Liver Responses to Repeated Doses of Carbon Tetrachloride in Mini and Wistar Rats

Hiroko Shimizu¹, Koji Uetsuka¹, Taro Okada¹, Hiroyuki Nakayama¹, and Kunio Doi¹

¹Department of Veterinary Pathology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1–1–1 Yayoi, Bunkyo-ku, Tokyo 113–8657, Japan

Abstract: The liver responses to repeated doses of CCl₄ (0.5 mg/kg b.w., twice weekly) were compared between Wistar and Mini rats, in which the expression of growth hormone (GH) gene is suppressed by the presence of an antisense gene, at 2, 4, and 6 weeks after treatment (WAT). Fibrosis started earlier and its degree was severer in Mini rats than in Wistar rats, and hepatocyte damage was also severer in Mini rats than in Wistar rats. This corresponded well with the changes in serum AST and ALP levels. Oval cell proliferation in the fibrous septa of the liver of CCl₄-treated Mini rats may also have some relations to such increase in ALP activity and fibrogenesis in Mini rats. The increase in the level of TGF-β₁ mRNA was more prominent in Mini rats than in Wistar rats at 4 and 6 WAT, and this corresponded to the strain difference in the degree of liver fibrosis. The present results indicate that the responsibility of the liver to repeated doses of CCl₄ was different between Mini rats and Wistar rats. Mini rats seem to be useful as a new tool in the investigation of regulatory mechanisms of GH on liver injury and regeneration. (J Toxicol Pathol 2002; 15: 79–83)

Key words: carbon tetrachloride, growth hormone, liver fibrosis, Mini rat, Wistar rat

Introduction

Nts:Mini rats of the Jcl:Wistar-TgN(ARGHGEN)1Nts strain (Mini rats) are a newly developed transgenic animal in which the expression of growth hormone (GH) gene is suppressed by the presence of an antisense transgene. The plasma GH level of Mini rats is reduced to approximately 60% and 80% in males and females, respectively, compared with those of matched sexes of Wistar/Jcl rats (Wistar rats), the parental strain of Mini rats. Previously Uetsuka et al. reported that Mini rats treated with D-galactosamine (GalN) showed a prolonged oval cell proliferation associated with an accumulation of extracellular matrices and an activation of Ito cells, and Tani et al. described that Mini rats treated with thioacetamide (TAA) developed hepatic cirrhosis more rapidly. Moreover, it was clarified that the content of cytochrome P450 (CYP) 2E1 in the liver is significantly higher in male Mini rats than in male rats of other strains. These findings suggest that the reduced level of GH may modify the liver pathology after chemical treatment.

In our previous paper, we reported that there was a difference in the localization of initial liver lesions between Mini and Wistar rats and the degree of liver lesion was more severe and its recovery was more delayed in Mini rats than in Wistar rats after a single injection of carbon tetrachloride (CCl₄). CCl₄ is a well known hepatotoxicant that induces fatty degeneration or coagulative necrosis of hepatocytes by a single injection and hepatic cirrhosis by repeated injections.

The purpose of this study is to compare the liver responses to repeated doses of CCl₄ between Mini and Wistar rats. The protocol of this experiment was approved by the Animal Care and Use Committee of Graduate School of Agricultural and Life Sciences, The University of Tokyo.

Materials and Methods

Animals

Twenty 4-week-old male Mini rats (Jcl:Wistar-TgN(ARGHGEN)1Nts strain) (NT Science Co., Tokyo) and 20 age-matched male Wistar/Jcl rats (Wistar rats) (Japan Clea, Tokyo) were used. The animals were housed using an isolator caging system (Niki Shoji Co., Tokyo) in an animal room controlled at 23 ± 2°C and 55 ± 5% relative humidity with 12 hr-light and 12 hr-dark cycle. They were fed commercial pellets (MF, Oriental Yeast Co., Ltd., Tokyo) and water ad libitum.
Treatments
Carbon tetrachloride (CCl4, WAKO Pure Chemicals Co. Ltd., Osaka) was dissolved in peanut oil (SIGMA Chemical Co., USA) and the concentration was adjusted to 0.5 mg/ml. Twelve rats of each strain were orally treated with 1 ml/kg b.w. of the above-mentioned CCl4 solution twice a week (on Monday and Thursday), and 4 rats of each strain were sacrificed under ether anesthesia at 2, 4, and 6 weeks after the first treatment (WAT), respectively. Four rats of each strain which were treated with peanut oil alone and killed at 0 and 6 WAT, respectively, were served as controls.

Blood biochemistry
Collected blood was centrifuged for 20 min at 4500 rpm, and serum was collected. Aspartate aminotransferase (AST) and alkaline phosphatase (ALP) activities were colorimetrically measured on each serum sample using an autoanalyser (Olympus Co., Tokyo) and test reagents (WAKO Pure Chemical Co.).

Competitive reverse transcription-polymerase chain reaction (RT-PCR)
For the quantification of transforming growth factor-β1 (TGF-β1) mRNA level, competitive RT-PCR method was used according to the report of Gilliland et al.9 In brief, total RNA was extracted from 0.5 g of liver tissue of each rat killed as scheduled. Next, 2 µg of total RNA was reverse transcribed to single-strand cDNA by incubation with reverse transcription mixture [1 µl Oligo(dT)12-18 Primer (Gibco/BRL Life Technologies Ltd., Paisley, UK), 2 µl 10 × PCR buffer (Gibco/BRL Life Technologies Ltd.), 2 µl MgCl2 (25 mM, Gibco/BRL Life Technologies Ltd.), 1 µl dNTP (10 mM, Gibco/BRL Life Technologies Ltd.), 2 µl DTT (0.1 M, Gibco/BRL Life Technologies Ltd.) and 1 µl Super script II reverse transcriptase (Gibco/BRL Life Technologies Ltd.).]

Competitive PCR was performed in a total volume of 50 µl [1 µl cDNA, 1 µl competitive DNA which was serially diluted, 36.75 µl UPDW, 5 µl 10 × PCR buffer which contains 100 mM Tris-HCl buffer, 500 mM KCl and 15 mM MgCl2 (Takara Shuzo Co., Ltd., Shiga), 5 µl dNTP (Takara Shuzo Co., Ltd.), 0.5 µl of 50 pmol/ml primer for TGF-β1 (sense), 0.5 µl of 50 pmol/ml primer for TGF-β1 (antisense) and 0.25 µl Taq polymerase (5 U/ml, Takara Taq, Takara Shuzo Co., Ltd.)] with Takara PCR Thermal cycler using the following program: 94°C for one min (denaturing), 63°C for one min (annealing), and 72°C for one min (extension). The TGF-β1 primers were designed as follows: GCCCTGGATACCAACTACTGCTTC (sense) and TCAGCTGCACTTGCAGGAGCGCACGATCAT (antisense). Amplification was carried out at 30 cycles. The amplified fragment of TGF-β1 cDNA was shorter than that of competitor (436 bp vs 399 bp). Coamplification of the two fragments occurred in a concentration-dependent manner.

For quantification, the products from the PCR were electrophoresed on a 2% agarose gel in Tris-borate EDTA buffer, stained with ethidium bromide and analyzed using UV-CCD video system (Epi-Light UV_FAI1100; AISIN COSMOS R & D Co., Ltd., Tokyo) and Luminous imager software (AISIN COSMOS R & D Co., Ltd.).

Histopathology
A part of the liver obtained from each rat was fixed in 10% neutral buffered formalin. Four-µm paraffin sections were stained with hematoxylin and eosin (HE) or by Watanabe’s silver impregnation (SI) method for histopathological examination.

Statistical analysis
Serum AST and ALP activities and TGF-β1 mRNA level were presented as mean ± standard error (SE). Statistical analysis was done using the Student’s t-test compared with the control values at 0 WAT, because there were no differences in the control values between 0 and 6 WAT.

Results
Clinical findings
The body weight gain in CCl4-treated Mini and Wistar rats became gradually depressed and the body weight of CCl4-treated rats was reduced to about 80% of controls in both strains at 6 WAT (Fig. 1).

Serum AST and ALP activities
Changes in the AST and ALP activities showed similar sequences in each strain and these activities increased more...
clearly and more sharply in Mini rats than in Wistar rats until 6 WAT (Figs. 2a and 2b).

**Changes in the expression of TGF-β1 mRNAs**

In Mini rats, the level of TGF-β1 mRNA increased at 2 WAT, peaked at 4 WAT (about 4 times higher than the control level) (Fig. 3), and then decreased at 6 WAT (about 2 times higher than the control level) (Fig. 3). On the other hand, the level of TGF-β1 mRNA showed a slight increase only at 2 WAT (about 2 times higher than the control level) in Wistar rats (Fig. 3).

**Histopathological findings**

In the liver of Mini rats, fibrosis started at 2 WAT, and fibrous components grew from the periportal area into the lobules (Fig. 4a). Formation of small-sized pseudolobules developed at 4 WAT (Fig. 4b), and progressed at 6 WAT. In some pseudolobules, fibrous components proliferated surrounding individual hepatocytes or small groups of hepatocytes (Fig. 4c). Fibrous septa surrounding pseudolobules generally contained many proliferated small epithelial cells at 4 and 6 WAT (Figs. 4c and 4d), and these small epithelial cells formed cluster, cord or bile ductule-like structures (Fig. 4d). Moreover, formation of regenerative nodules of hepatocytes developed at and after 4 WAT (Fig. 4c). Hepatocytes varied in their nuclear and cellular sizes at and after 2 WAT (Fig. 4d).

On the other hand, in the liver of Wistar rats, fibrosis started at 4 WAT (Fig. 5a). At 6 WAT, formation of pseudolobules surrounded by thin fibrous septa developed (Fig. 5b). Except for vacuolization, hepatocytes did not show prominent changes throughout the experimental period (Fig. 5b). Thus, the degree of liver lesions was more severe in Mini rats than in Wistar rats.

**Discussion**

The liver responses to repeated doses of CCl₄ was compared between Mini and Wistar rats.

In Mini rats, fibrosis started at 2 WAT and formation of pseudolobules with formation of regenerative nodules of hepatocytes developed at 4 WAT. On the contrary, fibrosis started at 4 WAT and formation of pseudolobules surrounded by thin fibrous septa was detected at 6 WAT in Wistar rats. Thus, the hepatic fibrosis was apparently severer in Mini rats than in Wistar rats as reported in the liver of Mini rats treated with repeated doses of TAA₄.

TGF-β1 is well known as a suppressor of mitosis for...
Fig. 4. Histopathological findings of the liver of Mini rats. (a) Fibrous components grow from periportal areas into lobules at 2 WAT. SI method, ×50. (b) Formation of small-sized pseudolobules is seen at 4 WAT. SI method, ×50. (c) Individual hepatocytes and small groups of hepatocytes are surrounded by fibrous components including small epithelial cells at 6 WAT. A regenerative nodule is also seen. SI method, ×100. (d) Small epithelial cells form bile ductule-like structures, and hepatocytes vary in size at 4 WAT. HE, ×260.

Fig. 5. Histopathological findings of the liver of Wistar rats. (a) The onset of fibrosis is detected at 4 WAT. SI method, ×50. (b) Formation of pseudolobules surrounded with thin fibrous septa is seen at 6 WAT. SI method, ×50.
preventing uncontrolled hepatocyte proliferation\textsuperscript{10,11} and as an accelerator of extracellular matrix expression\textsuperscript{12,13}. Thus TGF-\(\beta\) is implicated as a central role in tissue regeneration.

In the present study, the increase in the level of TGF-\(\beta\) mRNA following CCl\(_4\)-treatment was more prominent in Mini rats than in Wistar rats at 4 and 6 WAT, and this corresponded to the above-mentioned difference in the severity of liver fibrosis.

In the present study, the ALP activity was considerably greater in Mini rats than in Wistar rats after repeated doses of CCl\(_4\), suggesting that biliary system was more severely affected in Mini rats than in Wistar rats. As described in the liver of TAA-treated Mini rats\textsuperscript{4}, prominent proliferation of small epithelial cells (i.e. oval cells) which formed biliary ductule-like structures in the liver may also have some relations to such increase in ALP activity in Mini rats.

In conclusion, liver fibrosis started earlier and its degree was considerably severer in Mini rats than in Wistar rats, and hepatocyte damage was also severer in Mini rats than in Wistar rats following repeated doses of CCl\(_4\). Together with the previous reports of GalN- and TAA-induced liver injuries, it is reasonable to consider that GH-suppressed Mini rats respond to hepatotoxicants in a different manner from rats with normal GH level. Mini rats are considered to be useful as a new tool in the investigation of regulatory effects of GH on liver injury and regeneration.

References