Dig Dis Sci (2008) 53:2222–2232
DOI 10.1007/s10620-007-0112-y

ORIGINAL PAPER

Pathophysiological Characteristics of Dimethylnitrosamine-Induced Liver Fibrosis in Acute and Chronic Injury Models: A Possible Contribution of KLF5 to Fibrogenic Responses

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Received: 25 June 2007 / Accepted: 27 October 2007 / Published online: 20 December 2007

Abstract Dimethylnitrosamine administration induces a rapid increase in collagen deposition with concomitant proliferation of hepatic stellate cells in rats. Here, we investigated the pathophysiological profiles of acute and chronic hepatic fibrosis states and attempted to determine the possible role of Kruppel-like factor-5 (KLF5) in this model. In acute study using a single drug injection, we observed a rapid transient increase of ALT and mRNA levels of KLF5 followed by increases in fibrosis-related genes. Repeated administration of dimethylnitrosamine once a week caused early damage with severe fibrosis and sustained hepatocyte injury, while intermittent injections at 2-week intervals induced only modest fibrosis from 3 weeks. Weekly administration also induced profound upregulation of collagen I, α-smooth muscle actin, and KLF5 mRNA. In contrast, such continued augmentation was not observed after intermittent injections; KLF5 increased only after 3 weeks. These results suggested that dimethylnitrosamine induced a rapid hepatic fibrogenic response with a possible participation of KLF5.

Keywords Dimethylnitrosamine · Liver fibrosis · Hepatic stellate cell · Kruppel-like factor-5 · Transdifferentiation

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Introduction

Liver fibrosis is a consequence of chronic liver damage from a variety of causes, including hepatitis virus infection, alcohol, and autoimmune disease [1]. Such fibrosis involves the marked accumulation of extracellular matrix (ECM) components like collagen, the activation of cells producing such ECM components, and tissue remodeling [2, 3]. Hepatic stellate cells (HSCs) are the primary fibrogenic mediators, playing several important roles in the pathophysiology, including retinoid storage, ECM remodeling, growth factor production, and contraction of the sinusoidal lumen [4–6].

Hepatic stellate cells exist in two distinct states, a quiescent state and an activated state [7, 8]. HSCs are normally quiescent, producing only a small number of ECM components for maintenance of the basement membrane. When liver injury occurs, quiescent HSCs undergo transdifferentiation into the activated form, which displays a proliferative, contractile, and fibrogenic myofibroblast-like phenotype [5, 9]. HSC activation is regulated by both paracrine and autocrine stimulation with growth factors

and cytokines [2, 5]. Recently, multiple transcription factors have been recognized to express in HSCs and alter their expression patterns during activation, although the precise mechanism of transdifferentiation is not well understood [10, 11].

Among the transcription factors expressed by HSC, KLF6 (Kruppel-like factor-6, also known as zf9) is thought to be one of the key regulatory factors functioning during HSC activation, and this protein is rapidly induced during liver injury, likely due to transactivation of the collagen and TGF-β1 promoters [12, 13]. The Kruppel-like factor (KLF) families are highly related to zinc-finger transcription factors and have essential functions in the regulation of cell differentiation and proliferation [14]. Another KLF member, KLF5 (also known as BTEB2), is an important regulator of the phenotypic modulation of mesenchymal cells, including smooth muscle cells (SMC) [15], cardiac fibroblasts [16], and adipocytes [17] in response to external stresses. KLF5 has a critical role in SMC transdifferentiation from the mature "contractile" phenotype to the de-differentiated "synthetic" phenotype; the latter SMC subtype is capable of proliferation and migration, as well as the production of extracellular matrix-related factors [15]. KLF5 expression is downregulated in mature vascular smooth muscle cells, but markedly re-induced in activated SMCs and myofibroblasts during atherosclerosis and cardiovascular remodeling [18]. HSCs transform from quiescent to activated myofibroblast-like cells during liver injury, which appears to be a similar process to SMC transdifferentiation. Although few studies have reported the participation of KLF5 in liver, KLF5 may play a certain role in the activation of HSCs.

In rats, dimethylnitrosamine (DMN)-induced liver fibrosis is a well-established, reproducible animal model with severe hepatic necrosis and formation of septa with micronodular cirrhosis after 3 weeks of treatment [19–21]. DMN causes excessive deposition of extracellular matrix proteins, especially collagen [22–25], and a rapid increase of the proportion of proliferative HSCs in rat liver [12, 26, 27], which produces profound liver fibrosis over a short period. Therefore, this model is considered to be appropriate for investigation of the contribution of HSC activation to the early events in the development of fibrosis.

In this study, we examined the time course of events in DMN-induced liver injury and the subsequent development of fibrosis in both acute and chronic liver injury to characterize its model profile. To clarify the fibrogenic mechanisms and other contributing factors in chronic fibrosis, we compared the pathophysiological events and expression profiles of KLF5 and other fibrosis-related genes in chronic liver injury models using weekly and intermittent administration of DMN.

Materials and methods

Animals

All animal studies were approved by the Animal Ethics Committee at Pfizer Inc.'s PGRD Nagoya Laboratories. Male 7-week-old Wistar rats were purchased from Nihon SLC (Shizuoka, Japan). All animals were housed under constant temperature (23 ± 2°C) and humidity (55 ± 15%) conditions on a 12-h light/dark cycle (lights on 7:00 a.m.). Animals received ordinary laboratory food and water ad libitum and were housed under these conditions for 1 week prior to experimentation.

Experimental procedures

(1) Acute liver injury induced by single DMN injection: As the study of acute liver injury, rats were injected with a single intraperitoneal (i.p.) dose of DMN (Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan), diluted in saline at doses of 30 or 40 mg/kg/2 ml. Control rats received injections of 2 ml/kg of saline without DMN. On the 3rd and 5th day for the 30 mg/kg dose and on the 3rd, 5th, and 8th day for the 40 mg/kg dose, rats were anesthetized with inhaled isoflurane, and blood samples were collected from the inferior vena cava (IVC) into 15-ml tubes with a 1/10 volume of 3.13% citric acid solution. Liver tissue specimens were then excised from these rats for mRNA measurements.

(2) Chronic liver injury and liver fibrosis induced by repetitious DMN injections: In the first study, liver fibrosis was induced by three times injection of DMN once a week (30 mg/kg/week, i.p.) at 0, 1, and 2 weeks. Seven days after each DMN injection as the evaluation at 1, 2 and 3 weeks and 14 days after 3rd DMN injection as that at 4 weeks, respectively, a subset of rats was anesthetized with inhaled isoflurane. Blood samples were then collected from the IVC, and liver tissue specimens were excised. The left lobes of isolated livers were divided into approximately 5-mm sections, two of which were frozen at −80°C for hydroxyproline content determination and RNA isolation. In this study, the sections for histology were excised from other rats studied in the same protocol and were immersed in 4% paraformaldehyde-phosphate buffer at 4°C for later histological analysis as described below. In a second study, rats received two times injections of DMN intermittently every 2 weeks (30 mg/kg/2 weeks, i.p.) at 0 and 2 weeks. Blood samples and liver tissue specimens were acquired at 1, 2, 3, and 4 weeks, comprising samples taken 7 and 14 days after each DMN injection. With the exception of DMN injection frequency, all procedures were as described above.

Biochemical analysis and measurement of blood cell counts

Plasma alanine aminotransferase (ALT) and total protein (TP) levels were measured by the laboratory method using a Picollo® Point-Care Chemistry Analyzer (Abaxis Inc., CA, USA). In the second study of a chronic model utilizing intermittent DMN administration, plasma ALT activity was measured by standard photometric method using a Transaminase CII Test Wako kit (Wako, Osaka, Japan). The numbers of white blood cells and platelets were counted by Sysmex KX-21NV (Sysmex Inc., Hyogo, Japan).

Measurement of hepatic hydroxyproline content

To determine hydroxyproline content, a modification of the method described by Reddy et al. was used [28]. Approximately 40 mg frozen liver tissue was hydrolyzed in 2 N NaOH for 10 min at 65°C. Samples were first incubated for 20 min at 120°C, then in 6 N HCl for 20 min at 120°C. The addition of activated charcoal solution (10 mg/ml in 4 N NaOH) and 2.2 M acetic acid/0.48 M citric acid buffer (pH 6.5) generated a liquid of pH 7–8. After centrifugation, we added 45 mM chloramine T solution to the supernatant for 25 min at room temperature. After addition of 1 M Ehrlich's solution, samples were incubated for 20 min at 65°C. Absorbance was measured at 560 nm. Hydroxyproline content is expressed as μg/g wet liver weight.

Quantification of hepatic mRNA levels

RNA was extracted from frozen liver tissues using ISOGEN Reagent (Nippongene Inc., Toyama, Japan) according to the manufacturer's protocol. After determining RNA concentrations, 5 μg RNA was reverse transcribed using the SuperScript 3™ First-Strand Synthesis System (Invitrogen Corporation, CA, USA). cDNA from each sample (50 ng) was analyzed by quantitative PCR using Platinum® Quantitative PCR Supermix-UDG with ROX (Invitrogen Corporation, CA, USA) on an ABI PRISM ™ 7900 Sequence Detector (Applied Biosystems, CA, USA). FAM-labeled probes and primers specific for each gene were purchased from Applied Biosystems: B2m (Rn00560865_m1), collagen type 1 alpha 1 (Rn00801649_g1), TGFβ1 (Rn00572010_m1), KLF5 (Rn00821442_g1), and CTGF (Rn00573960_g1). Reagents specific for α-smooth muscle actin (α-SMA) were made by ordering the forward (CGAAGCGCAGAGCAAGAGA) and reverse (CATGTCGTCCCAGTTGGTGAT) primers. Relative gene expression was normalized to B2m as an internal control by calculating $2^{-\Delta Ct}$, the difference in Ct values between B2m and each target gene.

Histopathology

Histology sections were prepared at Applied Medical Research, Inc. (Osaka, Japan). After fixation in 4% paraformaldehyde phosphate buffer, liver sections were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (HE) and Sirius red to detect collagen. α-Smooth muscle actin (α-SMA) was detected by immunohistochemistry using a modified method of EnVision (DAKO, Kyoto, Japan) as reported [29].

Statistics

Results were expressed as means with SEM. Significant differences were evaluated using the unpaired Student's *t*-test between two groups and followed by closed testing among the analysis of three groups. Statistical differences between mRNA levels were analyzed by Wilcoxon's rank sum test using the SAS analysis software (version 8.0, SAS Institute Inc.). *P* values less than 0.05 were considered to be significant.

Results

Acute responses after single injection of DMN over 1 week

A single injection of 30 or 40 mg/kg DMN resulted in significant decreases in body weight on days 3 and 5; after 7 days, rat body weight in the group treated with 40 mg/kg DMN returned to near-baseline levels (Table 1). The number of white blood cells decreased on day 3 in comparison to the saline-treated control group; however, significant increases were observed at day 5 in both the 30 and 40 mg/kg DMN-treated groups (Table 1). The number of platelets dramatically decreased following DMN treatment; slight recovery was observed 1 week after administration in the 40 mg/kg DMN-treated group (Table 1).

Plasma ALT activity, an indicator of liver cell damage, was increased significantly at day 3, then rapidly decreased, returning to baseline levels at day 8 in the 40 mg/kg DMN-treated group (Fig. 1). Significant decreases in plasma TP levels were observed throughout the experimental period in both the 30 and 40 mg/kg DMN-treated groups (Table 1).

Intrahepatic mRNA was quantified by real-time PCR. Collagen I mRNA levels increased gradually, reaching levels 3.5 and 7.5-fold greater than baseline at day 5 in the 30 and 40 mg/kg DMN-treated groups, respectively (Fig. 1). By day 8, the levels of collagen I mRNA in the

Table 1 Influence of single injection with DMN for 7 days

		Body weight (g)	WBC (ml)	Platelets ($\times 10^4$/ml)	TP (g/l)
Day 3	Control	212.9 ± 2.2	3666.7 ± 284.8	46.1 ± 0.3	4.7 ± 0.0
	DMN (30 mg/kg)	192.0 ± 4.1**	2780.0 ± 326.2	1.5 ± 0.2**	3.2 ± 0.1**
	DMN (40 mg/kg)	186.5 ± 5.5**	1720.0 ± 139.3**	1.8 ± 0.2**	3.0 ± 0.2**
Day 5	Control	221.3 ± 2.1	5100.0 ± 152.8	46.0 ± 1.2	4.8 ± 0.1
	DMN (30 mg/kg)	202.7 ± 3.7**	8220.0 ± 920.5*	5.2 ± 0.6**	4.0 ± 0.2**
	DMN (40 mg/kg)	180.9 ± 6.9**	7700.0 ± 753.0*	2.7 ± 0.6**	3.8 ± 0.3*
Day 8	Control	227.9 ± 4.2	5133.3 ± 290.6	46.0 ± 2.9	5.0 ± 0.1
	DMN (40 mg/kg)	210.9 ± 8.2	11125.0 ± 1899.3	15.7 ± 1.4**	3.8 ± 0.1**

WBC: white blood cell, TP: total protein. * $P < 0.05$, ** $P < 0.01$ versus control by unpaired Student's *t*-test followed by closed testing procedure. Values are mean ± SEM ($n = 3$–5)

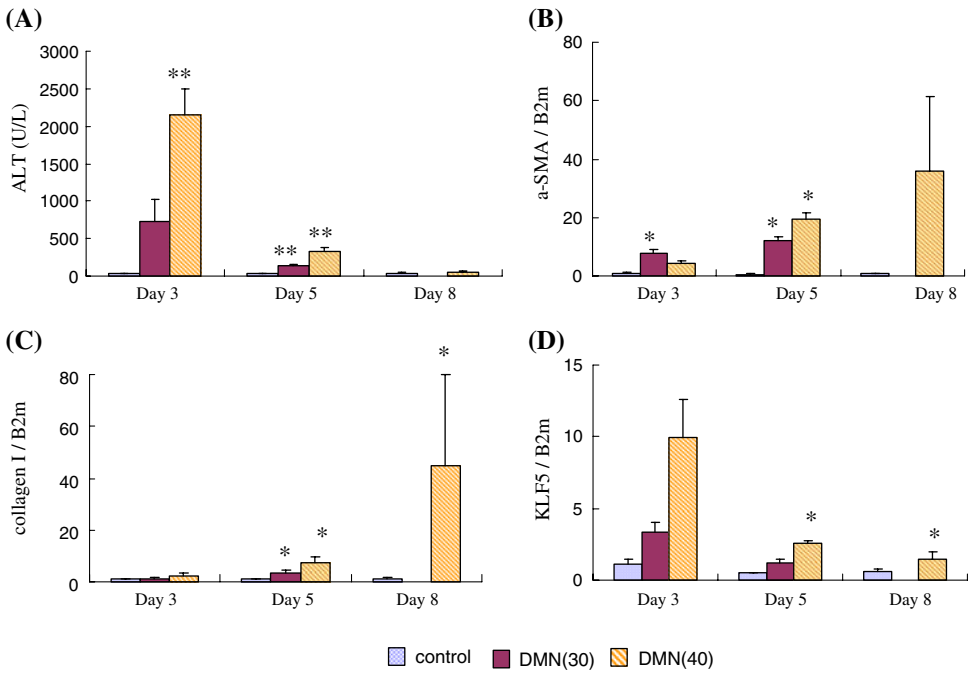

Fig. 1 Plasma ALT levels (**a**) and intrahepatic mRNA expressions of collagen I (**b**), α-SMA (**c**), and KLF5 (**d**) on days 3, 5, and 8 after a single administration of DMN (30 or 40 mg/kg; i.p.). In rats given 30 mg/kg DMN, data were obtained on days 3 and 5. * $P < 0.05$, ** $P < 0.01$ versus control using the unpaired Student's *t*-test followed by closed testing procedure. Values are the means ± SEM ($n = 3$–5)

40 mg/kg DMN-treated group increased by 44.8-fold in comparison to the control group, although there was individual variation among rats (Fig. 1). In another experiment, administration of 30 mg/kg DMN also induced further increases in collagen I mRNA levels at day 8 compared to day 5 (data not shown). Expression of α-SMA, a marker of activated HSCs, increased gradually throughout the experimental time course, reaching 12.0- and 19.4-fold that of control rats at day 5 in the 30 and 40 mg/kg DMN-treated groups, respectively, and 36.1-fold at day 8 in rats given 40 mg/kg DMN (Fig. 1). KLF5 mRNA levels increased by 3.3- and 9.9-fold at day 3 in the 30 and 40 mg/kg DMN-treated groups, respectively; these levels then declined to basal levels during the experimental period (Fig. 1). TGF-β1 mRNA expression levels in rats treated with 30 and 40 mg/kg DMN at day 3 were 2.1- and 3.0-fold greater than control rats, respectively, which was sustained almost equivalent levels throughout the experimental period (data not shown).

Chronic liver injury and general conditions induced by weekly DMN injections

DMN was administered once a week, indicating three times in total during the first 3 weeks at 30 mg/kg/week. DMN injection affected body weights at all time points, with significant decreases observed at 2 and 3 weeks to 83.7% and 78.9% of control values, respectively (Table 2). The livers of DMN-treated rats were hard and reduced in size; the ratio of liver weight to body weight was significantly lower in DMN-treated rats in comparison to control rats at

Table 2 Influence of three times continuous administrations with DMN once a week for 4 weeks

		Body weight (g)	WBC (ml)	Platelets ($\times 10^4$/ml)	TP (g/l)
1 Week	Control	218.3 ± 6.2	5,100.0 ± 694.0	51.5 ± 1.1	4.9 ± 0.1
	DMN	200.1 ± 5.0	8,550.0 ± 771.5*	36.5 ± 5.9	4.6 ± 0.1*
2 Weeks	Control	234.7 ± 3.3	7,733.3 ± 783.9	46.1 ± 6.6	5.6 ± 0.1
	DMN	196.5 ± 6.6**	8,950.0 ± 970.8	36.9 ± 10.6	4.5 ± 0.2**
3 Weeks	Control	253.6 ± 8.1	5,825.0 ± 295.5	63.8 ± 2.3	5.3 ± 0.1
	DMN	200.3 ± 10.7**	13,120.0 ± 1213.8**	15.4 ± 4.8**	4.2 ± 0.2**
4 Weeks	Control	268.1 ± 9.1	5,950.0 ± 202.1	59.8 ± 1.2	5.2 ± 0.1
	DMN	218.0 ± 24.8	6,300.0 ± 584.5	18.9 ± 7.0**	4.1 ± 0.4

WBC: white blood cell, TP: total protein. * $P < 0.05$, ** $P < 0.01$ versus control by unpaired Student's *t*-test followed by closed testing procedure. Values are mean ± SEM ($n = 3$–6)

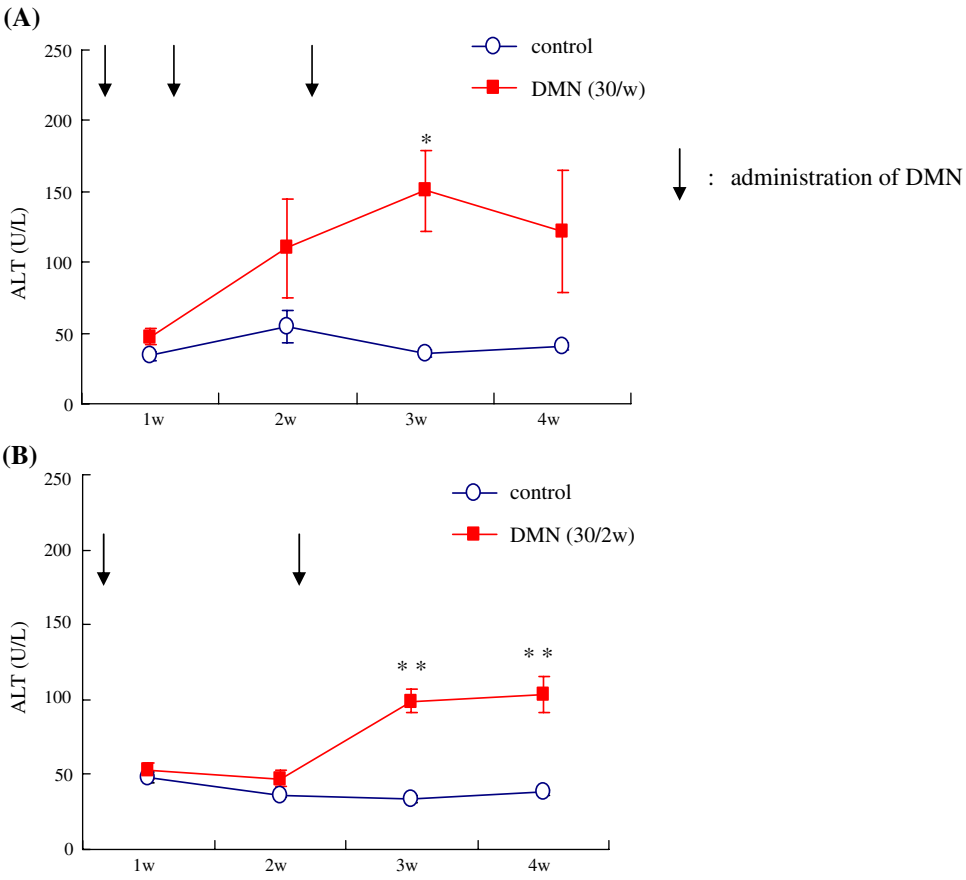

Fig. 2 Plasma ALT levels at 1, 2, 3, and 4 weeks during repeated DMN treatment in rat model of liver fibrosis. (**a**) DMN was injected once a week (30 mg/kg/week, i.p.) at 0, 1, and 2 weeks. Blood samples were collected 7 days after each DMN administration, and 14 days after the 3rd injection. (**b**) DMN was administered intermittently at 2-week intervals (30 mg/kg/2 weeks, i.p.); blood samples were collected at 1, 2, 3, and 4 weeks, 7 and 14 days after the each DMN injection. Arrows indicate the times of DMN administration. * $P < 0.05$, ** $P < 0.01$ versus control using the unpaired Student's *t*-test. Values are the means ± SEM ($n = 4$–6)

3 weeks (data not shown). Although none of the rats died during the first 2 weeks, survival rates decreased to markedly below 50% after 3 weeks of treatment; therefore, the 4th injection of DMN was given up in this study.

The numbers of white blood cells in the DMN-treated groups were higher than those seen in the control groups, peaking at 2.3-fold greater than controls at 3 weeks; at 4 weeks, however, white blood cell numbers were equivalent (Table 2). The numbers of platelets were significantly reduced at 3 and 4 weeks of treatment to 75.8% and 68.3% of control levels, respectively (Table 2).

Plasma ALT levels increased gradually, plateauing at 3 weeks of treatment (Fig. 2). Plasma aspartate aminotransferase levels in the DMN-treated groups increased in a similar fashion to the ALT levels (data not shown). Plasma TP levels decreased significantly at 1 week, which was sustained throughout the experimental period (Table 2).

Liver fibrosis and mRNA expressions in rats given weekly DMN injections

Remarkable liver fibrosis, as determined by hepatic hydroxyproline content, was observed after repeated DMN injection once a week. Hydroxyproline content in DMN-treated rats increased significantly at all time points (Fig. 3). Maximum hydroxyproline content measured at 4 weeks in DMN-treated rats was 1,208.3 ± 227.0 μg/g wet tissue in comparison to 338.2 ± 14.7 μg/g wet tissue in control rats, a 3.6-fold increase.

Intrahepatic mRNA expression of collagen I increased markedly following DMN treatment. An approximately 50-fold increase was observed in the DMN-treated groups at 3 weeks, which decreased minimally at 4 weeks (Fig. 4). Expression of α-SMA mRNA increased linearly over time; significant increases were observed from 1 to 3 weeks in treated rats (Fig. 4). Although KLF5 mRNA levels did not differ between the control and DMN-treated groups at 1 week, they were augmented approximately ten-fold after 2 weeks of treatment, which was sustained throughout the remainder of the experimental period (Fig. 4). This upregulation of KLF5 mRNA levels was reproducible in additional experiments. The extent of TGF-β1 mRNA expression in DMN-treated groups was approximately three-fold greater than that seen in control groups at all time points (data not shown). The expression of CTGF, a profibrogenic factor functioning in TGF-β(signaling, was also increased by approximately five– to eight-fold, although there was individual variation among rats at 4 weeks (data not shown).

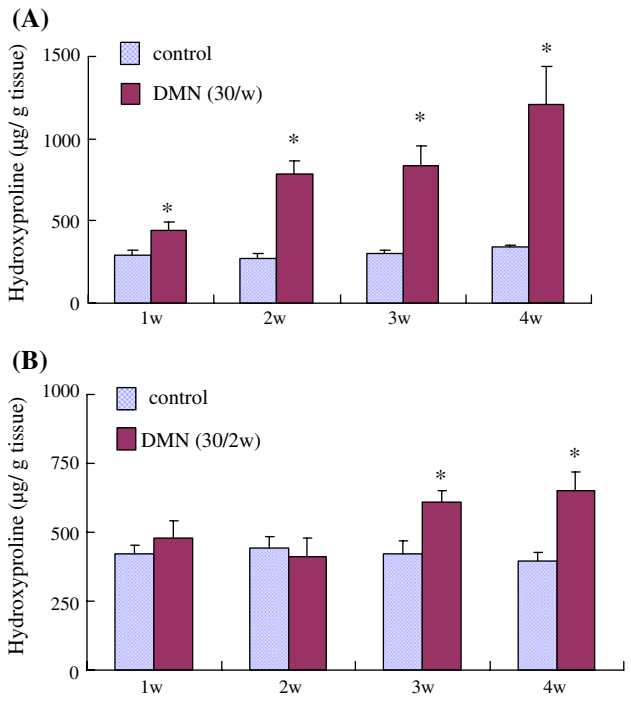

Fig. 3 Hydroxyproline content in liver tissues at 1, 2, 3, and 4 weeks during repeated DMN treatment in rats. (**a**) DMN was administered once a week (30 mg/kg/week, i.p.) for 3 weeks. Liver specimens were taken 7 days after each DMN administration, and 14 days after the 3rd injection. (**b**) DMN was administered at 2-week intervals (30 mg/kg/2 week, i.p.); liver specimens were taken at 1, 2, 3, and 4 weeks, 7 and 14 days after the each DMN injection. Arrows indicate the time of DMN administration. * $P < 0.05$, ** $P < 0.01$ versus control using the unpaired Student's *t*-test. Values are the means ± SEM ($n = 4$–6)

Chronic liver injury and general conditions in rats treated with intermittent DMN at 2-week intervals

DMN was administered at 2-week intervals for 4 weeks, with a total of two injections with DMN (30 mg/kg/2 week) over the 4-week period. DMN administration induced decreases in body weight to approximately 90% of control values at all time points (Table 3). No changes in the ratio of liver weight to body weight were observed (data not shown). One of the rats died in the group treated with DMN for 2 weeks; all of the rats in the 3- and 4-week treatment groups survived.

The numbers of white blood cells and platelets in DMN-treated groups were significantly altered at 1 and 3 weeks, but approached control levels at 2 and 4 weeks (Table 3).

Plasma ALT levels were significantly increased at 3 and 4 weeks of treatment; these values, however, were less than those seen following weekly DMN treatment as described above (Fig. 2).

Liver fibrosis and mRNA expressions in rats treated intermittently with DMN at 2-week intervals

While hydroxyproline content was not influenced by treatment with DMN at 1 and 2 weeks, significant increases were obtained at 3 and 4 weeks to 608.1 ± 45.3 μg/g wet liver and 650.2 ± 69.8 μg/g wet liver in comparison to control values of 420.5 ± 49.6 μg/g wet liver and 395.6 ± 32.0 μg/g wet liver, respectively (Fig. 3). A maximum increase of 1.6-fold increase was seen at 4 weeks, which was comparatively lower than that seen following repeated weekly DMN administration.

Parallel changes were observed in the intrahepatic mRNA expression of collagen I and α-SMA. In contrast to the results seen in rats treated with weekly DMN, no upward trend in upregulation of mRNA levels was observed over the 4-week experimental period. The expression of both mRNAs at 2 and 4 weeks was lower than that seen at 1 and 3 weeks, respectively (Fig. 5). Thus, the marked upregulation seen at 7 days in both the 1st and

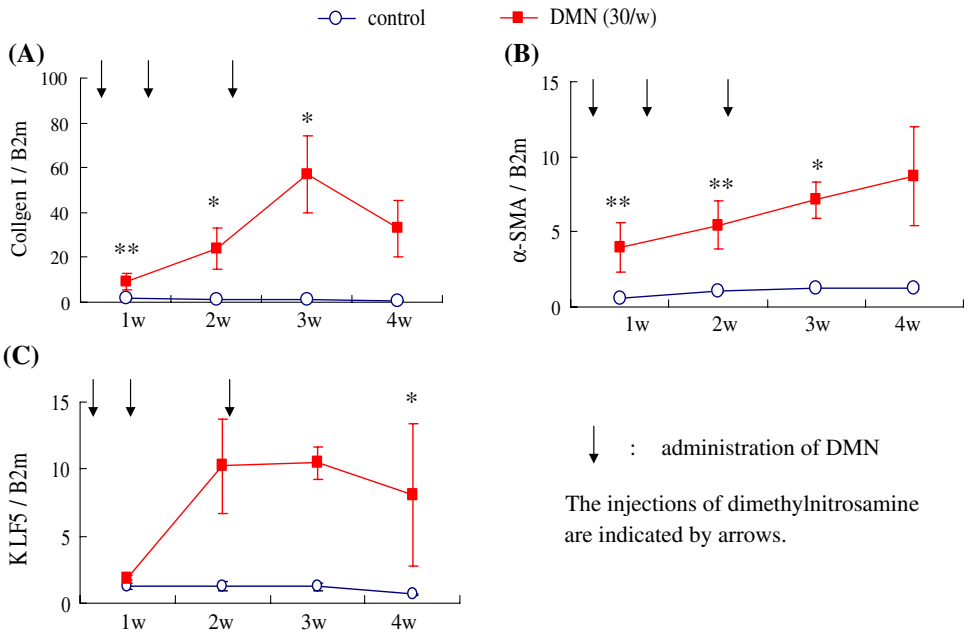

Fig. 4 Intrahepatic mRNA expression of collagen I (**a**), α-SMA (**b**), and KLF5 (**c**) at 1, 2, 3, and 4 weeks during weekly treatment with DMN in rat model of liver fibrosis. DMN was administered once a week (30 mg/kg/week, i.p.) for the first 3 weeks. Liver specimens were acquired 7 days after each DMN administration and 14 days after the 3rd injection. mRNA levels were quantified using real-time PCR. Arrows indicate the times of DMN administration. * $P < 0.05$, ** $P < 0.01$ versus controls using the Wilcoxon's rank sum test. Values are the means ± SEM ($n = 3$–6)

Table 3 Influence of intermittent administrations with DMN at 2 week's interval for 4 weeks

		Body weight (g)	WBC (ml)	Platelets ($\times 10^4$/ml)
1 Week	Control	226.4 ± 5.6	6,260.0 ± 557.3	53.6 ± 0.9
	DMN	210.0 ± 3.7*	9,140.0 ± 954.3*	35.5 ± 4.7*
2 Weeks	Control	242.7 ± 3.9	6,340.0 ± 318.7	51.0 ± 3.1
	DMN	224.2 ± 4.5*	6,850.0 ± 751.1	54.2 ± 3.8
3 Weeks	Control	258.8 ± 4.3	5,860.0 ± 525.9	45.6 ± 6.3
	DMN	229.8 ± 3.4**	9,000.0 ± 796.9*	22.3 ± 7.2*
4 Weeks	Control	267.5 ± 8.1	5,460.0 ± 504.6	53.9 ± 1.5
	DMN	237.9 ± 10.0	5,220.0 ± 536.1	46.0 ± 2.6*

WBC: white blood cell. * $P < 0.05$, ** $P < 0.01$ versus control by unpaired Student's *t*-test followed by closed testing procedure. Values are mean ± SEM ($n = 4$ or 5)

2nd DMN injections was not sustained to 14 days. The expression of KLF5 mRNA, however, was significantly upregulated at both 3 and 4 weeks, although the extents were less than those seen following repetitive weekly DMN injection (Fig. 5).

Histopathology of liver sections from rats in the chronic studies

In rats weekly administered DMN, we observed hemorrhage necrosis with fiber formation in the necrotic areas, anisokaryosis, and mitotic figures were observed as early as 1 week. Further injury was observed at 3 weeks, with more prominent pleomorphism and variation in cellular and nuclear size; both bridging necrosis and dense fiber formation linking central veins were observed by Sirius red staining, indicating the development of fibrosis (Fig. 6). α-SMA positive cells were present adjacent to areas of fiber formation by HE and Sirius red staining. At 3 weeks, a larger number of α-SMA positive cells were observed throughout the sinusoidal areas (Fig. 6).

In rats treated intermittently with DMN every 2 weeks, the histopathologic figures seen by HE staining at 1 week persisted thereafter; however, additional development to the extent described above for rats given repeated weekly DMN injections were not seen (data not shown). Although fiber formation, as measured by Sirius red staining, was observed at 1 week at levels exceeding controls, the extent of fiber development was less prominent than that seen in rats treated with DMN weekly (data not shown). Similar results were observed for α-SMA immunohistochemistry. Fiber formation, as detected by Sirius red staining, and the proportion of α-SMA-positive cells in sinusoidal areas were evidently increased at 1 and 3 weeks, but they were observed only slightly at 2 and 4 weeks (Fig. 7). Positive

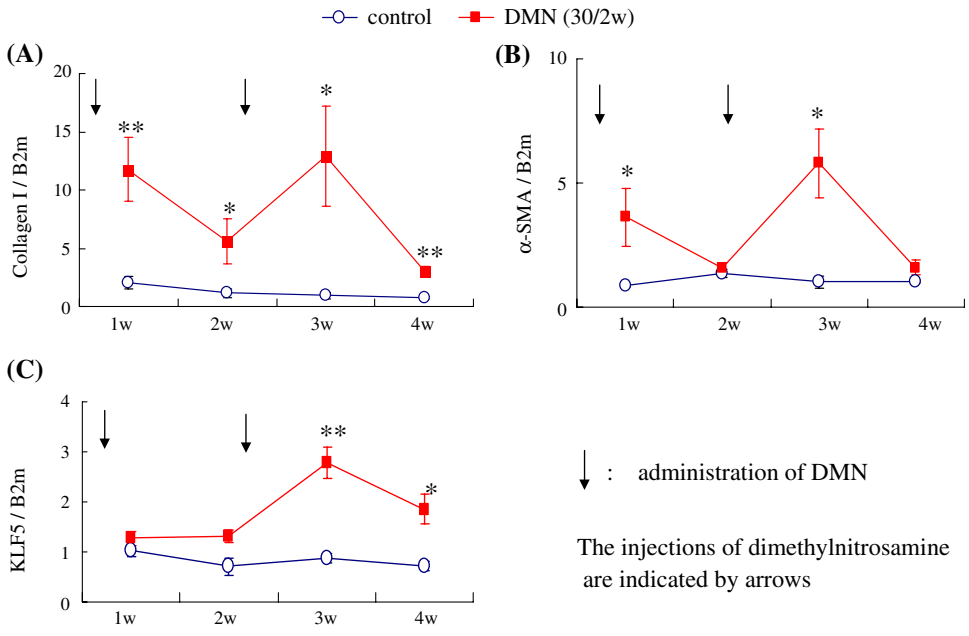

Fig. 5 Intrahepatic mRNA expression of collagen I (**a**), α-SMA (**b**), and KLF5 (**c**) at 1, 2, 3, and 4 weeks during intermittent treatment with DMN in rats. DMN was administered at 2-week intervals (30 mg/kg/2 week, i.p.). Liver specimens were taken at 1, 2, 3, and 4 weeks, which corresponded to 7 and 14 days after each DMN injection. Arrows indicate the times of DMN administration. mRNA levels were quantified by real-time PCR. * $P < 0.05$, ** $P < 0.01$ versus control using the Wilcoxon's rank sum test. Values are the means ± SEM ($n = 4$–5)

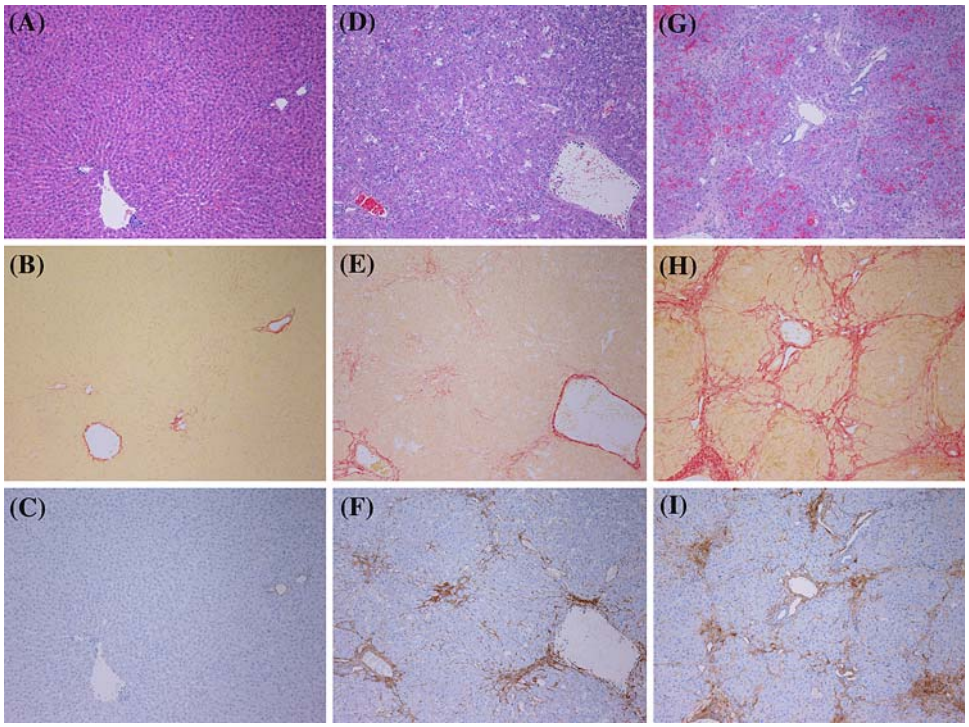

Fig. 6 Histological features of liver sections obtained from control rats (**a**–**c**), DMN-treated rats at 1 week (**d**–**f**), and DMN-treated rats at 3 weeks (**g**–**i**). DMN was administered once a week (30 mg/kg/week, i.p.). **a**, **d**, **g**: Hematoxylin and eosin (HE) staining; **b**, **e**, **h**: Sirius red staining; **c**, **f**, **i**: immunostaining for α-SMA (magnification: 100×)

Sirius red staining and α-SMA positive cells inducing bridging formation were prominently observed at 3 weeks.

Discussion

Activation of HSC during liver injury mediates several pathological functions, including HSC proliferation, secretion of proinflammatory cytokines, and increased deposition of ECM components, ultimately leading to liver fibrosis. In this study, we utilized DMN to induce marked hepatocyte injury and fibrogenic stimulation. Repeated administration of DMN promoted significant fibrosis with an increasing number of activated HSCs in approximately 3 weeks. These results indicate that DMN treatment provides a reproducible liver fibrosis model [19, 20, 25] suitable for the study of liver fibrosis with accelerated HSC activation in such a short period of time [12, 25–27].

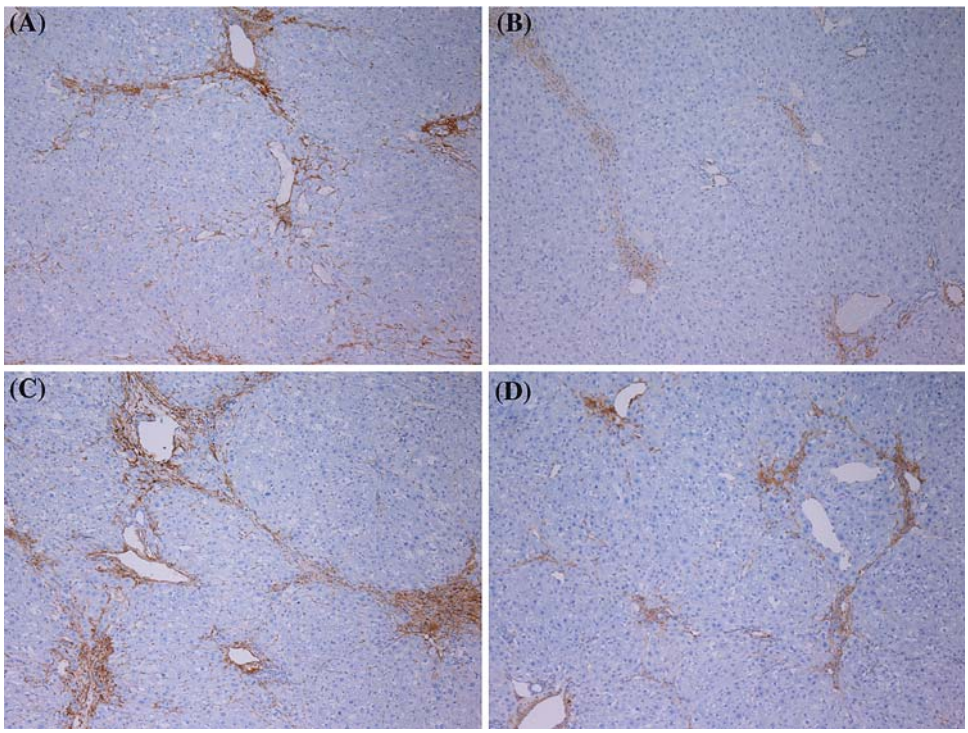

Fig. 7 Immunostaining for α-SMA in liver sections from rats treated intermittently with DMN at (**a**) 1 week, (**b**) 2 weeks, (**c**) 3 weeks, and (**d**) 4 weeks (magnification; 100×). DMN was administered at 2-week intervals (30 mg/kg/2 week, i.p.). Liver specimens were taken at 1, 2, 3, and 4 weeks, which corresponded to 7 and 14 days after each DMN injection

Furthermore, this DMN-induced model served as an effective forum in which to elucidate the relationship between liver cell injury and acute and chronic hepatic fibrosis with the underlying molecular mechanisms of fibrogenic responses.

In our examination of acute liver injury, a single injection of DMN caused marked hepatic cell injury, as measured by ALT elevation at day 3, followed by increases in collagen I mRNA levels. These results agree with those of Shiba et al. examining the influence of a single DMN administration on rats over a 42-day period [24]. In our study, inflammation and functional failure were clearly observed by alterations in white blood cell counts, platelets levels, and plasma TP levels during the acute phase. The mRNA levels of collagen I and α-SMA, a marker of HSC activation, increased in a time-dependent manner following the peak of ALT levels, suggesting that the pathological process in this animal model accurately reflects human pathology, such as infection with hepatitis viruses in which fibrogenic responses are associated with hepatocyte injury [30, 31]. KLF5 mRNA expression increased prior to the fibrogenic response, peaking at day 3. These results suggest that liver cell damage, inflammatory responses, and loss of liver function are early events after DMN treatment, and besides, expression of KLF5 may be triggered by hepatocyte cell injury or inflammation to stimulate fibrogenic responses.

Weekly administration of DMN had a deleterious influence on liver function and cell viability, which was sustained or increased throughout the experimental period. We observed that increasing fiber deposition was also documented by marked increases in hydroxyproline contents and Sirius red histology, as well as remarkable augmentation of fibrogenic responses, including elevated collagen I mRNA levels and HSC activation. This weekly DMN injection induced a similar, or more intense, feature than that seen following a single DMN injection, which was previously reported to induce hepatocyte necrosis, rapid excessive deposition of collagen fibers, and increased HSC activation [24, 32]. In contrast, intermittent administration of DMN caused a modest and sustained hepatocyte cell injury, and development of fibrosis was mild in its onset at 3 weeks in comparison with that in weekly treatment study. In addition, the expression levels of collagen I and α-SMA mRNA increased 7 days after each DMN administration, but returned to lower levels by 14 days. Collagen I and α-SMA mRNA exhibited parallel changes in expression levels, suggesting that collagen synthesis may correlate well with HSC activation in this DMN-induced liver fibrosis model. Upregulation of KLF5 mRNA expression was observed 2 weeks after weekly administration of DMN and 3 weeks after intermittent injection. It is noteworthy that the onsets of this upregulation were almost consistent with the observed increases in ALT levels and hydroxyproline content. In addition, in weekly DMN administration study, upregulation of KLF5 mRNA expressions was sustained even at 4 weeks, which was 14 days after the last DMN injection and which was correlated with still remaining higher levels of collagen I and α-SMA mRNA as well as a further increase of

hydroxyproline content. As the incremental rise in KLF5 mRNA expression was an early transient event after single DMN injection, sustained augmentation of KLF5 expression by repeated administration with DMN may further accelerate collagen synthesis via HSC activation and the subsequent rapid development of liver fibrosis. Although the precise mechanisms were not elucidated in this study, and further investigations are required, these results suggest that KLF5 induction may be relevant to liver cell injury or other deteriorative events, playing an important role in fibrogenic responses by inducing HSC activation.

KLF5 is a key transcriptional factor in the transdifferentiation of mesenchymal cells, including vascular smooth muscle cells and myofibroblasts; its expression is upregulated in vascular remodeling and atherothlerosis, leading to the phenotypical differentiation of SMCs into a "synthetic" form that is capable of proliferating, migrating, and producing ECM components [15, 18]. In KLF5$^{+/-}$ mice, the cardiac hypertrophy and fibrosis induced by angiotensin II were significantly reduced [33]; these mice also exhibited diminished arterial-wall thickening in a cuff-induced vascular hyperplasia model [34]. In this study, intrahepatic KLF5 mRNA levels correlated, at least in part, with the development of fibrosis. HSC activation shares several features with SMC and myofibroblasts, such as the transdifferentiation into a myofibroblast-like cell that produces ECM [5] and the expression of cytoskeleton-related genes, such as α-SMA [7, 35, 36]. Although further studies will be required, it is suggested that the transcriptional regulation of HSC activation, promoting transdifferentiation into a myofibroblastic phenotype, may be a key step in fibrogenic promotion in this model, and this pathological process may be regulated in a similar fashion as seen for KLF5 in SMC transdifferentiation.

Besides, KLF5 is a key regulator of adipocyte differentiation and an important component of the transcription factor network in these cells. In the signaling cascade controlling differentiation from a preadipocyte into a mature adipocyte, C/EBPβ/δ(induces KLF5 expression, and subsequently, KLF5 acts in concert with C/EBPβ/δ(to activate PPARγ2 [17, 37]. Tsukamoto et al. hypothesized that the differentiation of HSC is similar to that of adipocytes [38]. Adipogenic transcriptional factors, including C/EBP$\alpha/\beta/\delta$,(PPARγ, SREBP-1c, and LXRα, are essential for the maintenance of adipocytes and the quiescent phenotype of HSC [11, 39]. The transdifferentiation of adipocytes and HSC has several similar features: both adipocytes and quiescent HSCs store lipids; activated HSC and preadipocytes produce excessive collagen; factors that activate HSC, such as TGF-β and leptin, also regulate adipocyte differentiation; PPARγ? serves as a key transcriptional factor in HSC quiescence and adipogenesis [38, 39]. Although there has been no report describing a role for KLF5 in HSC activation or transdifferentiation, these studies may support the hypothesis that KLF5 can be induced by liver cell damage or certain impaired hepatic function, triggering HSC activation via transdifferentiation, as seen for adipocytes and SMC, and causing subsequent fibrogenic responses.

In conclusion, this study demonstrates the pathophysiological profiles of liver fibrosis induced by DMN administration in rats using three experimental protocols with single or repeated administrations of DMN. Intrahepatic expression of KLF5 mRNA was detected in this model, supporting a role for this protein in fibrogenic responses. Although further studies will be needed, we hypothesize that KLF5 may be induced by external stresses, including liver cell damage, and cause HSC transdifferentiation into activated phenotype, resulting in fibrogenic responses in this DMN-induced model of liver fibrosis.

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