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The Decrease in Total Collagen Fibers in the Liver by Hepatocyte Growth Factor after Formation of Cirrhosis Induced by Thioacetamide

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ABSTRACT. Liver cirrhosis is an inveterate disease accompanying fibrosis, hepatocyte damage, and liver dysfunction. In this study, the therapeutic effects of recombinant human hepatocyte growth factor (rhHGF) on liver cirrhosis were examined in rats administered thioacetamide (TAA). Repeated administration of TAA for 10 weeks to rats induced liver cirrhosis with collagen nodes and pseudo-lobe generation, a condition that was pathologically similar to that in humans. Administration of rhHGF after the formation of liver cirrhosis markedly decreased the incidence of pathological fibrosis and the degree of fibrosis as measured by a computed image analysis system. Continuous administration of rhHGF by infusion pump was more effective than bolus administration. Northern blotting analysis showed that rhHGF reduced mRNA levels of procollagen α2(I), α1(IV), and transforming growth factor-β1 (TGF-β1) that were stimulated in the TAA-treated liver. The labeling index of hepatocytes increased following administration of rhHGF in this model. These observations suggest that the pathological recession of liver fibrosis is the result of the reduction of TGF-β1 and collagen synthesis and, in part, of the stimulation of mitosis of hepatocytes directly by rhHGF and indirectly by TGF-β1 reduction in the cirrhotic liver. These results demonstrate the usefulness of rhHGF as a therapeutic agent in liver cirrhosis. BIOCHEM PHARMACOL **59**;6:681–690, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. rhHGF; TAA; collagen α2(I); collagen α1(IV); TGF-β1; cirrhosis

Liver cirrhosis is frequently found in chronic liver diseases where the hyperaccumulation and disorder of fibrous connective tissue and overgrowth of hepatic non-cells represent major phenomena. It is often accompanied by hypoalbuminemia, ascites, and esophageal varices, and leads to severe hepatic failure or hepatocellular carcinoma. Despite the large numbers of patients with liver cirrhosis, there is no effective treatment which can reduce the fibrosis in this disease. In these patients, it is necessary to inhibit the onset of liver cirrhosis and to reduce the extracellular fibrous components already formed. Many studies have revealed that hepatic fibrosis is a complex process that involves marked accumulation of extracellular matrix components, activation of cells capable of producing matrix materials, cytokine release, and tissue remodeling. The major extra-cellular matrix components are interstitial collagens (types I and III) and basement membrane collagen (type IV) [1]. Stellate cells (hepatic lipocytes, Ito cells, or fat-storing cells) are known as the primary cellular source of the matrix components in liver injury [2]. Stellate cell activation, characterized by stimulated proliferation and fibrogenesis [1, 3], is modulated by the most potent fibrogenic cytokine, TGF-β1‖ [4]. In mice and rats with experimentally induced hepatic fibrosis, fibrotic lesions are associated with increases in procollagen α1(I), α2(I), α1(III), α1(IV) and TGF-β1 mRNA levels [5–7]. In patients with chronic hepatitis and cirrhosis, the level of TGF-β1 mRNA is correlated with that of collagen α1(I) mRNA, the serum level of procollagen type III peptide, and the histological activity index [8].

Several studies have shown that HGF, originally identified as a potent mitogen for hepatocytes, has multiple biological properties [9–11] and is one of the strongest hepatotrophic factors for liver regeneration [10, 12, 13]. It has been reported that HGF accelerates hepatic regeneration and function in 70% hepatectomy model rats [14, 15] and acute hepatitis caused by hepatotoxin [16, 17]. Some recent studies have shown that HGF prevents the formation of liver fibrosis/cirrhosis caused by DMN [18, 19].

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‖ *Abbreviations:* TGF-β1, transforming growth factor-β1; HGF, hepatocyte growth factor; rhHGF, recombinant human hepatocyte growth factor; DMN, dimethylnitrosamine; TAA, thioacetamide; CBB, Coomassie brilliant blue; NT group, non-treated rats injected with saline instead of TAA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; BrdU, 5-bromo-2′-deoxyuridine; and IgG, immunoglobulin G.

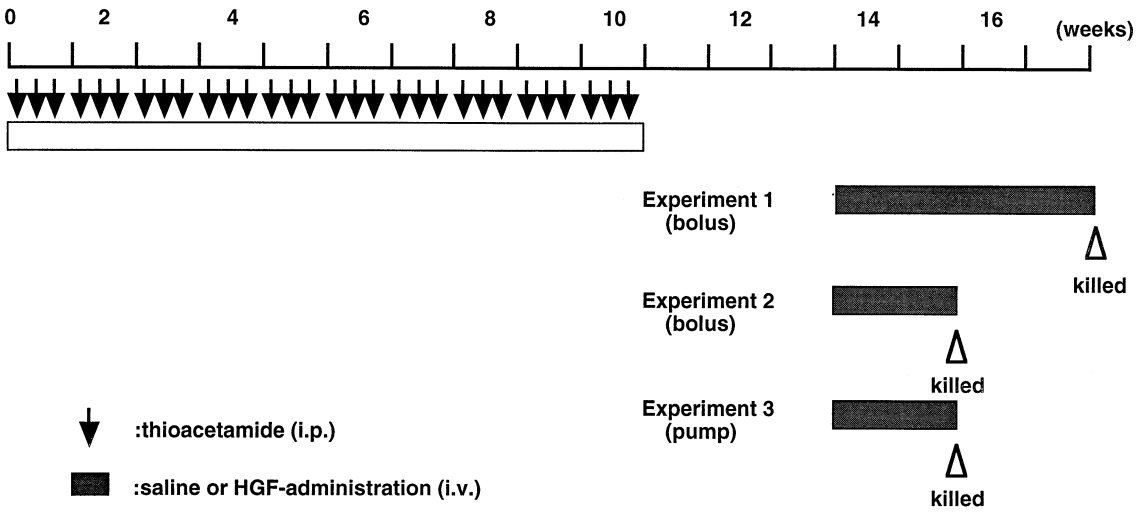

FIG. 1. Schematic representation of the development of liver cirrhosis by TAA administration. TAA was injected i.p. three times a week for 10 weeks (arrows). After the termination of TAA administration, rats were kept without TAA for 3 weeks. In Experiment 1, rhHGF (0.3 mg/kg/day) or saline was administered i.v. in a bolus daily from the 14th to the 17th week (closed box). In Experiment 2, rhHGF (0.3 mg/kg/day) or saline was administered in a bolus daily from the 14th to the 15th week (closed box). In Experiment 3, rhHGF (0.3 mg/kg/day) or saline was administered i.v. with an infusion pump (0.5 μL/hr) from the 14th to the 15th week (closed box).

Biochemical abnormalities induced by DMN resemble hepatic cirrhosis in man, but its pathological aspect does not. HGF prevented the onset of liver fibrosis/cirrhosis in previous studies, but it was not clear whether HGF could improve the damaged liver after complete formation of liver cirrhosis.

In this study, we examined whether rhHGF improves the liver fibrosis induced by TAA after the formation of cirrhosis. Numerous animal models of cirrhosis have been developed that more or less resemble the human disease. The slowly developing TAA-induced rat cirrhosis has proven to be morphologically well defined and uniform [20] and to resemble the major features of the human cirrhotic pathology, although the lack of both visible hepatocellular damage and of a decline of liver function parameters in serum are characteristic of this model [21, 22]. We used Northern blotting analysis and histological staining to elucidate whether rhHGF improves the liver cirrhosis induced by TAA administration. We showed here that exogeneously injected rhHGF prevents the *de novo* synthesis of collagens type I and IV in the cirrhotic liver in the posttranscriptional stage and reduces the amount of fiber in the whole liver. Recombinant hHGF accelerates recovery from cirrhosis by decreasing extracellular fibers and TGF-β1 mRNA expression and by stimulating the growth of hepatocytes.

MATERIALS AND METHODS

Preparation of Recombinant HGF

Recombinant human HGF was purified from conditioned medium of stable transformants of Chinese hamster ovary cells transfected with expression vector containing rhHGF cDNA, as previously described [23]. The purity of rhHGF exceeded 95%, as determined by SDS–PAGE and CBB staining. The biological activity of rhHGF was measured by the growth-stimulating activity in a primary culture of adult rat hepatocyte [24]. The activity was the same as previously described [24]. In all experiments, rhHGF was used from the same preparation.

Animal Model

Liver fibrosis was induced in male Sprague–Dawley rats (6 weeks of age; Charles River Japan) by i.p. injection of 4% TAA (Sigma Chemical Co.) solution at a dose of 0.2 g/kg/day 3 days a week for 10 weeks. Non-treated rats were injected with saline instead of TAA (NT group). TAA-treated rats were kept for 3 weeks (11th to 13th week) to eliminate acute or direct effects of TAA; on the first day of the 14th week, rats were divided into three groups in terms of their body weight under fasting (Experiments 1, 2, and 3). To address the question of whether rhHGF promotes recovery from cirrhosis, 0.3 mg/kg/day of rhHGF in PBST buffer (0.15 M NaCl, 0.005% Tween 80, 10 mM phosphate buffer pH 7.4) was i.v. injected daily in bolus in a volume of 1 mL/kg from the 14th to the 17th week (rhHGF bolus 4 weeks) (Fig. 1; Experiment 1), in bolus in a volume of 1 mL/kg from the 14th to the 15th week (rhHGF bolus 2 weeks) (Fig. 1; Experiment 2), or with an infusion pump (Alzet 2002, 0.5 μL/hr) through the jugular vein from the 14th to the 15th week (rhHGF infusion 2 weeks) (Fig. 1; Experiment 3). We tested the effects of administration of various amounts of rhHGF (0.1, 0.3, and 1.0 mg/kg body weight/day) to 70% hepatectmized rats, and found significant improvement of hepatic function in a dose-dependent manner.* In this study, we used the middle dosage (0.3

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mg/kg/day) in this study. The TAA-treated rats in each control group (control bolus 4 weeks, control bolus 2 weeks, and control infusion 2 weeks) received only PBST buffer instead of rhHGF in bolus or with the infusion pump. The NT group received only PBST buffer in a bolus or with the infusion pump in the same manner. At the end of the 17th (Experiment 1) and 15th week (Experiments 2 and 3), rats were killed and liver tissue was collected. A section of the tissue was fixed for histological analysis, and the remainder was stored at −80° until used for mRNA preparation. The present experiments were performed in accordance with the National Research Council's criteria for the care and use of laboratory animals.

Histology

Liver tissue was fixed in 10% buffered formalin, embedded in paraffin, and stained with Azan-Mallory. All the sections were examined qualitatively in a blind fashion by pathologists.

Measurement of Hepatic Collagen Content

Fiber content in the liver was measured by using an image analysis system. Computer image analysis was performed on the specimens stained with Azan in the following manner. The area of fibrosis was measured using a computerized image analysis system (Mac SCOPE Version 2.00, Mitani-Shoji, Tokyo, Japan). The ratio of the area of fibrosis to the area of the tissue specimen was calculated.

RNA Extraction and Northern Blotting Analysis

Total RNA was isolated from livers of the rats using the acid guanidium thiocyanate-phenol-chloroform extraction method as described by Chomczynski and Sacchi [25]. Poly (A) + RNA was prepared using Oligotex-(dT) 30 super according to the manufacturer's protocol (Japan Roche, Tokyo, Japan). Aliquots of 5 μg of poly (A) + RNA were denatured with glyoxal, electrophoresed on 1.0% agarose gels, and transferred on Biodyne filters (Nihon PALL Ltd.). The membranes were hybridized with cDNA fragments: chicken GAPDH [26], rat collagen α2(I) [27], mouse collagen α1(IV) [28], and mouse TGF-β1 [29]. cDNAs were labeled by ^{32}P-dCTP using the random priming method. After hybridization, the membranes were washed with 2 × SSC (1 × SSC = 15 mmol/L sodium citrate, 150 mmol/L sodium chloride)/0.1% SDS at 20°, followed by 0.1 × SSC/0.1% SDS at 60°. The membranes were then subjected to autoradiography using a Fuji imaging plate for 2 hr at room temperature and analyzed by BAS2000 (Fujix). The relative abundance of mRNA was calculated by dividing the signal strength of collagens or TGF-β1 by that of GAPDH.

Measurement of the BrdU Labeling Index

To determine the mitotic index of hepatocytes, 36 rats were administered TAA as described above for 6 weeks. Half the animals were given a single i.v. injection of 0.3 mg/kg of rhHGF on the first day of the 7th week, and the other half were injected with saline. Each rat was i.p. injected with 100 mg/kg of BrdU (Amersham International plc) dissolved in saline 24, 36, and 48 hr after rhHGF injection. The rats were killed 1 hr after BrdU injection on each day. The liver was excised and fixed in 10% buffered formalin, then embedded in paraffin. Liver sections were deparaffinized, washed, allowed to react with anti-BrdU monoclonal antibody and then with peroxidase-conjugated rabbit anti-mouse IgG as described previously [30]. Diaminobenzidine was used to visualize peroxidase deposits, and the BrdU labeling index was determined from 1500 hepatocytes in 5 different lobes in each of 6 animals.

Statistical Methods

The data are expressed as mean values ± SEM. Differences between the NT group, and control group and rhHGF group in the area of fibrosis, liver weight, hydroxyproline, and mRNA level were identified by Student's *t*-test. The histological data were analyzed by Armitage χ^2 test, and the BrdU labeling index was identified by the Mann–Whitney U-test.

RESULTS

To investigate whether rhHGF could improve already formed liver cirrhosis, rhHGF was administered to rats treated with TAA, a suitable model of the pathology of hepatic cirrhosis. The typical appearance of time-dependent TAA-induced liver fibrosis in our system is shown in Fig. 2 (A–D). In detail, after 3 weeks of TAA treatment, there was no typical figure of fibers (Fig. 2A), while after 6 weeks, connective tissues in the liver spread out radially between central veins and Glisson's sheathes (Fig. 2B). After 8 to 10 weeks, severe fibrosis with bridging of collagen fibers was observed and formed pseudo-lobes (Fig. 2, C and D). No significant hepatocellular damage was seen in any of the cases. Figure 2E shows the typical histological aspects of cirrhotic liver. On the other hand, rhHGF administration by infusion for 2 weeks after TAA treatment significantly ameliorated the degree of fibrosis (Fig. 2F) compared to the control group (Fig. 2E). Table 1 summarizes the results of histological analyses of the effects of rhHGF on TAA-induced liver fibrosis. In all experiments the degree of liver fibrosis was decreased by rhHGF administration. The administration of rhHGF for 2 weeks by infusion pump (Experiment 3) showed a significant reduction in liver fibrosis, while bolus administration (Experiments 1 and 2) showed a slight but not significant reduction. These results

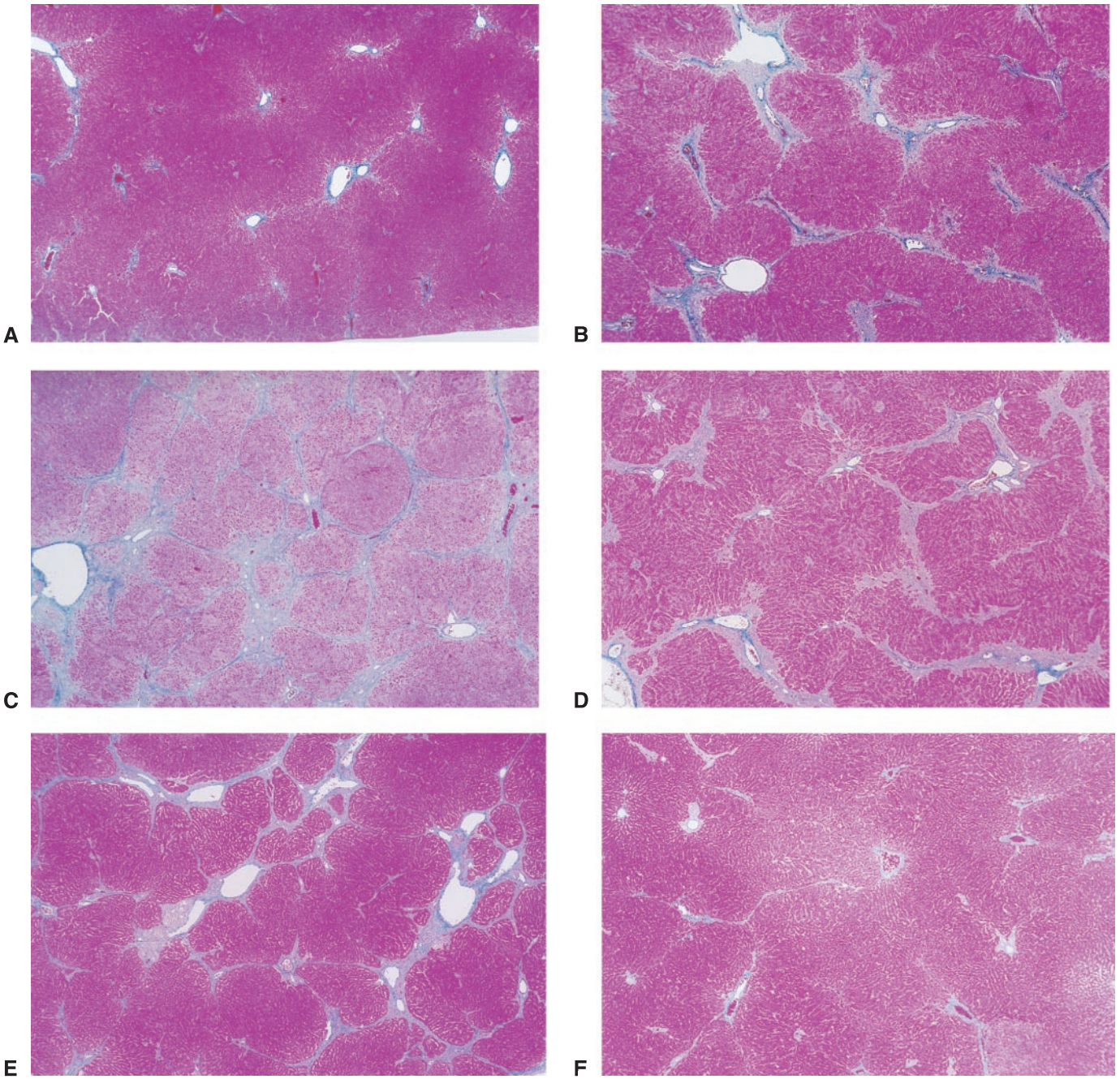

FIG. 2. Liver histology. Liver fibrosis was induced in male Sprague–Dawley rats by administration of TAA. Liver tissue was fixed in 10% neutral-buffered formalin, embedded in paraffin, and stained with Azan (magnification ×64). Appearance of the liver of rats after TAA administration for 3 weeks (A), 6 weeks (B), 8 weeks (C), and 10 weeks (D). Connective tissues in the liver began to spread out after TAA treatment for 6 weeks. (E) and (F) show the appearance of the livers of rats at the end of the 15th week in Experiment 2 without and with injection of rhHGF at 0.3 mg/kg/day, respectively.

indicated that rhHGF pathologically ameliorated liver fibrosis caused by TAA and that administration by infusion pump was more effective than bolus administration.

Next, we measured the area of hepatic fibers of the bolus group (Experiment 1) and the infusion group (Experiment 3) from liver sections by image processing. At the end of the 10th week, the area of fibers was 17.51 ± 2.58% (data not shown). As shown in Fig. 3, the area of fibers was reduced by 34% by rhHGF administration in Experiment 1, and was significantly reduced by 43% in Experiment 3. There were no significant differences in liver weight between control and rhHGF groups. These results indicated that the content of fibers in the whole liver was reduced by rhHGF, especially when it was injected by infusion pump.

To clarify the effects of rhHGF on collagen reduction in the cirrhotic liver, we analyzed the mRNA level of collagen α2(I) and α1(IV) in TAA-treated rat liver by Northern blotting analysis in Experiments 1 and 3. In both experi-

TABLE 1. Summary of histological analysis of the effects of rhHGF on TAA-induced liver cirrhosis

Experiment 1				Experiment 2				Experiment 3			
Group	NT	Control	HGF	Group	NT	Control	HGF	Group	NT	Control	HGF
N	10	12	12	N	10	12	12	N	10	11	11
Fibrosis				Fibrosis				Fibrosis			
—	10	0	0	—	10	0	1	—	10	0	2
+	0	6	9	+	0	7	8	+	0	5	7
++	0	6	3	++	0	5	3	++	0	6	2*

*$P < 0.05$ in comparison with control group by Armitage χ^2 test. Abbreviations: —, no abnormalities detected; +, moderate; ++, severe.

ments, the level of collagen α2(I) mRNA in rat liver was significantly increased by TAA treatment up to 2.8- and 3.8-fold, respectively (Fig. 4B). The administration of rhHGF in Experiments 1 and 3 significantly decreased the level of collagen α2(I) mRNA down to 46% and 55% of the level in the TAA-treated control groups, respectively (Fig. 4B). The mRNA level of collagen α1(IV) in rat liver was also increased 1.8- and 4.8-fold by TAA treatment in Experiments 1 and 3, respectively, and its expression in each experiment was significantly decreased down to 56% and 47% (Fig. 4C). These results showed that the neogenesis of collagens was suppressed by rhHGF.

We then measured the level of TGF-β1 mRNA in TAA-treated rat liver, since TGF-β1 is the most potent cytokine involved in collagen synthesis [5]. In Experiments 1 and 3, the level of TGF-β1 mRNA was significantly increased 2- and 4-fold by TAA treatment, while it was significantly decreased to 57% of the control level by administration of rhHGF (Fig. 4D). These observations indicated that the elevation of the TGF-β1 mRNA level by TAA treatment in the cirrhotic liver was significantly suppressed by administration of rhHGF.

Next, we measured the BrdU labeling index in the cirrhotic liver with or without rhHGF to clarify whether single administration of rhHGF can trigger hepatocyte DNA synthesis in cirrhosis *in vivo*. As shown in Fig. 2, B–D, liver cirrhosis was already formed in the rats after 6 weeks of TAA treatment. The labeling index of hepatocytes in the rhHGF-injected group was significantly higher than that of the control at 24 hr following rhHGF injection (Fig. 5A). An increase in the number of BrdU-positive hepatic nuclei was randomly observed in the liver treated with rhHGF (Fig. 5B). These results showed that single administration of rhHGF induces the BrdU labeling index in hepatocyte in the cirrhotic liver induced by TAA.

DISCUSSION

TAA-induced hepatic cirrhosis in rats is very similar histologically to human cirrhosis [20]. To evaluate the effects of rhHGF on TAA-induced liver cirrhosis, we investigated the pathology of TAA-treated liver tissue as well as collagen and TGF-β1 mRNA expression in the

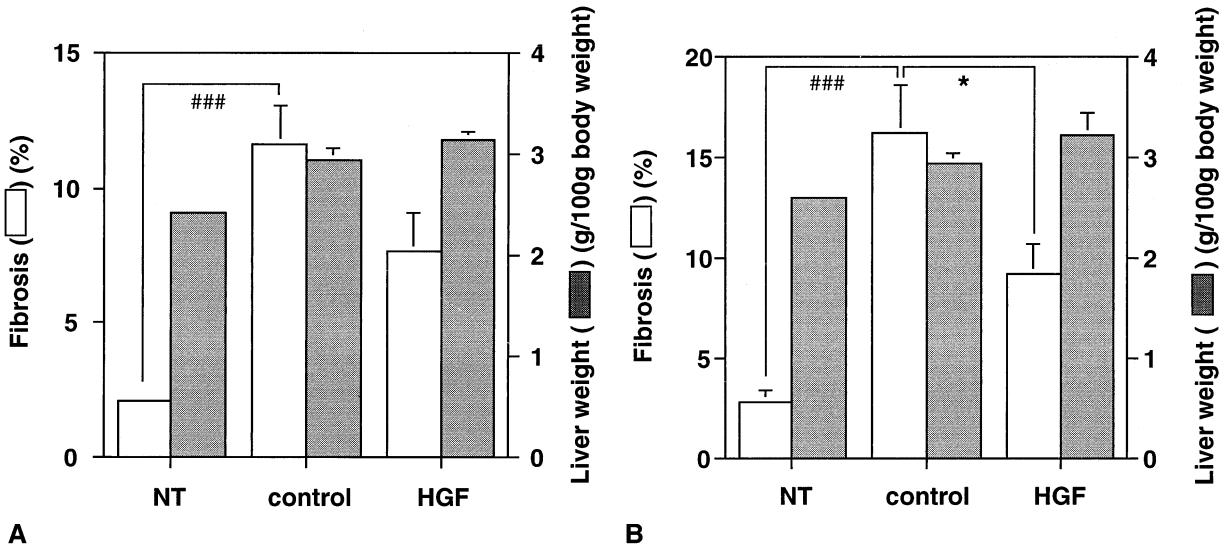

FIG. 3. Effects of rhHGF on fibrosis and liver weight in TAA-administered rats. Rats were treated with TAA for 10 weeks. Recombinant hHGF (0.3 mg/kg/day) or saline was intravenously administered in a bolus from the 14th to the 17th week (A. Experiment 1: NT, N = 10; control, N = 12; HGF, N = 12) or by an infusion pump from the 14th to the 15th week (B. Experiment 3: NT, N = 10; control, N = 11; HGF, N = 11). White bars indicate the area of fibrosis in liver sections measured by Mac SCOPE, Version 2.00. Hatched bars indicate liver weight. Each value represents the mean ±SEM. $^{###}P < 0.001$ in comparison with NT rats; $^{*}P < 0.05$ in comparison with control rats, by Student's *t*-test.

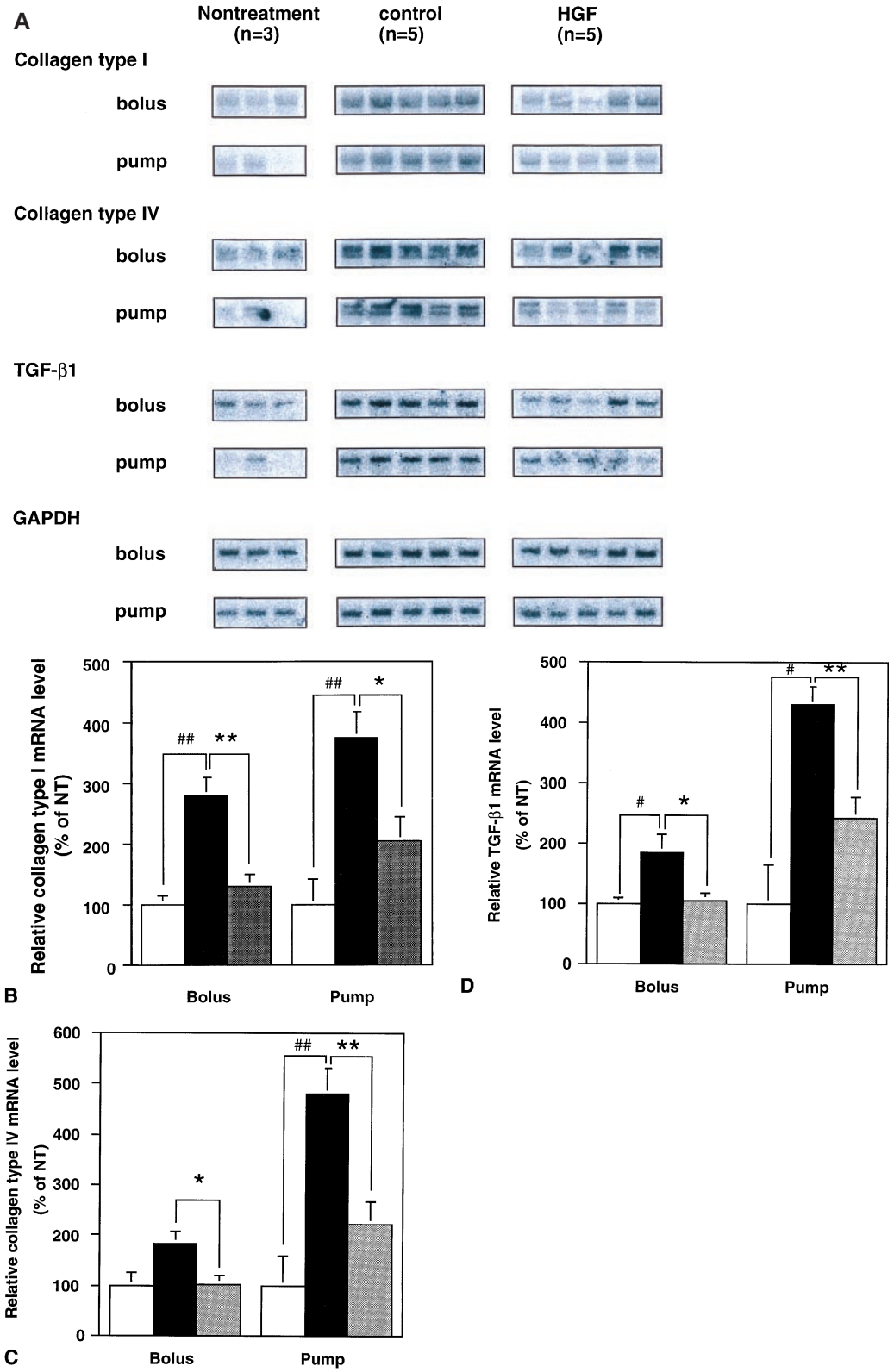

FIG. 4. Northern blotting analysis of liver poly (A) +RNA in Experiment 1 (bolus) and Experiment 3 (pump). (A) Collagen α2 (I), collagen α1 (IV), TGF-β1, and GAPDH mRNA expression. Aliquots of 5 μg of poly (A) + RNA from HGF-administered rats (N = 5), control rats (N = 5), and NT rats (N = 3) were electrophoresed in 1% agarose gels and transferred onto nylon membranes. The membranes were hybridized ^{32}P-labeled collagen α2 (I), collagen α1 (IV), TGF-β1, and GAPDH cDNA probes. After hybridization, the membranes were washed and exposed to imaging plate and analyzed using a BAS2000. The levels of (B) collagen α2 (I) mRNA, (C) collagen α1 (IV) mRNA, and (D) TGF-β1 mRNA expression shown in (A) were normalized relative to the GAPDH mRNA level. Results are expressed as means ±SEM and as percentages of the mean value of the NT group (100%). White, black, and hatched bars indicate mRNA from NT, control, and rhHGF-administered rats, respectively. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ in comparison with NT rats; $^{*}P < 0.05$ and $^{**}P < 0.01$ in comparison with control rats, by Student's *t*-test.

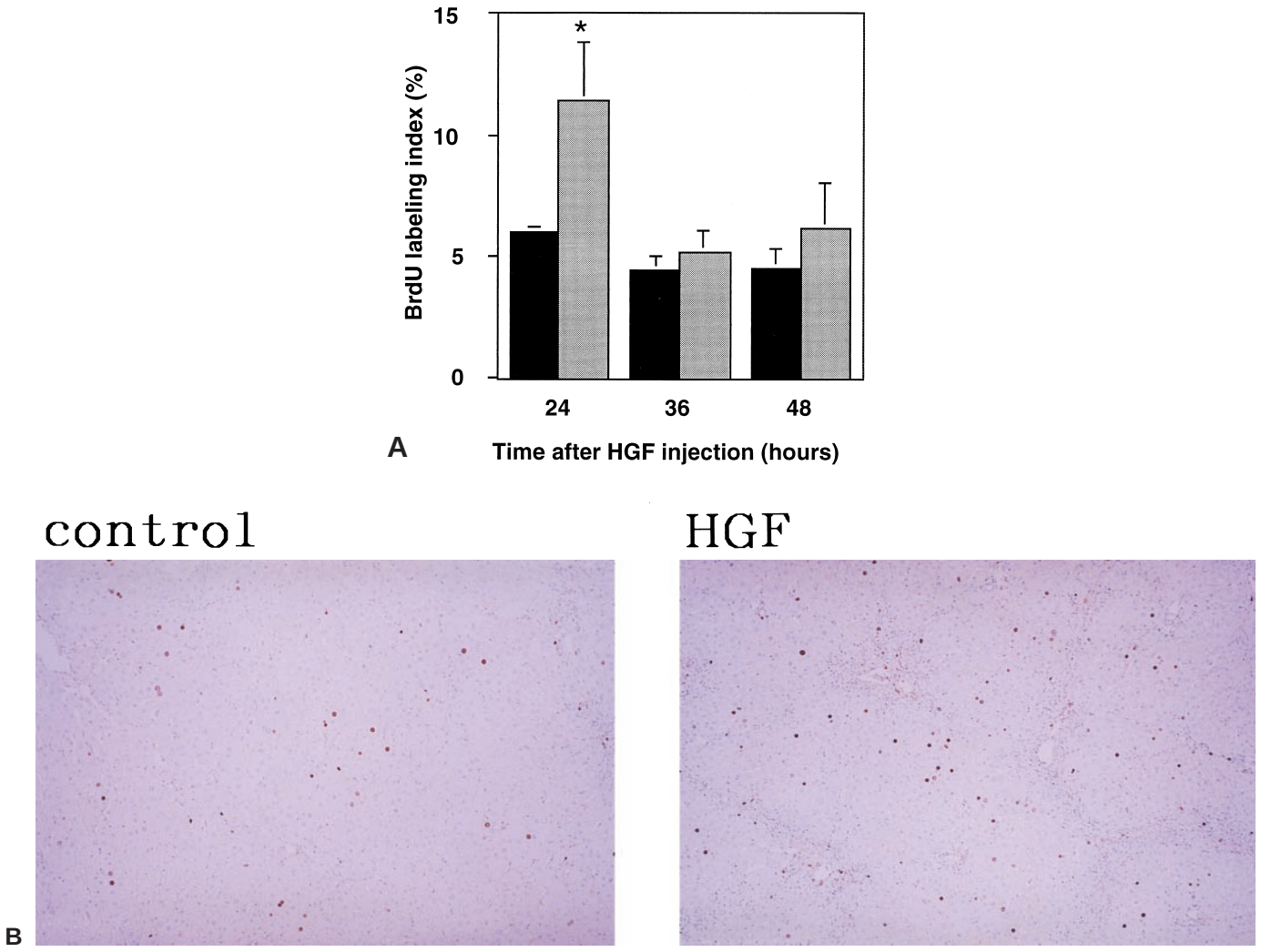

FIG. 5. Effects of a single i.v. injection of 0.3 mg/kg of rhHGF on the BrdU labeling index. (A) The labeling index in control rats (black bars) and in rhHGF-administered rats (hatched bars) indicates the percentage of BrdU-positive hepatocyte nuclei determined from 1500 hepatocytes in 5 different lobes of 6 rats. *$P < 0.05$ in comparison with TAA-treated control rats by Mann–Whitney U test. (B) Light micrographs of liver sections from TAA-treated control rats or TAA-treated rhHGF-administered rats 24 hr after buffer or rhHGF administration. The rats were administered buffer or rhHGF, and pulsed with BrdU *in vivo*. Liver sections were stained with anti-BrdU antibody. (Magnification ×90).

TAA-treated liver with or without rhHGF. The administration of rhHGF significantly ameliorated liver fibrosis histologically (Fig. 2). The area of fibrosis in the liver measured by an image analysis system was reduced to 57% compared to the control group by rhHGF infusion and was significant, whereas the increase in liver weight/body weight was only 9% compared to the control group. These results indicated that the total amount of fiber in the whole liver was reduced by rhHGF. Some studies have shown that HGF suppresses the formation of liver fibrosis when administered concurrently with some hepatotoxins which cause hepatic cirrhosis [8, 19, 31]. Yasuda *et al.* reported that HGF reduced hydroxyproline content/g liver to about half of that in the control rat liver when HGF was administered after the formation of liver cirrhosis by DMN. However, the liver weight of HGF-administered rats was approximately double that of control rats [19]. These results indicate that HGF administration did not reduce the fiber contents in the whole liver. In the present study, we demonstrated that rhHGF ameliorates liver cirrhosis even if administered after complete formation of cirrhosis and reduces the amount of fiber in the whole liver.

We investigated i.v. administration of rhHGF in bolus and/or by infusion to compare these two methods. From the results of the histological analysis, amelioration of liver cirrhosis by rhHGF appears to be more effective in the infusion group (Experiment 3) than in the bolus group (Experiments 1 and 2) (Table 1). We speculated that the reason was as follows. The half-life of rhHGF in rat was very short (about 4 min) and rhHGF showed rapid clearance from rat plasma when administered in bolus [32]. On the other hand, the concentration of rhHGF in plasma was calculated to be sustained at 3.7 ng/mL when rhHGF was injected by infusion pump at the concentration of 100

μg/kg/day.* It is reported that the half-maximal mitogenic activity of rhHGF *in vitro* is at the concentration of 3.5 ng/mL [33]. These results suggest that liver is exposed to sufficient rhHGF for a longer period in the infusion than in the bolus group. Uematsu *et al.* reported that HGF concentration in liver was sustained longer when administered by infusion than by i.v. bolus [34]. These observations suggest that continuous injection of rhHGF could be more effective on liver cirrhosis when used clinically.

Many studies have shown that levels of collagen type I, III, and IV increase during liver fibrosis/cirrhosis [35]. It has also been reported that TGF-β1 up-regulates these procollagen mRNA levels [6] and activates stellate cells [4, 36]. Therefore, we investigated the effects of rhHGF on the expression of collagen and TGF-β1 mRNAs in the bolus and infusion groups. TAA treatment increased, while rhHGF significantly reduced, collagen α2(I) and α1(IV) mRNA expression. These results reflected the histological observations (Fig. 2, E and F). We also found that TGF-β1 mRNA expression was clearly decreased by rhHGF administration after TAA pretreatment, as shown in Fig. 4D. These observations suggested that rhHGF ameliorates liver fibrosis by reducing *de novo* synthesis of collagens. The mRNA level of collagens and TGF-β1 in controls was higher in the infusion group (Experiment 3) than in the bolus group (Experiment 1) (Fig. 4, B–D). This might result from the difference of time interval after TAA treatment. The liver in Experiment 3 was collected at the end of the 15th week and that in Experiment 1 at the end of the 17th week, 2 weeks later than in Experiment 3. It is tempting to speculate that since cirrhosis induced by TAA was gradually ameliorated after the cessation of TAA treatment, the mRNA level of collagens and TGF-β1 in Experiment 1 was lower than that in Experiment 3. A similar result showed that the fibrosis area was smaller in Experiment 1 than in Experiment 3 (Fig. 3, A and B). We also detected a reduction in TGF-β1 mRNA by rhHGF administration in DMN-induced liver cirrhosis (data not shown). The reduction in TGF-β1 mRNA was reproducible. Future work should attempt to confirm the posttranslational level of TGF-β1 reduction by rhHGF.

To determine whether rhHGF stimulates regeneration of the cirrhotic liver is important for an evaluation of its clinical potency. We showed here that rhHGF stimulates DNA synthesis of hepatocytes in liver cirrhosis (Fig. 5). In this study, even a single administration of rhHGF in bolus showed a significant peak of the BrdU labeling index at 24 hr in cirrhotic liver. If rhHGF is administered repeatedly, the peaks of the labeling index appear intermittently, i.e. continuous stimulation of DNA synthesis will occur in hepatocytes. Kaibori *et al.* also reported that rhHGF stimulates the DNA synthesis of hepatocytes after partial hepatectomy of the cirrhotic liver caused by TAA injection for 10 weeks [37]. These results indicated that rhHGF could be a trigger for regeneration of the cirrhotic liver. They also indicated that fibers in the liver were somewhat reduced by the increase in normal hepatocytes by rhHGF. We propose two mechanisms for the reduction of fibrosis by rhHGF administration: a decrease in fibrogenesis through the down-regulation of collagen and TGF-β1 expression at the transcriptional and/or posttranscriptional level; and an increase in hepatic regeneration induced directly by rhHGF and indirectly by TGF-β1 reduction, since TGF-β1 has been shown to inhibit the proliferation of rat hepatocytes *in vitro* [38] and *in vivo* [39]. It has not yet been confirmed how rhHGF reduces TGF-β1 expression. To clarify this point, cell fractionation and/or *in situ* hybridization will be useful, since the major sources of TGF-β1 in the cirrhotic liver are thought to be Kupffer cells [40], activated stellate cells [4], endothelial cells [41], and infiltrating inflammatory cells rather than hepatocytes.

Some recent studies have shown that tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) plays an important role in fibrosis [42]. Our preliminary study showed, however, that TIMP-1 mRNA expression was not affected by rhHGF administration in this system (data not shown). In another report, Matsuda *et al.* showed that HGF stimulated collagenase activity in DMN-treated rat liver [18]. Our preliminary studies in the TAA-treated rat liver showed that the matrix metalloproteinase-2 (MMP-2) mRNA level was suppressed by rhHGF administration (data not shown). To clarify whether collagenase activation by rhHGF causes the amelioration of fibrosis, time–course studies of mRNA expression levels and the activities of collagenases and TIMP during rhHGF administration will be useful.

In conclusion, rhHGF suppressed neogenesis of collagens at the level of mRNA expression. It was possibly regulated by the reduction in TGF-β1 mRNA expression. Recombinant hHGF also stimulated hepatocyte growth in the cirrhotic liver. We speculate that the growth stimulation was directly induced by rhHGF itself, as well as partly and indirectly by the decrease in TGF-β1. Through these effects of rhHGF, completed fibrosis in the cirrhotic liver was significantly ameliorated histologically. Recombinant hHGF also improves various hepatic functions such as protein synthesis and serum content of liver-specific cytosolic enzymes *in vivo* [15, 16, 18, 37]. We have detected the formation of an rhHGF and anti-rhHGF antibody complex in plasma when rhHGF was administered to rats.† The effects of rhHGF might be reduced by the formation of the immune complex in rats. Although we showed that 0.3 mg/kg/day of rhHGF was effective in ameliorating liver cirrhosis in rats in the present study, it is expected that rhHGF is effective at a lower dose in human than in rats. All these results suggest that rhHGF may be clinically useful for treatment of hepatic cirrhosis.

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