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AT1A-deficient mice show less severe progression of liver fibrosis induced by CCl_4

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Abstract

The renin–angiotensin system has been shown to contribute to fibrogenesis in varieties of organs, including the liver. Here, we investigated whether the angiotensin II type 1A receptor (AT1A) is implicated in the development of liver fibrosis, using AT1A-deficient and wild-type (WT) mice. After single dose of carbon tetrachloride (CCl_4), there were no significant differences between two groups with regard to hepatic inflammation and necrosis. After 4 weeks of treatment with CCl_4, histological examination revealed that AT1A-deficient mice showed less infiltration of inflammatory cells and less severe progression of liver fibrosis compared with WT mice. These findings were accompanied by the hepatic content of hydoxyproline and the expression of α-smooth muscle actin (αSMA). The level of transforming growth factor-β1 (TGF-β1) messenger RNA was markedly higher in WT mice when compared with AT1A-deficient mice. These results confirm that signaling via AT1A plays a pivotal role in hepatic fibrogenesis.

Keywords: Liver fibrosis; Renin–angiotensin system; Carbon tetrachloride; Transforming growth factor-β; Angiotensin II type 1 receptor; Tumor necrosis factor-α; Knockout mouse; Hepatic stellate cells; Carbon tetrachloride; α-Smooth muscle actin

A growing number of studies have suggested that the renin–angiotensin system (RAS), an important factor in regulating the blood pressure and body fluid homeostasis, is also involved in hepatic fibrogenesis. Patients with chronic liver disease, such as cirrhosis, show an increase of plasma renin activity [1,2]. Under these conditions, hepatic stellate cells (HSCs), which are key producers of the extracellular matrix in liver injury, proliferate and acquire the characteristics of contractile cells (myofibroblasts) [3]. Moreover, blockers of the action of angiotensin (Ang) II, such as angiotensin-converting enzyme (ACE) inhibitors or Ang II receptor antagonists, induce regression or prevent the development of hepatic fibrosis in some animal models [4,5]. In vitro studies have shown that AT1 receptors are expressed in the activated human HSCs and have indicated that Ang II induces the contraction and proliferation of HSCs [6].

Ang II receptors can be pharmacologically differentiated into two distinct types, which are designated type 1 (AT1) and type 2 (AT2). Most of the well-known effects of Ang II on the cardiovascular system and renal function are mediated through the AT1 receptor [7]. There are two subtypes of this receptor (AT1A and AT1B) in humans, rats, and mice. Tissue distribution studies have revealed that only the AT1A subtype is expressed in the liver [8,9].

Mouse models in which a single receptor is completely eliminated by gene targeting provide a new approach to the investigation of receptor regulation and function. The advantage of using an AT1A-deficient mouse model is that absence of the receptor means complete and specific blockade of AT1A-mediated signaling. So far, AT1A-deficient mouse models have provided considerable information in the cardiovascular field [10–12], but have not been used to study hepatic fibrogenesis. Accordingly, the present study examined the influence of the AT1A receptor on liver fibrogenesis using AT1A-deficient mice.

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Materials and methods

Animals. AT1A-deficient mice were established by Sugaya et al. [12], and a kind gift from Tanabe Seiyaku (Osaka, Japan) and C57BL/6 mice were obtained from Hiroshima Jikken Doubutsu (Hiroshima, Japan). Both strains of mice have the same genetic background and animals aged 6–8 weeks were used in the present study. The mice were allowed free access to food and water, and were housed at a constant temperature with a 12-h light/dark cycle during the study. To assess the necrotic and inflammatory changes caused by acute exposure to CCl_4 (Wako Pure Chemical Industries, Osaka, Japan), a single intraperitoneal injection of 1.0 ml/kg (1:1 in mineral oil) was administered. Blood was collected by cardiac puncture after 24 h for examination of transaminases and tumor necrosis factor-α (TNF-α) [13,14], while liver samples were harvested after 72 h [15]. Liver fibrosis was induced by the subcutaneous injection of CCl_4 (1 ml/kg) twice weekly for 4 weeks and mice were killed 3 days after the last injection. Liver samples were harvested and rapidly frozen in liquid nitrogen for storage at −80 °C until assay or were fixed with periodate-lysine-4% paraformaldehyde (PLP) solution for histological examination. All animal procedures were done according to our institutional guidelines.

Histological examination. Liver samples were fixed in PLP solution by perfusion fixation. After successive transfer into solutions of 10–20% sucrose in 0.1 mol/L phosphate buffer, the samples were embedded in paraffin. Then, 5-μm thick sections were cut and processed for staining with hematoxylin and eosin (H–E) or Azan–Mallory stain. To evaluate the portal inflammation after chronic treatment with CCl_4, the following scoring system was used as previously described [4]: grade 0, none; grade 1, mild portal inflammatory infiltration; grade 2, moderate inflammation expanding portal areas; and grade 3; marked portal inflammation with spill over into acini. Six separated specimens were scored. Immunohistochemistry for αSMA was carried out using a monoclonal mouse anti-human smooth muscle actin antibody (Dako, Japan) and the Vector M.O.M. Immunodetection Kit (Vector Laboratories, USA) according to the manufacturer's instructions. For histological examination, several fields per slide were randomly selected and representative results from three animals are shown.

Hydroxyproline content. The hepatic hydroxyproline (HP) content was measured using the Kivirikko's method [16] with some modifications. Briefly, liver tissue (50 mg) was hydrolyzed in 6 mol/L HCl at 110 °C for 24 h in a glass test tube. After centrifugation at 3000 rpm for 10 min, 2 ml of the supernatant was neutralized with 8 N KOH. Subsequently, 2 ml of the resultant solution was stirred with 2 g KCl and 1 ml of 0.5 mol/L borate buffer for 15 min at room temperature and for an additional 15 min at 0 °C. Freshly prepared chloramine T solution was added and stirred at 0 °C for 1 h, followed by the addition of 2 ml of 3.6 mol/L sodium thiosulfate. The solution was incubated at 120 °C for 30 min and was then stirred with 3 ml toluene for 20 min at room temperature. After centrifugation at 2000 rpm for 5 min, 2 ml of the supernatant was mixed with 0.8 ml Ehlich's buffer and incubated for 30 min. Then, the sample was transferred to a flat-bottomed microtiter plate and the absorbance was measured at 560 nm. Results are shown as the -fold increase relative to the average HP of liver samples from untreated mice.

TNF-α ELISA. TNF-α was measured using a commercial Enzyme-linked Immunosorbent Assay Kit (BioSource International, USA) according to the manufacturer's instructions.

Reverse transcriptase-polymerase chain reaction. The steady-state level of transforming growth factor-β1 (TGF-β1) messenger RNA (mRNA) was assessed by a semiquantitative PCR using β-actin as the housekeeping gene. Messenger-RNA was isolated with an RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Then, single-stranded complementary DNA (cDNA) was synthesized from 1 μg of mRNA using 0.5 nmol of each random primer and was subjected to the polymerase chain reaction. Subsequently, the synthesized cDNA was amplified using specific sets of primers for AT1 receptor (forward: GAGTCCTGTTCCACCCGATCACCGATCAC and reverse: GGATGACGCCCAGCTGAATCAGCACATCC) [11], TGF-β1 (forward: GTGACGAGGCCCAGAGCAAGAG and reverse: GCAGGAGCGCACAATCATGTT) [17], and β-actin (forward: TGTACGTAGCCATCAGGCT and reverse: TTCTCCAGGG AGGAAGAGGA) [18]. The PCR procedure consisted of 30 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 58 °C (AT1 receptor), 60 °C (TGF-β1) or 55 °C (β-actin), and extension for 90 s at 72 °C, with initial denaturation of the cDNA for 5 min at 94 °C before starting the PCR and additional extension for 10 min after completing the cycles. An aliquot (10 μl) of each PCR product was loaded onto 2% agarose gel, which was stained with ethidium bromide.

Assay of serum transaminases. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using standard method.

Statistical analysis. Results were expressed as means ± SD. Statistical analysis was performed using one-way analysis of variance (ANOVA) and $P < 0.05$ was considered to indicate significance.

Results

AT1 mRNA expression

To confirm the absence of AT1 receptors in AT1A-deficient mice, RT-PCR was performed to evaluate hepatic mRNA levels of AT1. The expression of AT1 mRNA was not observed in the liver of AT1A-deficient mice, whereas it was seen in that of WT mice (Fig. 1).

Parameters of necrosis/inflammation

To determine whether absence of the AT1A receptor had any influence on necrosis and inflammation caused by acute exposure to CCl_4, serum transaminases and the proinflammatory cytokine TNF-α were measured. There were no significant differences of transaminases and TNF-α between WT and AT1A-deficient mice (Fig. 2).

Histological findings after a single dose of CCl_4

The acute effects of CCl_4 administration included pericentral necrosis in WT mice as well as AT1A-defi-

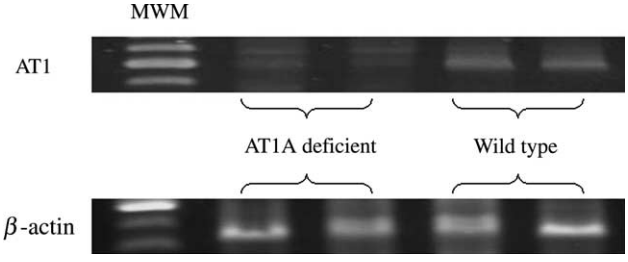

Fig. 1. Steady-state hepatic AT1 mRNA level in WT and AT1A-deficient mice. An aliquot of each PCR product was loaded onto 2% agarose gel and was stained with ethidium bromide. Amplification of β-actin was shown to confirm the evenness of mRNA in each sample.

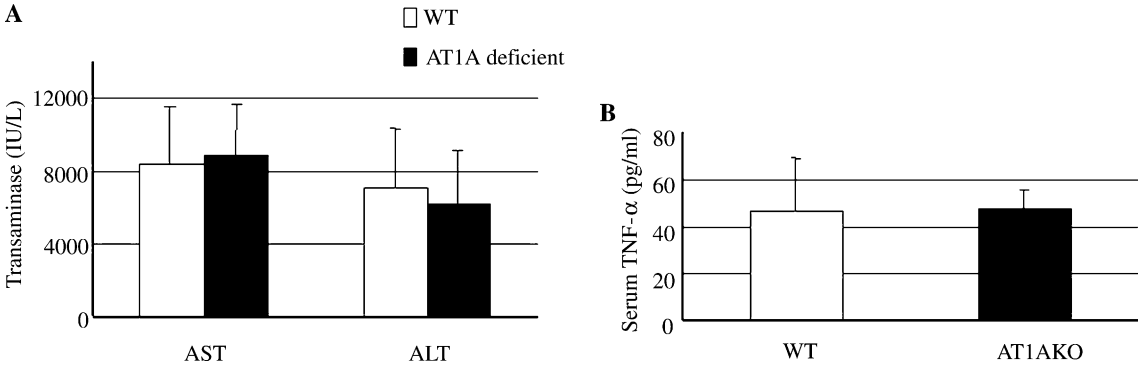

Fig. 2. Effect of a single dose of CCl_4 on the serum levels of transaminases (A) and TNF-α (B) in WT and AT1A-deficient mice. Serum samples were collected 24 h after single treatment with CCl_4. Each value represents the mean ± SE of seven mice per group. No significant differences of the serum transaminase and TNF-α levels were found between two groups.

cient mice. In addition to necrosis, inflammatory cell infiltrates were seen in the centrilobular region. The extent of necrosis and inflammatory infiltration was similar in the two groups (Fig. 3). These findings agreed with the data on serum parameters of inflammation/necrosis and suggested that the absence of AT1A receptors had little influence on the hepatic response to a single dose of CCl_4.

Histological findings after chronic CCl_4 administration

After administration of CCl_4 for 4 weeks, inflammatory cell infiltrates were evident around the portal tracts. Mononuclear cells represented the major infiltrating cell type and were more prevalent in WT mice than in AT1A-deficient mice (Figs. 4A and B). The mean inflammatory score in AT1A-deficient mice

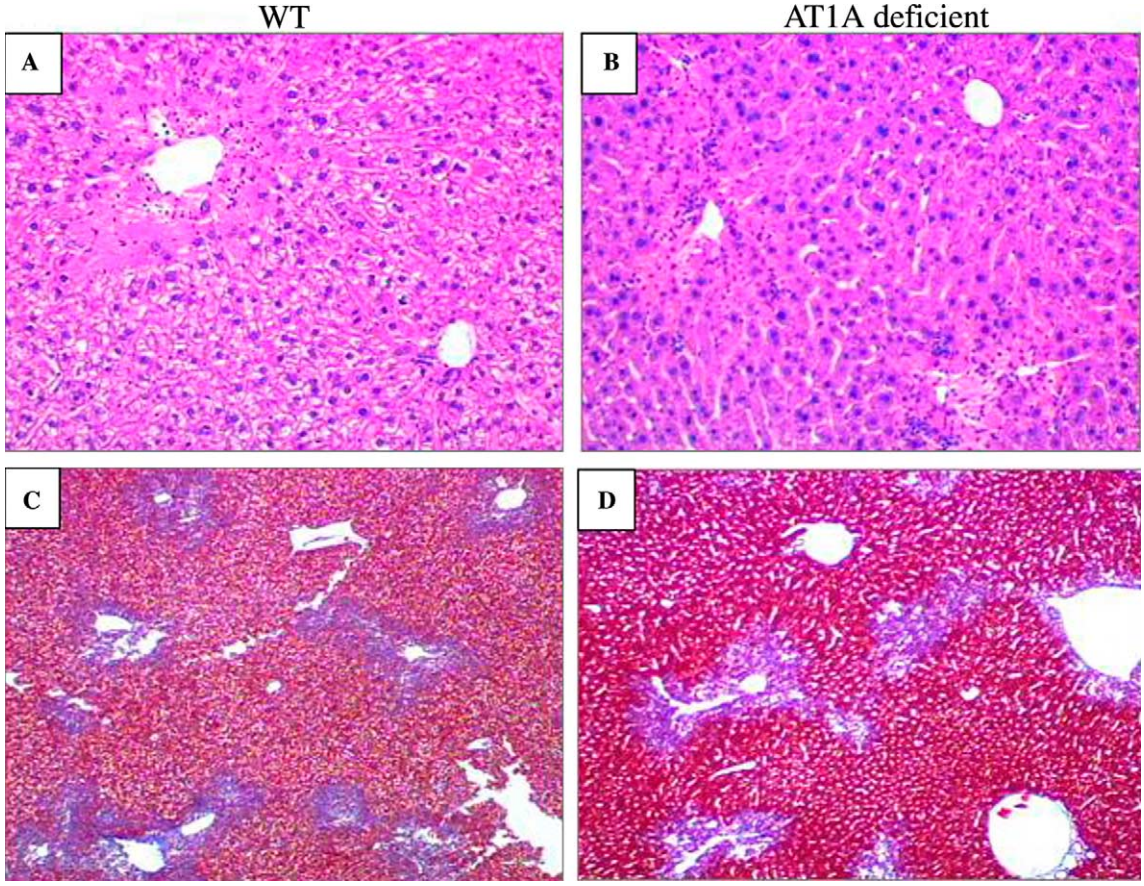

Fig. 3. Representative photomicrographs of the liver at 72 h after a single dose of CCl_4 in WT (A,C) and AT1A-deficient mice (B,D). Paraffin-embedded sections were processed for H–E staining (A,B, original magnification: 100×) or Azan–Mallory staining (C,D, original magnification: 40×).

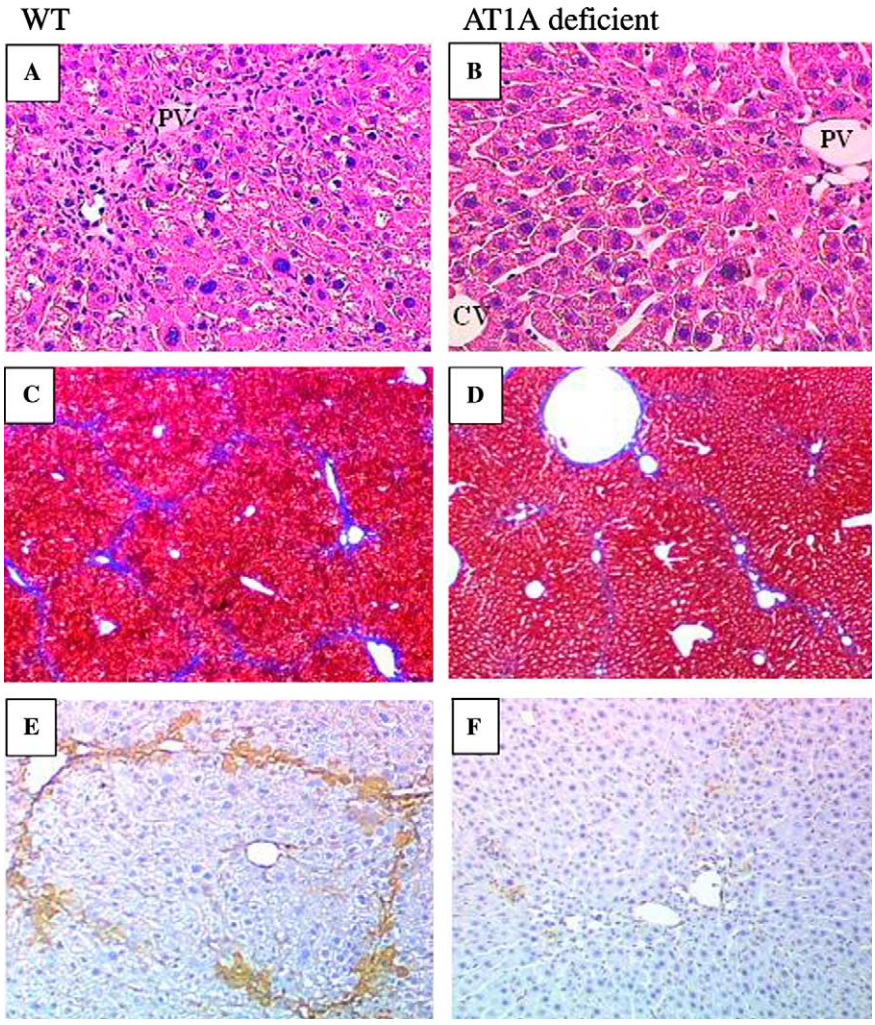

Fig. 4. Representative photomicrographs of the liver after 4 weeks of treatment with CCl_4 (1.0 ml/kg) in WT (A,C,E) and AT1A-deficient mice (B,D,F). Sections were processed for H–E staining (A,B, original magnification: 200×), Azan–Mallory staining (C,D, original magnification: 40×) or αSMA immunostaining (E,F, original magnification: 100×). PV, portal vein and CV, central vein.

showed significantly lower when compared with WT mice (1.1 ± 0.4 vs. 2.7 ± 0.5). In addition, the livers of WT mice showed formation of nodules and bridging fibrosis, whereas minimal fibrosis was found in the AT1A-deficient mice (Figs. 4C and D). Expression of αSMA, an indicator of HSC activation, was strongly detected in the fibrotic septa of WT mice, whereas it was negligible in AT1A-deficient mice (Figs. 4E and F).

Hydroxyproline content

Since the HP content of the liver has been shown to parallel the extent of fibrosis, the increase of HP was measured after chronic CCl_4 administration. WT mice treated with CCl_4 for 4 weeks showed a 6.1-fold increment compared with untreated WT mice, whereas AT1A-deficient mice only showed a 2.6-fold increment compared with untreated AT1A-deficient mice (Fig. 5).

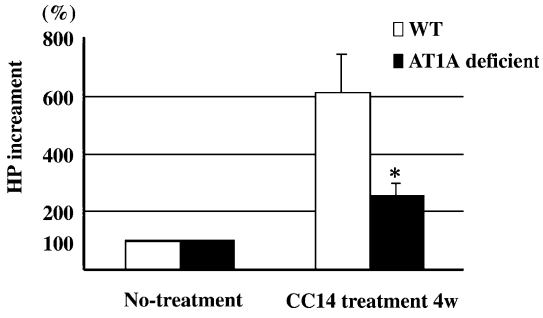

Fig. 5. Hydroxyproline content after 4 weeks of treatment with CCl_4. The -fold change was calculated on the basis of the average HP content of untreated livers from WT and AT1A-deficient mice. Each value represents the mean ± SE of four mice per group.

TGF-β1 mRNA expression

As TGF-β1 appears to be one of the central fibrogenic factors involved in collagen synthesis, TGF-β1

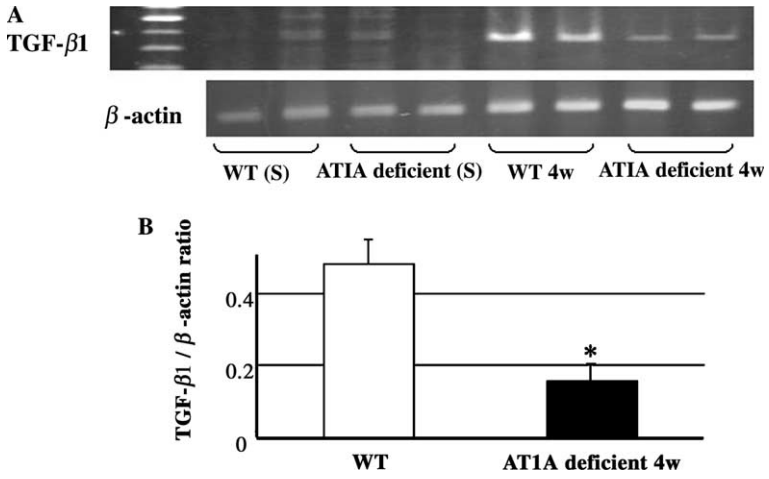

Fig. 6. Steady-state hepatic TGF-β1 mRNA level in WT and AT1A-deficient mice after 4 weeks of CCl_4 administration. (A) Ethidium bromide-stained 2% agarose gels of the RT-PCR products are shown. Gels were scanned with a digital image analysis system, the products were quantified, and results are shown relative to housekeeping gene β-actin (B). Each value represents the mean ± SE of four mice per group. *$P < 0.01$ compared with WT mice.

mRMA expression was examined after chronic CCl_4 treatment. The steady-state level of TGF-β1 mRNA was significantly higher in WT mice when compared with AT1A-deficient mice (Fig. 6).

Discussion

In the cardiovascular system and the kidneys, blockade of Ang II has been shown to prevent fibrosis and inflammatory cell infiltration [19–22]. Ang II could also be involved in the process of fibrogenesis because of its action as a proinflammatory cytokine, participating in various steps of the inflammatory response. Interestingly, systemic infusion of Ang II results in significant cardiac and renal fibrosis, but no fibrotic response occurs in the liver [23].

Previous in vitro studies have revealed that activated human [6] and rat [24] HSCs express AT1 receptors, and that Ang II induces the contraction and proliferation of HSCs [6]. To date, it is controversial whether blockade of Ang II can attenuate the development of hepatic fibrosis in animal models. In a rat bile duct ligation model, administration of captopril caused a decrease of TGF-β1 and collagen gene expression and delayed the progression of hepatic fibrosis [5]. However, irbesartan did not cause a significant reduction of matrix deposition in the liver, although it suppressed the overexpression of TGF-β1 and type I collagen gene [25]. In a rat model of pig serum-induced fibrosis, administration of perindopril and candesartan blocked hepatic fibrosis and decreased the expression of αSMA [4]. These conflicting findings could be attributed to differences in the method of fibrogenesis or in the drugs tested. In human clinical study with early stages of chronic hepatitis C, losartan reduced plasma TGF-β1 and serum type IV collagen, but had no significant effect on histological scores [26]. In the present study, we examined the role of AT1A signaling in the development of hepatic fibrosis using AT1A-receptor-deficient mice. The histological findings and changes of HP indicated that AT1A-deficient mice showed a marked reduction of collagen accumulation when compared with WT mice. These results confirm that AT1A signaling is responsible for regulating the development of hepatic fibrosis caused by CCl_4.

The hepatotoxicity of CCl_4 depends upon its metabolism in hepatocytes by cytochrome P450 2E1 (CYP2E1), which generates highly reactive trichloromethyl free radicals, leading to lipid peroxidation and membrane damage [27]. Kupffer cells are activated by free radicals and produce proinflammatory mediators, resulting in the triggering of an inflammatory cascade [18]. Thus, the fibrogenic response is a wide-ranging and dynamic process that includes hepatocyte necrosis followed by macrophage activation, the proliferation and activation of HSCs, and the release of fibrogenic mediators. A growing number of studies have focused on various steps in this process.

Tumor necrosis factor-α, mainly produced by monocytes and activated macrophages, is defined by its participation in inflammation, and it is instrumental in the process of hepatic necrosis. In previous studies [14], which focused on early fibrogenesis, TNF-α receptor-deficient mice showed less hepatic inflammation and fibrosis after treatment with CCl_4. Ang II activates inflammatory cells by direct chemotaxis as well as by promoting the production of proinflammatory mediators [28]. In this study, we examined the effect of the AT1A on the early necrotic and inflammatory response. Nevertheless, we found that the serum TNF-α level of AT1A-deficient mice was comparable to that of WT mice after a single dose of

CCl_4. Histological analysis also showed no significant difference of hepatic infiltration between the two groups. These results suggested that the AT1A had little involvement in the early necrotic and inflammatory response to CCl_4.

As shown in Fig. 3, mononuclear cell infiltration was observed in the liver after chronic CCl_4 administration and was more extensive in WT mice compared with AT1A-deficient mice. Stellate cells can reportedly amplify inflammation through the release of neutrophil and monocyte chemoattractants, such as colony-stimulating factor and monocyte chemoattractant protein (MCP)-1 [29,30], and the up-regulation of such adhesion molecules further amplifies inflammation during liver injury [31]. Consistent with these previous findings, the expression of αSMA, a marker of activated HSCs, was prominent in WT mice, whereas it was negligible in AT1A-deficient mice. Accordingly, AT1A signaling might have a pivotal role in regulating the proliferation of HSCs and in the subsequent enhancement of chronic inflammation. Taken together, AT1A signaling is not involved in the acute necroinflammatory response, but possibly modulates subsequent moderate hepatic inflammation and fibrogenesis.

Consistent with previous reports, TGF-β1 [25] was induced in the livers of mice treated with CCl_4. However, AT1A-deficient mice showed significantly lower expression of TGF-β1 when compared with WT mice, suggesting that absence of the AT1A reduced the production of TGF-β1. In cultured mesangial cells, Ang II increases TGF-β mRNA expression and conversion to its active form, while neutralizing antibodies to TGF-β causes marked reduction of Ang II-induced ECM production [32]. In addition, systemic infusion of Ang II caused a significant increase of TGF-β expression in the heart, which was correlated with cardiac fibrosis [23]. In vitro studies have shown that Ang II directly stimulates the proliferation of cardiac fibroblasts and production of ECM proteins [33,34]. These results suggest that there is a close relationship between Ang II and TGF-β.

In conclusion, AT1A-deficient mice show less severe progression of liver fibrosis as well as the decreased expression of TGF-β1 and αSMA. These results confirm that inhibition of the RAS, especially AT1A blockade, might be a useful form of therapy for liver fibrosis. It also seems that the AT1A might not be involved in the acute necroinflammatory response, but participates in subsequent chronic inflammation.

Acknowledgments

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