

# Study of plasma protein C and inflammatory pathways: Biomarkers for dimethylnitrosamine-induced liver fibrosis in rats

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## Abstract

The present investigation was designed to identify potential biomarker(s) and assess the involvement of inflammatory pathway in dimethylnitrosamine (DMN)-induced liver fibrosis in rats. Following DMN-treatment (10 mg/ml/kg, i.p., given three consecutive days each week for 4 weeks) body and liver weights were significantly decreased concurrent with increasing severity of liver damage assessed by bridging fibrosis, a histopathologic assessment and characteristic of human liver disease. Protein C along with albumin, C-reactive-protein (CRP), haptoglobin and total protein were significantly reduced and correlated with changes in liver histopathology. Biochemical markers of liver functions were significantly increased and correlated with changes in liver histopathology and plasma levels of protein C. Soluble intracellular-adhesion-molecule-1 (sICAM-1) levels were increased significantly but were poorly correlated with histopathology and protein C levels. Inflammatory chemokines and other analytes, monocyte-chemoattractant-protein-1 and 3 (MCP-1 and MCP-3), macrophage-colony-stimulating-factor (M-CSF) were significantly increased during the disease progression, whereas macrophage-derived-chemokine (MDC) and CRP were significantly suppressed. Circulating neutrophils and monocytes were also increased along with disease progression. The differential changes in sICAM-1, hyaluronic acid, gamma-glutamyltranspeptidase (GGT), neutrophil and other inflammatory chemokines suggest the involvement of inflammatory pathways in DMN-induced liver fibrosis. In conclusion, the progressive changes in protein C along with other noninvasive biochemical parameters whose levels were significantly correlated with disease progression may serve as biomarkers for pharmacological assessment of targeted therapy for liver fibrosis.

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## 1. Introduction

Fibrosis is a common ultimate consequence of injury in almost all the vital organs and notably hepatic fibrosis is the ultimate result of a variety of chronic liver diseases. Liver fibrosis reflects imbalance between the synthesis and breakdown of extracellular connective tissue components of the liver (Bataller and Brenner, 2005; George and Chandrakasan, 2000). Loss of normal function of liver caused by disorganized over-expression and excessive accumulation of extracellular matrix (ECM) including collagen may lead to liver fibrosis (Tao et al., 2003). In humans, histological evaluation of liver biopsy is regarded as gold standard

for assessing liver fibrosis, necrosis and inflammation (Poynard et al., 2000; Rockey and Bissell, 2006). In contrast, this procedure is invasive, hazardous, painful to the patient, and has potential complications, high risk of sampling error including hemorrhage, or even death (Rosenberg et al., 2004); whereas, plasma and/or serum markers may be more reflective of the status of liver pathology (Rosenberg et al., 2004; Rockey and Bissell, 2006).

Several animal models for liver fibrosis such as bile duct ligation (BDL) and chemical-induction models with carbon-tetrachloride (CCl<sub>4</sub>) and dimethylnitrosamine (DMN) have been described (Ala-Kokko et al., 1987; George and Chandrakasan, 2000; George et al., 2001, 2004; Parola et al., 1996; Saito and Maher, 2000). These models feature pathological conditions found in human liver fibrosis and have been used to test preclinical compounds (George and Chandrakasan, 2000; Jenkins et al., 1985). However, there is a lack of biomarkers to identify the progression of liver fibrosis in those animal models

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necessitating further studies to identify novel biomarkers. Ideally, the biomarkers should be measurable in accurate and reproducible tests that can be performed repeatedly and quickly without causing harm to the patient, and should be readily available (Rockey and Bissell, 2006). Therefore, identification of noninvasive biomarkers correlated with disease progression would be highly beneficial to patients.

Involvement of inflammatory chemokine, monocyte-chemoattractant-protein-1 (MCP-1) in chronic liver injury, its correlation with intracellular-adhesion-molecule (ICAM), and presence of high serum levels of sICAM in severe liver damage have formed the basis of the inflammatory mechanism of liver injury in humans (Panasiuk et al., 2004, 2005; Ross et al., 1998). Increased liver tissue expression of ICAM was reported in an animal model of liver fibrosis; however no statistical correlation with histopathology was available (Kornek et al., 2006). Hyaluronic acid is an important high molecular weight polysaccharide component of extracellular matrix in many connective tissues.

Both human and animal studies have suggested that hyaluronic acid has significant correlation with the liver disease and can be used as sensitive marker (George et al., 2004; Montazeri et al., 2005). Protein C is a plasma protein and its activated form, activated protein C, is a potent anticoagulant (Grinnell and Joyce, 2001) and may have a cytoprotective effect in liver and other diseases (Yoshikawa et al., 2000). In advanced hepatic patients plasma protein C levels are significantly depressed (Al Ghumlas et al., 2005) and have been used as a marker of hepatocellular disease in alcoholic liver damage patients (Kloczko et al., 1992). However, plasma levels of protein C and its correlation with liver damage in animal models of liver fibrosis has not been investigated previously.

Several biomarkers for human use were proposed but none of them have the precision to track liver fibrosis, and ultimately, their utility in diagnosing the disease is incomplete (Rockey and Bissell, 2006). Several biomarkers were also identified in animal studies, but no statistical correlations with liver histopathology are available. Further characterization of most of the potential biomarkers as well as identifying noninvasive biomarkers and its correlation with histopathology in a single study, to assess the severity of liver fibrosis, would be highly desirable and beneficial. Therefore, the present study aimed first, to validate a rodent model of liver fibrosis which is reflective of human disease. Second, provide further characterization of biochemical markers and study the association of inflammatory mediators with liver fibrosis. Third, evaluate protein C as a novel plasma biomarker during fibrogenesis and, along with other noninvasive biochemical parameters, determine their correlations with liver histopathology. In the present study we selected the previously described DMN model of liver fibrosis in rats to explore the identification of novel biomarkers and involvement of inflammatory components.

## 2. Materials and methods

### 2.1. General procedure and animal maintenance

Experiments were carried out in male Sprague–Dawley rats (Charles River, Portage, MI) weighing 325–375 g. Following

shipment all animals were acclimatized in the animal care facility for at least 1 week prior to any studies. The rats were maintained on a regular 12 h dark/light cycle (6 pm to 6 am) with access to food and water *ad libitum*. The protocol for this study was approved by the Institutional Animal Care and Use Committee (IACUC) of Eli Lilly and Company (Indianapolis, IN).

### 2.2. Induction of hepatic injury

The protocol used for inducing the liver injury by the chemical DMN is a modified version of that originally described by Jezequel et al. (1987). In brief, prior to the start of the study rats were randomized on the basis of their body weight and assigned them to two groups such that each group had similar distribution of body weights and subsequently received vehicle (saline) or dimethylnitrosamine (DMN). The animals were given intraperitoneal injections of either vehicle or DMN (10 mg/kg) for three consecutive days per week for 4 weeks. Details of the study protocol are schematized in Fig. 1. To determine the severity of liver injury by histopathology score (vehicle- and DMN-treated) several animals were sacrificed at the end of each week for 4 consecutive weeks. Body weights were measured each day prior to the injections of DMN. Blood and tissue samples for biochemical and pathological examinations were collected from the animals at sacrifice at the end of each week, as described specifically below.

### 2.3. Histopathology of liver tissues

At the end of weekly intervals rats were euthanized (deep anesthesia with isoflurane followed by cervical dislocation) and the abdomen opened immediately by a midline incision. The whole liver was isolated and weighed. A small portion of the liver tissue was isolated immediately and rinsed in cold saline before being placed in 4% formaldehyde. The fixed tissue was subsequently processed, microtomed, stained with both H&E and Masson's Trichrome and examined by light microscopy.

The disease severity from these liver sections was assessed by a certified pathologist. In humans, chronic liver disease does not affect the liver in a homogeneous pattern and sample variability is often high as in this animal model. Use of whole cross sections of the liver in this model together with the following scoring system alleviated much of the variability. The histologic scoring system used was as follows: Grade

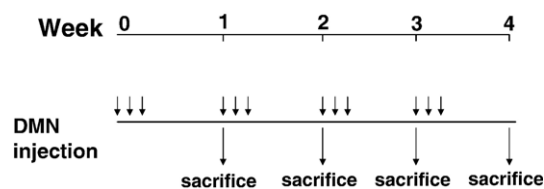


Fig. 1. Schematic representation of the liver fibrosis induction protocol in rats. Rats received DMN (10 mg/kg, i.p.) or vehicle (upper arrows) for three consecutive days each week for 4 weeks. Body weights were taken at each injection day prior to the injections. At the end of each week (4 days after the last dose), several rats from both vehicle- and DMN-treatment groups were sacrificed.

0=histologic characteristics included no inflammatory infiltration with preserved lobular architecture and little or no fibrosis. Grade 1=histologic characteristics included minimal inflammatory infiltration with preserved lobular architecture and little fibrosis. Fibrosis expansion seen in the portal area of 25% or less of the total portal triads. Grade 2=histologic characteristics included minimal inflammatory infiltration with preserved lobular architecture and little to moderate fibrosis. Moderate fibrosis expansion seen in the portal area of 25% to 50% of the total portal triads. Grade 3=histologic characteristics included minimal inflammatory infiltration with preserved lobular architecture and little to moderate fibrosis. Moderate fibrosis expansion seen in the portal area of 50% to 75% of the total portal triads. Bridging fibrosis seen in approximately 25 to 33% of the total liver histology section. Grade 4=histologic characteristics included minimal inflammatory infiltration with preserved lobular architecture and little to moderate fibrosis. Moderate fibrosis expansion seen in the portal area of 75% or more of the total portal triads. Bridging fibrosis seen in approximately 35% or more of the total liver histology section.

#### 2.4. Determination of plasma protein C levels

Measurement of rat plasma protein C levels was established in our laboratories as described earlier (Heuer et al., 2004). Recombinant mouse and rat protein Cs were prepared essentially as described previously (Berg et al., 2003), and mouse protein C was used to obtain rabbit anti-mouse protein C polyclonal antibody that was cross-reactive to rat protein C and used in a standard enzyme-linked immunosorbent assay (ELISA). In brief, 9 parts whole blood was collected into 1 part 3.8% sodium citrate/500 mM benzamidine-HCl, mixed briefly, centrifuged and plasma isolated for analysis. Plasma from each animal was diluted and assayed in triplicate in this ELISA; recombinant rat protein C was used as the reference standard.

#### 2.5. Determination of hematologic parameters

Peripheral hematologic parameters were determined by using an automated blood cell counter, Hemavet 850 (CDC Technologies, Oxford, CT) calibrated with MULTI-TROL reference control as per company set protocol prior to each use. After anesthesia, blood samples were collected by cardiac puncture into EDTA-washed 5 ml syringes (0.5 M EDTA, pH 8.0, GIBCO, Invitrogen Corporation, Grand Island, NY) and transferred immediately into 1 ml Microtainer brand tube with EDTA (Becton Dickinson and Company, Franklin Lakes, NJ). The tubes were then placed on an automatic roller (Easy-Mixer, SKRM-1, Seo Kwang Scientific Co, Korea) for 10 min prior to evaluation by the Hemavet. The hematologic parameters included total white blood cell, lymphocytes, neutrophils, monocytes, hematocrit values and hemoglobin concentrations and were measured in each animal.

At the end of the studies, rats were anesthetized under isoflurane (2.5% in 1.5% medical oxygen) constantly delivered through a Vetamac Dual Flow Meter (MGX-VAD) Research Machine (Vetamac, Inc., Rossville, IN). Blood samples were

collected by cardiac puncture for a variety of biochemical analyses. Samples were collected in EDTA-washed 5 ml syringes (see above) by cardiac puncture and transferred immediately into a 5 ml Monoject blood collection tube containing 7.5 mg EDTA (Sherwood Medical, St. Louis, MO). Blood samples were then centrifuged at 2500 g for 10 min at 4 °C and plasma was harvested following standard protocols established in our laboratory. For collection of serum samples, blood was collected by cardiac puncture without the use of anticoagulant and was allowed to clot for 1 h at room temperature before centrifugation at 5000 g for 10 min. The serum was collected and placed in separate tubes for biochemical analysis.

#### 2.6. Measurements of biochemical parameters

Serum samples were used for the determination of several biochemical parameters such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl-transpeptidase (GGT), alkaline phosphatase (ALP), total bilirubin, albumin and total protein by a Hitachi 912 analyzer (Roche Diagnostics, Indianapolis, IN). Plasma samples were used for determination of various chemokines and other analytes namely: C-reactive protein (CRP), haptoglobin, macrophage chemoattractant protein-1 and 3 (MCP-1, MCP-3), macrophage colony stimulating factor (M-CSF) and macrophage derived chemokine (MDC). These were measured in a single plasma sample from each rat using mouse multiplex kits (cross-reactive to rat plasma samples) by Rules Based Medicine (RBM Inc., Austin, TX).

#### 2.7. Measurements of plasma hyaluronic acid (HA) and sICAM-1

Plasma hyaluronic acid levels were determined by Quantitative HA Test Kits (Corgenix Inc., Westminster, CO). This is an Enzyme-linked Binding Protein Assay kit using hyaluronic acid binding protein (HABP) as the capture agent in 96-microwell plates following instructions provided by the manufacturer. In short, diluted rat plasma was incubated into plate for 1 h followed by the addition of horseradish peroxidase conjugated HABP for 30 min and TMB/H<sub>2</sub>O<sub>2</sub> substrate for 30 min. Finally 100 ul of stopping solution (0.36 N sulphuric acid) was added to the wells and the resulting optical densities were read using a Spectramax-190 microplate reader (Molecular Devices, Sunnyvale, CA) at 450 nm. Values for hyaluronic acid concentrations were computed from the standard curve prepared from the hyaluronic acid reference solution provided with the kit.

Plasma sICAM-1 levels were determined by quantitative sandwich enzyme immunoassay technique (Rat sICAM-1 CD54 kit, R & D Systems, Minneapolis, MN). The assay was performed following the instructions provided with the kit which used a monoclonal antibody specific for rat sICAM-1 pre-coated onto microplates. The sample values are read using a Spectramax-190 microplate reader (Molecular Devices, Sunnyvale, CA) set to 450 nm. Standard curve was created by using the reference sICAM-1 solution and the concentrations from the test samples were calculated from the standard curve.



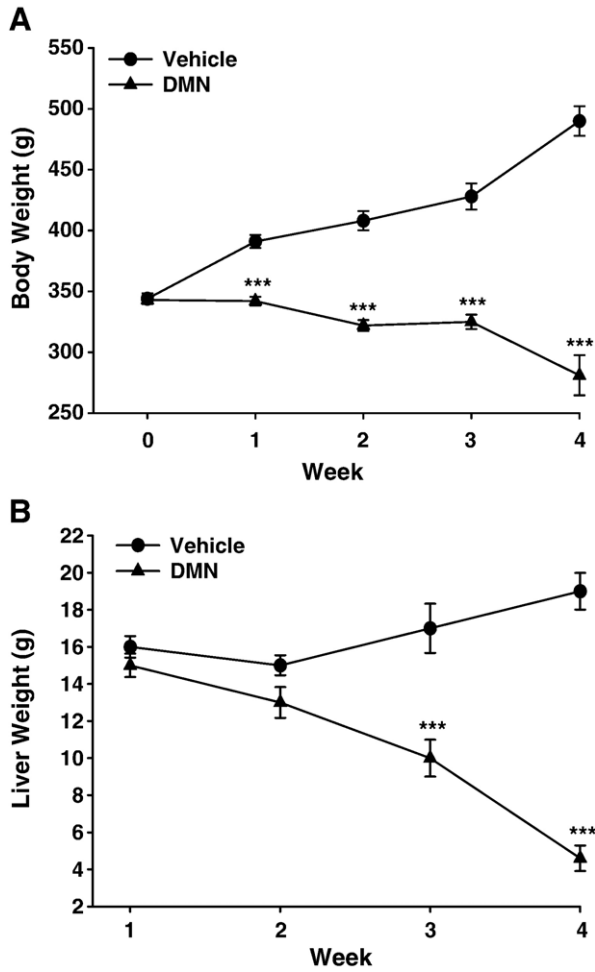


Fig. 2. Changes in (A) body and (B) liver weights during vehicle or DMN-treatment. Body and liver weights were measured throughout the study, the values presented are the body and liver weights at the end of each week at the time of sacrifice. Results are analyzed by two-way (group and time as factors) ANOVA and are presented as mean±SEM ( $n=5-6$  rats for vehicle and  $n=8-10$  rats for DMN at each interval). \*\*\*,  $P<0.001$  vs. vehicle group.

2.8. Materials

Dimethylnitrosamine (DMN) was from Sigma-Aldrich, Inc, St. Louis, MI and Isoflurane (IsoFlu) was from Abbott Laboratories, North Chicago, IL. Solutions of DMN for injections were prepared by diluting the concentrated DMN in normal saline. The diluted solution was stored at room temperature and was used for the entire study period (4 weeks).

2.9. Data analysis

Statistical significance was determined by Analysis of Variance (ANOVA) from SAS (Version 8.2) statistical analysis program (SAS Institute, Cary, NC). Box Cox transformation was applied to several parameters since their data distributions were skewed. To correlate liver fibrosis with various biochemical markers, Spearman's Correlation (Rank correlation) and Logistic Regression analysis are applied. Correlation  $r$  was considered strong correlation when  $1>r\geq 0.8$ , moderate when  $0.8>r\geq 0.5$  and weak when  $0.5>r\geq 0$ . Data comparison was

considered significant when the  $P$  value was  $<0.05$ . Results are pooled data from two independent studies with similar outcome and are expressed as mean±SEM.

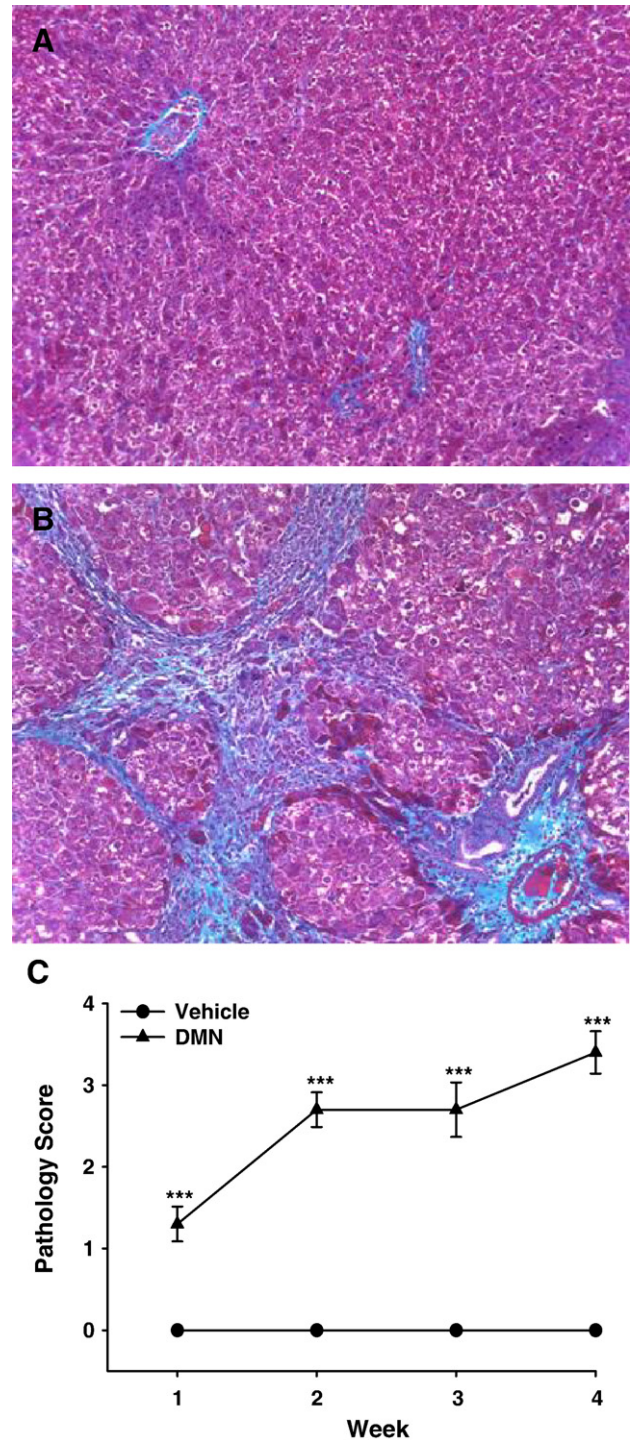


Fig. 3. Representative light photomicrographs of liver sections from (A) vehicle- and (B) DMN-treated (2 weeks) rats stained with H & E and Masson's trichrome. In (C) quantitative analysis of the extent of liver fibrosis (graded scoring of histopathological changes) induced by DMN are also shown. In contrast to the vehicle group the liver sections from DMN-treated rats showed pathological changes (interlobular fibrous bands, degenerated hepatic cells and increased inflammatory cells). Results are analyzed by two-way (group and time as factors) ANOVA and are presented as mean±SEM ( $n=5-6$  rats for vehicle and  $n=8-10$  rats for DMN at each interval). \*\*\*,  $P<0.001$  vs. vehicle.

### 3. Results

#### 3.1. Effect of DMN on body and liver weights

Body weights were measured each day prior to the injection of DMN over 4 weeks. Vehicle-treated rats gained body weight significantly in a time-dependent manner, reaching up to a 1.5-fold increase at week 4 from the baseline level (day 0) prior to the onset of treatment (Fig. 2A). DMN-treated rats failed to gain body weight at any time, indeed in contrast, the body weight in DMN-treated rats decreased slowly over the 4 week period (Fig. 2A). Total body weight at week 4 after DMN-treatment was significantly lower than the baseline value (day 0) and was about 2-fold less compared to vehicle-treated rats. DMN also resulted in changes in liver weight. As shown in Fig. 2B, although the liver weight loss in DMN-treated animals was not significantly different from the vehicle-treated group for the first 2 weeks, it became significant during the subsequent 2 weeks. At week 4 it was more than 4-fold less than vehicle-treated rats. All rats, both in vehicle and DMN treatment groups, remained alive for the first 2 weeks. During the following 2 weeks one rat each in week 3 and week 4 (1/10 and 1/9 respectively) died in the DMN-treatment group. In the vehicle treatment group none of the rats died during week 3 and week 4.

#### 3.2. Effect of DMN on liver histopathology

Liver specimens were evaluated for histopathological changes using both H & E and Masson's Trichrome staining procedures. The livers from vehicle-treated animals did not show any abnormalities at any time point during the 4 weeks. In contrast, in the DMN-treated (2 weeks) rats there was abundant bridging fibrosis (illustrated by the blue staining) and focal periportal fibrosis (localized blue staining around portal triad) (Fig. 3A & B). As indicated in Fig. 3C, vehicle-treated rats did

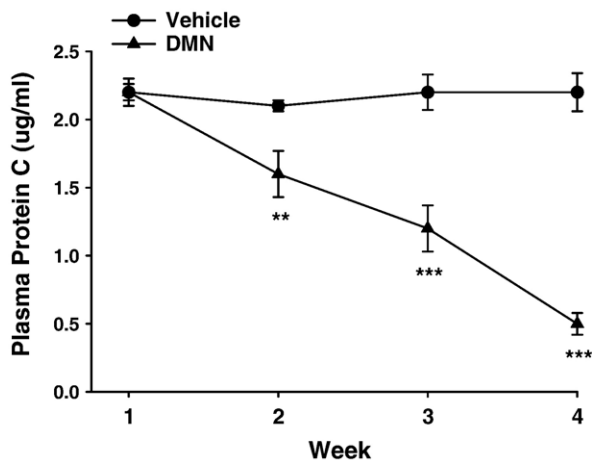


Fig. 4. Plasma levels of protein-C during development of liver fibrosis induced by DMN. Protein C levels decreased significantly beginning 2 weeks after initiation of DMN-treatment. Results are analyzed by two-way (group and time as factors) ANOVA and are presented as mean  $\pm$  SEM ( $n=5-6$  rats for vehicle and  $n=8-10$  rats for DMN at each interval). \*\*,  $P<0.01$ , and \*\*\*,  $P<0.001$  vs. vehicle.

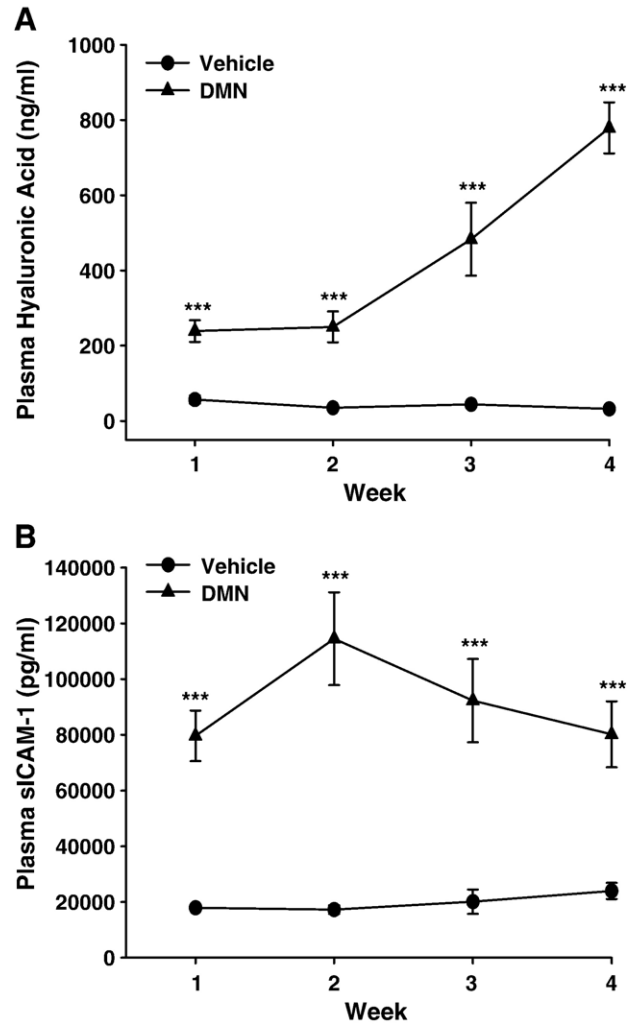


Fig. 5. Changes in plasma hyaluronic acid and sICAM-1 level during DMN treatment. (A) hyaluronic acid level increased significantly in DMN-treated rats beginning from week 1 and progressed with the advancement of disease severity during the subsequent week of DMN treatment. (B) sICAM-1 level also increased significantly beginning from week 1 of DMN-treatment but maintained a steady state level during the subsequent week of DMN treatment. In vehicle treated rats both hyaluronic acid and sICAM levels remained unchanged. Results are analyzed by two-way (group and time as factors) ANOVA and are presented as mean  $\pm$  SEM ( $n=5-6$  rats for vehicle and  $n=8-10$  rats for DMN at each interval). \*\*\*,  $P<0.001$  vs. vehicle.

not show any increase in their histopathology score (bridging fibrosis) while the DMN-treated rats showed a significant change in their score beginning from week 1 and progressing through week 4 of DMN-treatment.

#### 3.3. Effect of DMN on plasma levels of protein C

Protein C levels in the plasma of vehicle-treated and DMN-treated rats were determined at different time points after the start of DMN-treatment. Vehicle-treated rats did not show any appreciable changes from the baseline protein C levels at any time from week 1 to week 4. However, the DMN-treated rats showed a highly significant ( $P<0.001$ ) and progressive reduction in protein C levels in a time-dependent manner from control group beginning at week 2 of DMN-treatment such

Table 1  
Serum levels of liver enzymes and other biochemical parameters during development of liver fibrosis induced by DMN

Group	Week	AST (U/l)	ALT (U/l)	Albumin (g/dl)	Total protein (g/dl)	GGT (U/l)	ALP (U/l)	Bilirubin (mg/dl)
Vehicle	1	113.00±7.56	68.00±4.11	4.52±0.09	6.89±0.03	1.00±0.00	257.00±46.21	0.06±0.02
	2	107.83±3.94	56.83±2.74	4.45±0.07	6.77±0.12	1.00±0.00	255.33±47.38	0.04±0.07
	3	117.80±11.87	60.00±3.65	4.07±0.10	6.34±0.15	1.00±0.00	190.20±19.27	0.07±0.02
	4	107.50±7.09	52.00±2.78	4.34±0.12	6.67±0.17	1.00±0.00	170.17±18.89	0.07±0.02
DMN	1	159.78±23.02 <sup>a</sup>	58.11±6.50	4.35±0.07	6.54±0.13	1.22±0.22	322.70±25.87	0.14±0.01 <sup>b</sup>
	2	272.00±61.75 <sup>c</sup>	115.90±19.02 <sup>c</sup>	3.62±0.18 <sup>c</sup>	5.52±0.29 <sup>c</sup>	5.28±1.20 <sup>b</sup>	308.90±21.15	0.30±0.06 <sup>b</sup>
	3	258.67±29.97 <sup>c</sup>	161.89±17.98 <sup>c</sup>	3.14±0.12 <sup>c</sup>	4.74±0.18 <sup>c</sup>	5.34±1.33 <sup>b</sup>	388.44±43.05 <sup>b</sup>	0.88±0.28 <sup>b</sup>
	4	293.63±38.16 <sup>c</sup>	127.25±6.28 <sup>c</sup>	2.49±0.18 <sup>c</sup>	4.01±0.26 <sup>c</sup>	7.54±1.57 <sup>c</sup>	444.00±43.31 <sup>c</sup>	2.13±0.46 <sup>b</sup>

Results are analyzed by two-way (group and time as factors) ANOVA and are presented as mean±SEM ( $n=5-10$  rats per group). <sup>a</sup> $P<0.05$ ; <sup>b</sup> $P<0.01$ ; <sup>c</sup> $P<0.001$  vs. vehicle treatment. ALT=alanine aminotransferase; AST=aspartate aminotransferase; GGT=gama-glutamyltranspeptidase; ALP=alkaline phosphatase.

that by week 4 values were only 20% of those found at week 1 or in vehicle treated animals (Fig. 4).

### 3.4. Effect of DMN on plasma hyaluronic acid and sICAM-1

Plasma hyaluronic acid levels increased (more than 2 fold) within the first week of DMN-treatment, remained steady for the second week and then increased gradually up to the 4 week time point at which time it was an almost 9-fold increase over the vehicle-treated group (Fig. 5A). At all time points hyaluronic acid levels were significantly higher compared to the vehicle-treated rats. sICAM-1 levels were increased significantly (over 4 fold from baseline) compared to saline treatment within week 1 of DMN-treatment and remained elevated for the consecutive 3 weeks (Fig. 5B). Hyaluronic acid and s-ICAM-1 levels in vehicle-treated rats remained unchanged at all time points (Fig. 5A & B).

### 3.5. Effect of DMN on liver function markers

To further assess the impact of DMN on liver injury, several liver markers were determined. As shown in Table-1 a time-dependent increase in liver enzymes was noted, showing a gradual increase with the severity of the liver fibrosis as determined by histopathology score (Fig. 3). Both AST and ALT levels were increased significantly in a time-dependent manner beginning week 1 (AST) and week 2 (ALT) after DMN-treatment and remained significantly high in comparison to vehicle-treatment group at all subsequent time points. Alkaline phosphatase (ALP) levels followed a similar trend. On the other

hand, serum levels of albumin and total protein did not change significantly initially (week 1); however, they decreased significantly beginning from week 2 after the onset of DMN-treatment. GGT levels were increased as high as 5-fold compare to vehicle-treatment group within 2 weeks of DMN-treatment and progressed steadily up to week 4 of DMN-treatment, suggesting wide spread damage to the liver with chronic DMN-treatment (Table 1).

Serum levels of total bilirubin also increased gradually with the advancement of disease severity and became significantly higher than vehicle treated animals as early as week 1 after DMN. By week 4, DMN-treated rats had bilirubin levels that were 30-fold higher than vehicle-treated rats. Serum samples from vehicle treated rats did not show any change at any time point up to 4 weeks (Table 1).

### 3.6. Effect of DMN on plasma chemokines and other analytes

Plasma chemokines and other analytes were determined during vehicle- or DMN-treatment using a multiplex assay system and are presented in Table 2. CRP levels showed a gradual reduction to approximately 43% of the vehicle-treatment values, with the advancement of liver fibrosis. Haptoglobin levels followed a similar trend, but of greater magnitude, to approximately 14% of the vehicle-treated values by end of week 4 (Table 2). MCP-1 and MCP-3 levels showed a more than 2.5 and 2-fold increase respectively at week 1 after the onset of DMN-treatment. The MCP-1 and MCP-3 levels, however, were variable showing some changes but which were not significantly different from vehicle-treatment group, except

Table 2  
Plasma levels of various chemokines and other analytes during development of liver fibrosis induced by DMN

Group	Week	CRP (ug/ml)	Haptoglobin (ug/ml)	MCP-1 (pg/dl)	MCP-3 (pg/dl)	M-CSF (ng/ml)	MDC (pg/ml)
Vehicle	1	937.40±58.35	476.60±95.65	474.80±188.08	261.00±89.80	0.98±0.05	259.60±74.02
	2	1057.67±67.03	349.58±69.08	388.50±58.90	210.67±19.68	1.01±0.04	275.17±45.33
	3	1039.60±36.62	529.00±138.70	540.00±121.59	304.60±69.00	0.95±0.06	297.80±52.19
	4	1117.50±47.64	462.86±83.11	355.83±43.43	186.63±14.08	0.97±0.04	267.50±32.46
DMN	1	858.00±61.59	605.90±57.88	1184.70±206.59 <sup>a</sup>	633.70±81.00 <sup>a</sup>	1.78±0.03 <sup>c</sup>	249.70±35.22
	2	760.50±57.57 <sup>b</sup>	346.40±32.45	597.10±79.83	333.00±26.57 <sup>b</sup>	1.84±0.09 <sup>c</sup>	157.04±27.51 <sup>a</sup>
	3	596.22±75.81 <sup>b</sup>	167.19±43.92 <sup>b</sup>	969.56±230.87	413.89±69.43	1.85±0.05 <sup>c</sup>	139.69±15.39 <sup>b</sup>
	4	487.63±54.93 <sup>c</sup>	62.86±14.07 <sup>c</sup>	1196.25±334.71	487.88±126.67	1.96±0.08 <sup>c</sup>	196.25±18.62

Results are analyzed by two-way (group and time as factors) ANOVA and are presented as mean±SEM ( $n=4-5$  rats per group). <sup>a</sup> $P<0.05$ ; <sup>b</sup> $P<0.01$ ; <sup>c</sup> $P<0.001$  vs. vehicle treatment. CRP=C-reactive protein; MCP-1=macrophage chemoattractant protein-1; MCP-3=macrophage chemoattractant protein-3; M-CSF=macrophage colony stimulating factor; MDC=macrophage derived chemokine.



Table 3  
Differential blood cell counts during development of liver fibrosis induced by DMN-treatment in rats

Peripheral blood cells							
Group	Week	WBC (K/ul)	NE (K/ul)	LY (K/ul)	MO (K/ul)	Hb (g/dl)	HCT (%)
Vehicle	1	11.29±1.06	4.07±0.68	6.82±0.49	0.33±0.05	14.90±0.69	43.94±1.92
	2	10.23±1.41	3.13±0.44	6.68±0.94	0.37±0.06	15.85±0.13	46.80±0.88
	3	13.82±1.17	4.10±0.36	9.16±1.35	0.48±0.06	15.66±0.40	45.50±2.15
	4	11.90±0.80	4.19±0.49	7.29±0.64	0.35±0.06	16.03±0.27	46.02±1.32
DMN	1	21.22±1.35 <sup>c</sup>	11.89±1.05 <sup>c</sup>	8.03±0.35	0.65±0.06 <sup>b</sup>	14.03±0.33	41.22±1.24
	2	20.53±2.12 <sup>c</sup>	11.02±1.53 <sup>c</sup>	8.32±0.65	0.69±0.08 <sup>b</sup>	11.98±0.30 <sup>c</sup>	34.25±0.94 <sup>c</sup>
	3	19.08±1.37	11.53±1.10 <sup>c</sup>	6.73±0.52	0.40±0.05	11.83±0.47 <sup>c</sup>	35.82±1.58 <sup>c</sup>
	4	20.19±3.93 <sup>a</sup>	12.47±2.85 <sup>c</sup>	6.90±1.24	0.36±0.10	13.00±0.75 <sup>c</sup>	38.98±2.39 <sup>b</sup>

Results are analyzed by two-way (group and time as factors) ANOVA and are presented as mean±SEM ( $n=5-10$  rats per group). <sup>a</sup> $P<0.05$ ; <sup>b</sup> $P<0.01$ ; <sup>c</sup> $P<0.001$  vs. vehicle treatment. WBC=White blood cells; NE=Neutrophil; LY=Lymphocytes; MO=Monocytes; Hb=Haemoglobin; HCT=Hematocrit.

at week 1 for MCP-1 and week 1 and 2 for MCP-3 (Table 2). M-CSF levels were initially increased by 50% at week 1 after DMN-treatment and remained high at all time points up to week 4. MDC levels were, however, significantly reduced by 40% at week 2 and remained steady until week 3 after DMN. The MDC level tended to return to baseline level at week 4 after DMN (Table 2).

### 3.7. Effect of DMN on the blood components

Changes in circulating blood cell counts and hemoglobin and hematocrit levels in vehicle- and DMN-treated rats are shown in Table 3. Total white blood cell levels increased significantly, 2-fold over saline treatment, at all the time points after the start of DMN-treatment. This was accounted for primarily by a significant increase in neutrophils, which showed a more than 3-fold increase compared to vehicle-treatment at all time points. Lymphocytes were marginally increased at week 1 but thereafter were not significantly different from values in vehicle-treated rats. Both the hemoglobin and hematocrit values in the circulating blood were significantly decreased beginning at week 2 of DMN-treatment and remained steady for the rest of the DMN-treatment period.

### 3.8. Correlation of liver fibrosis with various biochemical markers

To correlate the severity of liver fibrosis, determined by histopathological changes (pathology score), with different biochemical parameters determined in the same animals, both Spearman rank correlation ( $r$  value) and also univariate ordinal logistic regression analysis ( $P$  values) were applied by considering liver fibrosis as either continuous or ordinal variable. Comparison of the histopathological changes with different variables were correlated with several noninvasive liver function markers such as GGT ( $r=0.77$ ), bilirubin ( $r=0.73$ ), AST ( $r=0.67$ ), hyaluronic acid ( $r=0.61$ ), haptoglobin ( $r=-0.64$ ), protein C ( $r=-0.71$ ), total protein ( $r=-0.73$ ), and albumin ( $r=-0.80$ ) with  $P$  values of  $<0.01$ . When protein C values were compared with different liver function markers, significant correlations were found with a number of markers including GGT ( $r=-0.74$ ), bilirubin ( $r=-0.92$ ), AST ( $r=-0.59$ ), ALT ( $r=-0.59$ ), hyaluronic acid ( $r=-0.85$ ), haptoglobin ( $r=0.89$ ), total protein ( $r=0.86$ ) and albumin ( $r=0.90$ )

with  $P$  values of  $<0.001$ . Plasma sICAM-1 level was weakly correlated ( $r=0.32$ ,  $P<0.01$ ) with histopathology. Additionally, the liver weight was highly correlated with protein C level ( $r=0.90$ ,  $P<0.001$ ) and also with histopathology score ( $r=-0.67$ ,  $P<0.001$ ). The acute phase protein (CRP) level was weakly correlated with the histopathology score ( $r=-0.34$ ,  $P<0.05$ ) but was highly correlated with protein C ( $r=0.72$ ,  $P<0.001$ ) level.

## 4. Discussion

In the present study we substantiate that dimethylnitrosamine induces liver fibrosis as evidenced by a graded increase in bridging fibrosis, a histopathologic marker of fibrosis that developed in a time-dependent manner. The histopathological damage was accompanied by a variety of changes in: 1) biochemical parameters such as hyaluronic acid, ALT, AST, GGT, bilirubin, albumin, sICAM-1, protein C and total protein, 2) hematological parameters such as white blood cell, neutrophil, monocytes, hemoglobin and hematocrit, 3) plasma chemokines and other analytes including MCP-1, MCP-3, M-CSF, MDC, CRP and haptoglobin, and 4) liver weight. From an in depth correlation analysis of histopathology score with various biochemical parameters, we demonstrated that graded severity of liver fibrosis was significantly correlated with total protein, albumin, GGT, bilirubin, AST, hyaluronic acid, and liver weight. The present study demonstrated for the first time in a rodent model of liver fibrosis that the plasma protein C levels were reduced significantly and were correlated with liver fibrosis, total protein, albumin, GGT, bilirubin, hyaluronic acid and liver weight. Considering the complexity of the disease and difficulty in establishing disease severity it is suggested that protein C along with a panel of established additional noninvasive biochemical parameters may provide a noninvasive assessment of disease progression/severity in hepatic disease.

Decreased plasma protein C has been reported in patients with chronic liver diseases and suggested to be a noninvasive marker of liver disease (Al Ghumlas et al., 2005; Bell et al., 1992; Kloczko et al., 1992; Viganò et al., 1985). Furthermore, in liver disease patients, protein C levels are significantly correlated with bilirubin and albumin (Bell et al., 1992; Kloczko et al., 1992) similar to the present observation. The reported relationship with GGT is conflicting; Kloczko et al. (1992) found a highly significant correlation with protein C

levels whereas Bell et al. (1992) did not find any correlation between protein C and GGT in patients with liver disease. While the liver is considered the primary site for protein C synthesis, we could find no data on protein C levels in animal models of liver disease. Recently, employing micro array analysis, Su et al. (2006) identified protein C as one of a large number of mRNAs altered in the liver tissue samples of a similar animal model. The present study successfully identified and determined protein C at the plasma protein level and provided correlation analysis with histopathologic scores and also with several inflammatory components of liver fibrosis. Our results describing significant correlation of protein C with histopathology suggest a possible utility for protein C levels in tracking liver pathology in preclinical models of liver disease and are in agreement with earlier observations in patients with liver disease (Bell et al., 1992; Kloczko et al., 1992; Vigano et al., 1985). In the present study, the graded reduction in protein C levels together with graded changes in total protein, albumin, GGT and hyaluronic acid levels and their high correlation with the severity of liver fibrosis further strengthen the utility of protein C as sensitive and accurate noninvasive novel biomarker. The significant reduction of plasma CRP levels, an exquisitely sensitive systemic marker of inflammation, synthesized mainly in the liver is similar to the previous findings described in patients with liver disease (Park et al., 2005). Decreased plasma protein C levels and increased levels of several inflammatory chemokines as observed in the present study clearly demonstrate the involvement of inflammatory component in the DMN-induced liver fibrosis and is similar to our previous observations in an inflammatory sepsis model (Heuer et al., 2004) that also manifest hepatic injury.

Chronic inflammation may lead to liver fibrosis via involvement of several cytokines and chemokines (Heydtmann et al., 2001; Zhang et al., 2004). Monocyte chemoattractant protein-1 and 3 (MCP-1 and MCP-3) are the most potent chemokines capable of regulating macrophages and monocytes and has been shown to play an important role in the recruitment and maintenance of the inflammatory infiltrate during liver injury (Muhlbauer et al., 2003; Tsuneyama et al., 2001; Tsuruta et al., 2004). Involvement of inflammatory chemokine MCP-1 in chronic liver injury and its correlation with ICAM, has been the basis of inflammatory mechanism of liver injury in patients (Panasiuk et al., 2004, 2005; Ross et al., 1998). The present study showed increased plasma levels of MCP-1, MCP-3 and M-CSF in parallel with an increased severity of liver fibrosis suggesting the involvement of inflammatory component in liver fibrosis. Neutrophils may stimulate stellate cells, may lead to neutrophilic inflammation and ultimately liver fibrosis (Ross et al., 1998). The findings that circulating neutrophil and sICAM-1 levels are significantly increased along with increased disease severity (present study); further support an inflammatory component in the DMN-induced liver fibrosis. Interestingly, MDC plays a central role in the systemic response to cecal ligation and puncture induced bacterial peritonitis (sepsis), a hallmark of which is liver injury due to excessive hepatic inflammation (Matsukawa et al., 2001; Simpson et al., 2003). Furthermore, MDC-neutralization by antibody causes increased

neutrophil infiltration and liver injury (Simpson et al., 2003) which is in line with the present observation in which MDC levels are decreased along with increased severity of liver injury. Therefore, the changes in plasma levels of chemokines may serve as biomarker of inflammatory pathway of liver fibrosis, and may also be novel therapeutic targets. Recent observation that anti-MCP-1 gene therapy is protective of DMN-induced liver fibrosis (Tsuruta et al., 2004) is consistent with this hypothesis.

sICAM-1 promotes intercellular adhesion, plays an important role in the inflammatory mechanism leading to tissue damage and are found elevated in liver disease patients (Capra et al., 2000; Panasiuk et al., 2004). A recent study in an animal model of liver fibrosis has reported an increased expression of ICAM in liver tissues but did not demonstrate any correlation with histopathology (Kornek et al., 2006). Accordingly, we explored changes in sICAM-1 in the progression of liver fibrosis in rats and found patterns of changes similar to previously reported changes in patients with liver disease (Capra et al., 2000; Panasiuk et al., 2004). In contrast to what has been reported in chronic liver disease patients (Abdalla et al., 2002) and despite substantial elevation in sICAM-1 levels as early as week 1 in the present study, sICAM-1 levels showed poor correlation with disease severity. However, this may reflect the nonlinear change in sICAM-1 level over the time course studied. An early increase in plasma levels of sICAM-1 in the rat model (present study), reflecting increased tissue levels is consistent with the increased accumulation of neutrophils leading to liver damage. Thus, sICAM-1 may be a potential and useful marker of liver fibrosis for preclinical monitoring of targeted therapy for liver fibrosis.

Hyaluronic acid, an important component of extracellular matrix, is a high molecular weight polysaccharide synthesized mainly in the liver fibroblasts and hepatic stellate cells (Saitou et al., 2005; Stickel et al., 2003). In severe liver fibrosis, hyaluronic acid clearance is diminished due to disturbed sinusoidal endothelial cell function resulting in elevated serum hyaluronic acid levels (McHutchison et al., 2000; Saitou et al., 2005; Stickel et al., 2003). Increased serum hyaluronic acid levels had more significant correlation with the severity of liver fibrosis and thus proposed as a noninvasive marker of liver fibrosis in humans (Montazeri et al., 2005; Saitou et al., 2005; Stickel et al., 2003). Several preclinical studies in animals have also demonstrated increased serum levels of hyaluronic acid due to increased synthesis by the activated stellate cells of animals with liver fibrosis although no correlation analysis was performed (George et al., 2004). The steady increase in hyaluronic acid levels and its significant correlation with the severity of liver fibrosis and also with protein C (present study) support hyaluronic acid as a noninvasive biomarker for liver fibrosis as described previously (Montazeri et al., 2005; Saitou et al., 2005; Stickel et al., 2003). Therefore, the present data clearly demonstrate the involvement of inflammatory pathways in the DMN-induced liver fibrosis. The identification of significant changes in protein C, an important component in the systemic inflammatory pathways, along with changes in various inflammatory components such as hyaluronic acid, sICAM-1, GGT, and graded severity in liver



histopathology as observed in the present study make protein C an important component in the pool of noninvasive biomarker of liver fibrosis.

In conclusion, the present study has identified plasma protein C as a novel noninvasive biomarker in the DMN-induced liver fibrosis model in rat based on its relationship and significant correlation with histopathology, various hematological and inflammatory components of liver function and body weight. Furthermore, the changes in sICAM-1 and several other biochemical parameters reflecting damage in structural integrity of the hepatic stellate cells may also serve as biomarkers of liver fibrosis. The demonstration of the differential changes in several biochemical parameters, circulating neutrophil, and several inflammatory chemokines along with progression of severity of liver fibrosis suggest the involvement of inflammatory components in this model of liver fibrosis. Accordingly, the noninvasive biomarkers identified in this DMN-induced liver fibrosis model would provide means for pharmacological assessment of targeted therapeutic agents for liver fibrosis.

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