

Relationship between the proliferative capability of hepatocytes and the intrahepatic expression of hepatocyte growth factor and c-Met in the course of cirrhosis development in rats

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Abstract. Liver cirrhosis is the fatal end stage of various chronic liver diseases. One of the most important causes of liver cirrhosis appears to be an impaired proliferative capability of hepatocytes caused by continuous hepatic damage. Hepatocyte growth factor (HGF) and its specific receptor, c-Met, play a pivotal role in hepatocyte proliferation. However, the relationship between the proliferative capability of hepatocytes and the intrahepatic expression of HGF and c-Met during the course of cirrhosis development has not been studied fully. In the present study, liver cirrhosis was produced in rats by intraperitoneally administering dimethylnitrosamine (DMN) and intrahepatic expression levels of HGF and c-Met were quantitatively estimated using a real-time reverse transcription-polymerase chain reaction (RT-PCR) method. Histological examination of liver sections and biochemical estimation of serum levels of transaminase revealed that the degree of hepatocyte destruction was elevated gradually during cirrhosis development in the DMN-induced rat cirrhosis model. The proliferative capability of hepatocytes estimated immunohistochemically by proliferating cell nuclear antigen staining was markedly increased at an early stage of cirrhosis development. However, it was gradually decreased thereafter and suppressed substantially at the time of cirrhosis manifestation. Intrahepatic HGF expression was also increased significantly during the course of cirrhosis development but decreased

significantly at the time of cirrhosis manifestation. Conversely, there was a tendency for the intrahepatic expression of c-Met to decrease from an early stage of cirrhosis development and intrahepatic c-Met expression was decreased significantly at the time of cirrhosis manifestation. These results suggest that the highly proliferative capability of hepatocytes at an early stage of cirrhosis development is induced by increased intrahepatic HGF expression. However, both HGF and c-Met expression levels in the liver are decreased significantly thereafter. Accordingly, the proliferative capability of hepatocytes is severely impaired and extracellular matrix components are deposited to retrieve space lost by the destruction of hepatic parenchyma, resulting in the establishment of liver cirrhosis.

Introduction

Any types of chronic liver injury, including viral hepatitis, alcoholic liver diseases, autoimmune hepatitis, metabolic disorders, parasitic diseases and non-alcoholic steatohepatitis, produce liver fibrosis whose end stage, liver cirrhosis, is a major public health problem worldwide owing to life-threatening complications of portal hypertension and liver failure. The overall 5-year survival rate of patients with cirrhosis is comparable to that of patients with cancer of various forms. For instance, the cumulative survival rate after the onset of ascites due to liver cirrhosis was shown to be 59.7% after 1 year, 44.5% after 2 years and 29.5% after 5 years (1). Furthermore, it was reported that the 3-year survival rate of cirrhosis was 16% and the 5-year survival rate was only 8% in a group of 308 patients after diagnosis (2). Liver cirrhosis is a pathologically defined entity which is associated with a spectrum of characteristic clinical manifestations. The cardinal pathological features reflect irreversible chronic injury of the hepatic parenchyma and include extensive fibrosis in association with the formation of regenerative nodules. These features result from hepatocyte necrosis, collapse of the supporting reticulin network with subsequent connective tissue deposition, distortion of the vascular bed and nodular regeneration of remaining liver parenchyma. Among them, one of the most

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important causes of cirrhosis appears to be an impaired proliferative capability of hepatocytes caused by continuous hepatic damage. Therefore, it is important to examine the mechanism of the impaired proliferative capability of hepatocytes.

Cell cycle-related molecules play essential roles in cell proliferation. Specifically, G1 phase-related cell cycle molecules are important because they play essential roles in the entry into the cell cycle, resulting in cell proliferation (3-5). We previously showed that G1 phase-related cell cycle molecules, the adaptor molecule, Shc, and the myristoylated alanine-rich C kinase substrate play important roles in the proliferation of hepatocytes and hepatocellular carcinoma cells (6-13). We have shown that decreased cyclin D1 expression and increased p15^{INK4b} and p16^{INK4a} expression caused an impaired proliferative ability of hepatocytes during the course of cirrhosis development in a rat model (10). Besides cell cycle-related molecules, a number of growth factors, such as hepatocyte growth factor (HGF), epidermal growth factor, basic fibroblast growth factor and transforming growth factor- β (TGF- β), have been shown to play important roles in hepatocyte proliferation (14,15), mainly in the setting of acute liver regeneration following partial hepatectomy. Among the various growth factors, HGF is believed to be the most important mitogen for hepatocytes *in vitro* and the humoral mediator of liver regeneration (16). HGF exerts the ability of hepatocyte proliferation through its receptor, c-Met, which is a receptor tyrosine kinase (17,18). HGF was shown to play a pivotal role in hepatocyte proliferation in the regenerating rat liver after partial hepatectomy (19). However, to the best of our knowledge, there has been no study of the sequential relationship between the proliferative capability of hepatocytes, and the intrahepatic expression of HGF and c-Met during the course of cirrhosis development. Therefore, in the present study, we compared the levels of hepatocyte proliferative capability with those of intrahepatic mRNA expression of HGF and c-Met in a rat cirrhosis model produced by intraperitoneal administration of dimethylnitrosamine (DMN).

Materials and methods

Animals and chemical treatment. Six-week-old male Sprague-Dawley rats were purchased from Japan SLC (Hamamatsu, Japan). Animals were kept under specific pathogen-free conditions at 24 \pm 2°C with a 12-h day/night light cycle and food and water available *ad libitum* throughout the experimental period. Animal experiments were performed with the approved protocols and in accordance with the institutional recommendations for the proper care and use of laboratory animals.

Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) or Wako Pure Chemical Co. (Osaka, Japan), unless otherwise mentioned. To develop cirrhosis in the rat livers, 1% DMN dissolved in phosphate-buffered saline (PBS) was given intraperitoneally at 1 ml per kg of body weight for 3 consecutive days per week. Two, 4 and 6 weeks after the initiation of DMN treatment, livers were isolated and perfused, as described previously (20). Briefly, rats were anesthetized with ether and the abdomen was opened. The portal vein was cannulated and approximate 5-ml blood samples were collected

from each animal. The livers were then cleared of blood with perfusion of 50 ml of PBS from the portal vein and removed for analysis of mRNA and protein expression. Untreated 8-week-old rats were also sacrificed as normal controls. Sera were obtained from the whole blood samples by centrifugation to examine the biochemical parameters of DMN-treated and untreated animals. Each group consisted of 5 animals.

Histological and immunohistochemical analysis. For histological analysis, approximately half the volume of the left-lateral hepatic lobe from each animal was fixed in 10% neutral-buffered formalin, embedded in paraffin and sliced into 4- μ m-thick sections. For examining liver damage, sections were stained with hematoxylin-eosin and, for evaluating the degree of liver fibrosis, sections were subjected to Azan-Mallory staining.

Liver sections were also immunohistochemically stained using the ABC method, as described previously (21). Briefly, the sections were placed in 10 mM citrate buffer (pH 6.0) and processed at 95°C for 10 min in a microwave oven. The sections were then deparaffinized with xylene, rehydrated through a graded series of alcohol solutions, and mixed with a solution containing 0.5% hydrogen peroxide to block endogenous peroxidase activity. After washing with PBS, the sections were processed for immunohistochemical staining by the ABC method. The sections were incubated overnight at room temperature with mouse monoclonal antibody anti-proliferating cell nuclear antigen (anti-PCNA) (Clone PC10, Dako, Glostrup, Denmark). The antibody was diluted at 1:200 before use. For signal amplification, the Renaissance tyramide signal amplification kit (MENTM life Science Products, Boston, MA, USA) was used, as described previously (22). Immunoreactive products were visualized using 3,3'-diaminobenzidine tetrahydrochloride and the sections were counterstained with Mayer's hematoxylin. The nuclear labeling index for PCNA-positive cells (positive nuclei/total counted nuclei) was determined, counting at least 1000 hepatocytes in randomly selected fields of each section.

RNA extraction and reverse transcription (RT). Total RNA was extracted from approximately 30 mg of liver tissues, using the RNeasy Protect Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized in a 30- μ l reaction volume. A sample of total RNA (2 μ g) was incubated at 65°C for 10 min, and then cooled for 2 min on ice. A sample was transferred to the tube of the Ready-To-Go You-Prime First-Strand Beads (Amersham Biosciences, Piscataway, NJ, USA) and incubated for 1 min at room temperature with the oligo-dT primers (Invitrogen, Carlsbad, CA, USA). The reaction was then incubated at 37°C for 60 min. The obtained cDNA was used for quantitative real-time polymerase chain reaction (PCR).

Quantitative real-time PCR. Quantitative PCR was performed using the real-time PCR system, LightCycler (Roche Diagnostics, Mannheim, Germany) with SYBR-Green I double strand DNA binding dye (Roche Diagnostics). The amplification of a target with the primers listed in Table I was carried out in a total volume of 20 μ l containing 0.3 μ M of each primer, 3 mM MgCl₂, 2 μ l of the master mixture

Table I. Primers used for real-time RT-PCR.

Gene	Primers
GAPDH	For: 5'-TGAACGGGAAGCTCACTGG-3' Rev: 5'-TCCACCACCCTGTTGCTGTA-3'
HGF	For: 5'-TTATGGGGAATGAGAAATGC-3' Rev: 5'-TCGAACAAAATACCAGGAC-3'
c-Met	For: 5'-AGTGGATGGCTTTAGAGAGT-3' Rev: 5'-ATCACTTCATACAAGGCGTC-3'

For, forward; Rev, reverse.

(LightCycler FastStrand DNA Master SYBR Green I) and 1 μ l of template cDNA. The reaction mixture was preheated at 95°C for 10 min, followed by 40 cycles at 95°C for 10 sec, 62°C for 10 sec and 72°C for 7 sec. Fluorescence data were collected after each extension step. Melting curve analysis was performed by heating the PCR product at 95°C, then cooling to 65°C and finally heating to 95°C with a 0.1°C/sec temperature transition rate while continuously monitoring the fluorescence. Fluorescence was analyzed using LightCycler Software Version 3.5 (Roche Diagnostics). The crossing point for each reaction was determined and manual baseline adjustment was made. To quantify and prove the integrity of isolated RNA, real-time RT-PCR analysis for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also carried out. Each run consisted of GAPDH as an internal standard and a negative control without samples.

Statistical analysis. All values are expressed as means \pm SD. All analyses were performed using the computer-assisted StatView program (SAS Institute, Gary, NC, USA). Standard descriptive statistics, Student's t-test and Welch t-test, were used according to the distribution of experimental values. A p-value of <0.05 was considered to indicate a significant difference between groups.

Results

Histological findings in the livers of rats treated with DMN. Intraperitoneal administration of DMN to rats is known to cause liver cirrhosis, characterized by hepatocellular necrosis, increased connective tissue and formation of regenerative nodules. Histological analysis by hematoxylin-eosin staining of liver sections of untreated normal controls revealed that there was no infiltration of inflammatory cells (Fig. 1A). Conversely, histological analysis of liver sections of rats treated with DMN revealed infiltration of inflammatory cells, including macrophages and neutrophils, and destruction of hepatic parenchyma. The hepatic damage became more severe according to the period of DMN treatment (Fig. 1B-D).

Serum transaminase levels of rats treated with DMN. To evaluate the degree of hepatocyte destruction during the course of cirrhosis development, blood samples were collected

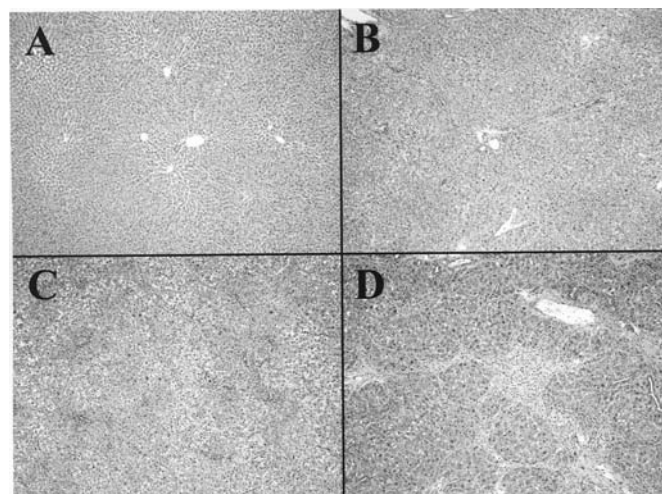


Figure 1. Hematoxylin-eosin staining of liver sections of rats treated with DMN. To develop liver cirrhosis in rats, DMN was given intraperitoneally for 3 consecutive days per week. Two, 4 and 6 weeks after the initiation of DMN treatment, livers were removed, sliced into 4- μ m-thick sections and stained with hematoxylin-eosin. There was no infiltration of inflammatory cells in the liver of DMN-untreated normal control animals (A). Conversely, histological analysis of the liver of rats treated with DMN for 2 (B), 4 (C) and 6 weeks (D) revealed infiltration of inflammatory cells including macrophages and neutrophils, and destruction of hepatic parenchyma. Hepatic damages became more severe according to the periods of DMN treatment. Representative pictures are shown in the figure. Original magnification x100.

from the portal vein when animals were sacrificed. The serum values of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), which are released from destructed hepatocytes, were determined. As shown in Fig. 2, the serum values of AST and ALT were elevated gradually according to the period of DMN treatment. The serum values of AST and ALT in animals treated with DMN for 6 weeks were significantly higher than those of both untreated, and 2- and 4-week-treated animals.

Development of liver cirrhosis in rats treated with DMN. To evaluate the amount of collagen fibers in the liver, liver sections collected from DMN-treated and untreated animals were stained with Azan-Mallory dyes. There were almost no fibers in the livers of untreated normal controls (Fig. 3A). In contrast, fine collagen fibers were observed only at periportal regions, known as Rappaport's zone 1, in the liver of rats treated with DMN for 2 weeks (Fig. 3B). Azan staining of liver sections of rats treated with DMN for 4 weeks demonstrated that the fibrotic septa were relatively thin and predominantly restricted to zone 1 (Fig. 3C). Azan staining of liver sections after 6-week DMN treatment revealed that cirrhosis had completely manifested by an increase and thickening of the fibrotic septa, traversing the lobular parenchyma by porto-venous or porto-portal bridging, thus promoting the formation of pseudobulbi (Fig. 3D).

Immunohistochemical staining for PCNA. To examine the proliferating capability of hepatocytes during the development of liver cirrhosis, liver sections were stained immunohistochemically with PCNA and the nuclei of hepatocytes positive for PCNA staining were counted. There were a few hepatocytes

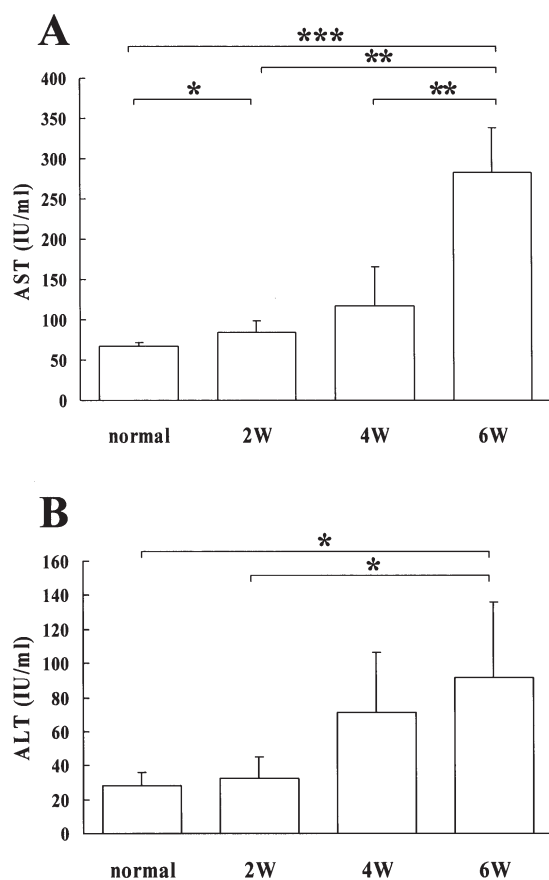


Figure 2. Serum transaminase levels of rats treated with DMN. To evaluate the degree of hepatocyte destruction during the course of cirrhosis development in DMN-treated rats, blood samples were collected from the portal veins when animals were sacrificed. Serum values of AST (A) and ALT (B), which are released from destructed hepatocytes, were elevated gradually according to the periods of DMN treatment. Both AST and ALT levels were significantly higher in animals treated with DMN for 6 weeks than in untreated control animals. Each bar represents the mean \pm SD of 5 animals. *0.01<p<0.05, **0.001<p<0.01, ***p<0.001.

positive for PCNA staining in the liver of untreated normal controls (Fig. 4A). In marked contrast, there were a large number of positive hepatocytes in the livers of rats treated with DMN for 2 weeks (Fig. 4B). Although hepatocytes positive for PCNA staining were decreased in the livers of animals treated with DMN for 4 weeks, a number were positive (Fig. 4C). Notably, hepatocytes positive for PCNA staining were decreased markedly in the livers of animals treated with DMN for 6 weeks (Fig. 4D).

We then quantitatively evaluated the number of hepatocytes positive for PCNA staining. As shown in Fig. 5, the number of positively-stained hepatocytes was significantly larger in rats treated with DMN for 2 weeks than in untreated and 6-week-treated rats. Although the number of positive cells decreased in animals treated with DMN for 4 weeks, the values were significantly higher compared with untreated or 6-week-treated animals. There were no significant differences between untreated and 6-week-treated animals.

Intrahepatic expression of HGF and c-Met during the course of cirrhosis development. To examine intrahepatic expression levels of HGF and c-Met during the course of cirrhosis develop-

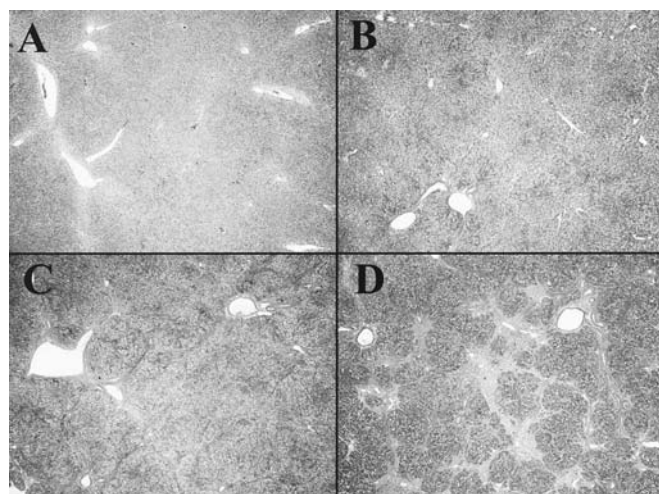


Figure 3. Azan-Mallory staining of liver sections of rats treated with DMN. To develop liver cirrhosis in rats, DMN was given intraperitoneally for 3 consecutive days per week. Two, 4 and 6 weeks after the initiation of DMN treatment, livers were removed, sliced into 4- μ m-thick sections and stained with Azan-Mallory dyes. There were almost no fibers in the livers of untreated normal controls (A). Fine collagen fibers were observed only at periportal regions in the livers of rats treated with DMN for 2 weeks (B). Azan staining of the livers of rats treated with DMN for 4 weeks demonstrated that the fibrotic septa were relatively thin and predominantly restricted to periportal regions (C). Azan staining of sections from livers after 6-week DMN treatment revealed that cirrhosis had completely manifested by an increase and thickening of the fibrotic septa, traversing the lobular parenchyma by porto-venous or porto-portal bridging, thus promoting the formation of pseudolobuli (D). Representative pictures are shown in the figure. Original magnification x40.

ment, total RNA was extracted from the livers of rats treated with DMN and mRNA levels were quantitatively evaluated using a real-time RT-PCR method. HGF expression in the liver increased according to the period of DMN treatment (Fig. 6). The intrahepatic mRNA levels of HGF of rats treated with DMN for 4 weeks were significantly higher than those of untreated controls and 2-week-treated animals. However, the mRNA levels of HGF decreased markedly in the livers of rats treated with DMN for 6 weeks. The levels of HGF expression were significantly lower in the livers of rats treated with DMN for 6 weeks than in those of both untreated, and 2- and 4-week-treated rats.

We then examined the mRNA expression levels of c-Met, the specific receptor of HGF, in the liver (Fig. 7). The mRNA expression levels of c-Met were decreased in the livers of rats treated with DMN for 2 and 4 weeks, although the differences were not statistically significant compared with untreated control animals. The level of c-Met expression in the livers decreased further thereafter, and intrahepatic c-Met expression was significantly lower in the livers of rats treated with DMN for 6 weeks than in those of untreated controls and 2-week-treated animals.

Discussion

Hepatocytes are believed to possess an extremely high proliferative capability. It has been shown that long-term hepatocyte replication was compatible with a differentiated phenotype by using cultured hepatocytes isolated from trans-

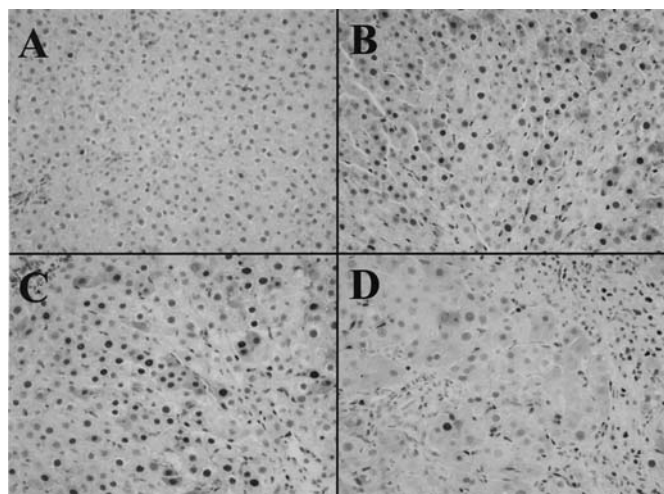


Figure 4. Immunohistochemical staining for PCNA of liver sections of rats treated with DMN. To examine the proliferating capability of hepatocytes during the course of cirrhosis development, liver sections were stained immunohistochemically with anti-PCNA antibodies and nuclei positive for PCNA staining were examined. There were a few hepatocytes positive for PCNA staining in the livers of untreated normal controls (A). In contrast, there were a considerable number of positive hepatocytes in the livers of rats treated with DMN for 2 weeks (B). Although hepatocytes positive for PCNA staining were decreased in the livers of rats treated with DMN for 4 weeks, there were still a number of positive cells (C). Hepatocytes positive for the staining were decreased markedly in the livers of animals treated with DMN for 6 weeks (D). Representative pictures are shown in the figure. Original magnification $\times 400$.

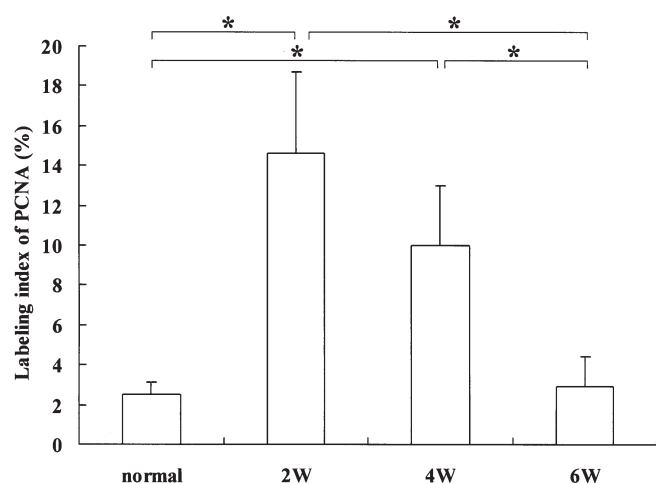


Figure 5. Quantitative estimation of hepatocytes positive for PCNA staining. The number of positively-stained nuclei of hepatocytes was significantly larger in rats treated with DMN for 2 weeks than in untreated and 6-week-treated rats. Although the number of positive cells was larger in animals treated with DMN for 4 weeks, the values were not significantly different from those of untreated and 6-week-treated animals. There were no significant differences between untreated and 6-week-treated animals. Each bar represents the mean \pm SD of 5 animals. $^{*}0.001 < p < 0.01$.

genic mice expressing TGF- α (23). Furthermore, hepatocyte transplantation experiments in urokinase-plasminogen activator transgenic mice (24) and fumarylacetoacetate hydrolase-deficient mice (25) demonstrated at least 12 and 70 rounds, respectively, of hepatocyte replication. In the serial transplantation experiments, there was no evidence that the repopulation capacity was dependent on stem cells. This

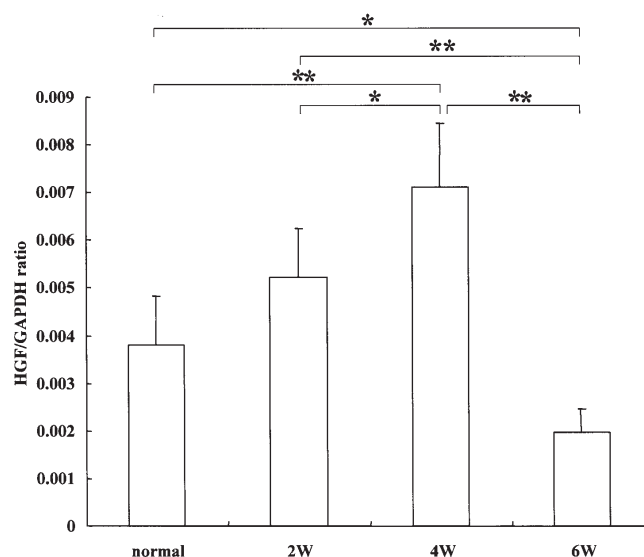


Figure 6. Intrahepatic mRNA expression of HGF during the course of cirrhosis development. To quantitatively estimate HGF mRNA expression in the liver during the course of cirrhosis development, a real-time RT-PCR method was employed. The value of each amplified message was standardized by the amount of GAPDH quantified from the same sample. mRNA levels of HGF were increased in the livers of rats treated with DMN for 2 and 4 weeks compared with untreated normal controls, although the differences were not statistically significant. mRNA levels of HGF were then decreased markedly in the livers of 6-week-treated rats. Statistically, mRNA levels of HGF were significantly lower in the livers of 6-week-treated animals than in untreated, and 2- and 4-week-treated animals. Each bar represents the mean \pm SD of 5 animals. $^{*}0.01 < p < 0.05$, $^{**}0.001 < p < 0.01$.

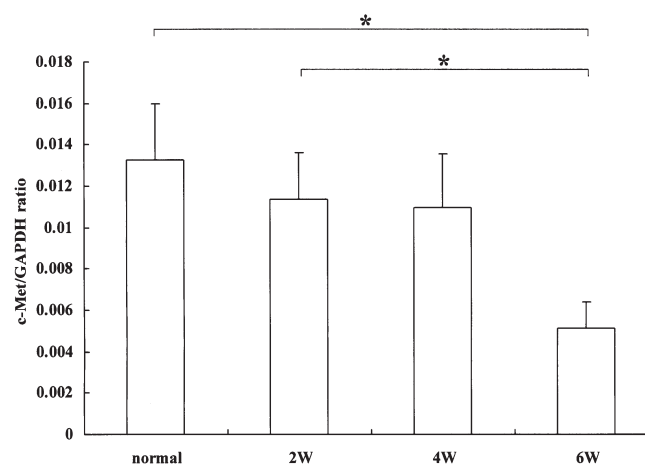


Figure 7. Intrahepatic mRNA expression of c-Met during the course of cirrhosis development. To assess expression levels of c-Met in the liver during the course of cirrhosis development, mRNA levels of c-Met were quantitatively evaluated using a real-time RT-PCR method. mRNA levels of c-Met were decreased in the livers of rats treated with DMN for 2 and 4 weeks compared with those of normal control rats, although the differences were not statistically significant. Intrahepatic expression levels of c-Met were significantly lower in animals treated with DMN for 6 weeks than in normal control animals. Each bar represents the mean \pm SD of 5 animals. $^{*}0.001 < p < 0.01$.

conclusion was also supported by data demonstrating that diploid, tetraploid and octoploid hepatocytes had roughly the same capacity to repopulate damaged livers (26). Thus, hepatocytes have an enormous proliferative potential that can be unleashed under certain conditions. Nevertheless, there is

evidence that the replicative activity of hepatocytes diminishes in advanced liver cirrhosis in humans and in chronic injury in mice, reaching a state of replicative senescence (27,28).

Thus, although hepatocytes are highly capable of proliferation, continuous liver damage caused by viral infection, alcohol, autoimmune-related diseases, metabolic disorders or hepatic toxins leads to cirrhosis. Liver cirrhosis is characterized by the formation of regenerative nodules of liver parenchyma that are separated by and encapsulated in thick fibrotic septa and is associated with major angio-architectural changes. TGF- β is believed to be a key molecule that activates hepatic stellate cells (HSCs), resulting in production of collagen fibers (29-31). We have shown that tissue inhibitor of metalloproteinase and vascular endothelial growth factor played important roles in the development of liver cirrhosis (32-37). In liver cirrhosis, fiber accumulation is induced to retrieve space lost by the destruction of hepatic parenchyma. Therefore, one of the most important causes of liver cirrhosis appears to be the impaired proliferative capability of hepatocytes.

Repeated intraperitoneal administration of DMN has been shown to cause hepatocyte destruction and production of extracellular matrix components mainly composed of collagen fibers, resulting in manifestation of liver cirrhosis (7,38-40). In the present study, we first examined the proliferative capability of hepatocytes during the development of liver cirrhosis, using an animal liver cirrhosis model induced by DMN treatment. PCNA is a stable cell cycle-related nuclear protein, 36 kDa in molecular weight, which is increasingly expressed in late G1 and throughout S phase of the cell cycle. Its rate of synthesis correlates with the proliferative rate of cells (41-45). Several studies comparing PCNA immunohistochemistry with established proliferation markers indicate that immunostaining of PCNA can be used to define and map proliferating cells in animal and human tissues and that this method represents a reliable marker for the determination of proliferative activity (46-49). Therefore, we employed an immunohistochemical PCNA-staining method to assess the proliferative capability of hepatocytes during the development of liver cirrhosis. At an early stage of the continuous hepatic damage caused by DMN, a number of hepatocytes with nuclei positive for PCNA staining were observed. However, PCNA-positive hepatocytes then decreased gradually and there were a very small number at the establishment of liver cirrhosis. These results indicate that, although hepatocytes can proliferate in response to hepatic damage for certain time periods, the proliferative capability of hepatocytes is exhausted during continuous hepatic damage. This exhaustion of the proliferative capability of hepatocytes is considered to be a main cause of the development of liver cirrhosis. Accordingly, the next important issue to be examined is why hepatocytes lose proliferative capability during the development of liver cirrhosis.

HGF and its specific receptor, c-Met, are believed to be the most important mitogen for hepatocytes. Therefore, it is considered that disruption of the regulation of the HGF and c-Met system contributes to the development of liver cirrhosis. However, little is known about the disproportion of these molecules during the development of liver cirrhosis. Hata *et al* (50) examined the expression of HGF mRNA in normal and DMN-induced cirrhotic rat livers by using an *in situ* RT-PCR method. The investigators have shown that HGF was expressed

in Kupffer cells and HSCs in normal rat livers while, in cirrhotic livers, HGF mRNA-positive cells were spindle-shaped and surrounded the hepatocytes located around the sinusoids, indicating that sinusoidal endothelial cells as well as Kupffer cells and HSCs produced HGF at the time of cirrhosis development. In the present study, we sequentially assessed intrahepatic expression levels of HGF during the course of cirrhosis development. The mRNA levels of HGF in the livers were increased in response to chronic liver injury induced by DMN. There was a tendency towards elevated HGF expression as fiber accumulation increased in the liver. However, HGF expression in the liver was then markedly decreased at the time of cirrhosis manifestation. Napoli *et al* (51) reported similar experimental results. They produced liver cirrhosis in rats by using a bile duct-ligated model. In their experiments, bile duct ligation led to liver cirrhosis in all rats by day 21. The intrahepatic expression of HGF was increased significantly at days 7 and 14 after bile duct ligation, and returned to control levels as cirrhosis developed. Taken collectively, these results suggest that intrahepatic HGF produced by Kupffer cells, HSCs and endothelial cells in the liver is increased for hepatocyte replication in response to hepatocyte damage. However, the ability to produce intrahepatic HGF is exhausted probably due to overloaded production, resulting in the manifestation of liver cirrhosis.

HGF exerts the ability of hepatocyte proliferation through its specific receptor, c-Met. Therefore, c-Met expression on hepatocytes is necessary for hepatocyte proliferation induced by HGF. In the present study, we assessed expression levels of c-Met in the liver during the course of cirrhosis development. In contrast to intrahepatic HGF expression, intrahepatic c-Met expression was not enhanced in response to liver damage induced by DMN. The expression levels of c-Met in the livers of rats treated with DMN for 2 and 4 weeks were even lower than those of normal control rats, although the differences were not statistically significant. Furthermore, c-Met expression became significantly lower in the livers of rats treated with DMN for 6 weeks than in those of untreated normal controls. These results indicate that intrahepatic c-Met expression was not enhanced in response to chronic liver damage caused by DMN and was reduced significantly at the time of cirrhosis manifestation. D'Errico *et al* (52) examined the proliferative capability of hepatocytes and hepatocellular carcinoma (HCC) cells in human liver specimens obtained from HCC, focal nodular hyperplasia, fulminant hepatitis and regenerated liver by immunohistochemical staining with cyclin A. They also immunohistochemically evaluated the expression levels of HGF and c-Met in the same specimens. It was shown that intrahepatic expression levels of HGF did not always correlate with hepatocyte proliferation, but intrahepatic expression levels of c-Met did. These results indicate that expression levels of c-Met on hepatocytes appear to be more closely related to the proliferative capability of hepatocytes compared with intrahepatic expression levels of HGF produced by various hepatic mesenchymal cells.

In conclusion, during the course of cirrhosis development, hepatocytes revealed high proliferative capability in response to hepatocyte damage. However, according to the time periods of continuous hepatic damage, hepatocytes gradually lost their proliferative capability, which appeared to be caused not only

by decreased intrahepatic expression of HGF but also by decreased intrahepatic expression of c-Met. Therefore, maintaining sufficient levels of intrahepatic HGF and c-Met expression may be an effective strategy for inhibiting the development of liver cirrhosis. In fact, there have been several reports demonstrating that the enhancement of HGF levels induced by administration of recombinant HGF protein (53) or transfection of the HGF gene (54) was a potent treatment strategy for the treatment of liver cirrhosis. However, the effectiveness of enhanced c-Met expression on hepatocytes for the treatment of liver cirrhosis has not yet been evaluated.

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