Enzyme-Linked Immunosorbent Assay for Serum Procollagen Type III Peptide in Rats with Hepatic Fibrosis

Jae-Jin CHO1,2) and Yong-Soon LEE1)*

1)Department of Veterinary Public Health and Laboratory Animal Science, College of Veterinary Medicine, Seoul National University, Suwon 441–744, Republic of Korea and 2)Department of Gastroenterology, Klinikum Benjamin Franklin, Free University of Berlin, Germany

(Received 19 June 1998/Accepted 12 July 1998)

ABSTRACT. The process of hepatic fibrosis, and the changes in contents of hepatic hydroxyproline (HYP) and serum procollagen type III peptide (PIIINP) were examined in two rat models for hepatic fibrosis, i.e. bile duct ligation/scission (BDL/s)- and dimethylnitrosamine (DMN)-induced models. In addition, an expression of type III collagen mRNA in the liver of BDL/s model was also examined. In BDL/s model, hepatic fibrosis started at 2 weeks after operation (WAO) and cirrhosis with prominent bile duct hyperplasia was detected at and after 5 WAO. Serum PIIINP content measured using a modified double armed inhibition enzyme-linked immunosorbent assay (ELISA) method proposed by us started to increase at 1 WAO and continued to increase thereafter. Hepatic HYP content measured colorimetrically started to increase at 3 WAO and it continued to increase until 7 WAO. An expression of type III collagen mRNA in the liver was enhanced at and after 2 WAO, especially at 4 and 5 WAO. In DMN model, marked hepatic fibrosis was detected at 1 week after the last DMN administration (WAA), and the degree of fibrosis was apparently reduced at 4 WAA. Serum PIIINP content prominently increased at 1 WAA and decreased at and after 3 WAA. Hepatic HYP content showed a marked increase at 1 WAA and decreased thereafter. The present results indicated that the sequences of hepatic fibrosis, hepatic HYP content and serum PIIINP content were well correlated with each other in both BDL/s and DMN models. In conclusion, ELISA system for the detection of serum PIIINP content is considered to be a reliable method for assessment of cirrhotic liver, and the present two rat models for liver fibrosis/cirrhosis seems to be a good tool for researching antifibrotic agents. — KEY WORDS: enzyme-linked immunosorbent assay, hepatic fibrosis model, rat, serum procollagen type III peptide.

Hepatic fibrosis and cirrhosis, the results of imbalance of fibrogenesis and fibrolysis, are characterized by an over-accumulation of extracellular matrix (ECM) in the liver [4, 15]. Unfortunately, proven therapies that stop progression of hepatic fibrosis or induce regression of established cirrhosis have not been developed yet. In addition, liver biopsy for diagnosis of hepatic fibrosis is an invasive procedure and impossible to be performed many times [21]. Therefore, there is need to find suitable noninvasive clinical markers useful for predicting synthesis and deposition of hepatic collagen (fibrogenesis) or its removal (fibrolysis) and for finding antifibrotic agents [9, 19, 26]. It was only recently that procollagen type III peptide (PIIINP) became to be considered as a useful serum marker of liver fibrogenesis [20, 26], and the stabilized radioimmunoassay (RIA) is said to be highly sensitive for serum PIIINP. However, non-radioactive method is also needed from practical viewpoints.

Concerning the methods to induce animal models for hepatic fibrosis, there are many animal reports [2, 5, 7, 16, 17]. In this study, we chose two models characterized by marked hepatic fibrosis with no prominent inflammation and hepatocyte necrosis. Namely, bile duct ligation/scission (BDL/s) is recommended as a useful method for producing progressive and reproducible biliary fibrosis in rats without prominent hepatocyte necrosis and severe inflammation [9, 19]. In addition, treatment with dimethylnitrosamine (DMN) is also known to have an advantage to induce fibrosis in rats in a relatively short period (3 weeks) with slight inflammation and hepatocyte necrosis [2, 26].

The aim of this study is to establish a non-radioactive method, i.e. enzyme-linked immunosorbent assay (ELISA) to evaluate serum PIIINP using BDL/s- and DMN-induced hepatic fibrosis in rats.

MATERIALS AND METHODS

Animals: Two hundred male Sprague-Dawley rats (11 weeks old) obtained from Laboratory Animal Center of Seoul National University in Korea were used. The animals were kept in an animal room maintained at 23 ± 2°C with 60 ± 10% r.h. with 12 hr-light and 12 hr-dark cycle, and were given normal pelleted diet (Shinchon, Korea) and water ad libitum.

Treatments: As to bile duct ligation/scission (BDL/s) model, the hepatic biliary system was occluded by distal and proximal ligation using 4–0 silk (Perma-hand, Ethicon, Germany) in 80 rats. Ten rats each were killed under ether anesthesia every week up to 8 weeks after operation (WAO), respectively. Forty-eight rats which were sham-operated...
served as controls and 6 rats each were killed in the same way.

As to dimethylnitrosamine (DMN) model, 40 rats was intraperitoneal injected with potent alkylating agent, DMN, at the dose level of 10 µl/kg body weight on the first 3 consecutive days of each week over a period of 3 weeks. Ten rats each were killed under ether anesthesia at 1, 2, 3 and 4 weeks after the last DMN administration (WAA), respectively. The remaining 32 rats which were given the same volume of saline served as controls, and 8 rats each were killed in the same way, respectively.

At necropsy, blood was obtained from the vena cava of each animal. The liver was weighed, and pieces of the left and the right lobes were stored in liquid nitrogen for hydroxyproline determination and Northern blot analysis. The remaining tissues were fixed in 10% neutral buffered formalin for histological examination.

Procollagen type III peptide (PIIINP) antigen: Purification of PIIINP was performed using modified method of Schuppan et al. [25]. In brief, 2 kg of rat skin were homogenized in 7 l preextracted buffer (100 mM tris, 4N NaCl, 1% Triton X-100, pH 7.4) and preextracted twice with protease inhibitor (PI, 20 mM phenylmethanesulfonylfluoride, 10 mM N-ethylmaleimide, 20 mM EDTA Na2, 0.2% NaN3). All procedures were performed at 4°C. The tissue residue was extracted twice with extraction buffer (50 mM Tris, 0.5N NaCl, PI, pH 7.4), followed by precipitation of procollagens and collagens from the supernatant by addition of ammonium sulfate to a final concentration of 30% of total volume. The pellet was redissolved in 1.4N NaCl and subjected to fractional salt precipitation. The fraction obtained at a concentration of 1.7N NaCl was dissolved in and dialyzed against 50 mM Tris, 0.3N NaCl, 2 M urea, PI, pH 8.6 and stirred with diethylaminoethylcellulose (DEAE, Whatman, Germany) to absorb acidic glycosaminoglycans interfering in later chromatographical steps. The supernatant containing unbound material was dialyzed in 50 mM Tris, 0.02N NaCl, 2 M urea, pH 7.6 and chromatographed on a column of DEAE-cellulose using a salt gradient of 0.02–0.5N NaCl. Fractions were desalted by extensive dialysis against 0.05N acetic acid containing 0.2 mg/l Pepstatin A (Sigma, U.S.A.) and lyophilized.

Preparation of antisera: One mg of PIIINP was mixed with an equal volume of complete Freund’s adjuvant (Difco, U.S.A.) and injected subcutaneously into New Zealand white rabbits. After 2 weeks, three booster injections were given twice a week using incomplete Freund’s adjuvant (Difco, U.S.A.). Then, blood was taken from the vena cava for obtaining antisera against PIIINP .

Serum biochemistry: For testing general liver function in rats with hepatic fibrosis, the activities of alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) and lactic dehydrogenase (LDH), and the contents of total bilirubin (TBIL), blood urea nitrogen (BUN) and creatinine (CREAT) were determined using an automated analyzer.

Hydroxyproline (HYP) contents in liver tissues: Hydroxyproline (HYP) was quantified colorimetrically in duplicates from 0.2 g of liver tissue using a modified method of Jamall et al. [10]. Briefly, the tissue was homogenized in 4 ml of 6N HCl and hydrolyzed at 110°C for 16 hr. The hydrolyzed material was filtered, 50 µl aliquots were evaporated under vacuum, and residual HCl was removed after addition of methanol. The sediment was dissolved in 1.2 ml of 50% isopropanol and incubated with 0.2 ml of 0.84% chloramine-T in 42 mmol/l sodium acetate, 2.6 mmol/l citric acid, 39.5% (vol/vol) isopropanol, pH 6.0, followed by incubation for 10 min at room temperature. Next, 1.0 ml of 12% Ehrlich’s reagent was added and the mixture was incubated at 50°C for 90 min. The optical density (OD) of the sample solution was measured at 558 nm of wave length. The OD value was obtained by subtracting OD of reagent blank from the OD of a sample. Then, hydroxyproline was quantitated from a standard curve with amino acid.

Enzyme-linked immunosorbent assay (ELISA): In order to measure serum PIIINP, a modified double armed inhibition ELISA which is based on competition of serial dilutions of the standard or sample with predetermined amount of rabbit antibodies to rat PIIINP was used. This method was developed by us by modifying the previous reports [14, 23, 24, 27]. In brief, uncompeteted antibodies were then bound to PIIINP immobilized on a 96-well microtiter plate and quantified using biotynylated goat anti-rabbit IgG followed by the addition of streptavidin-peroxidase (first arm) and finally peroxidase-rabbit-anti-peroxidase complex (second arm). The peroxidase of both arms yielded a color reaction, the intensity of which was inversely proportional to the amount of antigen present in the sample sera. Inter- and intra-assay variations were below 10%. PIIINP levels were expressed as ng/ml sample sera.

Northern blot analysis: Total RNAs in liver tissue of each rat in BDL/s- and control groups were extracted using the acid guanidinium thiocyanate-phenol-chloroform method [6]. The extracted total RNAs were loaded onto 1% (wt/vol) agarose-formaldehyde gels, transferred to activated membrane (Nytran, Schleicher & Schuell, Germany) for 2 hr. The cDNA probe (mouse type III collagen) [13] was labeled with [32P]-α-deoxyctydidine triphosphate using the random primer labeling method and was used for hybridization. After prehybridization in the prehybridization buffer for 2 hr at 42°C, the membrane was hybridized in the hybridization solution for 48 hr at 64°C. The membrane was washed with the first washing buffer (2 × SSC (0.15 M NaCl, 15 mM sodium citrate), 0.1% SDS) three times for 15 min at 56°C and then with the second washing buffer (0.1 × SSC, 0.1% SDS) two times for 10 min at 42°C. The membrane was exposed to X-ray film for 3 days at -70°C and then read.

Histology: Paraffin sections (4 µm) of formalin-fixed liver tissues were stained with hematoxylin and eosin (HE) and subjected to histological examination.

Statistical analysis: Data were presented as means ±
standard deviation (SD). Statistical analysis was done using general linear model (SAS Institute, Ver. 6.03) and Duncan multiple range test for differences between groups. Regression correlation coefficients were calculated using Spearman rank correlation.

RESULTS

Gross findings and liver weight: At necropsy, icterus was first found at 3 weeks after operation (3 WAO) in BDL/s-group and at 1 WAA in DMN-group, respectively. In addition, ascites was found in almost all rats at 5–8 WAO in BDL/s-group, and at 1 and 2 WAA in DMN-group, when the liver was enlarged in BDL/s-group while it was shrunken in DMN-group.

In BDL/s-group, the liver weight increased from 2 to 5 WAO, and finally, it was three times as much as that in control group. The liver/body weight ratio also increased, and it finally showed the level two times higher than that in control group (Fig. 1). In DMN-group, the liver weight decreased at 1 WAA but recovered at 4 WAA (Fig. 1).

Changes in hydroxyproline (HYP) contents: In BDL/s-group, total liver HYP contents started to increase from 3 WAO. From 5 to 8 WAO, it was five to seven times as much as that in control group (Fig. 2). In DMN-group, it showed a threefold increase at 1 WAA. Thereafter, it showed a little decrease at 3–4 WAA, but it was still significantly higher than that in control group (Fig. 2).

Changes in contents of serum PIIINP: In BDL/s-group, as shown in Fig. 3, serum PIIINP content began to increase at 1 WAO. It showed a slight decrease at 3 WAO, but it turned to increase again thereafter. Finally, the level of serum PIIINP in BDL/s-group was ten times higher than that in control group at 7 and 8 WAO.
In DMN-group, as shown in Fig. 3, the level of serum PIIINP was 5 to 6 times higher than that in control group at 1 and 2 WAA. The level slightly decreased at 3 and 4 WAA, but it was still significantly higher than that in control group.

Serum biochemical findings: In BDL/s-group, as shown in Table 1, ALT activity significantly increased at 2 to 6 WAO except for at 4 WAO. As to AST activity, it was significantly higher than that in control group throughout the experimental period. Total bilirubin content markedly increased at 1 and 2 WAO. Thereafter, it decreased at 4 and 5 WAO, but it increased again from 6 to 8 WAO. ALP activity increased 4 to 5 times as much as that in control group from 5 to 7 WAO. Other parameters did not show any differences between BDL/s- and control group (Table 1).

In DMN-group, as shown in Fig. 3, the level of serum PIIINP was 5 to 6 times higher than that in control group at 1 and 2 WAA. The level slightly decreased at 3 and 4 WAA, but it was still significantly higher than that in control group.

Serum biochemical findings: In BDL/s-group, as shown in Table 1, ALT activity significantly increased at 2 to 6 WAO except for at 4 WAO. As to AST activity, it was significantly higher than that in control group throughout the experimental period. Total bilirubin content markedly increased at 1 and 2 WAO. Thereafter, it decreased at 4 and 5 WAO, but it increased again from 6 to 8 WAO. ALP activity increased 4 to 5 times as much as that in control group from 5 to 7 WAO. Other parameters did not show any differences between BDL/s- and control group (Table 1).

In DMN-group, as shown in Table 2, ALT activity significantly increased at 1 WAA, turned to decrease at 2 and 3 WAA, and then increased again at 4 WAA. AST activity did not show a significant change from 1 to 3 WAA, but it was significantly higher than that in control group at 4 WAA. Except for at 2 WAA, ALP activity was

Table 1. Serum biochemical findings in rats of bile duct ligation/scission (BDL/s) group

<table>
<thead>
<tr>
<th>Weeks after operation</th>
<th>ALT (IU/L)</th>
<th>AST (IU/L)</th>
<th>BUN (mg/dl)</th>
<th>TBIL (mg/dl)</th>
<th>ALP (IU/L)</th>
<th>LDH (IU/L)</th>
<th>CREAT (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>34.88</td>
<td>73.85</td>
<td>18.69</td>
<td>0.17</td>
<td>59.43</td>
<td>536.07</td>
<td>0.55</td>
</tr>
<tr>
<td>±7.95 ±18.44</td>
<td>±24.68 ±23.09</td>
<td>±15.83</td>
<td>±174.67</td>
<td>±975.00 ±0.63</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>56.30 304.57*</td>
<td>21.94 ±21.74*</td>
<td>11.98</td>
<td>165.50</td>
<td>594.75 ±0.73</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>105.35* 435.20*</td>
<td>±2.31 ±2.23</td>
<td>±4.25</td>
<td>±41.16</td>
<td>±386.67 ±0.10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 ±28.73 ±0.00</td>
<td>23.48 5.74*</td>
<td>168.00</td>
<td>486.25 ±0.66</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 85.04* 244.23*</td>
<td>±5.67 ±115.75</td>
<td>±115.75</td>
<td>±392.98 ±0.21</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 71.38 252.30*</td>
<td>±2.96 ±171.00</td>
<td>±9.90</td>
<td>±503.10 ±0.43</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 105.84 261.53*</td>
<td>±2.96 ±171.00</td>
<td>±9.90</td>
<td>±253.41 ±0.19</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 ±80.38 ±142.09</td>
<td>±3.25 ±117.47</td>
<td>±238.48</td>
<td>±0.07</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 125.70 352.53*</td>
<td>±5.45 ±115.54</td>
<td>±0.04</td>
<td>±0.08</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 ±65.82 ±131.81</td>
<td>±7.52 ±305.00*</td>
<td>±358.60</td>
<td>±0.55</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 72.18 245.37*</td>
<td>±31.50 ±82.96</td>
<td>±10.55</td>
<td>±283.46 ±0.16</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 55.78 258.22*</td>
<td>±31.50 ±82.96</td>
<td>±10.55</td>
<td>±283.46 ±0.16</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11 ±20.59 ±123.91</td>
<td>±9.33 ±4.08</td>
<td>±94.34</td>
<td>±251.90 ±0.13</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PIIINP, procollagen type III peptide; ALT, alanine transaminase; AST, aspartate transaminase; BUN, blood urea nitrogen; TBIL, total bilirubin; ALP, alkaline phosphatase; LDH, lactic dehydrogenase; CREAT, creatinine. *, Significantly different from control group (p<0.05).
significantly higher than that in control group throughout
the experimental period. Other parameters did not show
any difference between DMN- and control groups.

Findings of Northern blot analysis: Northern blot analysis
showed very low level of collagen type III mRNA contents
at 1 WAO in BDL/s-group. At and after 2 WAO, collagen
type III mRNA contents prominently increased especially at
4 and 5 WAO (Fig. 4).

Histological findings: Rats in control groups exhibited
no hepatic lesions throughout the experimental period. In
BDL/s-group, hepatic fibrosis with weak bile duct
proliferation was seen as early as 1 WAO. This change
increased in a time-dependent manner until 5 WAO,
resulting in portal to portal connection with connective
tissues. Finally, advanced secondary biliary fibrosis with
prominent bile duct proliferation was observed at and after
6 WAO (Figs. 5a and b). On the other hand, in DMN-
group, more advanced fibrosis developed at 1 WAA,
resulting in portal to portal and portal to central vein
connection with connective tissues without apparent bile
duct proliferation (Fig. 5c). Fibrosis was greatly restored at
4 WAA (Fig. 5d).

Correlation between liver HYP and serum PIIINP
contents: Very good correlations were observed between
total liver HYP and serum PIIINP contents in both BDL/s-
and DMN-groups (BDL/s: r=0.78, DMN: r=0.85). On the
contrary, there were no reliable correlations between liver
HYP content and serum biochemical values except for ALT
activity (Table 3).

DISCUSSION

Liver cirrhosis is a terminal state of various chronic liver
diseases and is characterized by an over-accumulation of
ECM as a result of imbalance of fibrogenesis and fibrolysis
[18, 28]. Although several laboratory animal models for
hepatic fibrosis/cirrhosis have been used to find antifibrotic
agents, there have not been suitable models for testing of
antifibrotic drugs up to the present time. In this study, we
selected and compared two kinds of rat models for hepatic
fibrosis. One is BDL/s model and other is DMN-
administration model [3, 8]. In our study, both could
produce well established hepatic fibrosis with rare
inflammation and hepatocyte necrosis, and they were
considered to be useful models for hepatic fibrosis.

Contrary, there were no reliable correlations between liver
HYP content and serum biochemical values except for ALT
activity (Table 3).

Table 2. Serum biochemical findings in rats of dimethylnitrosamine (DMN) group

<table>
<thead>
<tr>
<th>Week after the last</th>
<th>ALT (IU/L)</th>
<th>AST (IU/L)</th>
<th>BUN (mg/dL)</th>
<th>TBIL (mg/dL)</th>
<th>ALP (IU/L)</th>
<th>LDH (IU/L)</th>
<th>CREAT (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>34.88 ± 7.95</td>
<td>73.85 ± 8.44</td>
<td>18.69 ± 5.77</td>
<td>0.17 ± 0.05</td>
<td>59.43 ± 24.48</td>
<td>536.07 ± 235.09</td>
<td>0.55 ± 0.15</td>
</tr>
<tr>
<td>1</td>
<td>89.18 ± 16.50</td>
<td>189.8 ± 7.3</td>
<td>320.67 ± 142.36</td>
<td>158.8 ± 140.4</td>
<td>353.20 ± 140.4</td>
<td>0.43 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>67.40 ± 26.09</td>
<td>140.78 ± 9.76</td>
<td>21.68 ± 9.76</td>
<td>0.24 ± 0.09</td>
<td>160.60 ± 92.00</td>
<td>148.33 ± 148.33</td>
<td>0.42 ± 0.13</td>
</tr>
<tr>
<td>3</td>
<td>57.55 ± 57.71</td>
<td>118.05 ± 95.99</td>
<td>23.43 ± 12.29</td>
<td>0.45 ± 0.63</td>
<td>276.75 ± 298.01</td>
<td>243.56 ± 243.56</td>
<td>0.50 ± 0.14</td>
</tr>
<tr>
<td>4</td>
<td>113.10 ± 262.62</td>
<td>18.72 ± 5.42</td>
<td>278.60 ± 136.16</td>
<td>0.50 ± 0.34</td>
<td>263.20 ± 260.88</td>
<td>0.07 ± 0.07</td>
<td></td>
</tr>
</tbody>
</table>

PIIINP, procollagen type III peptide; ALT, alanine transaminase; AST, aspartate transaminase;
BUN, blood urea nitrogen; TBIL, total bilirubin; ALP, alkaline phosphatase; LDH, lactic dehydro-
genase; CREAT, creatinine. *, Significantly different from control group (p<0.05).

Fig. 4. Changes in the expression of type III collagen mRNA (Col III, 1.8kb) in the liver of rats in
BDL/s- and control groups. C: control group; Wks: weeks after operation.
present histological findings confirmed such characteristics. Namely, BDL/s caused bile duct proliferation from 3 WAO and connective tissues increased surrounding the portal triads and bile ducts. Finally, fibrous septa were made up portal to portal and portal to central vein connection accompanying bile duct hyperplasia. On the other hand, DMN-administration induced an increase in connective tissues along sinusoids, resulting in portal to portal connection without apparent bile duct proliferation and parenchymal cell necrosis. In the previous reports of Jenkins et al. [11], fibrosis induced by DMN was irreversible for 24 weeks. In our study, fibrosis was however reduced at and after 3 WAA, suggesting DMN-induced fibrosis being reversible. Thus there was clear difference in the progression pattern of fibrosis between BDL/s and DMN models.

To diagnose liver fibrosis, the liver biopsy has been the almost only method available until now. However, because of several reasons, e.g. a significant sampling error [1] and an ethical problem of continuous biopsies [8], a reliable serum assay for hepatic fibrosis is needed urgently. For decades, many kinds of serum assay for ECM components have been studied and used to diagnose liver diseases. Since serum PIIINP has been considered as a useful marker of fibrogenesis [20], a reliable and sensitive radioimmunoassay for rat serum-PIIINP was developed [25]. There is a growing experimental evidence that PIIINP levels are primarily due to increased collagen type-III production. PIIINP reflects the cleavage of procollagen peptide from the N-terminal end of collagen type-III during fibril growth.


troenterology (Seitz H.K, and Kommerell B. eds.), Berlin: Springer.

10. Jamall, I. S., Finelli, V. N. and Que Hee. S. S. 1981. A simple method to determine nanogram levels of 4-hydroxy-

induced model of cirrhosis and portal hypertension in the rat. J. Hepatol. 1: 489–499.


