Changes in growth factor and cytokine mRNA levels after heptectomy in rat with CCl₄-induced cirrhosis

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LIVER SURGERY IS LARGELY used in humans as a consequence of the increased frequency of primary hepatocellular carcinoma (HCC) particularly in post-hepatitis C cirrhosis. Surgical resection is a potentially curative therapy, but the outcome of a radical hepatic resection is usually very poor, with a significant risk of liver failure (9). The postoperative recovery depends on the regenerative ability of the remaining liver. Previous studies (3, 18) have reported conflicting data regarding liver ability to regenerate after partial hepatectomy (PH) in animals and humans with cirrhosis. Recently, it has been reported (2, 21) that in rats with hepatic damage liver regeneration after resection did occur but was impaired and delayed compared with that observed in healthy animals. However, the mechanisms responsible for this impaired regeneration and the cross talk of the implicated factors still remain unclear.

Liver regeneration after PH is defined as an orchestrated response that is induced by specific stimuli, and it involves numerous and sequential changes in gene expression (8, 20). Many growth factors and cytokines, particularly hepatocyte growth factor (HGF) (23), transforming growth factor (TGF)–α (19, 30) and –β (28), interleukin (IL)–6, and tumor necrosis factor (TNF)–α (20) appear to play important roles in this process. Previous investigations have revealed that HGF is the most potent mitogen for normal hepatocytes, with early increased mRNA and plasma levels after liver injury and PH. Moreover, HGF elicits its biological effects through its binding to a specific receptor known as c-Met (36). It is also well established that in liver after various injurious processes cytokines are important components of the early signaling pathways leading to specific responses in neighboring or more distant target cells (10, 31). A recent study (25) has indicated the importance of these factors in the pathogenesis and progression of chronic liver diseases, with a significant activation of cytokines at the mRNA level in end-stage cirrhosis.

The aim of the present study was to quantify in rat mRNA levels for growth factors (TGF–α, HGF, and its receptor) and proinflammatory cytokines (IL–1β, IL–6 and TNF–α) to determine the relationships between these factors and to understand how they may account for the impaired regenerative process observed in cirrhotic liver.

MATERIALS AND METHODS

Animals. Adult male Sprague-Dawley rats (Charles River), weighing ~200 g were kept under a 12:12-h light-dark cycle and fed with standard laboratory chow and water ad libitum. They were kept at rest for at least 1 wk before the start of these studies. All animal manipulations were performed according to the recommendations of a local ethical committee and under the supervision of authorized investigators.

CCl₄-induced cirrhosis. Cirrhosis was induced according to a protocol modified from Proctor and Chatamra (27). Pheno- barbital at a concentration of 35 mg/dl was added to the drinking water because it has been shown to increase liver sensitivity to CCl₄ by increasing the activity of cytochrome P-450.

After a 2-wk exposure to phenobarbital for maximal induction of liver enzyme, CCl₄ administration was given weekly intrahepatically under light ether anesthesia. The vehicle was olive oil because, in acute CCl₄ poisoning, this oil is considered to be less harmful than other oils to the liver. The initial

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rats were submitted to a two-thirds hepatectomy. In hepatic fibrosis and/or cirrhosis was evaluated at the time of surgery by analysis of serial tissue sections of liver.

Surgical procedures. Rats were anesthetized with ether, and a two-thirds hepatectomy was performed after midline laparotomy according to the method of Higgins and Anderson (12). PH was performed by excision of the median and left lateral lobes of the liver. A biopsy specimen from the resected liver lobe was stored in a 10% buffered formaldehyde for subsequent histological examination.

Two experimental groups were studied. In group 1, healthy rats were submitted to a two-thirds hepatectomy. In group 2, PH was performed after 16 wk of CCl₄-induced cirrhosis. In both groups, four rats were killed at 6 h, 12 h, day 1, day 2, day 3, or day 6 after surgery. The liver was removed, one sample was stored at −80°C, and the nonparenchymal cells (NPC) were isolated from the residual liver.

Histopathological studies. Macroscopic appearance and microscopic examination of liver sections verified the presence of fibrosis and cirrhosis in rats treated with CCl₄ phenobarbital. After routine paraffin wax processing, trichrome blue-stained sections were prepared and the degree of liver damage was evaluated.

The biopsy specimens were examined by a liver pathologist who analyzed four main areas of fibrosis deposits as previously described by Chevallier et al. (4): centrilobular vein (CLV), perisinusoidal space (PS), portal tract (PT), and septa, taking into account the number of NS (normal) and WS (wine) of the septa. The histological score is detailed as follows: CLV, 0 = normal, 1 = moderately thickened, 2 = markedly thickened (annular aspect of vein wall with fibrosis extensions between hepatocytes); PS, 0 = normal, 1 = localized fibrosis, 2 = diffuse fibrosis; PT, 0 = normal, 1 = enlarged without septa, 2 = enlarged with septa, 3 = cirrhosis; NS, 0 = absence, 1 = <6 septa/10 mm, 2 = >6 septa/10 mm, 3 = nodular organization; WS, 1 = thin and/or incomplete, 2 = thick and loose connective matrix, 3 = very thick and dense connective matrix.

Fibrosis was graded on a scale of 0 to 2. Total score of hepatic fibrosis (SHF) was calculated according to

\[\text{SHF} = \text{CLV} + \text{PS} + \text{PT} + 2(\text{NS} + \text{WS})\]

For each biopsy, the selected value corresponded to the most representative lesion of the biopsy sample.

Preparation of NPC. Heparin (500 UI; Laboratoire Leo, Paris) in 1 ml of 0.9% saline solution was injected into the saphenous vein. The portal vein was then cannulated with a catheter, and the hepatic artery was ligated. The liver was immediately perfused with 10 ml of Gey's balanced salt solution (GBSS), excised, and removed from the peritoneal cavity. It was then transferred into a perfusion chamber to be kept in a cold chamber. The liver samples were washed with GBSS, and centrifuged at 600 g for 10 min. The final pellet contained ~0.5–1.10 × 10⁵ NPC.

Quantitative RT-PCR. Total RNA was extracted from a frozen liver sample or from NPC according to a one-step method. Briefly, cells were lysed with a denaturing solution containing 4 M guanidium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, and 100 mM 2-mercaptoethanol. Total cellular RNA was obtained by sodium acetate-phenol-chloroform extraction, and RNA concentration was measured by spectrophotometry at 260 nm. The RNA preparations were controlled by agarose minigel electrophoresis with visualization of 18S and 28S ribosomal RNA bands after ethidium bromide staining. Two micrograms of total RNA from each sample were reverse-transcribed in 60 µl containing 12 µl of 5× buffer, 6 µl dNTP (10 mM each), 120 units RNasin (Promega), 400 units of Moloney murine leukemia virus RT (Promega), and 500 pmol random hexamer primers (Pharmacia). The mixture was incubated for 60 min at 37°C, followed by heating at 95°C for 5 min. Ten microliters of the RT product were then used for PCR amplification. They were brought to a final volume of 50 µl containing 5 µl of 10× PCR buffer, 1 µl dNTP (10 mM each), 5 µl MgCl₂ (25 mM), 1.25 units Taq polymerase (Promega), 1 µCi [γ³²P]dATP, and 100 pmol of 3′- and 5′-specific primers (Eurogentec).

Primers were chosen to have 50–60% GC content. Primer sequences were chosen from separate exons of the rat genes so that RNA-associated PCR products could readily be distinguished from any PCR products induced by contaminating genomic DNA. These sequences and the sizes of the expected PCR products are summarized in Table 1. Preliminary experiments (data not shown) were made to select the number of cycles that would give a linear relationship between the number of cycles and the amount of PCR product.

After an initial denaturation at 94°C for 4 min, each PCR cycle consisted of a denaturation step at 94°C for 30 s, a primer annealing step at 58°C or 60°C for 1 min, and an elongation step at 72°C for 1 min in an Omnigene thermal cycle (Hybaid). The strictly prokaryotic chloramphenicol acetyltransferase (CAT) RNA was used as an exogenous control. A standard curve was constructed according to the protocol described by Jean et al. (13). Furthermore, an RT-PCR for the housekeeping β₂-microglobulin gene provided an endogenous standard to measure the loading homogeneity of different mRNA.

Aliquots of PCR products were electrophoresed in a 6% polyacrylamide gel and visualized under ultraviolet light after ethidium bromide staining. The gels were dried and exposed onto X-ray films. Autoradiograms were analyzed by computerized densitometric scanning with the use of Biocom equipment mostly composed of a charge-coupled device (CCD) camera (Canon) and Lechopt software. For amplification comparison, the values obtained by densitometric scanning were expressed in arbitrary units and normalized as a function of the amplified CAT signal.

SDS-PAGE and Western blot analysis. Frozen tissues were sliced very thinly and homogenized in lysis buffer with freshly added inhibitors. Lysates were clarified by centrifugation at 12,000 g for 20 min at 4°C. The supernatants were collected, and a protein assay was performed with the use of a Bio-Rad protein assay kit (Bio-Rad Laboratories). Twenty micrograms of total protein from each sample were loaded and separated onto 8% SDS-polyacrylamide gels. After electrophoresis, proteins were transferred to an Optitran nitrocellulose membrane (Schleicher & Schuell, Céa-lab, France) and analyzed by immunoblotting. Membranes were blocked with
Table 1. Oligonucleotide primers and sizes of expected PCR products  

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<td>GGT GAT ACT CCA TCT CCA AG</td>
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<td>Cyclin D1</td>
<td>GCT TCT CAT GAA TCA AG</td>
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<tr>
<td>Cyclin E</td>
<td>GAA ACA TCT TCC TAA AC</td>
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<td>TNF-α</td>
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<td>CAT</td>
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<td>β-2 Microglobulin</td>
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<td>β-2 Microglobulin</td>
<td>CTG TCT TCC TAC GCA</td>
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Histological findings. The condition of liver tissue was examined in all treated rats before PH. Macroscopically, the livers were inelastic and nodular. Microscopically, SHF was evaluated as described in MATERIALS AND METHODS.

In control animals, no abnormalities were seen (Fig. 1A) and the SHF was 0. A SHF between 2 and 4 revealed a moderate fibrosis of CLV and PT (Fig. 1B). Between 6 and 10, it corresponded to a strong fibrosis with a cirrhosis not fully developed (Fig. 1C) and at 10 the cirrhosis was complete, with regenerative nodules surrounded by broad strands of fibrous tissue (Fig. 1D).

As seen in Table 2, when we compared the SHF with the CCl4 intoxication period, we found that if 13 wk of treatment with CCl4 were necessary for the production of liver fibrosis (SHF = 4), 14–16 wk were required to induce an irreversible micronodular cirrhosis in >75% of treated rats. Therefore, we decided to perform the RT-PCR for cytokine and growth factor mRNA only from livers whose SHF was >6.

Markers of the cell-cycle progression as control of the impaired regeneration after PH in cirrhotic rats. The mRNA levels for cyclins B, D1, and E (Fig. 2) were determined in rat liver after a PH that was either preceded or not by a CCl4 treatment. Before hepatectomy, cyclins B, D1, and E were barely detectable in normal and cirrhotic rats. In normal rats, a strong induction of the all-cyclin mRNAs began at 12 h and peaked at day 1 posthepatectomy, whereas in cirrhotic rats a significant decrease in cyclin B, D1, and E mRNA levels was observed at 6 h, 12 h, and day 1, followed by a delayed peak at day 2. The difference was most pronounced for cyclin E, whose mRNA levels were significantly decreased in early times with a delayed peak at day 3. Interestingly, for thymidine kinase, a key regulatory enzyme of DNA synthesis, we observed in normal rats mRNA peaks at day 1 posthepatectomy, whereas in CCl4-treated rats this level was decreased, with a delayed peak at day 3 postresection.

Expression of growth factors during liver regeneration after PH in normal or cirrhotic rats. After PH, in NPC, HGF mRNA increased as soon as 6 h, remained high at 12 h and day 1, and then declined to the basal value at days 2–6. In CCl4-treated rats, we did not observe any significant difference for the expression of this growth factor (Fig. 3A).

In whole liver cells, expression of the c-Met mRNA increased at 6 h posthepatectomy and next decreased below the initial level from 12 h to day 6. In contrast, after CCl4 treatment, this mRNA level strongly decreased at 6, 12 h, and day 1 postsurgery, then transiently increased at day 2 and finally remained at a lower than normal level at days 3–6 (Fig. 3B).

TGF-α basal mRNA level in rats with a CCl4-induced cirrhosis was significantly increased before resection, then peaked early at 6 h posthepatectomy, and remained increased until day 6 (Fig. 3C) compared with levels noted after hepatectomy alone, whose peak occurred at day 1 with a decrease at days 2–6.

Expression of c-Met during liver regeneration after PH in normal or cirrhotic rats. To support the results observed at the mRNA level, we examined the expres-
sion of the c-Met receptor at the protein level by Western blot analysis with an antibody directed against the COOH terminus of c-Met p140 of mouse origin. As shown in Fig. 4, variable levels of 140-kDa c-Met protein were detected after hepatectomies performed at different times on healthy or cirrhotic rats. After CCl4 treatment, this protein level decreased at early times after surgery compared with levels observed after hepatectomy on healthy rats.

Fig. 1. Histopathological findings in liver biopsy from rats included in present study. Liver sections were stained with trichrome blue. Results from healthy rats (A), CCl4-treated rats with a moderate fibrosis of centrilobular vein and portal tract (B), and CCl4-treated rats with a strong fibrosis (C) or with a complete cirrhosis with regenerative nodules (D) are shown. Magnification = ×2.5.

Table 2. Score of hepatic fibrosis and CCl4 intoxication period

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<tr>
<td>SHF</td>
<td>4±0</td>
<td>16±5</td>
<td>12±2</td>
<td>19±4</td>
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Values are means ± SD. A score of hepatic fibrosis (SHF) between 2 and 4 revealed a moderate fibrosis, between 6 and 10 it corresponded to a strong fibrosis with a cirrhosis not fully developed, and at 10 the cirrhosis was complete. Fourteen to sixteen weeks of CCl4 treatment were required to induce irreversible micronodular cirrhosis.
Cytokine expression in NPC during liver regeneration after PH in cirrhotic rats. In control rats after hepatectomy, the mRNA levels for the proinflammatory cytokines increased, with a peak at 12 h for IL-1β (Fig. 5A) and IL-6 (Fig. 5B) and with a peak at 6 h for TNF-α (Fig. 5C), and then gradually decreased to the preoperative level at day 3 after surgery. In the CCl₄-treated animals, the mRNA levels of these three cytokine mRNAs were significantly lowered in the early times (6 h and 12 h posthepatectomy) with a delayed peak at day 1 for IL-1β and TNF-α and day 3 for IL-6. However, in the CCl₄-treated rats IL-1β mRNA levels remained strongly elevated at days 2 and 3.
DISCUSSION

To understand the mechanisms involved in cirrhotic liver regeneration after PH and control thereof by growth factors and cytokines, we have analyzed the mRNA levels of some of these mediators at different time points after surgery in rat with CCl4-induced cirrhosis.

Hepatotoxic agents commonly used for induction of liver fibrosis and cirrhosis include CCl4, dimethylnitrosamine, thioacetamine, and D-galactosamine. We have selected CCl4 because it has been reported to promote a cirrhosis that is remarkably similar to alcoholic cirrhosis in humans (2). However, numerous protocols that differ in intoxication times and/or administration routes have been described. The unpredictable response of animals to CCl4 makes it difficult to produce a consistent model of cirrhosis with this drug. Most often, CCl4 administration to rats must be maintained for up to 3 wk to produce a steatosis and for up to 10 wk more to

Fig. 3. Quantitative RT-PCR analysis of growth factor mRNA after a two-thirds hepatectomy performed on healthy (●) rats (lanes 1, 3, 5, 7, 9, 11, and 13) or cirrhotic (○) rats (lanes 2, 4, 6, 8, 10, 12, and 14) livers. A: hepatocyte growth factor (HGF). mRNA was extracted from nonparenchymal cells (NPC). B and C: c-Met and transforming growth factor-β1 (TGF-β1), respectively; mRNA were extracted from whole liver. Results are given in arbitrary units (AU) and normalized with exogenous standard CAT. Values are means ± SD of triplicate experiments with n = 4 rats per time point. *P < 0.05, **P < 0.01, and ***P < 0.001, respectively, between cirrhotic and normal rats (Student’s t-test).

Fig. 4. Top: representative Western blot of c-Met expression after hepatectomy performed at 6 h, 12 h, day 1, day 2, day 3, and day 6, on healthy (lanes 1, 3, 5, 7, 9, 11, and 13) or cirrhotic (lanes 2, 4, 6, 8, 10, 12, and 14) livers. Immunoreactive band indicates 140-kDa β-subunit of c-Met. Equal loading of gel was confirmed by staining membranes after Western blot analysis with ponceau S (data not shown). Bottom: densitometric values for MET protein expressed in arbitrary units on healthy (●) or cirrhotic (○) rats. For each time point, protein extracts (n = 4) were pooled and then used for analysis.
obtain fibrosis or cirrhosis. After 10–12 wk of CCl₄ treatment, liver regeneration after a resection has been described to be impaired and delayed (2, 18) as inferred from [³H]thymidine incorporation into DNA as a marker of cell proliferation. We then expected that this impaired regenerative process would be further reflected by an altered expression of cell cycle-associated genes, such as those for cyclins that are assumed to regulate cell cycle progression in the regenerating liver (16). Therefore, after 16 wk of CCl₄-induced cirrhosis as confirmed by histological examination, we studied the expression patterns of the cyclins B, D1, and E, which are the essential components of the cell cycle machinery. There was a delayed and decreased expression of the mRNA for these cyclins, notably for cyclin E. Likewise, the TK mRNA levels that are strictly proportional to the levels of TK activity during liver regeneration (33) showed an impaired expression after PH in our cirrhotic animals, with a delayed peak at day 3 postresection. Together, these results provide evidence for the impaired and delayed hepatic regeneration in our model of CCl₄-induced cirrhosis.

Before surgery, CCl₄-induced cirrhosis was associated with a significant increase in the intrahepatic mRNA level for TGF-α. This observation reflects the chronic hepatic regeneration that constitutes an important component of the cirrhotic process (25) and focuses on the role of this growth factor in the pathogenesis of hepatic regeneration in cirrhosis.

After PH in healthy rats, changes in intrahepatic HGF, TGF-α, and c-Met mRNA levels showed a classical pattern, as already described by several studies (8, 20). The peak of c-Met mRNA expression was earlier, at 6 h, preceding that of HGF.

In the CCl₄-treated rats after PH in NPC, no significant change of HGF mRNA level was observed compared with that observed in healthy controls. In contrast, the significant decrease of both mRNA and protein levels of the HGF receptor, i.e., c-Met, is correlated with the impaired regeneration of cirrhotic liver. Therefore, despite a normal expression of HGF, the delayed proliferative process might be related to an impaired c-Met expression.

Our results suggest that after hepatectomy on cirrhotic liver, local downregulation of c-Met mRNA and protein is crucial to regenerate because it decreases the activity of this receptor/ligand system with a lower targeting HGF action toward hepatocytes. Our observation is in complete agreement with a recent study that mentioned the lack of c-Met expression in fulminant hepatic failure livers associated with a poor response to exogenous HGF that normally promotes liver growth and regeneration (17). Other data have also been

Fig. 5. Quantitative RT-PCR analysis of proinflammatory cytokine mRNA in NPC after a two-thirds hepatectomy performed on healthy (●) rats (lanes 1, 3, 5, 7, 9, 11, and 13) or on cirrhotic (○) rats (lanes 2, 4, 6, 8, 10, 12, and 14). A: IL-1β. B: IL-6. C: tumor necrosis factor-α (TNF-α). Results are given in arbitrary units, and values are means ± SD of triplicate experiments with n = 4 rats per time point. *P < 0.05, **P < 0.01, and ***P < 0.001, respectively, between cirrhotic and normal rats (Student’s t-test).
be a critical factor in determining the site specificity of HGF, c-Met protooncogene seems the most active modifier of liver cell proliferation, and its induction might overexpression of c-Met in HCC and patient survival (26) has been noted, whereas there was no correlation between HGF concentration and this survival.

As also reported in humans by d’Errico et al. (6), our study suggests that, regardless of the level of liver HGF, c-Met protooncogene seems the most active modulator of liver cell proliferation, and its induction might be a critical factor in determining the site specificity of the receptor/ligand system and might be a useful prognostic marker for evaluating liver regeneration.

In the CCl$_4$-treated group after PH, we observed an early increase in TGF-$\alpha$ mRNA level that remained high up to day 6. Given that constitutive TGF-$\alpha$ expression in transgenic mice increases hepatocyte proliferation and leads to the development of hepatocellular tumors (35), the sustained high expression seen in cirrhotic rat after PH could likewise lead to the appearance of dysplasia or neoplasia in chronically regenerating liver. Our data support the hypothesis by Tomiya and Kenji (32) that TGF-$\alpha$ level may provide useful information for diagnosis of postcirrhosis HCC. Cytokines constitute a complex network of molecules involved in the regulation of the inflammatory response (22) and coordinate physiological and pathological processes in the liver, such as growth and regeneration (8), fibrosis, and cirrhosis (29).

In our study, a strong increase in the mRNA levels for IL-1$\beta$, IL-6, and TNF-$\alpha$, with a peak at 12 h, was observed in the isolated liver NPC after PH in control rats, which suggests that a local inflammatory response takes place in the liver after this injurious surgical process.

Moreover, hepatocyte DNA synthesis during liver regeneration has been found to be suppressed in IL-6-/- mice (5). It has also been proposed (20) that IL-6 and TNF-$\alpha$ are the major cytokines involved in liver regeneration and allow the G$_0$/G$_1$ and G$_1$/S transitions in the cell cycle. We did not find any modifications in the intrahepatic mRNA levels for IL-1$\beta$, IL-6, or TNF-$\alpha$ before PH in cirrhotic livers vs. controls. However, a previous study (11) has shown a significant increase in the plasma levels of cytokines, which was correlated with disease progress in liver cirrhosis and could be an early way to follow liver inflammation and fibrosis. Kamimura and Tsukamoto (14) has observed an expression of hepatic TNF-$\alpha$ mRNA that was three- to fourfold higher in ethanol-treated rats compared with controls. A similar observation was made by Napoli et al. (24) who found a significant activation of cytokines at the mRNA level in end-stage cirrhosis in humans. Our results may be explained by 1) our experimental model, which is quite different (CCl$_4$-induced cirrhosis), and 2) an analysis of isolated NPC instead of the whole liver cells. Therefore, the expression of cytokine mRNA in liver cirrhosis might depend on the status of liver disease at the time of evaluation and the origin of the secreted cytokines.

The IL-6 and TNF-$\alpha$ mRNA levels were significantly decreased and delayed in cirrhotic rats after PH. These significant intrahepatic cytokine decreases and the resulting delay in cell-cycle progression may account for the impaired regeneration observed in cirrhotic liver after surgery. In contrast, IL-1$\beta$, described as a downregulator of liver regeneration (1), did not show in cirrhotic livers a decrease of its mRNA level but only a delayed peak at days 1–3 compared with results observed after PH on normal livers. Thus the time course of IL-1$\beta$ expression is difficult to reconcile with the kinetics of regeneration.

In conclusion, our study suggests that, even though cytokines and growth factors play a role in the impaired regeneration of cirrhotic livers after PH, the low expression of the c-Met/HGF receptor along with a normal expression of its specific ligand may provide a further explanation for this delayed regenerative process. Our data now stress the role of the c-Met protooncogene as a critical partner of liver cell regeneration. This observation eventually could provide a basis for further investigations aimed at a therapeutic use of these factors.

REFERENCES


