Original

Effects of Exogenous Growth Hormone (GH)-Treatment on Carbon Tetrachloride (CCl₄)-Induced Acute Liver Injury in GH-Deficient Mini Rats

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Abstract: The effects of exogenous growth hormone (GH)-treatment on the development and regeneration of CCl₄-induced acute hepatic injury were examined in GH-deficient Mini rats. Alanin aminotransferase (ALT) and aspartate aminotransferase (AST) activities increased significantly at 24 and 48 hours (h) after CCl₄-administration in both GH-treated and non treated groups, and they tended to be suppressed by GH-treatment. The expression level of *insulin-like growth factor (IGF)-1* mRNA was higher in GH (+) CCl₄ (+) group than in GH (–) CCl₄ (+) group, especially at 72 h, and that of *hepatocyte growth factor (HGF)* mRNA was also higher in some points in the former group. Histologically, degenerative changes of hepatocytes observed at 24 and 48 h was less severe in GH (+) CCl₄ (+) group than in GH (–) CCl₄ (+) group. At 72 h, depositions of reticular fibers and extracellular matrix materials were less severe and the number of desmin- or α -SMA-positive cells was larger in GH (–) CCl₄ (+) group than in GH (+) CCl₄ (+) group. Proliferating cell nuclear antigen (PCNA)-labeling index of centrilobular hepatocytes increased at 48 h in both GH (+) CCl₄ (+) and GH (–) CCl₄ (+) groups, and it returned to the control level at 72 h in the former group while that in the latter group showed still higher level at 72 h. In this study, it was shown that CCl₄-induced acute liver injury in Mini rats can be restored by exogenous GH-treatment, and upregulation of *IGF-1* and *HGF* mRNAs may be involved in this phenomenon. (J Toxicol Pathol 2003; 16: 49–57)

Key words: liver injury, Mini rat, GH, CCl₄, IGF-1

Introduction

Growth hormone (GH) is a pituitary polypeptide hormone that plays a critical role in tissue growth, and it is also known to influence liver regeneration. For example, retarded liver regeneration was observed after 70% partial hepatectomy (PH) in hypophysectomized rats, and this was recovered by the treatment of exogenous GH^{1–4}.

Mini rat (Jcl:Wistar-TgN (ARGHGEN) 1Nts) is a Wistar-derived transgenic strain in which pituitary GH is suppressed by the presence of antisense transgene for the rat GH located downstream of the GH gene^{5,6}. The blood GH level of Mini rats is approximately 60 to 80 % of that of Wistar rats, and the body weight gain is significantly suppressed compared with that in age-matched Wistar rats^{5,6}.

The difference in the responses to chemical-induced hepatic injury between Mini rats and Wistar rats was reported by several researchers^{7–14}. After treatment with some hepatotoxicants (D-galactosamine, thioacetamide, and carbon tetrachloride (CCl₄)), Mini rats showed more severe parenchymal damages, delay in regeneration of hepatocytes, and prominent proliferation of mesenchymal cells and fibrosis compared with Wistar rats^{7–9,11,13,14}. In addition, Uetsuka et al. (1997) reported¹² that after PH, the labeling indice of 5-bromo-2'-deoxyuridine (BrdU) and proliferating cell nuclear antigen (PCNA), which represent cell proliferating activity, were significantly lower in Mini rats than in Wistar rats.

In this study, effects of the treatment with exogenous porcine pituitary GH (pGH) on CCl₄-induced liver injury and regeneration were investigated in GH-deficient Mini rats.

The protocol of this study was approved by the Animal Care and Use Committee of the Graduate School of Agricultural and Life Sciences, the University of Tokyo.

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Materials and Methods

Animals

Forty-two 4-week-old Male Mini rats weighing about 76.0g were obtained from NT Science Co., Tokyo, Japan. They were kept under controlled conditions ($23 \pm 2^\circ\text{C}$ and $55 \pm 5\%$ relative humidity with 14 hr-light and 10 hr-dark cycle) using an isolator caging system (Niki Shoji Co., Tokyo), and were fed commercial pellets (MF, Oriental Yeast Co. Ltd., Tokyo) and water *ad libitum* during acclimatizing and experimental periods.

Chemicals

Porcine pituitary GH (pGH) (Sigma Chemical Co., St. Louis, MO) was dissolved in physiologic saline containing 0.01% bovine serum albumin, and the concentration was adjusted to 4 IU/ml. CCl₄ (Wako Pure Chemicals, Osaka, Japan) was diluted twenty times with peanut oil (Sigma Chemical Co., St. Louis, MO).

Treatments

Experimental design is shown in Fig. 1. Animals were divided into four groups; 18 rats each for GH (+) CCl₄ (+) and GH (-) CCl₄ (+) groups and 3 rats each for GH (+) CCl₄ (-) and GH (-) CCl₄ (-) groups, respectively. Namely, 6-week-old Mini rats were injected intraperitoneally (i.p.) with pGH (20 IU/kg body weight) or vehicle 2 times per day (10:00 and 20:00) for 10 days. Rats were orally given a single dose of CCl₄ (0.5 ml/kg body weight) or vehicle immediately after the GH-treatment (at 10:00) on day 7. Fourteen rats each of GH (+) CCl₄ (+) and GH (-) CCl₄ (+) groups were sacrificed by heart puncture under ether anesthesia at 0, 6, 24, 36, 48, and 72 hours (h) after CCl₄-administration, respectively. In addition, 6 rats each of GH (+) CCl₄ (-) and GH (-) CCl₄ (-) groups were killed at 72 h after vehicle (peanut oil) administration, respectively. Then, the liver and the blood were collected from each rat.

Body weight changes and mortality

Clinical signs were checked and the body weight (BW) and mortality were recorded daily. The BW gain was expressed as the difference between BW on every experimental day and BW at the beginning of experiment.

Blood biochemistry

The activities of aspartate aminotransferase (AST) and alanin aminotransferase (ALT), indicators of hepatocyte damage, and alkaline phosphatase (ALP), an indicator of bile duct system damage, were measured at each blood sample with Hitachi Autoanalyzer 7250.

RNA extraction and semi-quantitative RT-PCR for growth factors

Liver tissues were snap-frozen immediately after autopsy and stored at -80°C for RNA extraction. Total RNA was isolated with ISOGEN (Nippon Gene, Toyama, Japan) from 1 g of liver tissue. To prepare cDNA, reverse transcription (RT) reaction was carried out with 40 μl of reaction mixture containing 10 μg total RNA, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 1 μg of Oligo (dT)₁₂₋₁₈ primer, 0.5 mM of dNTPs, and SUPERSRIPT™ II RNase H-Reverse Transcriptase (Invitrogen, Rockville, MO).

To detect the expression levels of *insulin-like growth factor (IGF)-1*, *hepatocyte growth factor (HGF)*, and *transforming growth factor (TGF)- β 1* mRNAs expression, PCR amplification was performed. *Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*, a housekeeping gene, was used as an internal standard. Structure, annealing temperature, and amplification cycles of each oligonucleotide primers described above are shown in Table 1. Chain reaction was carried out in 50 μl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 200 μM of each dNTP, 2 μM of sense and antisense primers, 1.25 units of *Takara Taq*™ (Takara Shuzo, Ohtsu, Japan) DNA

Fig. 1. Experimental protocols. White arrowhead: treatment with pGH or saline intraperitoneously (i.p.) twice a day (at 10:00 and 20:00) from day 1 to 10; Black arrowhead: treatment with CCl₄ or peanut oil at 10:00 on day 7; Arrow: 0, 6, 24, 36, 48, and 72 h after CCl₄ or peanut oil treatment, respectively.

Table 1. Oligonucleotide Primers Used in PCR

Gene	Forward (5'–3')	Reverse (3'–5')	T _m (°C)	Cycles
<i>GAPDH</i>	GAGTATGTCGTGGAGTCTACTG	GCTTCACCACCTTCTTGATGTC	58.0	24
<i>IGF-1</i>	CACTCTGCTTGCTCACCTTTACCA	GGTCTTGTTTCCTGCACCTTCCTCT	60.5	26
<i>HGF</i>	GGGTTCTTACTGCAAGGCAATGTT	CCTCACAAGGTCCTGATCCAATCT	60.5	34
<i>TGF β1</i>	TACGTCAGACATTCGGAAGCAGT	GGTTGTGTTGGTTGTAGAGGGCAA	60.5	28

Fig. 2. Changes in body weight gain in GH (+) CCl₄ (+) group (■) and GH (-) CCl₄ (+) group (□). Values represent mean $\pm$ SE of 3 rats. * p <0.05 and ** p <0.01: Significantly different between the two groups.

Fig. 3. Plasma ALT (A) and ALP (B) activities. ■: GH (+); □: GH (-). Data represent mean $\pm$ SE of 3 rats.

Fig. 4. The expression levels of *HGF* (A), *IGF-1* (B) and *TGF-β1* mRNAs (C) (ratio to GAPDH) after CCl₄- or vehicle-administration. ■: GH (+); □: GH (-). Data represent mean $\pm$ SE of 3 rats. * p <0.05 and ** p <0.01: Significantly different between GH (+) CCl₄ (+) and GH (-) CCl₄ (+) groups.

polymerase, and 1 μ l of sample cDNA. PCR mixtures were preheated at 94°C for 7 min before amplification (1 cycle: 94°C for 1 min, each annealing temperature for 1 min, 72°C for 1 min) was done for defined cycles. After the amplification cycles, samples were heated at 72°C for 3 min and cooled down to 4°C.

Four- μ l of each PCR products were electrophoresed in 1.2 % agarose S (Nippon Gene Co. Ltd., Toyama, Japan) in 1 $\times$ Tris-borate EDTA (TBE) buffer, and stained with etidium bromide (Gibco BRL, Grand Island, NY). Bands were detected by Fluorescence gel imaging using UV-CCD video system Fas-III (Toyobo, Tokyo, Japan) and fluorescence intensity was quantified by pdi Quantity One® Software, respectively. From obtained results, relative ratio to *GAPDH* was calculated.

Histology

The liver specimens were fixed in 10% neutral-buffered formalin for 24 h and embedded in paraffin, or directly embedded in O. C. T. compound (Sakura, Tokyo, Japan) and suddenly frozen with dry ice and acetone. Four- μ m paraffin sections were stained with hematoxylin and eosin (H&E). Some of them were stained by Watanabe's silver

impregnation method or by immunohistochemical staining. Five- μ m cryosections were also prepared for immunohistochemistry. To assess the changes in the area occupied by reticular fibers stained by Watanabe's silver impregnation method, the percentage of argyrophilic area to the total liver tissue area (%) was determined based on the observation from 10 randomly selected fields under 200-fold magnification per section per rat. A microscope TV system was used for generating images. The images were recorded with an interacting CCD (FUJIX HC-300Z FUJIFILM, Tokyo, Japan) camera. Scanned photographs were stored in a computer (Adobe Photoshop 5.0) for subsequent evaluation by image analyzer software (NIH image 1.61 soft) for quantification of argyrophilic area.

Immunohistochemistry

Immunohistochemical staining was done on the liver sections. Namely, immunohistochemical staining for proliferating cell nuclear antigen (PCNA) and alpha smooth muscle actin (α -SMA) was performed using 4- μ m paraffin sections, and that for desmin, collagen type 1 (Col (I)), and fibronectin, was done using 5- μ m cryosections. Mouse anti-PCNA monoclonal antibody (clone PC 10; 1: 100;

Fig. 5. Histopathology of the liver sections of GH (+) CCl₄ (+) and GH (-) CCl₄ (+) groups after CCl₄-administration. Left column: GH (+) CCl₄ (+) group; Right column: GH (-) CCl₄ (+) group. A and B: 24 h after CCl₄-administration. Vacuolar degeneration or necrosis in centrilobular to mid-zonal hepatocytes with inflammatory cell infiltration is observed. The changes are less severe in (A). C and D: 48 h after CCl₄-administration. The changes become less severe in both groups, especially in GH (+) group (C). E and F: 72 h after CCl₄-administration. Degenerative changes of hepatocytes almost disappear while an infiltration of small epithelial-like cells increases. G and H: 72 h after CCl₄-administration. Accumulations of reticular fibers are more prominent in (H) than in (G). A–F: H&E, $\times 80$. G and H: Watanabe's silver impregnation method, $\times 80$.

Novocastra Laboratories, Newcastle, UK), mouse anti-human α -SMA monoclonal antibody (clone 1A4; 1:50; DAKO, Denmark), rabbit anti-desmin polyclonal antibody (1:200; Dako), rabbit anti-Col (I) polyclonal antibody (1:1000; LSL Co., Japan), and rabbit anti-fibronectin polyclonal antibody (1:1000; LSL Co., Japan) were used as the primary antibodies, and biotin-labeled anti-mouse IgG polyclonal antibody (1:400; KPL, Guildford, UK) and biotin-labeled anti-rabbit IgG polyclonal antibody (1:400; KPL) were used as the secondary antibodies. Immunostaining was carried out by avidin-biotin-peroxidase complex (ABC) method using a commercial reagents (Vector Laboratories, Burlingame, CA), and the positive signals were visualized by peroxidase-diaminobenzidine reaction. The sections were counterstained with methylgreen.

Statistical analysis

Results were indicated as mean and standard error (SE). To determine the difference between GH (+) and GH (-) groups, unpaired Student's *t*-test was performed (Statcel software package, Seiun-sha, Tokyo, Japan).

Results

Body weight changes and mortality

No deaths occurred to all 4 groups throughout the experimental period. The degree of BW gain was GH (+) CCl₄ (-), GH (-) CCl₄ (-), GH (+) CCl₄ (-), and GH (+) CCl₄ (+) in descending order. Changes in BW gain are shown in Fig. 2. Until CCl₄-treatment, the increase in BW gain was larger in GH (+) CCl₄ (+) group than in GH (-) CCl₄ (+) group (Fig. 2). In addition, the recovery of BW gain after

CCl₄-administration was also somewhat greater in the former group (Fig. 2). In all four groups, the significant decrease in BW gain was observed at 1 day after CCl₄ or vehicle administration (Fig. 2).

Blood biochemical findings

Results of blood biochemistry are shown in Fig. 3A. The activity of ALT showed significantly higher values at 24 and 48 h after CCl₄-administration, in both GH (+) CCl₄ (+) and GH (-) CCl₄ (+) groups, and the ALT activity tended to be suppressed by GH treatment (Fig. 3A). The activity of AST also showed similar changes to that of ALT. In contrast, the activity of ALP showed no significant changes (Fig. 3B).

Expressions of IGF-1, HGF, and TGF- β 1 mRNAs

The expression level of IGF-1 mRNA was higher in GH (+) CCl₄ (+) group than in GH (-) CCl₄ (+) group although not significant until 48 h after CCl₄-treatment (Fig. 4B). At 72 h, the difference between two groups became significant. The expression level of HGF mRNA was also higher in GH (+) CCl₄ (+) group than in GH (-) CCl₄ (+) group, although not significant (Fig. 4A). On the other hand, the expression level of TGF- β 1 mRNA was significantly higher in GH (+) CCl₄ (+) group than in GH (-) CCl₄ (+) at 6 and 36 h after CCl₄-administration (Fig. 4C).

Histopathological findings

Histological pictures at 24, 48, and 72 h after CCl₄-administration are shown in Fig. 5. At 24 h, vacuolar degeneration in centrilobular to mid-zonal hepatocytes and necrosis of hepatocytes with inflammatory cell infiltration around the centrilobular vein were observed in both GH (-) CCl₄ (+) and GH (+) CCl₄ (+) groups, but the changes were less severe in GH (+) CCl₄ (+) group (Figs. 5A and 5B). At 48 h, the changes became less severe in both groups, especially in GH (+) CCl₄ (+) group (Figs. 5C and 5D). At 72 h, degenerative changes of hepatocytes almost disappeared while infiltration of small epithelial-like cells extended from centrilobular zone and/or periportal area into the lobules (Figs. 5E and 5F). In addition, an accumulation of argyrophilic reticular fibers was observed along with infiltration of small epithelial cell-like cells (Figs. 5G and 5H). These changes were less severe in GH (+) CCl₄ (+) group than in GH (-) CCl₄ (+) group. Figure 6 shows the percentage of the area occupied by argyrophilic reticular fibers in the whole liver, and the percentage was significantly smaller in GH (+) CCl₄ (+) group than in GH (-) CCl₄ (+) group at 72 h.

Immunohistochemical findings

At 72 h, deposition of collagen type 1 and fibronectin was seen in similar fashion to that of reticular fibers in both GH (+) CCl₄ (+) and GH (-) CCl₄ (+) groups, and it was stronger in the latter group (Figs. 7A–7D). Desmin- or α -SMA-positive cells were seen mainly around the central vein and along the argyrophilic reticular fibers in GH (+) CCl₄ (+)

Fig. 6. The percentage of argyrophilic area in GH (+) CCl₄ (+) group (■) and GH (-) CCl₄ (+) group (□). Data represent mean $\pm$ SE of 3 rats. * p <0.05 and ** p <0.01: Significantly different between the two groups.

and GH (-) CCl₄ (+) groups, and the number of them was larger in the latter group (Figs. 8A–8D).

Proliferating activity of hepatocytes

The PCNA-labeling index of hepatocytes following CCl₄-administration is shown in Fig. 9. At 0 h after CCl₄-administration, the labeling index in both centrilobular and periportal areas was higher in GH (+) CCl₄ (+) group than in GH (-) CCl₄ (+) group. In the centrilobular area where parenchymal damage was severer, the index was low (< 2%) from 6 to 36 h after CCl₄-administration in both groups, and it was significantly higher in GH (+) CCl₄ (+) group than in GH (-) CCl₄ (+) group at 36 h after CCl₄-administration. At 48 h, the index was high in both groups, and, at 72 h, it returned to the baseline level in GH (+) CCl₄ (+) group while it still maintained a high level in GH (-) CCl₄ (+) group, although not significant. In contrast, in the periportal area, the labeling index was higher at 48 h after CCl₄-administration in both GH (+) CCl₄ (+) and GH (-) CCl₄ (+) groups. The difference in the index between the two groups was noted only at 0 h after CCl₄-administration.

Discussion

In this study, the effects of exogenous GH-treatment on the development and regeneration of CCl₄-induced hepatic lesions were investigated in GH-deficient Mini rats.

A significantly higher increase in body weight gain was observed in GH-treated group than in GH-untreated group before CCl₄-administration, and the recovery of body weight gain after CCl₄-administration was also larger in GH-treated group than in GH-untreated group. A significant decrease in body weight gain at 1 day after CCl₄-administration seems to be due to physical stress by oral administration rather than toxicity of CCl₄, because a similar decrease was also observed in both CCl₄ (+) and CCl₄ (-) groups.

The increases in the activities of ALT and AST, which represent hepatic parenchymal damage, were suppressed by exogenous GH-treatment, and this suggests that CCl₄-

Fig. 7. Immunohistochemistry for collagen type 1 (A and B) and fibronectin (C and D) at 72 h after CCl₄-administration. Left column (A and C): GH (+) CCl₄ (+) group; Right column (B and D): GH (-) CCl₄ (+) group. Depositions of collagen type 1 and fibronectin around the central vein are stronger in B and D than A and C. $\times 100$.

induced hepatocyte damage in GH-deficient Mini rats may be protected by exogenous GH-treatment. In addition, degeneration of hepatocytes with infiltration of inflammatory cells was less severe in GH (+) CCl₄ (+) group than in GH (-) CCl₄ (+) group from 24 to 48 h after CCl₄-administration. Moreover, an accumulation of reticular fibers, an increase in number of small epithelial cell-like cells, i.e. hepatic stellate cells, and accumulations of type 1 collagens and fibronectins were suppressed in GH (+) CCl₄ (+) group compared with GH (-) CCl₄ (+) group. Thus, stellate cell proliferation and accumulation of extracellular matrix materials were depressed by exogenous GH-treatment in GH-deficient Mini rats.

In the liver, CCl₄ is metabolized by cytochrome P450 (CYP) 2E1 and resultant free radical products damage hepatocytes. GH affects the localization of CYP2E1 in the liver¹⁵, and the amount of hepatic CYP2E1 content in Mini rats is significantly higher than that of other strains of rats with normal GH levels¹⁰. In addition, distribution of CYP2E1 is different between Mini and Wistar rats in the

early stage after CCl₄-treatment⁷. Based on these findings, distribution of CYP2E1 in the liver after CCl₄-treatment was compared between GH-treated and nontreated groups, but no significant difference in CYP2E1 distribution was detected in the present study (data not shown).

Desmin-positive hepatic stellate cells are activated to α -SMA-positive cells which have myofibroblast-like character and play a pivotal role in hepatic fibrosis^{16,17}. In this study, proliferation of hepatic stellate cells was found almost in the centrilobular zone, where hepatocytes were mostly injured, and along the argyrophilic reticular fibers, and it was slighter in GH (+) CCl₄ (+) group than in GH (-) CCl₄ (+) group. IGF-1, which is upregulated in hepatocytes by GH^{18,19}, is said to stimulate the expression of HGF but not of TGF- β 1 in hepatic stellate cells²⁰. Further studies are needed to clarify the relationships between them.

From the results of expression analysis of growth factor-related genes mRNAs by RT-PCR, the expression levels of *IGF-1* and *HGF* mRNAs were generally higher in GH (+) CCl₄ (+) group with a few exceptions. This seems to

Fig. 8. Immunohistochemistry for desmin (A and B) and α -SMA (C and D) at 72 h after CCl₄-administration. Left column (A and C): GH (+) CCl₄ (+) group; Right column (B and D): GH (-) CCl₄ (+) group. Desmin- or α -SMA-positive cells are seen mainly around the central vein and along the argyrophilic fibers. $\times 100$.

Fig. 9. PCNA-labeling index of hepatocytes in centrilobular (A) and periportal (B) areas after CCl₄- or vehicle-administration. ■: GH (+); □: GH (-). Values represent mean $\pm$ SE of 3 rats. * $p < 0.05$ and ** $p < 0.01$: Significantly different between GH (+) CCl₄ (+) and GH (-) CCl₄ (+) groups.

be related to the difference in the severity of histological changes in the liver after CCl₄-administration between the two groups. This may be also related to the difference in the PCNA-labeling index between these two groups. In contrast, the expression level of *TGF-β1* mRNA detected in the present study had no relations to CCl₄-induced hepatic lesions and their regeneration. Further detailed study is needed to analyze this point.

In conclusion, it was shown that CCl₄-induced acute liver injury in GH-deficient Mini rats is able to be restored by exogenous GH-treatment. Upregulation of *IGF-I* and/or *HGF* mRNAs by GH-treatment may be involved in this restoration.

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