also been postulated for the fibrogenesis of the liver, but it is not yet fully understood.

In this study, we clarified the role of the tissue renin–angiotensin system in hepatic fibrogenesis by using an ACE inhibitor in a rat model of liver fibrosis induced by a treatment with chronic carbon tetrachloride ($CCl_4$). We also examined the role of Ang II in HSCs in vitro. The results showed that the renin–angiotensin system exacerbates hepatic fibrosis and that $CCl_4$-induced hepatic fibrogenesis was inhibited by an ACE inhibitor.

## 2. Materials and methods

### 2.1. Animals

All animal experiments were approved by the Animal Experimentation Committee of the School of Medicine, Keio University. Male Sprague–Dawley rats weighing 180–220 g were used throughout the experiments. All animals were housed under specific pathogen-free conditions in cages and were acclimated to the housing situation for one week before the experiments. Two rats were housed to a cage and given a commercial diet in pellet form.

### 2.2. Administration of $CCl_4$ and lisinopril

$CCl_4$ (ultra pure grade, Kanto Kagaku, Tokyo, Japan) was dissolved in olive oil at 50% (v/v), and was administered at a dose of 0.2 ml/kg of body weight twice a week i.p. as described previously [16]. Lisinopril (CV-11974, Takeda Chemical Industries, Tokyo, Japan), which is absorbed in active form and not metabolized in the liver, was used as the ACE inhibitor and is stable at least for a month once diluted in drinking water. Lisinopril was dissolved in the animals' drinking water every two weeks at a concentration of 50 mg/l and was given ad libitum by free drinking throughout the experiments. The volume of supplied drinking water was measured, and was found to be almost equal between the groups. On average, the rat drank the supplied water at a rate of $130 \pm 6$ ml/kg/day (or about 25 ml/day rat). The estimated amount of supplied lisinopril was 6.5 mg/kg/day. This amount was estimated to be about 120 mg/ml of serum concentration based on preliminary experiments. Ang II was purchased from Sigma Chemical Co. (St. Louis, MO).

### 2.3. Experimental groups

Thirty-nine rats were divided into four groups: the first group was given $CCl_4$ only ($n = 9$, $CCl_4$ only group); the second was given both $CCl_4$ and lisinopril simultaneously ($n = 11$, $CCl_4$/lisinopril group); the third was given lisinopril i.p. twice a week ($n = 5$, lisinopril only group); and the fourth was given only olive oil ($n = 5$, control group). The animals were sacrificed at 8 ($n = 3$, $CCl_4$ only group; $n = 2$ $CCl_4$/lisinopril group), 12 ($n = 3$, $CCl_4$ only group; $n = 4$, $CCl_4$/lisinopril group; $n = 2$, lisinopril only group; $n = 2$, control group) and 24 weeks ($n = 3$, $CCl_4$ only group; $n = 4$, $CCl_4$/lisinopril group; $n = 3$, lisinopril only group; $n = 3$, control group) after start of administration. Before the sacrifice, the rats were injected with 100 U of sterile heparin sulfate i.p. and 5 mg/ml of Nembutal (total of 20 mg in PBS). Blood was quickly collected, and separated plasma was stored at −20°C until use. The liver of each animal was also removed.

### 2.4. Histological study

After the sacrifice, their liver was cut into 1- to 2-mm thick slices and fixed in 10% neutral buffered formalin. The tissue was then processed through graded alcohol and xylene and embedded in paraffin. Sections of 4 μm thick were obtained from the paraffin blocks and stained with hematoxylin and eosin, and silver. The sections were coded and examined without knowledge of the animals' treatment in the study.

### 2.5. Measurement of the tissue hydroxyproline

Hydroxyproline content in the rat liver was measured according to the method described by Jamall et al. [17]. Briefly, 3 g of each rat liver was dissolved in 6N HCl, and incubated at 110°C for 16 h, then filtered and desiccated. The sample was then dissolved in 1.2 ml of 50% isopropanol, and then chloramine T solution (20% p-toluenesulfonchloramine) and Ehrlich's reagent solution (p-dimethylaminobenzaldehyde in 60% HCl) were added. It was incubated at 50°C for 90 min. Hydroxyproline was quantified by absorption at 558 nm.

### 2.6. Serum fibrosis markers and aspartate aminotransferase and alanine aminotransferase levels

Type IV collagen 7s in the serum was assayed as ng/ml using a radioimmunoassay (Nippon DPC Corp., Tokyo, Japan), and hyarulonic acid was measured as ng/ml by a sandwich binding protein assay [18]. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured using standard laboratory methods (Nippon DPC Corporation, Tokyo, Japan).

### 2.7. Purification of hepatic stellate cells

Male Sprague–Dawley rats were anesthetized with ether after a 24-h starvation, and HSCs were collected according to the method described by Knook et al. [19] with some modifications. Briefly, the rat liver was perfused with $Ca^{++}$- $Mg^{++}$-free Hanks' solution via the portal vein at 40 ml/min for 5 min and then with 0.05% collagenase (type I, Sigma Chemical Co.) in Hanks' solution at 25 ml/min for 7 min. Next, the liver was removed, minced, and filtered in Hanks' solution at 4°C, then centrifuged at $50 \times g$ for 3 min. The pellet was overlaid onto 7%, 13%, and 18% metrizamide solution at 20°C and centrifuged at $1000 \times g$ for 20 min. The HSC fraction was obtained as the top layer. Cell viability was estimated by the trypan blue dye exclusion test, and the purity was estimated by ordinary light and fluorescent microscopic examinations and by indirect enzyme immunoreactivity with anti-desmin antibody [20,21] (Dako, Versailles, France).

### 2.8. Effect of angiotensin II on hepatic stellate cells

The fractionated rat HSCs were dispersed into the wells of a 96-well microtiter plate at $2 \times 10^4$ cells/well in DMEM (Nissui Seiyaku, Tokyo, Japan) supplemented with 10% fetal calf serum (FCS, Gibco, Grand Island, NY). The cells were cultured with Ang II ($10^{-5}$, $10^{-7}$ and $10^{-9}$ mol/l) for 48 h, and [$^3$H]-thymidine uptake was measured.

HSCs were dispersed into culture dishes at $5 \times 10^5$ in DMEM supplemented with 10% FCS and cultured for 24 h. Ang II was then added to the culture at 0, $10^{-11}$ and $10^{-8}$ mol/l. The cells were further cultured for an additional 48 h. Total RNA was extracted from the cells before and after the culture and mRNA was purified using a mRNA isolation kit (Pharmacia Biotechnology, Uppsala, Sweden) and cDNA was synthesized by the Marathon cDNA Amplification Kit (Clontech Laboratories Inc., Palo Alto, CA). The cDNAs were ligated with Marathon Adaptor (Clontech Laboratories Inc.) at both the 5′ and 3′ ends. Five microliters of whole cDNA was used as the template and the cDNA was amplified by PCR with Advantage cDNA Polymerase Mix (Clontech Laboratories Inc.). The PCR conditions were; denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min for a total of 38 cycles. Five microliters of the PCR product were analyzed by 1.5% agarose gel electrophoresis. As a positive control, glyceraldehydes 3-phosphate dehydrogenase (G3PDH) cDNA (983 bp) was amplified by PCR using primers specific for the rat G3PDH mRNA (Clontech Laboratories Inc.) Primers specific for Marathon Adaptor (5′-CCA TCC TAA TAC GAC TCA CTA TAG GGC-3′) were used for the sense primers in order to amplify the Ang II receptor 1A and 1B cDNA. As the antisense primers, we used 5′-GAC TTT GAT GTG TGG ACT TGG GTA-3′ for the Ang II receptor 1A cDNA and 5′-ACA GTA AGA CAT TAT TCA CGA CAA GCT G-3′ for the Ang II receptor 1B cDNA. In order to amplify pro α (I) collagen cDNA (1165 bp), the following primers were arranged according to the reported sequence [22]: 5′-GGC AAG AAT GGC GAC CGT GGT GAA CCG-3′ (sense) and 5′-CAC CAA CGT CCA AGG GTG CCA CAT CG-3′ (antisense).

### 2.9. Northern blotting and densitometry

Northern blotting was performed according to the method described previously [23]. Probes that detect pro α (I) collagen, transforming growth factor (TGF) β, and G3PDH were labeled with α-[$^{32}$P] dCTP by random priming (Random Primed DNA Labeling Kit; Boehringer, Tokyo, Japan). After hybridization and washing, the nylon membranes were subjected to autoradiography. Bands were measured by densitometry and then amounts of mRNA were corrected by comparison to the housekeeping gene G3PDH mRNA.

### 2.10. Statistical analysis

Data were represented as the mean ± S.D. and the difference between two groups was analyzed by Student's *t*-test.

## 3. Results

### 3.1. In vivo experimental model

The body weight and the liver weight of each rat did not significantly differ between four experimental groups.

Histopathological comparison was made between the experimental groups. Fig. 1 shows Silver staining of the histology at 8, 12, and 24 weeks. No significant histological changes were detected in the lisinopril only group at any time during the experiment. A histological examination of livers obtained 8 weeks after the start of treatment showed diffuse fat deposition in hepatocytes and periportal fibrosis in the $CCl_4$ only group (Fig. 1A, silver staining), and elongation of fine fibers was noted around the portal area. Simultaneous treatment with lisinopril clearly reduced the fat deposition throughout the liver (Fig. 1B), but fine fibers were noted around the portal area as well as in the liver treated with $CCl_4$ alone.

Fig. 1C and D shows the typical histological appearance of the liver (silver staining) at 12 weeks in the $CCl_4$ only group and the $CCl_4$/lisinopril group. Expansion of the periportal fibrosis, elongation along the sinusoids, and some portal–portal bridgings and severe fat deposition were noted in the livers of the group treated with $CCl_4$ alone for 12 weeks. In the livers of the $CCl_4$/lisinopril, the bundles of fibrosis in portal areas and periportal regions were thinner, and there were fewer portal–portal bridgings than in

the $CCl_4$ only group. At 12 weeks, fat deposition was similar in both groups. No inflammatory cell infiltration was noticed in any group. Furthermore, neither apoptotic cells nor necrotic cells were detected.

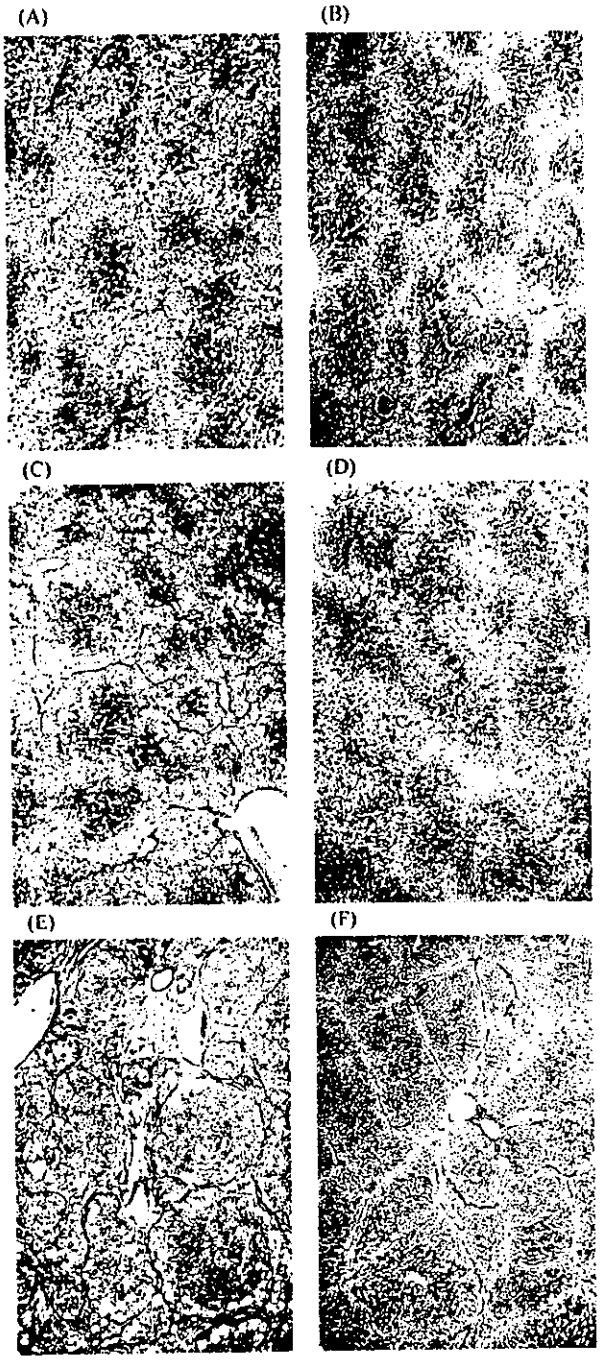


Fig. 1.

Fig. 1E and F shows the typical histological appearance of the liver (silver staining) at 24 weeks in both groups. The livers in the groups given $CCl_4$ alone for 24 weeks showed greater thickening of the fiber bundles between the portal areas than before, and ultimately regenerative nodules were observed. The livers of the $CCl_4$ only group in this period showed typical liver cirrhosis histologically (Fig. 1E). The severe fat deposition observed at 8 and 12 weeks had decreased during this period in both groups. Simultaneous lisinopril treatment for 24 weeks significantly reduced the thickness of the fiber bundles observed in the $CCl_4$ only group, and no obvious regenerative nodules were detected (Fig. 1F). No inflammatory cell infiltration was noticed in any group. Furthermore, neither apoptotic cells nor necrotic cells were detected.

### 3.2. *Hydroxyproline content in the liver*

Collagen production in the liver was assessed by the hydroxyproline content of the liver. The hydroxyproline content in the liver of each experimental group was summarized in Fig. 2. Although hydroxyproline levels in the liver were not significantly different in the $CCl_4$ only and $CCl_4$/lisinopril groups at 8 weeks, they were significantly higher in the $CCl_4$ only group at 12 and 24 weeks compared to the other groups. Thus, simul-

Fig. 1. Typical liver histology at 8, 12 and, 24 weeks. The rats were sacrificed 8 (A and B), 12 (C and D) and 24 weeks (E and F) after the beginning of the administration of carbon tetrachloride only (A, C and E) and lisinopril simultaneously (B, D and F), and the liver tissue were removed, fixed and stained with silver. Silver staining shows fine periportal fibrosis in both groups and no significant difference at 8 weeks (A and B). At 12 weeks, the construction of fine portal–portal fibers in the $CCl_4$ alone group is found, while no obvious portal–portal fibrosis is observed in the $CCl_4$/lisinopril group. Finally at 24 weeks, regenerative nodule formation in the $CCl_4$ alone group is found, but no severe fibrosis and regenerative nodules in the $CCl_4$/lisinopril group. In addition to the fibrotic change, marked steatosis is noted in the $CCl_4$ alone group, while fatty change is significantly reduced in the $CCl_4$/lisinopril group. Original magnification 200 ×.

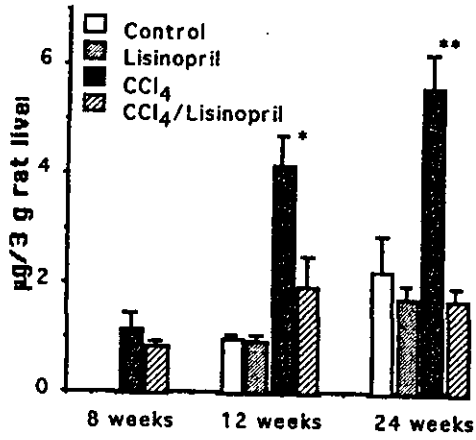


Fig. 2. Hydroxyproline content (μg/3 g of rat liver) in the livers of each group at 8, 12 and 24 weeks. Hydroxyproline in the livers was measured according to the method described by Jamall et al. ([17]). The control group and lisinopril only groups at 8 weeks were not tested. The number of examined livers were as follows: the $CCl_4$ only group at 8 weeks ($n=3$), 12 weeks ($n=3$), and 24 weeks ($n=3$); the $CCl_4$/lisinopril group at 8 weeks ($n=2$), 12 weeks ($n=4$), and 24 weeks ($n=4$); Control group at 12 weeks ($n=2$) and 24 weeks ($n=3$); lisinopril alone group at 12 weeks ($n=2$), and 24 weeks ($n=3$). *$P<0.05$ vs. control and $CCl_4$/lisinopril group, **$P<0.01$ vs. control and $CCl_4$/lisinopril group.

taneous treatment with lisinopril attenuated the $CCl_4$-induced overproduction of hydroxyproline in the liver.

### 3.3. Serum fibrosis markers

The serum type IV collagen 7s and hyarulonic acid levels were compared among the experimental groups. As shown in Table 1, no significant differences in the levels of serum type IV collagen 7s were detected. By contrast, the serum hyarulonic acid levels were significantly decreased by simultaneous treatment with lisinopril at 24 weeks (Table 2), indicating that local tissue fibrogenesis was inactivated by the simultaneous treatment with lisinopril.

### 3.4. Serum aspartate aminotransferase, alanine aminotransferase and albimin levels

The serum AST and ALT levels of the $CCl_4$ only group were elevated at 12 weeks instead of at 24 weeks, indicating that liver cell damage was significantly induced at 12 weeks. Simultaneous treatment with lisinopril did not statistically modify the levels at 12 weeks, but lisinopril did attenuate the $CCl_4$-induced elevation of the AST and ALT levels at 24 weeks (Fig. 3). Serum albumin levels were also measured by a standard laboratory method, and the levels in the $CCl_4$ only group were lower than in the other groups at 24 weeks (Fig. 4). These results suggest that lisinopril did attenuate the $CCl_4$-induced hepatic damage at 24 weeks and that lisinopril could suppress the $CCl_4$-induced decrease in albumin production attributable to the liver cell damage.

### 3.5. Effect of angiotensin II on rat hepatic stellate cells

Lipid droplets were observed in cytoplasm of HSCs obtained by the method described above one day after purification (Fig. 5A). Fig. 5B shows purified cells that had been cultured for seven days and stained by indirect fluorescence method with anti-desmin antibody. The cells were positive for desmin, and their morphologic characteristics suggest that the isolated cells were HSCs.

Table 1
Serum type IV collagen 7s levels in rat groups with various treatments[a]

| Weeks | Control | Lisinopril | $CCl_4$ | $CCl_4$/lisinopril |
|---|---|---|---|---|
| 8 | ND | ND | $4.7\pm0.3$ ($n=3$) | $4.8\pm0.1$ ($n=3$) |
| 12 | $4.3\pm0.1$ ($n=2$) | $4.2\pm0.3$ ($n=2$) | $4.2\pm0.3$ ($n=3$) | $5.2\pm0.6$ ($n=4$) |
| 24 | $4.1\pm0.2$ ($n=3$) | $4.4\pm0.3$ ($n=3$) | $4.8\pm0.5$ ($n=3$) | $4.4\pm0.2$ ($n=3$) |

[a] Each number shows mean ± S.D.; ND: not done, each value indicates mean ± S.D. No significant difference was noted between the groups.

Table 2
Serum hyarulonic acid levels in rat groups with various treatments[a]

| Weeks | Control | Lisinopril | $CCl_4$ | $CCl_4$/lisinopril |
|---|---|---|---|---|
| 8 | ND | ND | $21.2 \pm 5.3$ ($n=3$) | $23.0 \pm 4.9$ ($n=3$) |
| 12 | $4.2 \pm 1.4$ ($n=2$) | $10.5 \pm 3.8$ ($n=2$) | $104.6 \pm 81.7^{b,c}$ ($n=2$) | $128.6 \pm 68.3^{b,c}$ ($n=4$) |
| 24 | $9.1 \pm 5.0$ ($n=3$) | $12.7 \pm 0.5$ ($n=3$) | $205.0 \pm 90.6^{b,c}$ ($n=3$) | $12.3 \pm 4.4^{d}$ ($n=4$) |

[a] Each number shows mean ± SE; ND: not done, each value indicates mean ± S.D.
[b] $P<0.01$ vs. control group.
[c] $P<0.01$ vs. Lisinopril group.
[d] $P<0.05$ vs. $CCl_4$ only group.

The serum Ang II levels in this study did not differ between groups at any time points. In the in vitro study, no stimulatory effect of Ang II on the proliferation of rat HSCs was observed in the $^3$H-thymidine uptake test (data not shown). This result was not compatible with that described elsewhere [24]. Expression of pro α (I) collagen, Ang II receptor 1A and 1B mRNA in rat HSCs was assessed by RT-PCR. Fig. 6 shows the results of agarose gel electrophoresis of the RT-PCR products. The mRNAs of pro α (I) collagen and Ang II receptor 1A, but not 1B, were demonstrated in isolated rat HSCs.

After culturing HSCs with $10^{-5}$, $10^{-7}$, and $10^{-9}$ mol/l of Ang II for three days, the expression of pro α (I) collagen and TGF-β1 mRNA was examined by Northern blotting (Fig. 7A). The pro α (I) collagen and TGF-β1 mRNAs increased according to the increase in concentration of Ang II more than $10^{-7}$ mol/l. The relative expression corrected by comparative densitometry of housekeeping G3PDH mRNA (Fig. 7B) showed that more than $10^{-7}$ mol/l of Ang II increased the expression of pro α (I) collagen and TGF-β1 mRNAs in rat HSCs in vitro.

## 4. Discussion

The inhibition of hepatic fibrosis is ultimately beneficial in preventing the progression of chronic liver diseases such as chronic viral or alcoholic hepatitis and liver cirrhosis. Potential inhibitors of hepatic fibrosis, including dexamethasone [25], interferon γ [26], colchicines [27], and 16,16-dimethyl prostaglandin $E_2$ [28], have been reported, but none of them has been successfully used for the treatment of patients. One of the ACE inhibitors, captopril, has been well studied and reported to suppress rat hepatic fibrosis induced by pig serum [29], but its active mechanism of action is not fully understood. Additionally, whether the ACE inhibitors generally affect other models of hepatic fibrosis have not been understood. Hepatic fibrosis progressed during chronic inflammation in the liver may be induced by the production of several cytokines that are initially triggered by cell death. In this study, one of the ACE inhibitors, lisinopril, was found to inhibit liver cell damage that was signaled by the elevation of serum transaminases, inhibit the decrease in serum albumin levels, and inhibit hepatic fibrosis induced by low-dose chronic $CCl_4$ in rats. HSCs were also found to play a role in hepatic fibrogenesis via the locally excreted tissue Ang II. The serum Ang II levels in this study did not differ between the experimental groups but a significant difference in hepatic fibrosis was noted between the $CCl_4$ only group and the $CCl_4$/lisinopril group, indicating that the local tissue levels of Ang II are more important than the systemic level. Such a local renin–angiotensin regulation has already been demonstrated in the infarct heart and obstructive nephropathy [30,31]. Thus, blocking of the local renin–angiotensin system seems beneficial for patients with liver cirrhosis not only in terms of improving their hyperdynamic circulation [32] and portal hypertension [33], but also from the standpoint of non-hemodynamic mechanisms such as prevention of hepatic fibrogenesis.

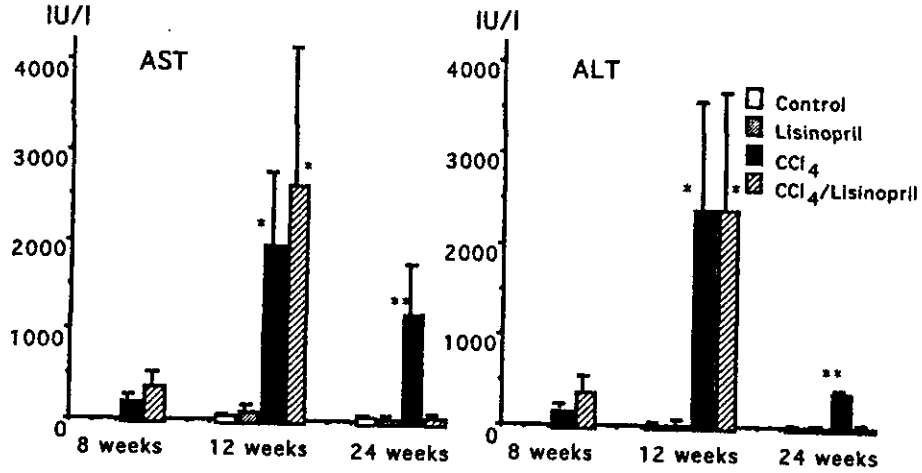


Fig. 3. Serum AST and ALT levels in each group at 8, 12, and 24 weeks. The control group and lisinopril only groups at 8 weeks were not tested. The number of examined rats were same as described in Fig. 2. $^{*}P < 0.05$ vs. control group, $^{**}P < 0.05$ vs. control and $CCl_4$/lisinopril group.

Although serum transaminase levels did not show significant difference between the $CCl_4$ only group and the $CCl_4$/lisinopril group at the 12th week, tissue hydroxyproline levels were decreased by simultaneous lisinopril administration. These results suggest that lisinopril directly inhibits hepatic fibrogenesis irrespective of the degree of reduction of hepatic damage.

After 24 weeks, simultaneous treatment with the ACE inhibitor significantly reduced not only the hepatic fibrosis but also the hepatic steatosis. Carbon tetrachloride is known to exert toxic effects on cells after being metabolized by cytochrome p-450, and thus $CCl_4$ may only damage hepatocytes by subjecting them to oxidative stress [34]. In addition to this $CCl_4$-induced oxidative stress, a recent transgenic approach has revealed that Ang II stimulates oxidative stress through endothelin 1 [35]. These mechanisms may induce lipid peroxidation in hepatocytes, and in turn, induce hepatic steatosis in this animal model. In the present study, the simultaneous treatment with an ACE inhibitor significantly reduced the $CCl_4$-induced fatty change, as shown in Fig. 1. These results suggest that treatment with ACE inhibitors may reduce hepatic steatosis by preventing oxidative stress.

The in vitro effects of Ang II on HSCs were examined in order to determine how ACE inhibitors reduce hepatic fibrosis. Ang II plays various physiological roles by binding to specific receptors on the cell membrane. At least two types (type 1 and 2) of the Ang II-receptor have been demonstrated, and there exists two subtypes (1A and 1B) of the type-1 receptor [36]. The type 1 receptor has been detected in vascular endothelium, lungs, heart, kidney, liver, brain, and adrenal glands. The type 1A receptor is localized

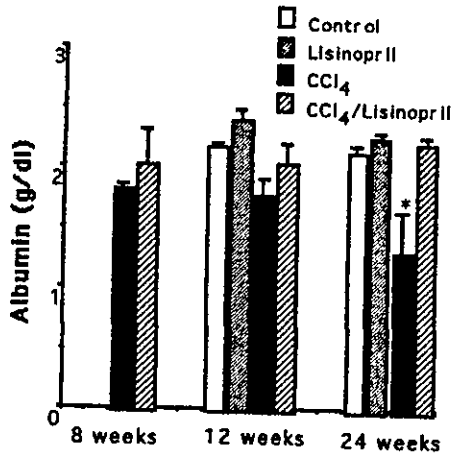


Fig. 4. Serum albumin levels in each group at 8, 12, and 24 weeks. The number of examined livers were same as described in Fig. 2. Only the levels of $CCl_4$ only group at 24 weeks was significantly reduced than those of other groups ($^{*}P < 0.05$).

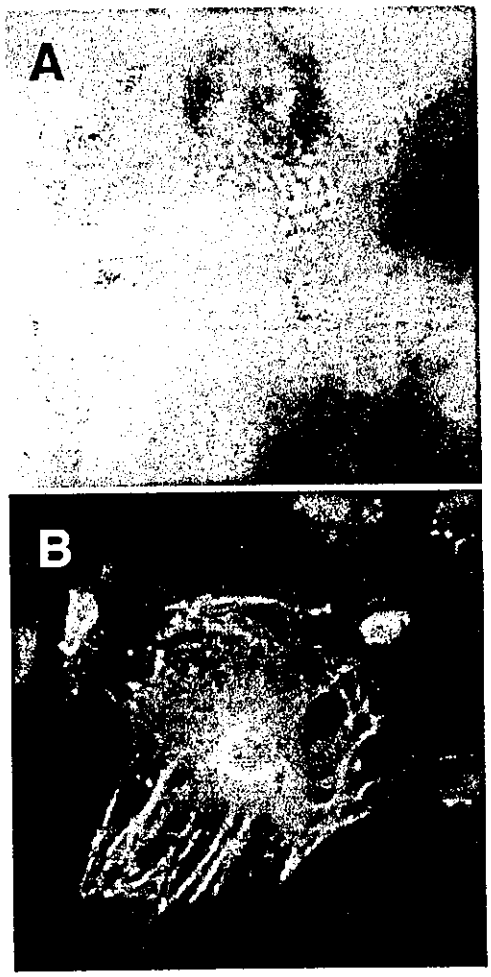


Fig. 5. Microphotographical demonstration of the purified HSC. The rat HSCs were isolated from the liver of a Sprague–Dawley rat according to the method described in the text. A: A rat HSC one day after the purification showing many lipid droplets in the cytoplasm. Original magnification 1000 ×. B: The purified rat HSC after being cultured for 7 days, showing desmin-positive by an indirect immunofluorescence method. Original magnification 1000 ×.

in the liver, heart and lungs, and type 1B is found in the adrenal glands [37]. In this study, only the mRNA of type 1A receptor, not 1B, was detected in the rat liver, indicating compatible results with these previous reports. This result suggests that Ang II affects HSCs.

During hepatic fibrogenesis, HSCs in the necrotic area are probably activated by locally excreted cytokines and/or conformational changes in Disse's cavity [12,38]. After activation, HSCs proliferate and transform into myofibroblasts [10,39]. Activating factors of HSCs already

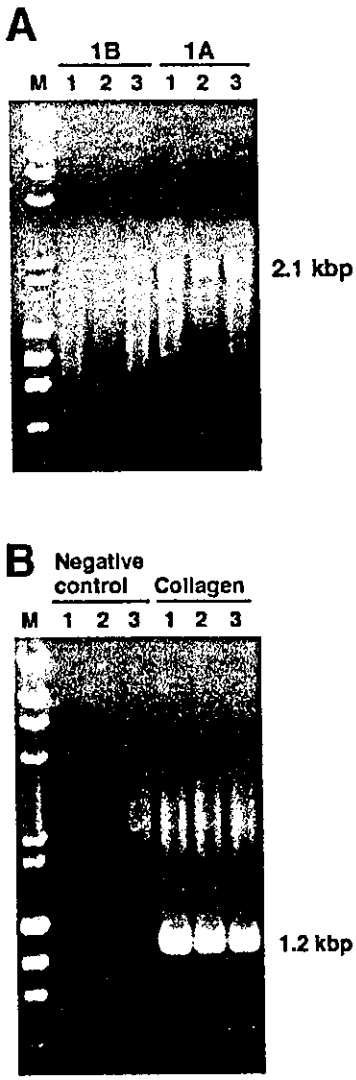


Fig. 6. RT-PCR analysis of Ang II receptor and pro α (I) collagen cDNA in the rat HSCs. A: Ang II receptor type 1A mRNA was positive in rat HSCs, while type 1B receptor was not detected by RT-PCR. Isolated rat HSCs were cultured with Ang II at $10^{-5}$ (lane 1), $10^{-7}$ (lane 2) and $10^{-9}$ (lane 3). M: molecular weight markers (λ-Hind III digest plus φχ174 Hae III digest). B: An RT-PCR product for pro α (I) collagen mRNA was positive in rat HSCs. Isolated rat HSCs were cultured with Ang II at $10^{-5}$ (lane 1), $10^{-7}$ (lane 2) and $10^{-9}$ (lane 3). RT-PCR products without using mRNA (negative control) show negative bands in the left 3 lanes.

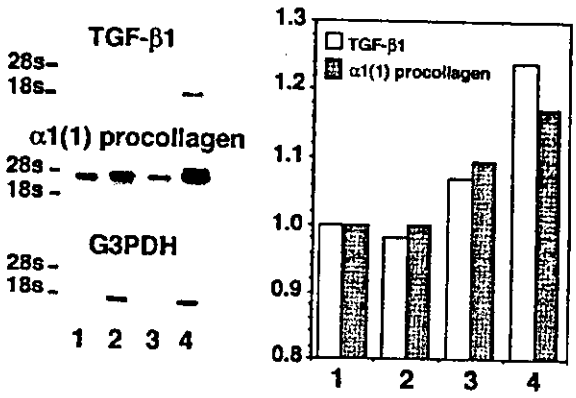


Fig. 7. Semi-quantitative comparison of mRNA amounts in pro α (I) collagen and transforming growth factor (TGF)-β1 in rat HSCs after the culture with Ang II at 0 (lane 1), $10^{-9}$ (lane 2), $10^{-7}$ (lane 3) and $10^{-5}$ (lane 4) for 3 days. A: Northern blot analysis of pro α (I) collagen and TGF-β1 mRNA. RNA was electrophoresed and blotted on a nylon membrane and the same membrane was used for rehybridization to each specific α-[$^{32}$P] dCTP-labeled probe after washing. G3PDH indicates glyceraldehyde-3-phosphate dehydrogenase. B: Each mRNA band was quantified by the densitometry. The horizontal axis indicates the concentration of Ang II added; 0 (lane 1), $10^{-9}$ (lane 2), $10^{-7}$ (lane 3) and $10^{-5}$ (lane 4).

demonstrated are TGF-β1 [40,41] and platelet derived growth factor [42]. TGF-β1 increases the production of the extracellular matrices of HSCs and also stimulates autocrine TGF-β1 excretion [40,43]. In this study, Ang II stimulated the production of collagen and TGF-β1 at the gene level [24]. The collagen and TGF-β1 mRNA was dose-dependently induced by Ang II, confirming its direct effects at the gene level. Whether collagen production is up-regulated by a direct action of Ang II, whether Ang II initially up-regulates TGF-β1 and then collagen is produced secondarily, or whether both mechanisms proceed simultaneously, were not resolved in this study. Ang II increases TGF-β1 production, and it secondarily causes overproduction of extracellular matrices in renal mesangial cells [44]. Thus, the role of TGF-β1 is postulated to be most important.

These results suggest that ACE inhibitors at least inhibit hepatic fibrogenesis at the gene expression level by blocking the local tissue renin–angiotensin system. Moreover, ACE inhibitors or the receptor blockers seem to have a clinical benefit when used in patients with chronic active hepatitis, because the drug concentration in vitro used in this study was compatible with that utilized clinically. However, hepatic fibrosis indicated by the tissue hydroxyproline level is possibly reduced by not only decreasing its production rate but also increasing degradation of the fiber. In the present study, we cannot determine which factor, active fibrogenesis or degradation, was predominantly involved in this model, because no definite pathway of the degradation of hepatic fibrosis has not been clarified. Further study is necessary for better understanding of these problems.

## Acknowledgements

This work was partially supported by a grant from the Japanese Ministry of Education, Science and Culture and from Keio University.

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