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Comparison of TGF-β, PDGF, and CTGF in hepatic fibrosis models using DMN, CCl₄, and TAA

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Abstract

Three chemotoxins including dimethylnitrosamine (DMN), carbon tetrachloride (CCl₄), and thioacetamide (TAA) are commonly used in hepatofibrotic models. We aimed to draw characteristics of histopathology and pro-fibrogenic cytokines including TGF-β, PDGF and CTGF among three models. Rats were divided into six groups and intra-peritoneally injected with DMN (10 mg/kg, for three weeks, three consecutive days weekly), CCl₄ (1.6 g/kg, for 10 weeks, twice weekly), TAA (200 mg/kg, for 12 weeks, twice weekly) or their corresponded treatment for each control group. The liver weights were decreased in DMN model, but not other models. Ascites were occurred as 3-, 2-, and 7-rats in DMN, CCl₄, and TAA model, respectively. The lipid peroxidation was highest in CCl₄ model, serum levels of liver enzymes were increased as similar severity. The hepatofibrotic alterations were remarkable in DMN and TAA model, but not CCl₄ as evidenced by the Masson trichrome staining and hydroxyproline. The immunohistochemistry for α-SAM showed that the DMN model was most severely enhanced than other models. On the other hand, hepatic tissue levels of pro-fibrogenic cytokines including TGF-β, PDGF, and CTGF were generally increased in three models, but totally different among models or measurement resources. Especially, serum levels of three cytokines were remarkably increased by CCl₄ injection and CTGF levels in both hepatic tissue and serum were highest in CCl₄ group. Our results firstly demonstrated comparative study for features of morphological finding and pro-fibrogenic cytokines in serum and hepatic protein levels among three models. Above results would be a helpful reference for hepatofibrotic studies.

Keywords

Animal models, chemotoxins, CTGF, liver fibrosis, PDGF-β, TGF-β

History

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Introduction

Chronic liver injury eventually leads to liver fibrosis and often progress into cirrhosis that is one of the most common cause of death worldwide (Leon & McCambridge, 2006). Scientists anticipate that the mortality by chronic liver disease will increase due to changing life style and its related habits including alcohol consumption, increase rate of obesity populations, and patients with metabolic syndromes (Kochanek et al., 2009; Poynard et al., 2010). However, no convincing therapy to treat liver fibrosis exists to date yet.

Whatever reasons of liver fibrosis, the final etiological characters are accumulation of extracellular matrix (ECM) in the liver tissues resulted from hepatic stellate cells (HSCs) activation (Lee & Friedman, 2011). The pro-fibrogenic cytokines are strongly associated with development of liver fibrosis including transforming growth factor beta (TGF-β), platelet-derived growth factor (PDGF), and connective tissue growth factor (CTGF) (Friedman, 2003; Tsukada et al., 2006). Among them, the PDGF-β plays major role as a potent mitogen and activator of HSCs, resulting in ECM productions. TGF-β not only leads to activate HSCs, but also increases the expression of PDGF-β and CTGF receptors, and induces apoptosis in hepatocytes (Kisseleva & Brenner, 2006). CTGF is a potent pro-fibrogenic factor which can mediate TGF-β-induced ECM synthesis during progression of liver fibrosis (Gressner & Gressner, 2008).

Various animal models have been used to study for revealing the pathological features of liver fibrosis and develop anti-fibrotic agents or therapies. Among them, three representative chemotoxins including dimethylnitrosamine (DMN), carbon tetrachloride (CCl₄), and thioacetamide (TAA) are most frequently used in anti-fibrotic studies due to their applicability, reproducibility and sufficient comprehension for progressing liver fibrosis (Tsukamoto et al., 1990). A sound understanding the features of animal models is essential for anti-fibrotic research, while no comparison study for pathological features among above three models has been reported till to date.

*These authors contributed equally to this work.

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Herein, thus, we examined the characteristics of three major pro-fibrogenic cytokines in terms of hepatic protein and serum levels as well as histopathological features via the three animal models simultaneously.

Materials and methods

Reagent and chemicals

Chloramines-T, thiobarbituric acid and TEP were purchased from Sigma-Aldrich (St. Louis, MO); perchoric acid was obtained from GFS Chemical Co. (Columbus, OH), and TBA was purchased from Lancaster Co. (Lancashire, UK). Histofine was from Nichirei Biosciences (Tokyo, Japan); hydrochloric acid and phosphoric acid were from Kanto Chemical Co., Inc. (Tokyo, Japan); n-butanol, isopropanol and hydrochloride (HCl) were purchased from J.T. Baker (Phillipsburg, NJ); dianimobenzidine (DAB) was from Abcam (Cambridge, UK); Mayer’s hematoxylin, methanol and isopropanol were obtained from Wako Pure Chemical Industries (Osaka, Japan); TRI reagent was obtained from Invitrogen (Carlsbad, CA); goat anti-human CTGF antibody, CTFG standard solution, rabbit anti-human CTGF antibody, and anti-rabbit IgG-HRP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Animals

Forty-eight of specific-pathogen-free male Sprague–Dawley (SD) rats (six-weeks old, 190–210 g) were purchased from Orient Bio (Gyeonggi-do, Korea). All of the rats were acclimated for seven days in an environmentally controlled room at 22 ± 2 °C, 55% ± 10% relative humidity, and a 12 h light/dark cycle. The rats were fed commercial pellets (Koatech, Kung-gi, Republic of Korea) and tap water ad libitum for one week. Experiments were designed and performed in strict accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1985), approved by the Institutional Animal Care and Use Committee of Daejeon University (Animal ethical clearance number: DJUARB 2011–015).

After seven days, all of the rats were randomly divided into four groups, each control group (n = 8, matched with each experiment), DMN injection group (n = 8), CCl4 injection group (n = 8) and TAA injection group (n = 8). The body weight changes were recorded twice weekly.

Study design

Each experiment was completed by different chemotoxins and durations, respectively. The rats in the control group were intraperitoneally administered saline at a dose of 10 mL/kg of body weights. The DMN group was injected DMN in saline via by intra peritoneal (10 mg/kg in saline, three consecutive days weekly) for three weeks. The CCl4 group was injected CCl4 (1.6 g/kg in 50% olive oil, twice weekly) for 10 weeks. The TAA group was injected TAA (200 mg/kg in saline, twice weekly) for 12 weeks. The injection volume was constant for all of the groups at 10 mL/kg of body weights (Table 1).

After each experimental period, rats were sacrificed under the ether anesthesia and total blood was isolated via the abdominal aorta after fasting for 12 h. The liver tissues were removed and weighed immediately. Liver tissues were fixed in 10% formalin solution, RNA or stored separately in 10% neutral formalin solution, RNA was isolated and weighed immediately. Liver tissues were fixed in 10% formalin solution was re-fixed in Bouin’s solution. The stained tissue slices were performed by conventional hematoxylin and eosin (H&E) staining and Masson’s trichome staining protocols. After sealing the slides containing the tissue slices with NeoMount® (Darmstadt, Germany) the stained tissue slices were microposcopically examined at 200× magnifications. These histopathologic changes for inflammation were scored on a scale from 0 to 3, where 0 = normal, absence of pathology (<5% of maximum pathology), 1 = mild (~10%), 2 = moderate (15–20%), and 3 = severe (>20%) (Brunt et al., 1999). For the histopathological evaluation, a portion of liver tissue in 10% formalin solution was re-fixed in Bouin’s solution. The paraffin-embedded liver tissue slices (4 μm thickness) were performed by conventional hematoxylin and eosin (H&E) staining and Masson’s trichome staining protocols. After sealing the slides containing the tissue slices with NeoMount® (Darmstadt, Germany) the stained tissue slices were microposcopically examined at 200× magnifications. These histopathologic changes for inflammation were scored on a scale from 0 to 3, where 0 = normal, absence of pathology (<5% of maximum pathology), 1 = mild (~10%), 2 = moderate (15–20%), and 3 = severe (>20%) (Brunt et al., 1999).

Table 1. Experiments schedules.

<table>
<thead>
<tr>
<th>Items</th>
<th>Control (n = 8)</th>
<th>DMN (n = 8)</th>
<th>CCl4 (n = 8)</th>
<th>TAA (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration</td>
<td>3 weeks</td>
<td>10 weeks</td>
<td>12 weeks</td>
<td></td>
</tr>
<tr>
<td>Route</td>
<td>i.p.</td>
<td>i.p.</td>
<td>i.p.</td>
<td>i.p.</td>
</tr>
<tr>
<td>Dose</td>
<td>10 mg/kg</td>
<td>1.6 g/kg</td>
<td>200 mg/kg</td>
<td></td>
</tr>
<tr>
<td>Frequency (weekly)</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

DMN; dimethylnitrosamine, CCl4; carbon tetrachloride, TAA; thioacetamide, i.p.; intra peritoneal. All of the rats were intra peritoneally injected with neutral saline or each chemotoxin for different durations depend on the chemotoxins’ characterstics.

Assessment of ascites index

We determined the formation of ascites previously described (Domenicali et al., 2009). Briefly, the volume of ascites was estimated at the time of sacrifice. The extents of ascites formation were graded as follows: 0 for no peritoneal fluid, 1 for mild ascites (less than 3 mL), 2 for moderate ascites (3 mL to less than 6 mL), and 3 for severe ascites (over than 6 mL), respectively.

Platelet counts and serum biochemical analysis

A portion of EDTA-mixed blood was used for platelet counts using an automatic blood cell counter (Hemavet; CDC Technologies Inc., Oxford, CT). Serum was separated by centrifugation (3000 × g, 15 min) following clotting of the remaining blood. Serum AST, ALT, ALP, total bilirubin, total protein, albumin and A/G ratio levels were determined using a Chemistry Auto Analyzer (Chiron, Emeryville, CA).

Histopathological evaluations and immunohistochemical staining

For the histopathological evaluation, a portion of liver tissue in 10% formalin solution was re-fixed in Bouin’s solution. The paraffin-embedded liver tissue slices (4 μm thickness) were performed by conventional hematoxylin and eosin (H&E) staining and Masson’s trichome staining protocols. After sealing the slides containing the tissue slices with NeoMount® (Darmstadt, Germany) the stained tissue slices were microposcopically examined at 200× magnifications. These histopathologic changes for inflammation were scored on a scale from 0 to 3, where 0 = normal, absence of pathology (<5% of maximum pathology), 1 = mild (~10%), 2 = moderate (15–20%), and 3 = severe (>20%) (Brunt et al., 1999). For the histopathological evaluation, a portion of liver tissue in 10% formalin solution was re-fixed in Bouin’s solution. The paraffin-embedded liver tissue slices (4 μm thickness) were performed by conventional hematoxylin and eosin (H&E) staining and Masson’s trichome staining protocols. After sealing the slides containing the tissue slices with NeoMount® (Darmstadt, Germany) the stained tissue slices were microposcopically examined at 200× magnifications. These histopathologic changes for inflammation were scored on a scale from 0 to 3, where 0 = normal, absence of pathology (<5% of maximum pathology), 1 = mild (~10%), 2 = moderate (15–20%), and 3 = severe (>20%) (Brunt et al., 1999). A METAVIR fibrosis score from 0 to 4 was used to differentiate the levels of liver fibrosis. Briefly, stage 0 meant no scarring, stage 1 = minimal scarring, stage 2 = meant scarring had occurred and extended outside the areas in...
the liver that contained blood vessels, stage 3 meant bridging fibrosis was spreading and connecting to other areas than contained fibrosis, and stage 4 meant advanced scarring of the liver or cirrhosis (Sahin et al., 2013).

Immunohistochemical analysis against α-SMA was also performed to investigate HSC activation. Briefly, liver tissue sections were deparaffinized, hydrated and heated in citrate buffer for antigen retrieval at 100 °C for 15 min, and then treated with control serum for 30 min. Next, the slides were treated with anti-α-SMA mouse mAb (1:200) overnight. After washing three times with PBS, tissues were stained with the secondary antibody, N-Histofine Simple Stain MAX PO (Nichirei Biosciences, Tokyo, Japan), and its substrate, 3,3-diaminobenzidine (DAB). After counterstaining with Mayer’s hematoxylin, the slides were examined under an optical microscope (Leica Microsystems, Wetzlar, Germany).

**Determination of hydroxyproline in liver tissues**

Hydroxyproline determination was performed according to the previous method (Fujita et al., 2003). Briefly, liver tissues (200 mg) were homogenized in 2 mL of 6 N HCl and incubated overnight at 110 °C. After acid hydrolysates through filter paper, 50 μL samples or hydroxyproline standards were air-dried. The dried samples were dissolved in methanol (50 μL), and then 1.2 mL of 50% isopropanol and 200 μL of chloramine-T solution were added, and followed by incubation at room temperature for 10 min. Ehrlich’s solution (1.3 mL) was added, and the samples were incubated at 50 °C for 90 min. The optical density of the reaction product was read at 558 nm using a spectrophotometer (Cary 50; Varian, Palo Alto, CA). A standard curve was constructed using serial dilutions of 1.0 mg/mL hydroxyproline solutions.

**Determination of malondialdehyde MDA in liver tissues**

Lipid peroxidation levels in liver tissues were determined by measuring concentrations of MDA, an end-product of lipid peroxidation, using the TBARS method (Mihara & Uchiyama, 1978). The concentration of TBARS was expressed as mol/g tissue using TEP as a standard. The absorbance of the upper organic layer was measured at 535 and 520 nm with a spectrophotometer, and compared with the value of freshly-prepared TEP as a standard.

**Determination of cytokine levels in the protein and serum levels**

Three major fibrotic cytokines were determined using TGF-β1, PDGF-BB and CTGF levels in hepatic tissues and serum samples were measured using commercial ELISA kits (BioSource, San Jose, CA; R&D Systems, Minneapolis, MN; PeproTech, Rocky Hill, NJ).

**Statistical analysis**

The results are expressed as the means ± standard deviations (SD). The statistical significance of differences among groups was analyzed by one-way analysis of variance (ANOVA) followed by post hoc multiple comparisons with Fisher’s LSD t-test using the IBM SPSS statistics software, ver. 20.0 (SPSS Inc., Chicago, IL). Differences at p<0.05, p<0.01, or p<0.001 were considered statistically significant.

**Results**

**Comparisons of final body and organ weights change and formation of ascites**

The body weights were significantly lowered about 0.7-, 0.9- and 0.7-fold by DMN, CCl4 and TAA injections compared with their control group (p<0.01 for all groups). The body weight changes were significantly different between CCl4 and DMN or CCl4 and TAA model (p<0.01). The absolute liver weight was decreased by DMN injection (p<0.01), but was increased in both CCl4 and TAA groups as compared with each control group (p<0.01). The relative liver weight was decreased approximately 0.5-fold due to DMN injection (p<0.01), whereas increased about 1.6- and 1.5-fold by CCl4 and TAA injection compared with each control group (p<0.01). The absolute and relative spleen weight was increased most significantly in DMN group approximately 1.9- and 2.6-fold, respectively. Ascites was occurred in all groups as 3-, 1-, and 7-rat by DMN, CCl4 and TAA injection (Table 2).

**Comparisons of serum biochemistries and platelet counts**

The serum AST levels were significantly increased by three chemotoxins injections including DMN, CCl4, and TAA as compared with their control group (1.60 ± 0.38 folds for DMN, p<0.01; 1.58 ± 0.54 folds for CCl4, p<0.05; 2.28 ± 1.14 folds for TAA, respectively). Each chemotoxins caused significant increases of serum ALT levels as compared with each control group (2.88 ± 0.16 folds for DMN, p<0.01; 2.70 ± 0.68 folds for CCl4, p<0.01; 2.33 ± 0.93 folds for TAA, p<0.01, respectively). Serum ALP levels was most significantly increased in DMN group (3.44 ± 1.42 folds, p<0.01), and CCl4 and TAA groups were also showed significant increases serum ALP levels as compared with each control groups (2.28 ± 0.75 folds for CCl4 group and 2.93 ± 0.93 folds for TAA group, p<0.01, respectively). Those serum levels of liver enzymes including AST, ALT, and ALP had no significant differences among three groups. All group showed significant increase of total bilirubin in serum levels (30.44 ± 13.42 folds for DMN, p<0.01; 1.50 ± 0.53 folds for CCl4, p<0.05; 3.59 ± 1.69 folds for TAA, p<0.01, respectively), and DMN injection most notably altered serum total bilirubin levels with significances compared with others (p<0.01 versus others). The platelets counts were significantly decreased in DMN group (0.73 ± 0.19 folds, p<0.05) as compared with control group, but not other groups (Table 3).

**Comparisons of hydroxyproline and MDA contents in hepatic tissue**

The hydroxyproline contents in the hepatic tissues were significantly increased by 3.1-, 2.2- and 3.6-fold by DMN, CCl4 and TAA injection compared with their control groups, respectively. The statistical difference significantly was observed between TAA and CCl4 groups...
Table 2. Comparisons of final body and organ weight changes, ascites scores.

<table>
<thead>
<tr>
<th>Items</th>
<th>Control (n=8)</th>
<th>DMN (n=8)</th>
<th>CCl4 (n=8)</th>
<th>TAA (n=8)</th>
<th>p Value*</th>
<th>Comparisons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute liver weight</td>
<td>1.00</td>
<td>0.85 ± 0.06##</td>
<td>0.67 ± 0.03##</td>
<td>p &lt; 0.01</td>
<td>DMN versus CCl4</td>
<td></td>
</tr>
<tr>
<td>Relative liver weight</td>
<td>1.00</td>
<td>1.59 ± 0.23##</td>
<td>1.45 ± 0.11##</td>
<td>p &lt; 0.01</td>
<td>CCl4 versus TAA</td>
<td></td>
</tr>
<tr>
<td>Absolute spleen weight</td>
<td>1.00</td>
<td>1.37 ± 0.13##</td>
<td>1.02 ± 0.30</td>
<td>p &lt; 0.05</td>
<td>DMN versus CCl4</td>
<td></td>
</tr>
<tr>
<td>Relative spleen weight</td>
<td>1.00</td>
<td>1.63 ± 0.24##</td>
<td>1.52 ± 0.46##</td>
<td>p &lt; 0.01</td>
<td>DMN versus CCl4</td>
<td></td>
</tr>
<tr>
<td>Ascites</td>
<td>0/0</td>
<td>3/9</td>
<td>1/2</td>
<td>7/18</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

All data were calculated as fold values compared with each control group, except the ascites scores. Values represent means ± SD (n = 8). #p < 0.05, ##p < 0.01 model group versus each control group.

*p Value represents the significance among three groups.

Table 3. Comparisons of serum biochemistries and platelet counts.

<table>
<thead>
<tr>
<th>Items</th>
<th>Control (n=8)</th>
<th>DMN (n=8)</th>
<th>CCl4 (n=8)</th>
<th>TAA (n=8)</th>
<th>p Value*</th>
<th>Comparisons</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST</td>
<td>1.00</td>
<td>1.60 ± 0.38##</td>
<td>1.58 ± 0.54#</td>
<td>2.28 ± 1.14#</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>ALT</td>
<td>1.00</td>
<td>2.88 ± 0.76##</td>
<td>2.70 ± 0.68##</td>
<td>2.33 ± 0.93##</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>ALP</td>
<td>1.00</td>
<td>3.44 ± 1.42##</td>
<td>2.28 ± 0.75##</td>
<td>2.93 ± 0.93##</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Total bilirubin</td>
<td>1.00</td>
<td>30.44 ± 13.42##</td>
<td>1.50 ± 0.53##</td>
<td>3.59 ± 1.69##</td>
<td>p &lt; 0.01</td>
<td>DMN versus CCl4</td>
</tr>
<tr>
<td>Total protein</td>
<td>1.00</td>
<td>0.89 ± 0.21</td>
<td>0.90 ± 0.07#</td>
<td>0.86 ± 0.03##</td>
<td>p &lt; 0.05</td>
<td>CCl4 versus TAA</td>
</tr>
<tr>
<td>Albumin</td>
<td>1.00</td>
<td>0.84 ± 0.22</td>
<td>0.90 ± 0.11#</td>
<td>1.01 ± 0.05</td>
<td>p &lt; 0.05</td>
<td>CCl4 versus TAA</td>
</tr>
<tr>
<td>A/G ratio</td>
<td>1.00</td>
<td>0.90 ± 0.09</td>
<td>0.98 ± 0.16</td>
<td>1.32 ± 0.11##</td>
<td>p &lt; 0.01</td>
<td>CCl4 versus TAA</td>
</tr>
<tr>
<td>Platelets</td>
<td>1.00</td>
<td>0.73 ± 0.19#</td>
<td>1.00 ± 0.23</td>
<td>1.07 ± 0.10</td>
<td>p &lt; 0.01</td>
<td>CCl4 versus DMN</td>
</tr>
</tbody>
</table>

A/G ratio; albumin per globulin ratio, ALP; alkaline phosphatase, ALT; alanine aminotransferase, AST; aspartate aminotransferase. All data were calculated as fold values compared with each control group. Values represent means ± SD (n=8). #p < 0.05, ##p < 0.01 model group versus each control group.

*p Value represents the significance among three groups.

(p < 0.05, Figure 1A). The levels of MDA content, a marker of lipid peroxidation, were 1.8-, 3.7- and 2.1-fold higher in DMN, CCl4 and TAA groups than each control group without statistical differences among three groups, respectively (Figure 1B).

Analysis of histopathological characters and immunohistochemical staining

The DMN, CCl4 and TAA group showed notable bridging necrosis, inflammation and wide infiltration of inflammatory cells around the central veins (Figure 2A and D). From the Masson’s trichrome staining, fibrotic changes (Figure 2B and E) were most considerable in DMN group, but mildest in CCl4 group. The similar patterns were shown in immunohistochemical staining against α-SMA, presenting the HSCs activation (Figure 2C and F).

Comparisons of pro-fibrogenic cytokines in hepatic tissue protein and serum level

The hepatic protein levels of TGF-β were significantly increased approximately 1.8- and 3.1-fold by DMN and TAA injections compared with their control groups, whereas CCl4 injection didn’t markedly affect. The serum levels of TGF-β were significantly high in CCl4 and TAA groups by 2.7- and 1.8-fold compared with their control groups. These alterations of TGF-β levels in hepatic protein were significantly different between DMN and TAA (p < 0.01) or CCl4 and TAA groups (p < 0.01), and serum protein levels of TGF-β also showed significant differences between DMN and CCl4 (Figure 3A). Hepatic protein levels of PDGF significantly increased in similar manner in all the three groups (1.8- to 1.9-fold). The serum levels of PDGF also significantly increased about 2.7- and 1.5-fold by CCl4 and TAA injections, while the DMN injection didn’t alter it. These changes of serum protein levels were significantly different among all the three groups (p < 0.01, Figure 3B). On the other hand, CTGF in both hepatic and serum protein levels were elevated in only CCl4 group by 2.2- and 2.3-fold as compared with its control group, and the statistical significance appeared mainly between CCl4 and others in hepatic protein (p < 0.05) as well as serum protein levels (p < 0.001, Figure 3C).
Figure 1. Quantification of collagen deposition and MDA contents in the hepatic tissues. The hydroxyproline concentrations (A), and oxidative stress markers (MDA) were determined in the liver tissues (B). Values represent means ± SD (n = 8). \#p < 0.05, \#\#p < 0.01 for model group versus each control group, and *p < 0.05 among three groups. DMN; dimethylnitrosamine, CCl₄; carbon tetrachloride, TAA; thioacetamide, MDA; malondialdehyde.

Figure 2. Histopathological and immunohistochemical examination. Staining with H&E (A, black arrows indicated the inflammatory cells infiltrations), Masson’s trichrome (B, yellow arrows presented the collagen fibers), and immunohistochemistry for α-SMA (C, blue arrows marked the α-SMA positive cells). All of the pathophysiological examinations were performed under light microscopy at 200× magnification. Scores of inflammation (D), collagen fiber (E), and quantification analysis of α-SMA positive cells (mean value ± SD, n = 8). \#p < 0.05, \#\#p < 0.01 for model group versus each control group, and *p < 0.05 among three groups. DMN; dimethylnitrosamine, CCl₄; carbon tetrachloride, TAA; thioacetamide, MDA; malondialdehyde, α-SMA; alpha-smooth muscle actin, H&E; hematoxylin and eosin.
Discussion

Various animal models have been developed for inducing liver fibrosis since the past few decades (Iredale, 2007; Tsukamoto et al., 1990; Weber & Wasmuth, 2010). Owing to their applicability and reproducibility, DMN, CCl4, and TAA are most frequently used as chemotoxins inducing the liver fibrosis models (George et al., 2001; Natarajan et al., 2006). The previous studies already reported the differences in the etiological features and fibrosis degree between pairs of models (Jang et al., 2008, Salguero Palacios et al., 2008). However, there is no comparative study showing the comprehensive features of above fibrosis models induced by above three chemotoxins simultaneously. Herein, we analyzed concurrently the histopathological aspects and especially three major pro-fibrogenic cytokines, TGF-β, PDGF, and CTGF in hepatofibrotic animal models induced by DMN, CCl4, or TAA injection.

Due to the dissimilar susceptibility against those chemotoxins, rats were intra-peritoneally injected with different doses and treatment durations of DMN, CCl4, or TAA (Table 1). As our expectation, all of the models showed fibrous septa bridging around portal triads, giving the METAVIR score of F3 (Figure 2B and E). However, the DMN model gave the most severe fibrosis based on the Masson’s trichome staining (Figure 2B and E) and hepatic hydroxyproline levels (Figure 1B). DMN injection induced hepatic shrinkage, splenomegaly, thrombocytopenia, and significant increased total serum bilirubin level concurrently (Tables 2 and 3). These alterations are typical features of DMN-induced liver fibrosis, and then this model was known to be similar to patients with early stage of liver cirrhosis, especially by alcoholic liver damages (George, 2006).

These three hepatotoxic agents have been well known to induce severe oxidative stress leading to hepatocyte injury (Poli, 2000). As a strongest free-radical generator, CCl4 treatment induced the highest level of hepatic lipid peroxidation and accumulation of fat droplet (Figures 1A and 2A); this might be associated with the induction of the hepatomegaly which was shown in CCl4 model (Panduro et al., 1988). In fact, progression of liver fibrosis and cirrhosis results in
shrinkage of liver in human (Wisse et al., 2008). Different from others, TAA injection resulted in no changes of absolute liver and spleen weights, but ascites formation was most frequent (seven of eight rats, Table 2). Liver enzymes are representative markers of inflammation in hepatic damages (Cheong et al., 2011), and then these enzymes, including AST, ALT and ALP were similarly increased by all chemotoxins (Table 3). The differences of pathophysiological features among histological aspects, oxidative stress, liver enzymes and fibrotic changes were attributed to distinct characters of each chemotoxins (Parola & Robino, 2001).

Hepatofibrosis generally results from the excessive productions of collagen by activated HSCs and uncompensated degradation of the ECM (Wight & Potter-Perigo, 2011). In our results, immunohistostaining analysis of α-SMA, a typical marker of HSCs activation, didn’t show notably different pattern among the three models (Figure 2C and F). Three cytokines vitally evoke fibrogenic signaling (TGF-β and CTGF) and growth factor signaling (mainly PDGF) to HSCs in autocrine or paracrine manners, respectively (Friedman, 2008). Accordingly, the above three cytokines themselves or their pathways have been focused on the therapeutic targets in anti-hepatofibrosis (Wang et al., 2010). Interestingly we found that activities of three cytokines were totally different according to chemotoxin injections and measurement resources. TGF-β in hepatic protein level was highest in TAA model (Figure 2A). On the other hand, the PDGF in hepatic protein levels were similarly increased in all three models, but CCl4 group showed most remarkable increases of CTGF in hepatic protein levels (Figure 3A–C).

The hepatic and serum levels of TGF-β, PDGF, or CTGF are constantly elevated in patients with hepatofibrotic disorders resulted from hepatitis B virus or hepatitis C virus infections or biliary atresia (Faiz et al., 2000; Hora et al., 2008). The measurement of these cytokines in the serum level is used as a non-invasion assessment for diagnosis of liver fibrosis (Hora et al., 2008; Weng et al., 2009). Our results showed that serum levels of three cytokines were highest in CCl4 model with the weakest fibrotic changes (Figure 2A–C). In addition, CTGF levels were markedly activated in CCl4 model rather than other models. CTGF works alone or as an enhancer of TGF-β, in activation of HSCs as well as a mediator of fiber to matrix interactions (Abou-Shady et al., 2000; Gressner & Gressner, 2008).

To date, no universally effective therapies for hepatofibrotic progression are available (Wang et al., 2012). Some scientists have reported the similarity of histopathological features, such as in the DMN model versus alcoholic cirrhosis or portal hypertension (George & Chandrakasan, 1996, 2000) and the CCl4 versus steatosis derived liver fibrosis (Parola & Robino, 2001).

**Conclusion**

Data from the present study firstly presented the pathological features of liver fibrosis using three different chemotoxins-induced animal models at the same time. Among them, the DMN produces features most resembling human liver fibrosis or cirrhosis in the relatively short term, compared with other models. Our results also demonstrated the strictly different profile of TGF-β, PDGF, and CTGF activity depending on measurement resources. These would be a helpful reference for hepatofibrotic studies using animal models, which researchers could consider for their study design and interpretation of the data.

**Declaration of interest**

The authors declared that there were no conflicts of interest in the present study. This research was supported Oriental Medicine Research and Development Project, Ministry of Health and Welfare, Republic of Korea (HI12C-1920-010014).

**References**


