MATERIALS AND METHODS

All study protocols were reviewed and approved by the University of Nagasaki Research Animal Resources and Animal Care Committee, and met both institutional and national guidelines.

Induction of fulminant hepatic failure

Male Lewis rats (SLC, Shizuoka) weighing 300 to 350g were used in this study. The animals were housed in plastic cages in a temperature and humidity-controlled room with 12-hour light/dark cycle, and were given standard rat chow and water. The fulminant hepatic failure rat was prepared according to the method described by one of the authors (S. E.) (21). Briefly, the common pedicle to the right liver lobes (24% liver) was ligated, and the two anterior liver lobes (68% liver) were removed using the standard Higgins and Anderson technique (22). After 18 hours, whole blood was harvested by aortic puncture, and FHF serum was collected. Normal serum was collected from non-operated rats. High levels of HGF and TGF-ß1 were shown in this model.

Hepatocyte isolation

Isolated hepatocytes were prepared by perfusion of inbred Lewis rats (150-200 g). Livers were treated with collagenase using a modification of the method described by Selgen (23). After enrichment through a Percoll gradient (Amersham Biosciences Corp., Piscataway, NJ), hepatocytes viability was nearly 90% by trypan blue exclusion.

Preparation of the bioreactor and perfusion model

$1x10^8$ hepatocytes were incubated with collagen-coated dextran microcarrier beads (Cytodex3, Amersham Biosciences) in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum at 37°C in 95% air and 5% CO_2. Four hours later, the microcarrier-attached hepatocytes were inoculated into the extra-fiber space of a hollow-fiber module (PE-G, Asahi Medical Co., Ltd, Tokyo). The hollow polyethylene fibers had an internal diameter of 330 µm, a pore size of 0.3 µm, and a membrane thickness of 50 µm. The system was composed of a pump, serum pool whose volume was 30ml, hollow fiber module, temperature controlled bath and oxygenator. The temperature of the circulated serum was maintained at 37°C.

Experimental design

Two BAL system groups were compared: the FHF BAL group (n=5 modules) and the control BAL group (n=5), in which either Lewis rat FHF serum or normal Lewis rat serum, respectively, were circulated through each module for 6 hours.

Isolation of RNA

Total ribonucleic acid (RNA) was extracted from the hepatocytes attached to the microcarriers before and after circulation, according to the instructions of the manufacturer of the Katorimox-14 RNA Isolation kit (Takara Bio Inc., Tokyo). Total RNA content was quantified by using a spectrophotometer (BioSpec-1600, Shimadzu Biotech, Kyoto).

Quantitative assessment of the relative expressions of mRNAs

For the reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, a one-step RT-PCR (Takara Bio Inc., Tokyo) kit was used. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal control. Albumin, glutathione S-transferase A1 (GST A1), cytochrome P450 1A2 (CYP 1A2), ornithine trans-carbamylase (OTC), and c-fos mRNAs were measured at the exponential portion of the curve, after PCR amplification. The optimal number of thermal cycles for GAPDH, albumin, GST A1, P450 1A2, OTC and c-fos mRNA was determined, respectively, 14, 12, 16, 20, 20 and 24. Forward and reverse primers are presented in Table II (24-29). A sample of mRNA (1 µg) was added to 5 mM $MgCl_2$, 1 mM dNTP, 0.8 U of RNase inhibitor, 0.1 U of AMV reverse transcriptase XL, 0.1 U of AMV-optimized Taq, and 0.2 µM 5' and 3' specific primers. PCR was performed using an automated thermal cycler (Gene Amp PCR System 9700, Applied Biosystems Japan Co., Ltd, Tokyo). The samples were denatured by heating to 94°C for 60 sec, annealed by cooling to 56-60°C for 60 sec, and extended by heating to 72°C for 90 sec. The PCR products were separated by 2.0% agarose gel electrophoresis and visualized by ethidium bromide staining. The optical density of various bands was quantified using the Scion image analysis package (Scion Corp., Frederick, MD).

Immunohistochemical staining

The hepatocytes attached to microcarriers were collected and 5 μm-thick paraffin sections were made for PCNA immunohistochemical staining with monoclonal anti-PCNA antibodies (PC-10, DakoCytomation, Carpinteria, CA) before and after serum exposure. The number of PCNA-positive hepatocytes per 100 hepatocytes on the microcarrier surface was determined.

Biochemical analysis

Serum aspartate aminotransferase (AST), total bilirubin (T. Bil), and ammonia (NH_3) were measured with commercial kits (Wako Pure Chemical Industries, Ltd., Osaka). Serum IL-1ß, IL-6, and TNF-α were measured using ELISA kits (BioSource International Inc., Camarillo, CA).

Statistical analysis

All data were expressed as a mean ± standard deviation (SD). Between-group comparisons of the data carried out by the Wilcoxon t-test. A p-value less than 0.05 was considered to indicate significant differences.

RESULTS

Cytokine levels

Apart from high levels of HGF and TGF-ß1 in the FHF rats (n=5) at 18 hours after induction, serum TNF-α (125.4 ± 27.7 pg/mL), IL-1ß (327.5 ± 160.2 pg/mL), and IL-6 (2597.8 ± 631.8 pg/mL) levels were markedly increased. In the normal rats (n=5), TNF-α, IL-1ß, and IL-6 levels were low or undetectable (Tab. I).

Liver support functions

The AST value was significantly higher after perfusion than before (3387.0 ± 94.2 IU/L, 2085.0 ± 135.4 IU/L, $p<0.05$), suggesting that hepatocyte damage had occurred in the module. In contrast, total bilirubin and ammonia levels were significantly lower after perfusion than before: T. Bil (1.31 ± 0.24 mg/dL, 1.77 ± 0.25 mg/dL, $p<0.05$) and NH_3 (294.6 ± 15.9 μg/dL, 346.4 ± 29.7 μg/dL, $p<0.05$) (Fig. 1).

mRNA expression in BAL hepatocytes

The oligonucleotide PCR primers were used to amplify rat albumin, GST A1, CYP 1A2, OTC, c-fos and GAPDH. As the housekeeping gene, GAPDH was used to determine the constitutive level of gene transcription and to control for variations in RNA recoveries from each specimen.

The albumin, GST A1, and CYP 1A2 levels decreased more after the perfusion of FHF serum than after the perfusion of normal serum. Mild decrease in OTC and c-fos expression was also seen (Fig. 2).

The mRNA expression (percent decrease) was calculated by densitometric evaluation. Even though the effect of FHF relative to normal serum on OTC (70.6 ± 20.5%, 60.0 ± 28.0%) and c-fos mRNAs (56.8 ± 12.9%, 58.6 ± 27.7%) was insignificant, albumin (88.0 ± 6.5%,

TABLE I - INFLAMMATORY CYTOKINE PROFILES[a] IN FHF RATS AND NORMAL RATS

Groups	TNF-α (pg/mL)	IL-1 ß (pg/mL)	IL-6 (pg/mL)
FHF (n=5)	125.4 ± 27.7	327.5 ± 160.2	2597.8 ± 631.8
Normal (n=5)	7.7 ± 4.9	N.D.	N.D.

In the FHF rats, serum levels of TNF-α, IL-1ß, and IL-6 were markedly increased. In contrast, in the normal rats TNF-α, IL-1ß, and IL-6 were low or undetectable. [a] 18 h after induction of FHF.

TABLE II - PRIMERS USED IN PCR REACTION

Gene	Sense primer (5'-3')	Antisense primer (5'-3')	Fragment size (bp)
Albumin	TTGCCAAGTACATGTGTGAG	TTGCCAAGTACATGTGTGAG	373
GST A1	ATGAGAAGTTTATACAAAGTCC	GATCTAAAATGCCTTCGGTG	213
CYP 1A2	GTCACCTCAGGGAATGCTGTG	GTTGACAATCTTCTCCTGAGG	236
OTC	GATAAGCATGGGACAAGAGG	CAGGTGAGTAGTCTGTCACG	247
c-fos	AGCCGACTCCTTCTCCAGCAT	CAGATAGCTGCTCTACTTTGC	298
GAPDH	TTCAACGGCACAGTCAAG	CACACCCATCACAAACAT	240

GST A1, glutathione S-transferase A1; CYP 1A2, cytochrome P450 1A2; OTC, ornithine transcarbamylase; GAPDH, glyceraldehydes 3-phosphate dehydrogenase.

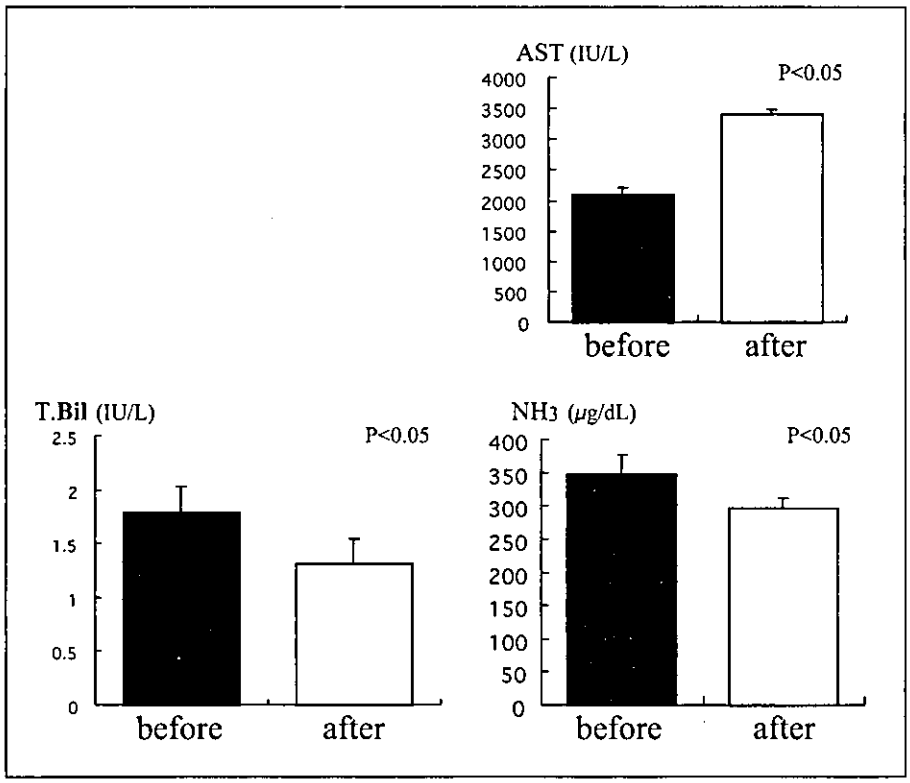

Fig. 1 - *Serum AST, T.Bil and NH_3 values before and after perfusion. The serum AST value increased significantly more after perfusion than before ($p<0.05$). In contrast, T.Bil and NH_3 values improved significantly more after perfusion than before ($p<0.05$). AST, aspartate aminotransferase; T.Bil, total bilirubin.*

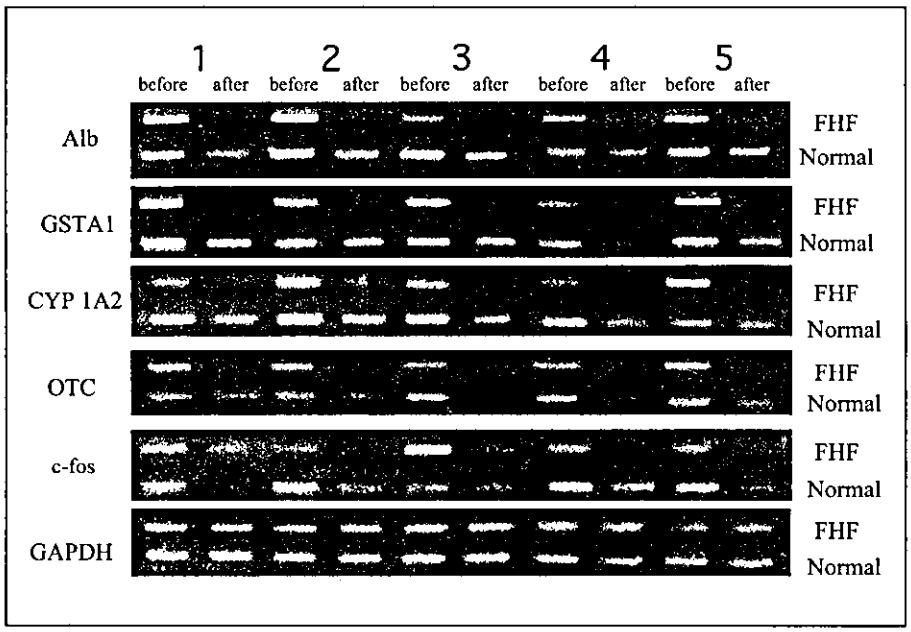

Fig. 2 - *Profile of mRNA expression, rat albumin, GST A1, CYP 1A2, OTC, c-fos, and GAPDH. The albumin, GST A1, and CYP 1A2 levels decreased more after the perfusion of FHF serum than after the perfusion of normal serum. Mild decreases in OTC and c-fos expression were also seen. Alb, Albumin; GST A1 glutathione S-transferase A1; CYP 1A2, cytochrome P450 1A2; OTC, ornithine transcarbamylase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; and FHF, fulminant hepatic failure.*

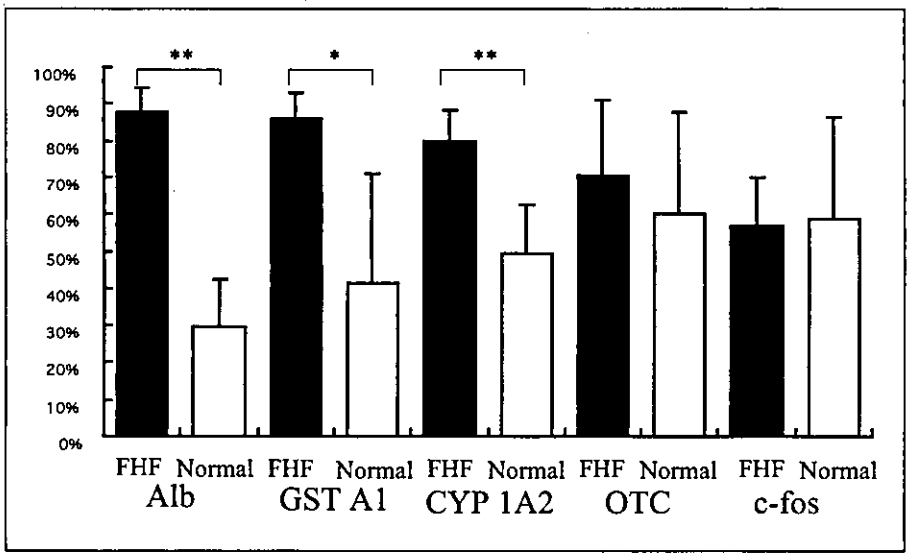

Fig. 3 - *The mRNA expression (percent decrease) was calculated by densitometric evaluation. The expressions of albumin, GST A1, CYP 1A2 were significantly decreased in hepatocytes treated with FHF serum compared to normal control cells, whereas those of OTC and c-fos were not different.*

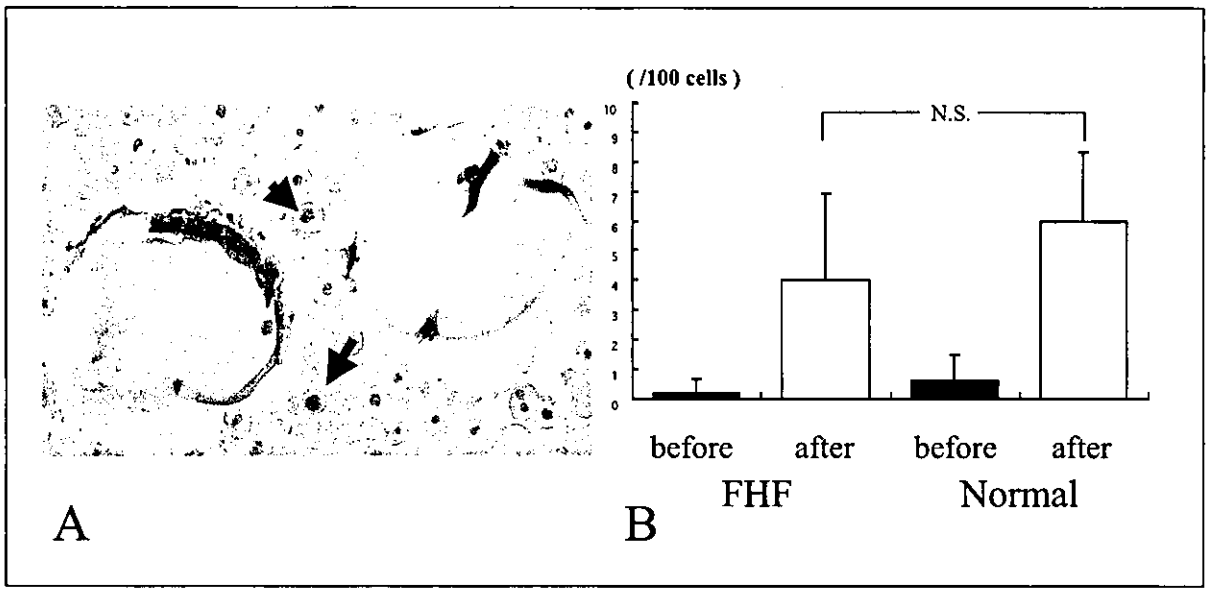

Fig. 4 - ***(A)*** *Immunohistochemical staining of hepatocytes using anti-PCNA antibody (original magnification x 100). Arrows represent positive staining.* ***(B)*** *PCNA positive cells were modestly increased after perfusion. However, the number of these cells was low and did not differ between FHF- and normal serum-exposed hepatocytes.*

29.6 ± 12.7%, p<0.01), GST A1 mRNA (86.0 ± 6.8%, 41.4 ± 29.3%, p<0.05) and CYP 1A2 (80.0 ± 8.4%, 49.2 ± 13.1%, p<0.01) levels were significantly decreased (Fig. 3).

Proliferating cell nuclear antigen (PCNA) labelling

PCNA positive cells were modestly increased after perfusion (i.e., 0.2 ± 0.4 cells before FHF vs 4.0 ± 2.9 cells

after FHF, and 0.6 ± 0.9 cells before normal serum vs 6.0 ± 2.3 cells after normal serum). However, no difference in these increases was found between the FHF and normal serum treated groups (Fig. 4).

DISCUSSION

Two opposite effects of FHF serum on hepatocytes have been reported. One report indicates that hepatocyte proliferation is increased and the other indicates it is suppressed (18, 19).

HGF, a strong growth factor of hepatocytes, is excessively increased in FHF serum. However, in patients with FHF, liver regeneration is hardly seen and also levels of HGF are reported to correlate inversely with patient prognosis (30). This finding implies the presence of some growth inhibitory factor antagonistic to HGF in FHF serum. We reported TGF-ß1 was increased in FHF patient's blood (14) and one of our animal studies showed a similar profile for HGF and TGF-ß1 and less liver regeneration in FHF than normal animals (21). Theoretically, when cells begin DNA synthesis, cell-specific functions are down-regulated during liver regeneration, while in FHF livers, neither proliferation nor specific functions are maintained. This discrepancy suggests that another factor other than HGF or TGF-ß1 is mediating this cellular derangement.

Several putative factors (we focused on inflammatory cytokines) increase in FHF livers. In fact, our model showed higher levels of TNF-α, IL-1ß, and IL-6. It was reported that inflammatory cytokines rapidly lowered the expression of major cytochrome P450 enzymes and decreased the transcription of rGST A2 *in vitro* (15, 16).

For example, IL-6 modulates hepatocyte proliferation through the signal transducer and activator of transcription (Stat3) (31). It also directly suppresses albumin secretion (17). It has been suggested that IL-6 causes hepatocytes to switch proliferation and performing other cellular functions. In an animal model of FHF, Stat3 activation suppressed an early phase of the cell cycle by inducing Stat3 inhibitors, resulting in impaired proliferation (32). In the present study, it was demonstrated that PCNA labeling is low despite the fact that immediate early gene (c-fos) expression is well preserved. The disruption of Stat3 signaling is caused by negative feed back mechanisms (32). Thus, cell proliferation is interrupted before DNA synthesis. We speculate that elevated IL-6 suppressed liver specific functions initially allow hepatocytes to enter the proliferating process in normal regeneration. In contrast, under FHF conditions, this process is interrupted by IL-6 itself and finally liver loses both its ability to proliferate and perform other functions.

In several inflammatory conditions, OTC is consistently expressed. It could be regulated by another signal pathway that does not directly connect to proliferation. In a phase I clinical trial of a BAL system at Cedars-Sinai Medical Center, treatment resulted in significant reduction of ammonia but had no effect on albumin (10). Our experiment showed similar results.

Some liver specific functions were consistently expressed in any situation; however, the majority of hepatocyte-specific functions was impaired by imbalance between growth factors and inflammatory cytokines, especially inflammatory cytokines. This imbalance may play a key regulatory role in the switch between performing cellular functions and undergoing proliferation.

To eliminate external cytokine stimuli, activated charcoals and hemodiafiltration have been developed; however, clinical outcome of patients treated by these methods is unsatisfactory (33, 34). The IL-6 pathway is considered to involve a soluble receptor that is necessary to transduce the signal (35). Such receptors are usually high molecular weight molecules that are difficult to remove from circulation using current modalities of cytokine reduction. Therefore, we consider it is important to create new hepatocyte systems that are functionally stable and hyposensitive to extra-cellular stimuli. Recent advances in cellular technology are promising.

In conclusion, several specific functions of hepatocytes were down-regulated after perfusion with FHF serum, even though the BAL system showed a better elimination capacity. Our findings suggest that the exposure of hepatocytes to hypercytokinemia, including inflammatory cytokines and positive and negative growth factors, causes a loss in liver specific functions and fails to support liver regeneration.

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Original Paper

Selective Suppression of Initial Cytokine Response Facilitates Liver Regeneration after Extensive Hepatectomy in Rats

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ABSTRACT

Background/Aims: After extensive hepatectomy, the cytokine network plays an important role in injury to the remnant liver and subsequent impairment of liver regeneration. Tumor necrosis factor α (TNF α) and interleukin 1β (IL-1β) are thought to be the initial cytokines associated with liver injury as well as with regeneration. We investigated the effect of the suppression of these cytokines on liver function and on liver regeneration after subtotal hepatectomy in rats.

Methodology: Following 90% hepatectomy, rats were divided into two groups. Animals in the FR group received intraperitoneal FR167653, a selective inhibitor of TNF α and IL 1β, while those in the Control group received vehicle only. Liver chemistry and serum levels of TNF α and IL-6 were measured serially. Liver specimens were obtained 48 hr after surgery and regenerative activity assessed by proliferating cell nuclear antigen (PCNA) expression and remnant liver weight.

Results: The survival rate was significantly better in the FR group (76.4±11.7 hrs) than in the Control group (26.8±4.3 hrs, $p=0.0014$). Liver enzyme and blood sugar levels after surgery were higher in the FR group compared to the Control group ($p=0.03$ or less). Changes in serum levels of both TNF α and IL-6 were suppressed in FR group rats after surgery. Microscopically, hepatocellular damage and steatosis was less prominent in FR group livers. PCNA labeling index and residual liver weights were higher in the FR group ($p<0.001$).

Conclusions: Following extensive hepatectomy in rats, suppression of early cytokine induction improved liver function and facilitated liver regeneration. Suppression of selective cytokine responses could allow extended liver resection and reduced risk of liver failure.

KEY WORDS: FR167653; Extensive hepatectomy; TNF α; IL-6; Liver regeneration

ABBREVIATIONS: Tumor Necrosis Factor α (TNF α); Interleukin 1β (IL-1β); Proliferating Cell Nuclear Antigen (PCNA); Ischemia/Reperfusion (I/R); Intracellular Adhesion Molecule-1 (ICAM-1); Nuclear Factor κB (NFκB); Aspartate Transaminase (AST); Alanine Transaminase (ALT); Lactate Dehydrogenate (LDH)

INTRODUCTION

Extensive resection of the liver, especially the cirrhotic liver, is frequently associated with postoperative liver failure with sustained portal hypertension and hepatic congestion by microcirculatory disturbance (1). Cytokines have been reported to play key roles in mechanisms of liver damage (2).

Tumor necrosis factor α (TNF α) and interleukin-1 (IL-1) are recognized as initial-phase cytokines in the inflammatory response following systemic infection or injury (3). They also appear to play pivotal roles in liver disease following hepatectomy or ischemia/reperfusion (I/R) injury. TNF α released from Kupffer cells and/or hepatic sinusoidal endothelial cells stimulates the expression of chemo-attractants (4,5) such as intracellular adhesion molecule-1 (ICAM-1) (6) and induces neutrophil accumulation in hepatic sinusoids. Adherence of these neutrophils to endothelial cells causes microcirculatory disturbances (6), with subsequent neutrophil elastase release and accelerated I/R injury. In addition, several cytokines are induced by TNF α through nuclear factor κB (NFκB) activation (7). Recent evidence suggests that TNF α affects IL-6 expression downstream in the cytokine cascade through NFκB activation. While IL-6 is a strong promoter of liver regeneration in rats treated with 70% hepatectomy (8), other investigators including ourselves (9) have demonstrated excessive IL-6 production in extensively (90%) hepatectomized rats, which was associated with adverse effects on hepatic microcirculation and liver regeneration.

FR157653 is a selective inhibitor of IL-1β and TNF α production in monocytes and macrophages (10-12). Using 90% hepatectomized rats, we investigated whether the blockade of the initial cytokine response by FR167653 could modulate the cytokine cascade, resulting in improved hepatocyte protection and proliferation.

METHODOLOGY

Animal studies were performed in compliance with the Nagasaki University guidelines for humane care of experimental animals.

Animals

Adult male Wistar rats weighing 200-250g were obtained from Ohtsubo Co. Ltd. Nagasaki, Japan, and

Hepato-Gastroenterology 2004; 51:701-704

acclimatized to our laboratory conditions for 1 week prior to use in experiments. Animals were housed in climate-controlled (21°C) conditions under a 12-hr light/dark cycle and given tap water and commercial rat chow *ad libitum*. Surgical procedures were performed under ether inhalation anesthesia in sterile conditions between 9 am and 12 am. After surgery, the animals were warmed externally and given a single bolus of 15mL of 10% glucose in normal saline subcutaneously to avoid dehydration.

Surgical Procedures

Under ether inhalation anesthesia, the abdomen was entered by median laparotomy. Nearly 90% hepatectomy was archived using Gaub's technique (13). Prior to resection, vascular pedicles of the right and median lobes were ligated and resected. Remnant livers consisted of two omental lobes that represented approximately 10% of the whole liver mass.

Experimental Design

FR167653, a potent suppresser of IL-1 and TNF α production, was provided by Fujisawa Pharm. CO. Ltd (Osaka, Japan) (10-12). Animals were divided into two groups, FR and Control. Each animal in the FR group received FR167653 intraperitoneally at 10mg/kg twice, 1 hour prior and immediately after surgery. Each animal in the Control group received the same volume of saline intraperitoneally.

Assessments

Blood samples were obtained postoperatively from the inferior vena cava 2, 6, and 12 hr after surgery. Aspartate transaminase (AST), alanine transaminase (ALT) and lactate dehydrogenate (LDH) levels in the blood samples were then measured using commercially available kits. Blood glucose was also measured to evaluate the severity of liver failure. TNF α and IL-6 levels were measured 2, 6, and 12 hr after surgery using a rat TNF α immunoassay kit (TECHNE corporation, Minneapolis, MN) and an IL-6 ELISA kit (WAKO corporation, Osaka, Japan), respectively.

Fifteen animals from each group that underwent 90% hepatectomy were observed for survival until death, after which the residual omental liver lobes were harvested, weighed and fixed in 10% neutral formalin.

At 48 hr after hepatectomy, animals were killed and the residual livers weighed to assess liver regeneration, and stained with hematoxylin-eosin to evaluate histological liver damage. Hepatocyte proliferation was also assessed by proliferating cell nuclear antigen (PCNA) staining. The PCNA-labeling index was defined as PCNA-positive cells per 1,000 hepatocytes from 10 high power fields (x400).

Statistical Analyses

StatView-J 4.5 software was used for statistical data analysis. Student's *t* and Mann-Whitney's U tests were performed and results expressed as a mean ± standard deviation (SD). Survival rates were compared by the Kaplan-Meier method. *P* values less than 0.05 were considered significant.

RESULTS

FR treatment did not affect systemic hemodynamics, and no obvious side effects were observed in the FR group. All animals tolerated surgery well and recovered uneventfully from anesthesia.

Survival

The survival rate in the FR group was significantly higher compared to the Control group ($p=0.0014$) **(Figure 1)**.

Blood Chemistry

FR group rats exhibited lower serum ALT levels at 2, 6, and 12 hr after surgery compared to Control group rats ($p=0.015$ at 12 hr) **(Figure 2)**. Changes in AST and LDH levels after surgery were similar to that observed for ALT (data not shown). Blood sugar levels decreased gradually after surgery. Control group rats demonstrated severe hypoglycemia (<50mg/dL) 12 hr after surgery, whereas FR group rats exhibited significantly higher serum blood sugar levels, maintained at least 12 hr after surgery ($p=0.03$) **(Figure 2)**.

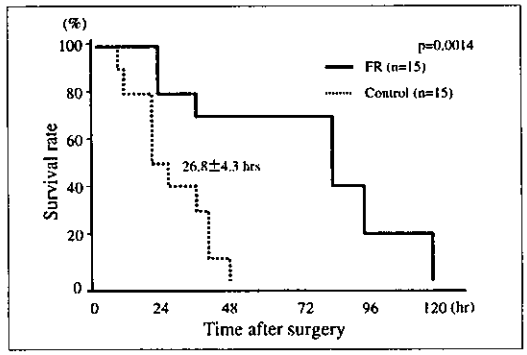

FIGURE 1 All FR group rats survived over 24 hours after surgery, and had significantly better survival compared to Control group rats (76.4±11.7 hrs *vs.* 26.8±4.3 hrs, respectively, $p=0.0014$).

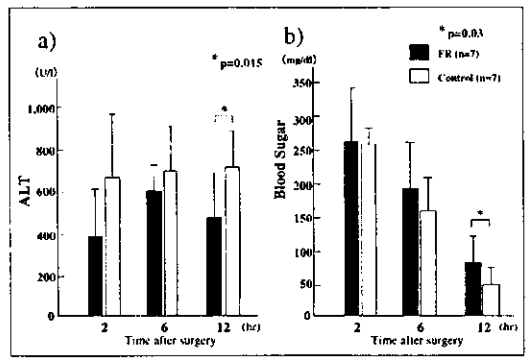

FIGURE 2 (a) FR group rats exhibited lower serum ALT levels at 2, 6, and 12 hr after surgery compared to Control group rats ($p=0.015$ at 12 hr). Changes in serum LDH and AST levels after surgery were similar to ALT. **(b)** Blood sugar levels decreased gradually after surgery. Control group rats demonstrated severe hypoglycemia (<50mg/dL) 12 hr after surgery, whereas serum blood sugar levels FR group rats 12 hr after surgery remained significantly higher ($p=0.03$).

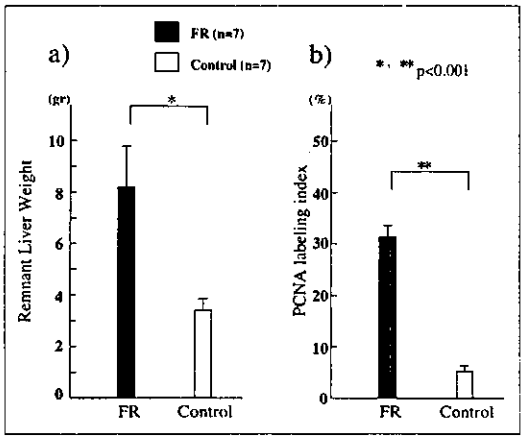

FIGURE 3 (a) Mean remnant liver weight in the FR group was greater than in the Control group (3.2±0.4g *vs.* 8.2±1.6g, respectively, $p<0.001$). **(b)** The PCNA-labeling index for FR treated rats was significantly higher than for Control group rats ($p<0.001$).

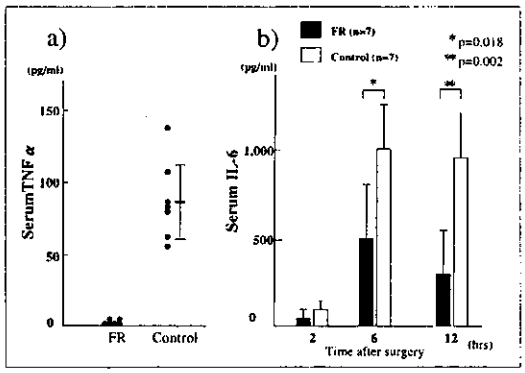

FIGURE 4 (a) Serum TNF α levels in Control group rats 2 hr after surgery were elevated (80±9.2pg/mL), but undetectable in FR group rats. **b)** Changes in IL-6 serum levels were suppressed in FR group rats at each time point after surgery. At 6 and 12 hr after surgery, serum IL-6 levels in the FR group was significantly lower than in the Control group ($p=0.002$ or less).

Liver Regeneration

Mean remnant liver weights were 3.2±0.4g in Control group rats and 8.2±1.6g in FR group rats ($p<0.001$). The PCNA labeling index of the FR group was significantly higher compared to the Control group ($p<0.001$) **(Figure 3)**.

TNF α and IL-6 Serum Levels

At 2 hr after surgery, serum TNF α levels in Control group rats increased (80±9.2pg/mL), while TNF α remained undetectable in FR group rats. Serum IL-6 values in the FR group were significantly lower than in the Control group at each time point after surgery ($p=0.002$) **(Figure 4)**.

Histological Findings

The degree of histological liver damage was less prominent in the FR group than in the Control group. While Control group liver tissues exhibited hepatocellular damage, steatosis, and marked sinusoidal neutrophil infiltration, FR group livers demonstrated much less hepatocellular damage and sinusoidal neutrophilic infiltration **(Figure 5)**.

DISCUSSION

Following extensive hepatectomy, congestion of the remnant liver leads to portal hypertension followed by hepatic injury due to gut-derived endotoxins (14). This results in severe liver dysfunction, hypoglycemia and poor liver regeneration, and is often fatal (13). Free radicals released following reperfusion stimulate the production of TNF α and IL-1 from nonparenchymal liver cells (15,16). Both TNF α and IL-1 stimulate the production of each other and further amplify the inflammatory response. Our results demonstrated that the inhibition of these initial key cytokines prevented neutrophil accumulation and the inflammatory response in the remnant liver.

Recent reports have suggested that TNF α affects IL-6 expression in the cytokine cascade, and that IL-6 is associated with hepatoprotective effects. IL-6 is an acute reactant cytokine with anti-inflammatory properties (17,18), which has been found to prevent injury in a murine model of acute hepatitis through down-regulation of TNF α (19). Other positive effects of IL-6 have also been reported, including that recombinant IL-6 (rIL-6) was able to reduce acute inflammation in endotoxin-induced tracheal injury and sepsis in rats (20,21). Camargo *et al.* (8) also reported that IL-6 has hepatoprotective effects through the prevention of TNF α expression in rats.

With regard to the enhanced liver regeneration by FR167653 in our study, the suppression of IL-6 expression by blocking TNF α production may have played a role. While IL-6 has hepatoproliferative effects that stimulate hepatocyte and bile duct cell proliferation *in vitro* and *in vivo* (22,23), perioperative administration of IL-6 lowered hepatocyte prolifera-

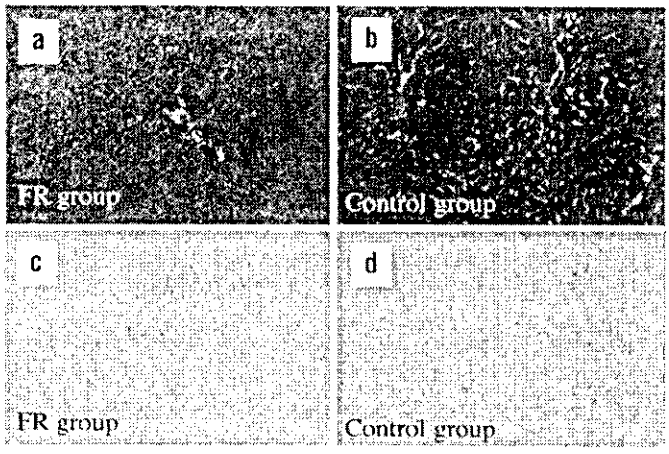

FIGURE 5 (a, b) Control group rat livers showed marked neutrophilic infiltration and parenchymal hemorrhage of the sinusoids as well as severe steatosis of the parenchymal cells. In contrast, FR group rats exhibited neither significant hepatocellular damage nor neutrophilic infiltration of the sinusoids. **(c, d)** PCNA-positive cells were more prominent in FR group rats than in Control group rats.

tion in rats with 70% hepatectomy. In our previous study, serum IL-6 levels in rats after conventional 70% partial hepatectomy decreased gradually, while in rats that had undergone subtotal hepatectomy, levels were persistently elevated and associated with poor proliferative activity of the liver cells with transcriptional derangement. It is possible that excessive enhancement of cytokine levels may cause intracellular missignaling, such that the hepatocytes are no longer able to proliferate and lose function. Therefore, it appears that control of cytokine levels is vital. To suppress initial key cytokines, we used FR167653, a dual synthetic inhibitor of TNF α and IL-1, probably through suppression of the mitogen-activated protein kinase p38 pathway (12). Beneficial effects of FR167653 have been reported in I/R injury of the lung (24,25), liver (26) and pancreas (27) as well as cardiac transplantation (28) in rats. For the liver, improvement of both proliferation and function has been reported (24,26).

In our study, the production of IL-6, a secondary cytokine, was suppressed compared to controls. Initially activated transcription factors, such as NF-κB, may be involved through TNF α/IL-1β suppression. It is also possible that suppression of initial cytokine stimulation may inhibit the inflammatory response, allowing the proper elevation of IL-6, and contributing to hepatocyte proliferation.

In conclusion, suppression of the initial cytokine response by FR167653, a dual TNF α and IL-1 inhibitor, in rats that had undergone subtotal hepatectomy facilitated liver regeneration and exhibited hepatoprotective effects with improved hepatocyte proliferation. Thus, suppression of the initial cytokine response after massive hepatectomy may improve the outcome of major hepatic resection.

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Enhanced expression of growth factors and imbalance between hepatocyte proliferation and apoptosis in the livers of rats fed a choline-deficient, L-amino acid-defined diet

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Abstract

In a rat model of hepatocarcinogenesis induced by a choline-deficient, L-amino acid-defined (CDAA) diet, hepatocellular carcinoma (HCC) occurs in conjunction with fatty liver, hepatocyte injury and regeneration, fibrosis and cirrhosis. This is similar to human HCC development with cirrhosis. The aim of this study is to clarify sequential changes in the expression of growth and growth inhibitory factors, and hepatocyte proliferation and apoptosis during development of preneoplastic nodules in rats fed a CDAA diet. The expression of hepatocyte growth factor was stimulated at about the same time as CDAA diet-induced liver injury within 1 week. Hepatocyte growth factor reached a maximum level of expression from 4 to 8 weeks. Transforming growth factor (TGF)-α expression increased from 4 to 40 weeks. Although TGF-β, a growth inhibitory factor for hepatocytes, was also expressed with a peak from 4 to 8 weeks followed by a gradual decrease until 48 weeks, expression of cyclin D1 and hepatocyte proliferation continued to be stimulated throughout the experimental period. Additionally, the number of apoptotic hepatocytes was markedly reduced after peaking at 8 weeks. These results suggest that some hepatocytes in the livers of rats fed a CDAA diet may escape from TGF-β-induced growth inhibition and apoptosis, leading to development of preneoplastic nodules.

Keywords: Hepatocarcinogenesis; Hepatocyte growth factor; Transforming growth factor-α; Heparin binding-epidermal growth factor-like growth factor; Transforming growth factor-β; Cell proliferation; Apoptosis

1. Introduction

The development of hepatocellular carcinoma (HCC) is a lengthy process, and is strongly associated with liver cirrhosis or fibrosis. These preconditions are most often a consequence of chronic viral hepatitis due to hepatitis B virus or hepatitis C virus infection. Although viral proteins have been reported to play a role in hepatocarcinogenesis [1,2], this is not sufficient to explain all mechanisms of HCC development, indicating the involvement of endogenous factors, such as prolonged exposure to growth factors due to repeated hepatocyte injury. Recently, Nakae et al. have reported a rat model of hepatocarcinogenesis induced by a choline-deficient, L-amino acid-defined (CDAA) diet. This system is an appropriate in vivo experimental model, in which HCCs develop without exposure to exogenous carcinogen [3]. In this rat model, HCCs occur in conjunction with fatty liver, hepatocyte death and subsequent regeneration, fibrosis, and eventual cirrhosis. This is similar to the histopathological sequence of human HCC development with cirrhosis [4]. The repeating cycles of hepatocyte injury and regeneration [5], inhibition of apoptosis [6], oxidative stress [7], hypomethylation of DNA and RNA [8], and chronic activation of protein kinase C [9] are known to be involved in hepatocarcinogenesis induced by choline-deficiency.

Several growth factors play an important role in liver regeneration. Once hepatic injury occurs, liver regeneration is stimulated by hepatocyte growth factor (HGF), transforming growth factor (TGF)-α, and heparin binding-epidermal

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growth factor-like growth factor (HB-EGF). These growth factors induce hepatocyte proliferation mainly through the mitogen-activated protein kinase pathway and G1 cyclins [10]. On the other hand, TGF-β1 has been shown to be involved in the development of cirrhosis [11–14], and to inhibit proliferation of hepatocytes [15–18]. TGF-β1 suppresses the growth factor-induced signals which stimulate cyclin D1 and cyclin E, thereby resulting in the inhibition of DNA synthesis [10]. These growth and growth-inhibitory factors act in concert to regulate proliferation of hepatocytes precisely during liver regeneration in vivo, and the impaired cooperation of growth factors and the escape by some initiated cells from growth inhibitory stimuli are hypothesized to be contributing factors in HCC development.

In this study, we clarify the relationship between the expression of growth factors and hepatocyte proliferation following CDAA diet-induced hepatic injury. We have investigated the sequential changes in blood and serum markers, expression of growth and growth-inhibitory factors, and proliferation and apoptosis of hepatocytes during the development of cirrhosis and preneoplastic nodules.

2. Materials and methods

2.1. Animals

Six-week-old male Fischer 344 rats were obtained from Kyushu Experimental Animal Supply (Kumamoto, Japan). The animals were maintained under constant room temperature (25 °C) and provided free access to water and the indicated diet throughout the study. The protocol for these animal studies was approved by the ethical committee of Miyazaki Medical College (Miyazaki, Japan).

After a 1-week acclimation period on a standard diet, the rats were switched to a CDAA diet (Dyets Inc., Bethlehem, PA), and sacrificed at pre-determined intervals over the following 48 weeks. Blood was obtained from the bifurcation of the abdominal aorta. The number of platelets, serum alanine amintransferase (ALT), and albumin were determined. The liver and spleen were immediately excised, and the wet weight of these organs was determined. Samples were subjected to histological analysis or frozen in liquid nitrogen and stored at −80 °C until analysis.

2.2. Northern blot analysis

Total RNA was extracted from liver tissues of rats fed a CDAA diet by the acid guanidinium thiocyanate:phenol: chloroform method. Growth factor mRNAs were detected using the following PCR-amplified cDNA probes: the 2274-bp fragment corresponding to bases 20–2306 of rat hepatocyte growth factor/scattering factor (HGF/SF) cDNA [19], the 240-bp fragment corresponding to bases 57–297 of rat transforming growth factor (TGF)-α [20], the 801-bp fragment corresponding to bases 690–1498 of rat heparin binding-epidermal growth factor like growth factor (HB-EGF) [21] and the 438-bp fragment corresponding to bases 518–956 of rat TGF-β1 [22]. Cyclin D1 transcript was detected using a 427-bp rat cyclin D1 cDNA [23].

2.3. Histopathological and immunohistochemical analysis

Two 5 mm thick slices from the two major liver lobes (left lateral and median lateral lobes) were fixed in 10% formalin and embedded in paraffin. Four or five serial 4 μm sections were prepared from each fixed liver slice. The first was stained with hematoxylin and eosin (HE) or azan for histological examination. The remaining three sections were subjected to immunohistochemical analysis. After boiling in distilled water for 10 min, slides were incubated with a rabbit polyclonal antibody against the placental form of rat liver glutathione *S*-transferase (GST-P) (Medical and Biological Laboratories, Nagoya, Japan), an anti-proliferating cell nuclear antigen (PCNA) monoclonal mouse antibody (Dako Japan, Kyoto, Japan) or a rabbit polyclonal antibody against single-stranded DNA (Dako Japan) [23]. Goat anti-mouse or anti-rabbit IgG was then applied followed by an avidin–biotin–peroxidase complex and chromatin 3′,3′-diaminobenzidine. The PCNA labeling indices were calculated as percentage of labeled hepatocytes counted by light microscope. The number of apoptotic cells, which were stained with the anti-single-stranded DNA antibody, was analyzed.

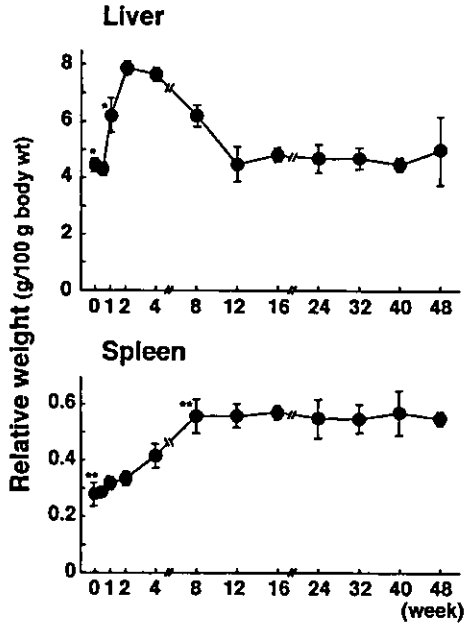

Fig. 1. Changes in the relative liver and spleen weights during CDAA diet administration. The ratios of liver and spleen weight to body weight (g/100 g body weight) in rats fed a CDAA diet were determined at the indicated time points (n = 5 or 10). Administration of a CDAA diet-induced a transient increase in liver weight from 2 to 4 weeks, and gradual enlargement of the spleen until 8 weeks ($^{*}P = 0.001$, $^{**}P < 0.001$).

2.4. Statistical analysis

Unless otherwise specified, data are expressed as mean ± S.D. Statistical parameters were ascertained using Statview J-4.5 software (Abacus Concepts Inc., Berkeley, CA). Differences between means were assessed by the unpaired Student's *t*-test. The significance level was set at $P < 0.05$.

3. Results

3.1. Sequential changes in the weight of the liver and spleen during CDAA diet-induced cirrhosis development

The weight of the liver and spleen was measured periodically during CDAA diet administration (Fig. 1). The relative liver weight increased with statistical significance at 1 week ($P = 0.001$), and reached a peak weight from 4 to 8 weeks. After this, the liver weight gradually decreased until after 12 weeks the liver was the same weight as before CDAA diet feeding. Histologically, fat deposition was observed in zones 2 and 3 of hepatocyte lobuli 3 days after the start of the CDAA diet, and quickly expanded, resulting in diffuse fatty liver at 1 week (Fig. 2A and B). Collagen fibers began to extend at 4 weeks, followed by development of cirrhosis (Fig. 2C, E and F). GST-P positive nodules were observed in some rats fed a CDAA diet for 4 weeks (Fig. 2D). Administration of the CDAA diet for 8 weeks resulted in a gradual increase in the relative spleen weight ($P < 0.001$) (Fig. 1). Persistent enlargement of the spleen was observed in rats fed a CDAA diet from 8 to 48 weeks. These results indicate that an increase in the liver and spleen

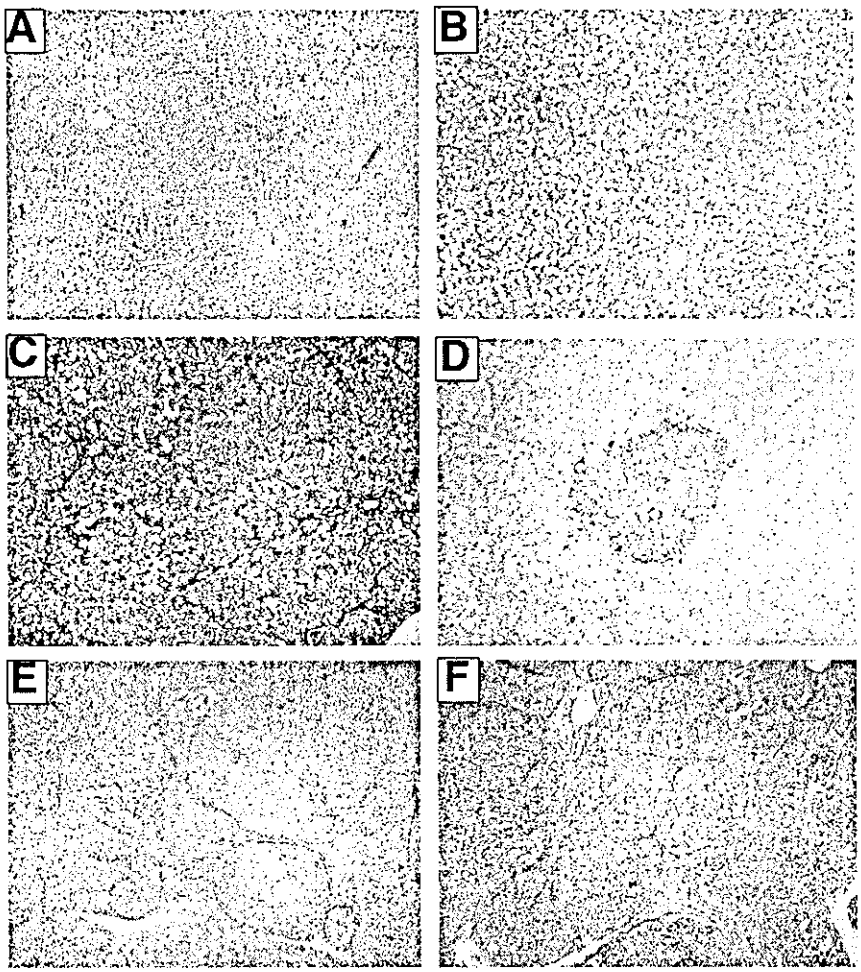

Fig. 2. Sequential histological changes in the livers of rats fed a CDAA diet. Liver tissue was obtained from rats fed a CDAA diet for 3 days (A), 1 weeks (B), 4 weeks (C and D), 12 weeks (E), and 24 weeks (F), and stained with HE (A, B, E and F), azan (C) or anti-GST-P antibody (D), as described in Section 2. Fat deposition was observed in zones 2 and 3 of hepatocyte lobuli on day 3, and then expanded. Collagen fibers began to extend starting at 4 weeks, followed by development of cirrhosis. GST-P-positive nodules were observed in a few rats fed a CDAA diet for 4 weeks [magnifications 40× (A–C, E and F) and 100× (D)].

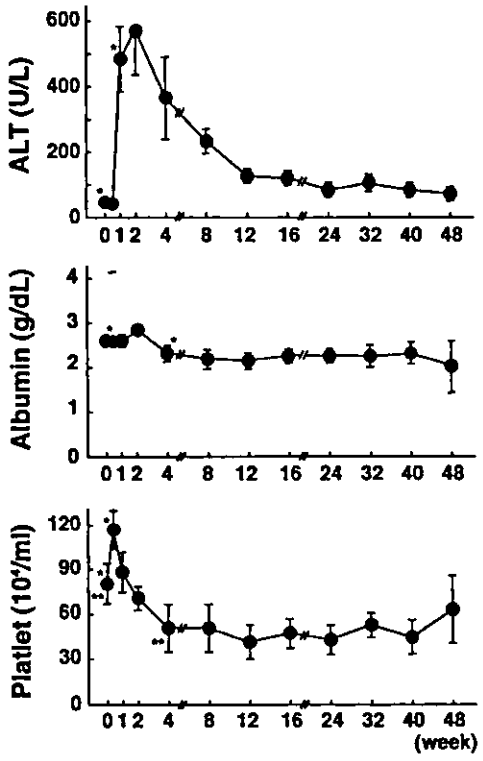

Fig. 3. Changes in serum levels of ALT and albumin and the number of platelet in rats fed a CDAA diet. A transient increase in serum ALT was observed from 2 to 4 weeks of administration of a CDAA diet ($n = 5$ or 10) ($^*P < 0.001$). The level of serum albumin was reduced 8 weeks after feeding the CDAA diet ($n = 5$ or 10) ($^*P < 0.001$). The number of platelets continued to show a decrease from 4 to 48 weeks following a transient increase on day 3 ($n = 5$ or 10) ($^*P = 0.005$, $^{**}P < 0.001$).

weights are associated with development of fatty liver and cirrhosis.

3.2. Hepatocyte injury was induced in the early weeks of CDAA diet administration, followed by development of cirrhosis

To evaluate the liver damage during CDAA diet feeding, we examined sequential changes in serum ALT, a sensitive indicator of hepatocyte injury (Fig. 3). Rats fed a CDAA diet for 1 week showed a marked increase in serum ALT levels ($P < 0.001$), although this effect was not observed after 3-day administration of the CDAA diet. The serum ALT levels reached a peak at the 2nd week, and then gradually decreased during the following 10 weeks. A mild elevation of serum ALT levels continued from 12 weeks throughout the experimental period. Although serum albumin, a marker for biosynthetic function of the liver, showed a slight transient increase in rats fed a CDAA diet for 2 weeks, serum albumin then decreased with statistical significance to lower levels than were present at the start of administration of the CDAA diet ($P < 0.001$) (Fig. 3). These decreased levels continued from 4 to 48 weeks. In addition, platelets, which are reduced in parallel with development of cirrhosis, increased to a maximum level after 3 days of a CDAA diet administration ($P = 0.005$), and then rapidly decreased within 4 weeks (Fig. 3). The numbers of platelets from 4 to 40 weeks was reduced to 50–60% of the levels observed in rats before CDAA diet feeding ($P < 0.001$). These results indicate that CDAA diet-induced hepatic injury occurs mainly in the early phase of diet administration. This damage is followed by the development of cirrhosis, resulting in a decrease in serum albumin and platelets.

3.3. Increased expression of growth factors and cyclin D1 in the livers of rats fed a CDAA diet

To examine growth stimuli following CDAA diet-induced liver injury, we evaluated the expression of growth factors and cyclin D1, a growth factor-dependent G1 cyclin (Fig. 4). Low-level expression of HGF mRNA was observed in normal liver tissue. However, expression of HGF was stimulated in rats fed a CDAA diet for 3 days. The level of HGF expression increased to a maximum level after 4 weeks of treatment, and then decreased until week 12. In contrast, expression of TGF-α mRNA, which was also detected in normal liver tissues, gradually increased beginning at 2 weeks, and reached a peak from 24 to 40 weeks. HB-EGF transcripts were barely detectable in normal liver tissues. However, low-level expression of HB-EGF was detected after 1 week of CDAA diet feeding, and moderately increased from 4 to 8 weeks, followed by low expression. In parallel with expression of these growth factors, cyclin D1 expression was significantly stimulated 1 week after CDAA diet administration, and enhanced expression of cyclin D1 continued throughout the experimental period.

Expression of TGF-β1, which plays an important role in hepatic fibrosis [11–14] and is an inhibitory growth factor for hepatocytes [15–17], also increased in rat livers treated with a CDAA diet for 1 week. Expression of this mRNA reached a maximum level from 4 to 8 weeks of treatment, followed by a gradual decrease until 48 weeks. Collagen fibers begin to extend after 4 weeks of CDAA diet feeding (Fig. 2C), resulting in the development of cirrhosis until 30 weeks [24,25]. Therefore, the sequential changes in TGF-β1 expression correlate closely with this development of cirrhosis.

3.4. Accelerated proliferation and decreased apoptosis of hepatocytes during administration of a CDAA diet

Next we examined the proliferation of hepatocytes in GST-P-positive nodules, which were considered to be precancerous lesions, and the surrounding GST-P-negative areas of livers from rats fed the CDAA diet (Fig. 5A). The number of hepatocytes staining positive for PCNA, an S-phase marker, in GST-P-negative areas increased with sta-

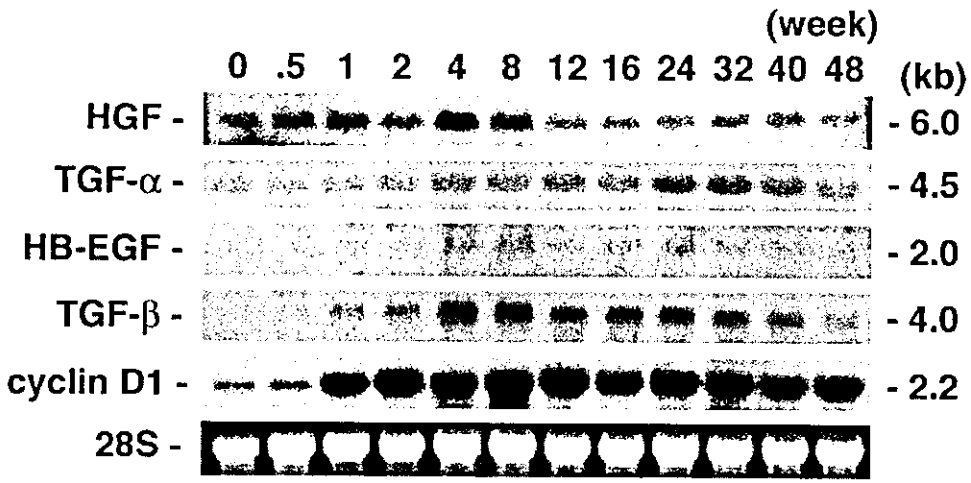

Fig. 4. Enhanced expression of growth factors and cyclin D1 in the liver tissues of rats fed a CDAA diet. Northern blot analysis demonstrated the expression of growth factors and cyclin D1 in the liver of rats fed a CDAA diet at the indicated time-points. Total RNA (10 μg) was analyzed as described in Section 2 for HGF, TGF-α, HB-EGF, TGF-β1, and cyclin D1. HGF expression was rapidly stimulated after CDAA diet feeding (from day 3 to 8 weeks), whereas stimulation of TGF-α expression was observed mainly in the late phase. Expression of HB-EGF increased slightly from 4 to 8 weeks. TGF-β1 expression was stimulated highly from 4 to 8 weeks, followed by a gradual decrease until 48 weeks. Enhanced expression of cyclin D1 was observed from 1 to 48 weeks. The expression pattern of each transcript was confirmed by several experiments using different individual animals at selected time points.

tistical significance 4 weeks after the start of the CDAA diet ($P < 0.001$) (Fig. 5B). Furthermore, the PCNA-positivity was much higher in GST-P-positive nodules than in the surrounding GST-P-negative areas at 8 weeks ($P < 0.001$), and an increase in PCNA-positive hepatocytes in both areas continued throughout the experimental period with a peak at 24 weeks (Fig. 5B).

Since a choline-deficient diet has been demonstrated to induce p53-independent programmed cell death [26], we immunohistochemically examined apoptosis in hepatocytes using an anti-single-stranded DNA antibody (Fig. 6). Although the number of apoptotic hepatocytes was much lower than the number of PCNA-positive cells (Fig. 5B), the number of apoptotic hepatocytes increased slightly 3 days after the start of the CDAA diet. This was followed by a marked increase at 4 weeks. After a peak at 8 weeks, the number of apoptotic cells rapidly decreased until 16 weeks ($P < 0.001$), and only a few were detected at 48 weeks.

4. Discussion

Early changes in rats fed a CDAA diet are due to oxidative injury to DNA and other subcellular components and result in the elevation of serum ALT and liver triglyceride [27,28]. The most abundant oxidative DNA damage, caused by 8-hydroxydeoxyguanosine (8-OHdG), has previously been detected after 1 day of feeding the CDAA diet. In this study, the level of serum ALT was markedly elevated beginning 1–4 weeks after CDAA diet administration, meaning that CDAA diet-induced hepatic injury mainly occurred in the early phase of this treatment. The number of platelets was significantly increased by day 3, while an increase in serum ALT was not observed at this point. We are not able to fully explain this phenomenon. Cytokines, such as interleukin (IL)-6, which is also known to play an important role in liver regeneration, may cause the transient increase in platelet levels [29]. Although platelets are known to release reactive oxygen species (ROS) [30], Hensley et al. have reported that administration of a CDAA diet caused complex 1 dysfunction and increased H_2O_2 generation in liver mitochondria [31].

The relative liver weight increased from 2 to 4 weeks of CDAA diet feeding, then decreased until 12 weeks, and consequently became similar sized to livers of untreated rats. These findings may be explained by the histopathological changes observed in this study as well as the data reported in previous investigations [24,25]. In the livers of rats fed a CDAA diet, fat deposition quickly expands, leading to a diffuse fatty liver within a week. In addition, an increase in hepatocyte death was detected from 4 weeks. In contrast, the relative weight of the spleen gradually increased 8 weeks after the start of CDAA diet administration in parallel with a decrease in platelet levels, and enlargement of the spleen continued throughout the experimental period. These symptoms progress in conjunction with cirrhosis development.

Following CDAA diet-induced hepatocyte injury, the expression of growth and growth-inhibitory factors was stimulated (Fig. 3). HGF expression was upregulated in the early phase of CDAA diet feeding, followed by enhanced expression of TGF-α in the late phase. These results indicate that hepatocytes are persistently exposed to growth stim-

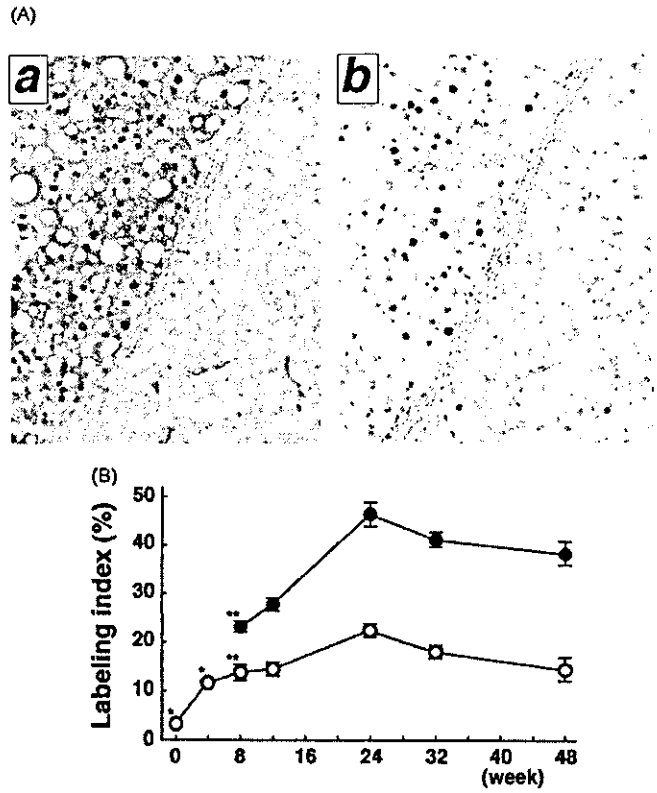

Fig. 5. Administration of a CDAA diet induced accelerated proliferation of hepatocytes throughout the experimental period. Proliferation of hepatocytes in the GST-P-positive nodules and the surrounding GST-P-negative areas was evaluated immunohistochemically using anti-PCNA antibodies as described in Section 2. (A) The same sections of liver tissue were stained with anti-GST-P or anti-PCNA antibody (a or b, respectively) [original magnification 100×]. (B) The number of PCNA-positive cells per 3×10^3 hepatocytes in both GST-P-negative areas (open circle) and GST-P-positive nodules (closed circle) was indicated as a PCNA-labeling index ($n = 3$). The PCNA-positivity reached a peak at 24 weeks, and was much higher in GST-P-positive nodules than in the surrounding GST-P-negative areas ($^*P < 0.001$, $^{**}P < 0.001$).

uli during exposure to a CDAA diet. Conversely, expression of TGF-β1, a growth-inhibitory factor for hepatocytes [15–17], was also stimulated during this treatment. TGF-β1 has been demonstrated to inhibit growth factor-induced cyclin D1 expression and DNA synthesis in primary cultured rat hepatocytes [10]. TGF-β1 also induces hepatocyte apoptosis both in vitro and in vivo [32,33]. Recent investigations have reported that the sensitivity of hepatocytes to TGF-β-mediated growth inhibition is modulated by the expression levels of TGF-β receptors during liver regeneration [34,35]. Furthermore, hepatocytes that adapted to survive in low choline were resistant to TGF-β1 [6,26]. Additionally, other cytokines, such as IL-1α and IL-1β also play a role in terminating DNA synthesis in hepatocytes induced by partial hepatectomy [36]. In this study, despite stimulation of TGF-β1 expression, enhanced expression of cyclin D1 and an increase in PCNA-positive hepatocytes were observed during CDAA diet administration, and the number of apoptotic hepatocytes markedly decreased following a peak at 8 weeks. Although we did not examine expression of TGF-β receptors and other cytokines in the rat livers, continuous stimulation of growth and growth inhibitory factors induced by prolonged exposure to a CDAA diet may affect TGF-β receptor expression and its signaling pathway directly or indirectly, leading to modulation of susceptibility of hepatocytes to TGF-β1.

Recent reports have demonstrated that the cells existing in CDAA diet-induced preneoplastic nodules escaped from TGF-β-mediated growth inhibition and have a selective growth advantage. This indicates the possibility that the TGF-β1 signaling pathway is disturbed in preneoplastic nodules induced by a CDAA diet [37,38]. TGF-β1 is produced as a small, biologically inactive, latent complex, which consists of mature TGF-β1 and latency-associated peptide (LAP). The latent TGF-β binding protein (LTBP) facilitates fixation of latent TGF-β1 in the extracellular matrix, resulting in a reservoir of latent TGF-β1 [39]. In contrast to diet-induced hepatocarcinogenesis, expression of TGF-β1 mRNA and latent TGF-β1 protein was observed in nonparenchymal cells surrounding or within the preneoplastic nodules or HCCs in a rat model of chemical hepatocarcinogenesis, however, mature TGF-β1 protein was detected only

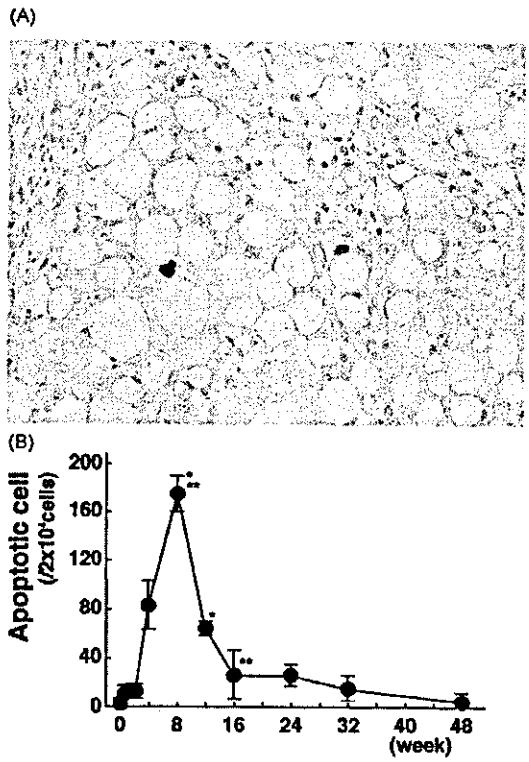

Fig. 6. Hepatocyte apoptosis was decreased following a peak in the early weeks of CDAA diet feeding. (A) Apoptosis of hepatocytes was examined by immunohistochemistry using an anti-single-stranded DNA antibody [23] as described in Section 2 [original magnification 400×]. (B) The number of apoptotic cells per 2×10^4 hepatocytes was counted ($n = 3$). The number of apoptotic hepatocytes rapidly decreased after a peak at 8 weeks of CDAA diet administration, and only a few apoptotic cells were detected at 48 weeks ($^*P = 0.004$, $^{**}P < 0.001$).

in nonparenchymal cells and connective tissues associated with HCCs, but was not observed in preneoplastic nodules [40]. In this study, the number of PCNA-positive hepatocytes continued to increase throughout the experimental period, and the PCNA-positivity in GST-P-positive nodules was much higher than that in GST-P-negative areas. Although the difference between GST-P-positive and GST-P-negative cells, both of which are positive for PCNA, is not fully understood and, also, details of the activation process of latent TGF-β in liver have not been elucidated, the regulatory effect of TGF-β1 on growth of GST-P-positive cells may contribute to the development of HCCs.

Following CDAA diet-induced hepatocyte injury in the early phase, both growth and growth-inhibitory stimuli were induced, and accelerated proliferation and decelerated apoptosis of hepatocytes was also observed. These results allow us to hypothesize that altered hepatic microenvironments in the early phase of a CDAA diet administration may contribute to development of preneoplastic nodules. In this context, we screened genes differentially expressed in rat livers treated with a CDAA diet for 8 weeks, and isolated osteoactivin (OA) cDNA [41]. Expression of OA was stimulated in rat livers during CDAA diet-induced cirrhosis development and in human HCCs. Further experimentation is underway to clarify the role of OA in the lengthy process of cirrhosis-associated hepatocarcinogenesis.

In this study, we have demonstrated that expression of growth and growth-inhibitory factors was stimulated following CDAA diet-induced liver injury, and that hepatocyte proliferation was accelerated during CDAA diet feeding, while hepatocyte apoptosis was decelerated. Although further investigations are necessary to understand the impaired mechanisms of liver regeneration following repeated hepatocyte injury induced by a CDAA diet, the results presented here indicate the possibility that imbalance between proliferation and apoptosis of hepatocytes, which are exposed to growth and growth-inhibitory stimuli over lengthy periods, is involved in CDAA diet-induced hepatocarcinogenesis.

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Pharmacokinetic study of recombinant human hepatocyte growth factor administered in a bolus intravenously or via portal vein

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Abstract

Hepatocyte growth factor (HGF) stimulates liver regeneration and has the potential to be a therapeutic agent for fatal liver diseases, including fulminant hepatic failure and liver cirrhosis. In this study, we investigated the pharmacokinetics of recombinant human HGF, which will be soon available for clinical applications. When recombinant human HGF (0.1 mg/kg) was administered intravenously to normal rats, serum levels of human HGF increased to 89.7 ± 20.6 ng/ml 5 min after the bolus injection, followed by a decrease with a half-life of 2.4 min. Recombinant HGF administered intravenously was distributed primarily to the liver and induced c-Met tyrosine phosphorylation in liver tissues. In comparison, rats given recombinant human HGF via the portal vein exhibited lower serum HGF and an increase in hepatic distribution. Additionally, when compared with normal rats, those with 70% partial hepatectomy or liver cirrhosis showed an increase in serum levels of human HGF with a prolonged half-life. These results suggest that, despite a short half-life, bolus injection of recombinant human HGF may induce therapeutic effect in patients with fatal live disease, and that the dose of this recombinant protein should be modulated according to the degree of liver injury.

Keywords: Hepatocyte growth factor; Pharmacokinetics; Tissue distribution; Serum HGF

1. Introduction

Hepatocyte growth factor (HGF) was originally purified from the plasma of patients with fulminant hepatic failure [1,2], and is one of the major agents that promote hepatocyte proliferation. HGF also functions as a pleiotropic factor, acting as a mitogen, motogen, and morphogen for a variety of epithelial cells (e.g. kidney, intestinal and bronchial cells) as well as endothelial cells by ligation of the c-Met receptor at the cell membrane [3–8]. Additionally, recent investigations have demonstrated that HGF inhibits apoptosis [9–15], and that fibrotic changes in various organs, including the liver and kidneys, were ameliorated by treatment with HGF [16–21]. Thus, HGF plays an important role in the regeneration and repair of injured tissues.

We have previously established an enzyme-linked immunosorbent assay (ELISA) to measure human HGF in serum, and reported that levels of serum human HGF increased in patients with various liver diseases [22]. We have also demonstrated that the measurement of serum HGF is useful for outcome prediction in fulminant hepatic failure [23]. Recombinant human HGF will soon be available for patients with fatal liver diseases, including fulminant hepatic failure, small-for-size grafts in living donor liver transplantation (LDLT), and liver cirrhosis. Since HGF functions as an anti-

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