

Portacaval shunt causes apoptosis and liver atrophy in rats despite increases in endogenous levels of major hepatic growth factors

Chandrashekhar R. Gandhi^{1,*}, Noriko Murase¹, Vladimir M. Subbotin², Tadahiro Uemura¹, Michael Nalesnik¹, Anthony J. Demetris¹, John J. Fung¹, Thomas E. Starzl¹

¹Thomas E. Starzl Transplantation Institute, University of Pittsburgh, E-1540 BST, 200 Lothrop Street, Pittsburgh, PA 15213, USA

²Mirus Corporation, Madison, WI, USA

Background/Aims: The response to the liver damage caused by portacaval shunt (PCS) is characterized by low-grade hyperplasia and atrophy. To clarify mechanisms of this dissociation, we correlated the expression of ‘hepatotrophic factors’ and the antihepatotrophic and proapoptotic peptide, transforming growth factor (TGF)- β , with the pathologic changes caused by PCS in rats.

Methods: PCS was created by side-to-side anastomosis between the portal vein and inferior vena cava, with ligation of the hilar portal vein. Hepatic growth mediators were measured to 2 months.

Results: The decrease in the liver/body weight ratio during the first 7 days which stabilized by day 15, corresponded to parenchymal cell apoptosis and increases in hepatic TGF- β concentration that peaked at $1.4 \times$ baseline at 15 days before returning to control levels by day 30. Variable increases in the concentrations of growth promoters (hepatocyte growth factor, TGF- α and augments of liver regeneration) also occurred during the period of hepatocellular apoptosis.

Conclusions: The development of hepatic atrophy was associated with changes in TGF- β concentration, and occurred despite increased expression of multiple putative growth promoters. The findings suggest that apoptosis set in motion by TGF- β constrains the amount of hepatocyte proliferation independently from control of liver volume.

© 2002 European Association for the Study of the Liver. Published by Elsevier Science B.V. All rights reserved.

Keywords: Portacaval; Liver; Atrophy; Apoptosis; Growth factor

1. Introduction

Portacaval shunt (PCS, also known as Eck’s fistula [1]) and partial hepatectomy (PH) are experimental operations that have opposite effects on liver size. Eck’s fistula has been a model of liver atrophy for more than 100 years [2]. The shrinkage of the liver is accompanied by a tripling of steady state hepatocyte renewal that stabilizes after 4–7

days in rats, dogs, pigs and baboons [3]. Interestingly, however, the side effects of PCS (originally termed ‘meat intoxication’ [2]) that cause the death of dogs, baboons and swine within several weeks are much less marked, or frequently may not be clinically apparent, in rats and humans [3–6]. In contrast to the hepatocyte atrophy of PCS, PH causes acute hypertrophy and high grade hyperplasia of hepatocytes of the residual fragment and has been used to study liver regeneration in a variety of species [7,8] ever since the procedure was standardized in rats by Higgins and Anderson [9].

Despite the ostensibly great differences between PCS and PH, both operations set in motion interactions between most, if not all, the same ‘hepatotrophic’ factors: i.e. hepatocyte growth factor (HGF), transforming growth factor- α (TGF- α), insulin-like growth factor (IGF)-II, augments of liver regeneration (ALR) and insulin [10]. All five of these endogenous factors prevent the atrophy of PCS when

Received 14 November 2001; received in revised form 11 April 2002; accepted 2 May 2002

* Corresponding author. Tel.: +1-412-648-9316; fax: +1-412-624-6666.

E-mail address: gandhics@msx.upmc.edu (C.R. Gandhi).

Abbreviations: ALR, augments of liver regeneration; ELISA, enzyme-linked immunosorbent assay; HGF, hepatocyte growth factor; HR, hepatic regeneration; IGF, insulin-like growth factor; PCS, portacaval shunt; PH, partial hepatectomy; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; TGF, transforming growth factor; TUNEL, TdT-mediated dUTP nick end labeling.

infused in appropriate doses into the detached portal vein at the hilum following complete PCS [11]. As was first demonstrated with insulin [12], these molecules initiate proliferation in the Eck fistula liver beyond the preexisting low-grade hyperplasia resembling in magnitude the burst of regeneration that follows PH. The hepatotrophic effects of active molecules are so reliably reproducible that an Eck fistula infusion assay has been used to screen the same liver growth factors that can be identified more laboriously with assays based on PH [10–12]. Importantly, both Eck fistula and PH assays also recognize the only known endogenous antihepatotrophic molecule transforming growth factor- β (TGF- β) [10,11,13].

Except for insulin, all of the endogenous hepatotrophic molecules, as well as TGF- β , have multiple sites of synthesis, including the liver [7,8]. Thus, it is conceivable that the liver could autoregulate its size or regeneration by the autocrine or paracrine actions of these peptides. This view is consistent with, but not proven by, the changes that occur after PH in the hepatic mRNA and protein expression of HGF [14,15], TGF- α [16,17] and ALR [18]. Furthermore, it has been reported that the peak increases in TGF- β mRNA coincide with the termination of regeneration prompting speculation that this molecule is the prime determinant of the reconstituted liver's ultimate size [7,8,19]. Because members of the TGF- β superfamily are proapoptotic [20–23], we have postulated that programmed cell death is an important mechanism in regulating liver growth and regeneration [18].

We report here peptide concentrations and the mRNA levels in the liver of the hepatotrophic molecules HGF, TGF- α and ALR following PCS. The patterns of change were similar to those caused by PH. However, because the increases in TGF- β peptide concentration corresponded closely to apoptosis of the atrophic liver cells, the apoptotic effects of TGF- β (and presumably the TGF- β superfamily [20–23]) were correlated with hepatic proliferation and occurred independently of liver volume.

2. Materials and methods

The protocols were approved by the IACUC, University of Pittsburgh in accordance with the guidelines of the NIH. Inbred male Lewis rats (200–250 g), purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN, USA), were maintained in a laminar-flow, pathogen-free animal facilities at the University of Pittsburgh. Animals were kept at 20°C with a 12:12 h light–dark cycle, and fed ad libitum.

2.1. Surgical procedures

Surgical procedure was performed on preweighed rats under methoxyflurane anesthesia. Laparotomy was performed and hepatoesophageal plexus was ligated. Side-to-side anastomosis was created between the portal vein and inferior vena cava using 10–0 Novafil suture as described by Lee and Fisher [24]. After the shunt was examined to ensure its patency, the portal vein was carefully separated from hepatic artery and ligated at the hepatic hilum to create total PCS.

2.2. Sample preparation

One, 2, 4, 7, 15, 30 and 60 days after the creation of PCS, rats were weighed and sacrificed (three for each time point). The liver was excised after laparotomy, weighed, rinsed in ice-cold phosphate-buffered saline, and processed as follows: a portion of the right lobe was preserved in OCT compound (Miles Inc., Elkhart, IN, USA) and a portion in buffered formalin. The rest of the liver was snap-frozen in liquid nitrogen, crushed and saved in portions at -80°C .

2.3. Histological examination and determination of apoptosis

Hematoxylin and eosin-stained sections of the liver were examined for histological changes. An ApopTag[®] peroxidase kit (Intergen Company, New York, NY, USA) was used for determination of apoptosis. A total of 45–50 fields (about 50 hepatocytes per field) were examined to count apoptotic nuclei in the liver sections.

2.4. Determination of growth mediators

2.4.1. TGF- β

The liver was homogenized in 2.5 volumes of 2.5 N acetic acid containing 10 M urea, 1 mM phenylmethane sulfonyl fluoride (PMSF) and 10 $\mu\text{g}/\text{ml}$ pepstatin A. After 20 min at room temperature, the pH was adjusted to 7.4 with 2.7 N NaOH/1 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid (HEPES). The samples were centrifuged at 20,200 $\times g$ for 20 min, and the supernatants were used in the enzyme-linked immunosorbent assay (ELISA; R and D Systems Inc., Minneapolis, MN, USA) for TGF- β quantification.

2.4.2. HGF

The liver was homogenized in five volumes of ice-cold Tris-HCl, pH 7.4, containing 2 M NaCl, 0.1% Tween-80, 1 mM PMSF and 1 mM ethylenediamine-tetraacetic acid (EDTA). The homogenate was centrifuged for 30 min at 20,000 $\times g$ at 4°C, and the supernatant was used in the ELISA (R and D Systems).

2.4.3. TGF- α

The liver was homogenized in five volumes of 1% trifluoroacetic acid, and centrifuged at 17,000 $\times g$ for 20 min at 4°C after 2 h at room temperature for 2 h. The supernatant was applied to C-18 reverse phase column (Waters Corp., Milford, MA, USA) prewashed with 2 ml of 60% acetonitrile in 1% trifluoroacetic acid (one wash), and 2 ml of 1% trifluoroacetic acid (three washes). Following three washes with 1% trifluoroacetic acid, TGF- α was eluted with 3 ml 60% acetonitrile in 1% trifluoroacetic acid. The organic solvents were dried under N_2 , and the residual aqueous material was lyophilized and reconstituted in radioimmunoassay (RIA) buffer for determination of TGF- α (Peninsula Laboratories Inc., Belmont, CA, USA).

2.4.4. ALR

ALR was extracted and its concentration determined by ELISA essentially as described previously [18].

2.5. Determination of mRNA for HGF, TGF- α , TGF- β and ALR

Total RNA was isolated using TRI reagent (Molecular Research Center Inc., Cincinnati, OH, USA), and mRNA was extracted using a PolyAT-tract[™] mRNA isolation system (Promega, Madison, WI, USA). Twenty micrograms of total RNA (TGF- β , HGF and ALR) or 3 μg of the mRNA (TGF- α) was denatured at 55°C for 15 min and electrophoresed on a 1% agarose/formaldehyde gel and transferred to a Hybond N+ membrane (Amersham-Pharmacia, Piscataway, NJ, USA). The membranes were hybridized with ³²P-labeled cDNA probes in a solution of 50% formamide,

5X standard saline citrate (SSC), 10 mM sodium phosphate (pH 6.8), 0.5% sodium dodecyl sulfate (SDS), 5X Denhart solution and 20 µg/ml herring sperm DNA at 42°C overnight. The membranes were washed twice with 2X SSC containing 0.1% SDS at room temperature for 30 min, twice with 0.2X SSC containing 0.1% SDS at 55°C for 30 min, and exposed to Kodak XOMat film (Eastman Kodak Co., Rochester, NY, USA). The probes were radiolabeled by random priming method using multiprime DNA labeling kit (Amersham-Pharmacia) in the presence of [³²P]dCTP (NEN, Boston, MA, USA). A 0.99 kb fragment of rat TGF-β1 cDNA was purchased from ATCC (Rockville, MD, USA). The cDNA probes for HGF, TGF-α and reduced form of GAPDH were kindly provided by Dr George Michalopoulos, Department of Pathology, University of Pittsburgh and Drs H. Miyazaki and T. Tsuji, Department of Cellular Biology, Okayama University Medical School, Okayama, Japan. GAPDH mRNA expression was determined as an internal control.

2.6. Isolation and culture of hepatocytes

Hepatocytes were prepared by collagenase digestion of the liver followed by purification on a percoll gradient as described previously [25]. Purity was determined by immunohistochemical markers for various non-parenchymal cells and biliary epithelial cells [25]. The cells obtained thus showed >90% viability (trypan blue exclusion) and were more than 97% pure. Cells were washed twice and suspended in William's medium E containing 2 mM L-glutamine, 250 units/ml penicillin, 250 µg/ml streptomycin, 10% fetal bovine serum, 10⁻⁶ M insulin (Eli Lilly Co., Indianapolis, IN, USA) and 20 mM HEPES. Cells were plated in 24-well plates (0.125 × 10⁶ cells/well) and the medium was renewed 3 h later. After overnight incubation, cells were placed in serum-free William's medium E containing 0.1% bovine serum albumin (BSA), with or without insulin and the growth mediators were added at the indicated concentrations. Following a 24 h, [³H]thymidine (1 µCi/ml) was added to the wells and incubation was continued for 4 h. The medium was aspirated, the cells washed with ice-cold Hanks' balanced salt solution (HBSS) and treated with 10% ice-cold trichloroacetic acid (TCA) twice before determination of TCA-precipitable radioactivity.

2.7. Statistical analysis

The results are expressed as averages ± SEM. Statistical significance was derived by non-parametric Mann-Whitney two-tailed variance test using an SPSS program. A *P* value of <0.05 was considered statistically significant.

3. Results

3.1. Liver/body weight ratio after PCS

There was a rapid decline in liver/body weight ratio after PCS (Fig. 1A), most of which had occurred by day 7 with only a further 10% decrease by day 60.

3.2. Hepatocellular apoptosis after PCS

Apoptosis was rarely observed in the control liver. After the PCS, there was a rapid increase in the number of apoptotic nuclei in hepatocytes that peaked between day 4 and day 7, and then declined sharply to the basal level on day 15 (Figs. 1B and 2). A similar pattern was also observed for other apoptotic bodies that could not be distinguished as hepatocytes. These apoptotic bodies were more numerous than hepatocytes at all time points, and their number did not return to the control value during the time course of the

experiment. Necrosis of hepatocytes or non-parenchymal cells was not observed at any time except in the arteries (see below).

3.3. Arterial remodeling after PCS

Arterial remodeling was evidenced by endothelial cell damage. Unlike the hepatocytes and non-parenchymal cells, patchy necrosis, mostly of smooth muscle cells, was found in small arteries on day 2 (Fig. 3). Necrosis was reduced on day 4, and by day 7, there was thickening of arterial walls and some increase in the size of the lumens that persisted throughout the time course of the experiment (Fig. 3). These observations are consistent with earlier descriptions of thickened arterioles and compensatory increases in hepatic arterial blood flow in rats with congenital portal-systemic shunts [26].

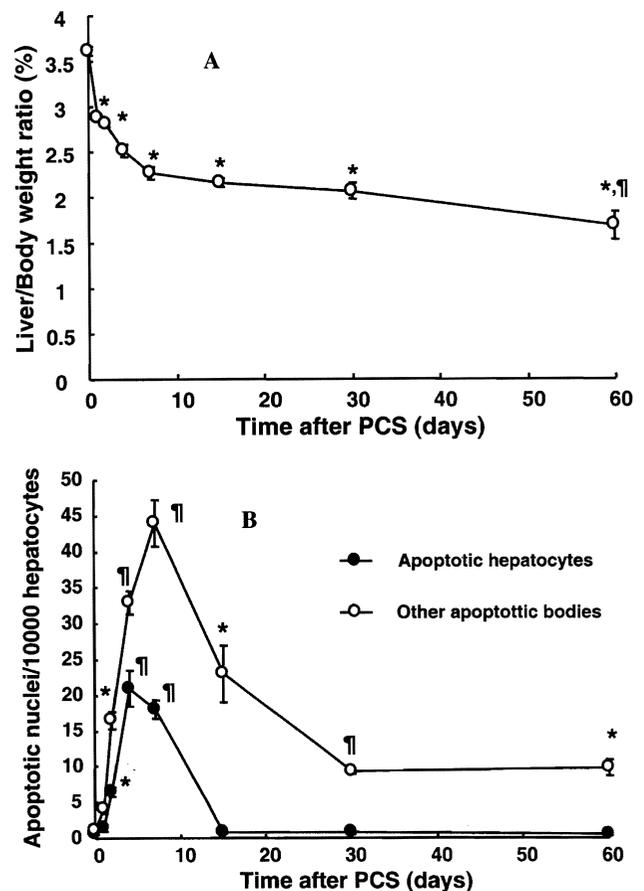


Fig. 1. (A) Liver/body weight ratio and (B) hepatocellular apoptosis at successive interval after PCS. (A) At the indicated times post-PCS, the rats were sacrificed and the respective weights determined. Values represent averages of three determinations ± SEM. **P* < 0.001 vs. '0' time; ¶*P* < 0.05 vs. '7-day' time point. (B) Apoptosis was quantified using both TUNEL assay and the morphological manifestations of apoptotic figures. Each point represents averages of the values from three rats (45–50 fields per section) ± SEM. **P* < 0.005 vs. '0' time; ¶*P* < 0.001 vs. '0' time and <0.01 vs. '2-day' time point.

3.4. Hepatic TGF- β after PCS

The concentration of TGF- β mRNA increased at 24 h after PCS and remained elevated until day 15, but the increase was not statistically significant relative to the control value (Fig. 4A). The protein concentration of TGF- β increased sharply (140% increase) at 24 h after PCS, remained at about this level until day 15, before declining to the basal value at 30 days (Fig. 4B).

3.5. Hepatic HGF and TGF- α after PCS

HGF mRNA levels doubled between days 1 and 4 after PCS, and declined thereafter to the basal value by day 30 (Fig. 5A). In contrast to the doubling of its mRNA tran-

script, HGF protein increased only about 12% between days 1 and 4 after PCS (Fig. 5B), before rising to 45–50% over the basal value between days 7 and 15. Return to the basal level occurred by postoperative day 60.

The concentration of TGF- α mRNA decreased somewhat after PCS and did not return to the normal, even on day 60 (Fig. 6A). However, the decrease was not statistically significant compared to the control. Paradoxically, TGF- α protein concentration doubled by 4 days, and decreased to the basal level by day 30 (Fig. 6B).

3.6. Hepatic ALR after PCS

ALR mRNA concentration declined, but not significantly, after PCS for 4 days, and returned to the normal

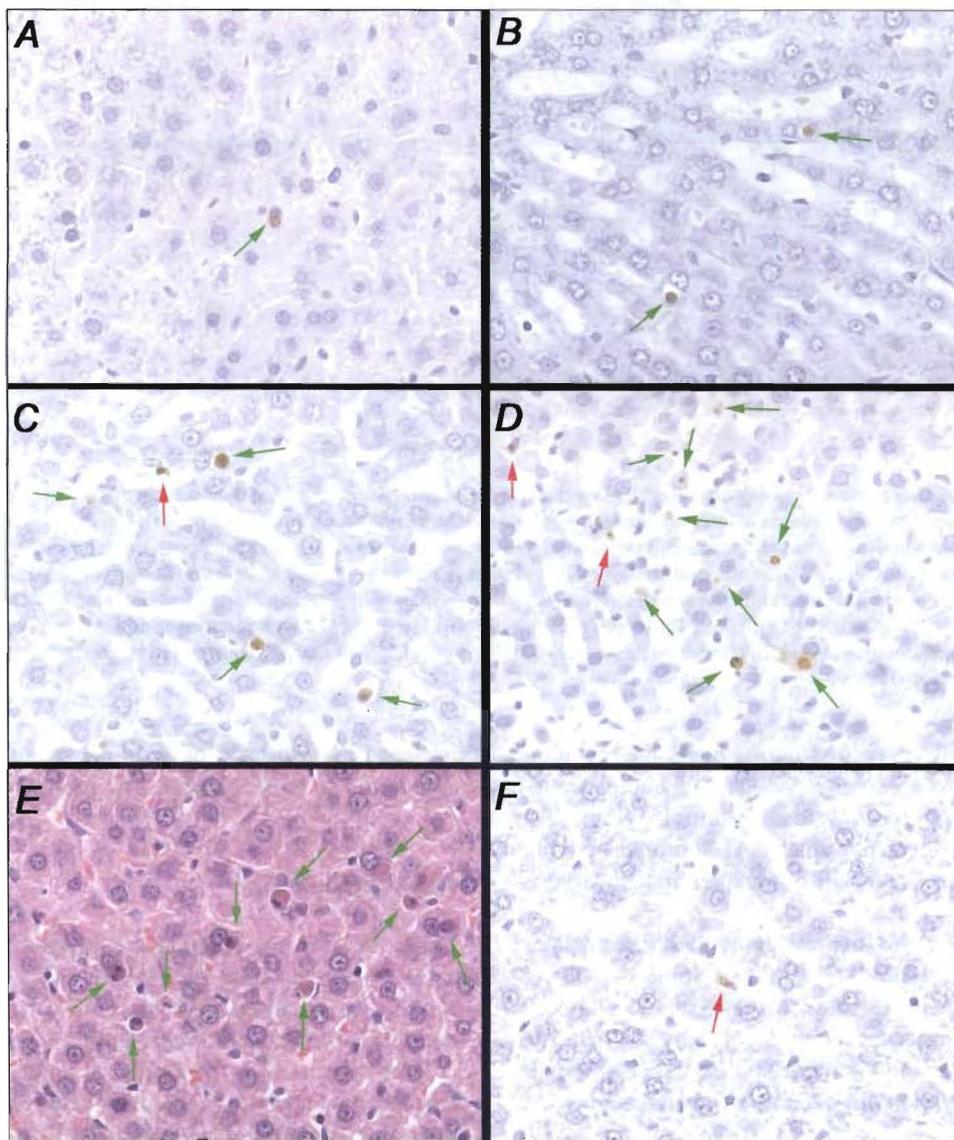


Fig. 2. Histopathology showing hepatocellular apoptosis after PCS. The sections were processed for TUNEL assay to demonstrate apoptosis. In addition to their presence in the parenchymal cells (green arrows), apoptotic bodies that cannot be distinguished as belonging to any specific cell type (red arrows) were also observed from day 2 (B). (C) day 4; (D) day 7; (F) day 30. Hematoxylin/eosin-stained section of the liver on day 7 after PCS (E) demonstrates removal of apoptotic cells by 'dropout'. On day 30, apoptotic bodies were found very rarely. Original magnification $\times 400$.

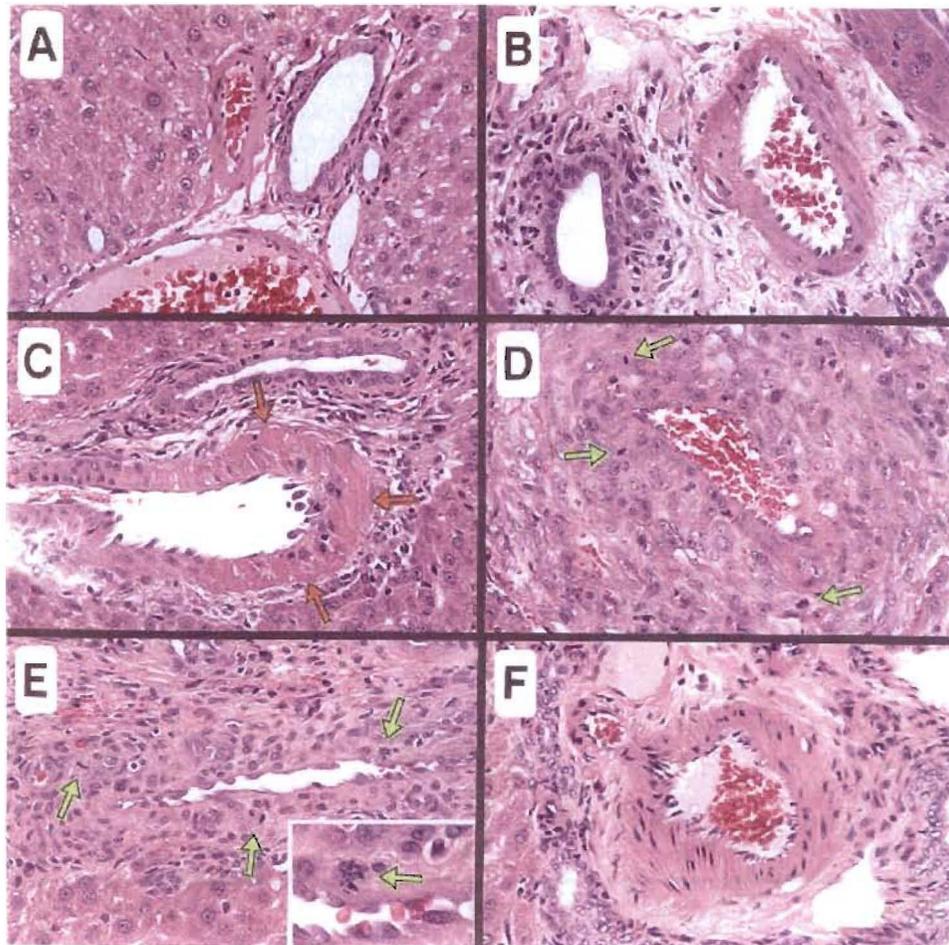


Fig. 3. Histopathology showing arterial remodeling after PCS. Sections of the normal liver (A) and of the livers on day 1 (B), 2 (C), 4 (D), 7 (E) and 30 (F) after PCS are shown. On day 1, edema associated with minimal macrophage infiltration was noted in periarterial space around some hepatic arteries. On day 2, the edema was decreased and macrophage infiltration became more evident. Patchy necrosis, predominantly of the smooth muscle cells was also seen (red arrows). Necrosis was minimal on day 4 (D), and thickening of the arterial walls due to smooth muscle cell proliferation (green arrows) and macrophage accumulation around the arteries occurred. On day 7, macrophage accumulation was minimal, but thickness of the arterial wall was still significantly increased due to increase in smooth muscle cells. The latter showed high number of mitotic figures (green arrows, inset). Arterial morphology was similar and close to normal on days 30 and 60 (not shown), except that the walls were thicker and the lumen size increased. Original magnification $\times 400$.

level by day 10 (Fig. 7A). In contrast to its mRNA transcript, ALR protein increased by 44% at 24 h before gradually declining to the basal value by day 15 (Fig. 7B). Changes in hepatic concentration of ALR protein without significant alterations in its mRNA expression have been reported previously [18].

Interestingly, the mRNA expression of all of these mediators, with the exception of TGF- α , was somewhat lower than the control value on day 30 although it was still above or at near normal levels on day 15 and day 60. The significance of this finding is unclear since the corresponding protein concentration did not exhibit similar trends.

3.7. Effect of TGF- β on HGF-induced DNA synthesis

In cultured hepatocytes TGF- β completely inhibited HGF-stimulated [3 H]thymidine incorporation, both in the

presence and absence of insulin (Table 1). These results are similar to those described by Nakamura et al. [27].

4. Discussion

The liver of rats previously subjected to PCS retains its capacity to regenerate after PH, but only to the preexisting shrunken size caused by the portal diversion [6,28]. Regeneration is itself hepatotrophic in that glycogen and hepatocyte size as well as a variety of organelles temporarily normalize when PH is performed on the atrophic Eck fistula liver, but only during the period of regeneration [29]. Moreover, a full recovery (including its weight) of the liver that has undergone PCS-induced atrophy, occurs promptly after restoration of normal portal venous blood circulation [30,31]. The results of such experiments have suggested

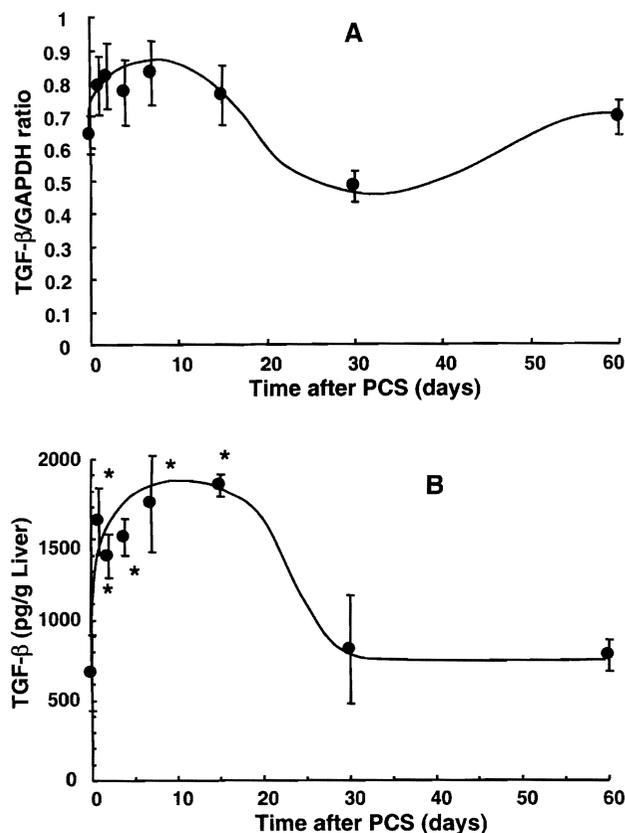


Fig. 4. Effect of PCS on hepatic TGF- β (A) mRNA and (B) protein. The mRNA expression and the protein levels of TGF- β were determined in the liver samples from three rats for each time point. For each rat duplicate determinations were made. Values are averages of such determinations \pm SEM. * $P < 0.05$ vs. '0' time.

that endogenously produced extra-hepatic growth factors, of which insulin is the most readily identifiable [10,12,32], are essential for the maintenance of hepatic size, structure and function. Other studies have shown that these same factors are required for maintenance of the full capacity for regeneration, but not of regeneration per se [29,33,34].

In screening for candidate growth modulating factors by infusing test substances directly into the tied off central portal vein, two growth inhibitory (antihepatotrophic) factors have been identified that make the hepatocyte atrophy of the PCS liver more extreme and inhibit its characteristic low-grade hyperplasia: TGF- β [11,13] and the T cell-directed immunosuppressant rapamycin [35]. In contrast to the potentially artifactual conditions of the PCS infusion experiments (e.g. excessive doses of the test molecules), the studies reported here were of the physiologic expression of endogenous growth modulating peptides after PCS in rats, with particular reference to TGF- β . The protein concentration of the assayed growth mediators (i.e. HGF, TGF- α , ALR and TGF- β) increased in a time-dependent manner. The increases were of lesser magnitude and of longer duration than those that have been reported after PH [7,8,14–17,19], and did not correlate precisely with

the corresponding changes in the respective mRNA transcripts. It is likely that for such small increases in the protein concentrations enhanced translational activity may be adequate, and similar increases in the mRNA levels are not necessary. Alternatively, stabilization of the mRNA may be a mechanism of smaller increases of the protein expression of the growth mediators after PCS. Despite increases in the hepatotrophic peptides, the liver underwent atrophy and apoptosis that correlated with the prompt and sustained increase in TGF- β . In several animal models, including the rat, PCS causes low-grade hyperplasia that stabilizes after 4–7 days after the operation [3]. Therefore, near stabilization of the liver size 15 days after PCS suggests that the two processes (apoptosis and hyperplasia) may have reached equilibrium.

It has been shown that primary cultures of hepatocytes exposed to TGF- β [20–22] and activin A [23] (a member of the TGF- β superfamily), undergo apoptotic death and that activin A administration causes significant cell loss from apoptosis localized primarily around the central vein in rodent livers [23]. Moreover, TGF- β also induces apoptosis of non-parenchymal cells (e.g. the stellate cells [36]).

The strong antagonism of TGF- α actions by TGF- β has

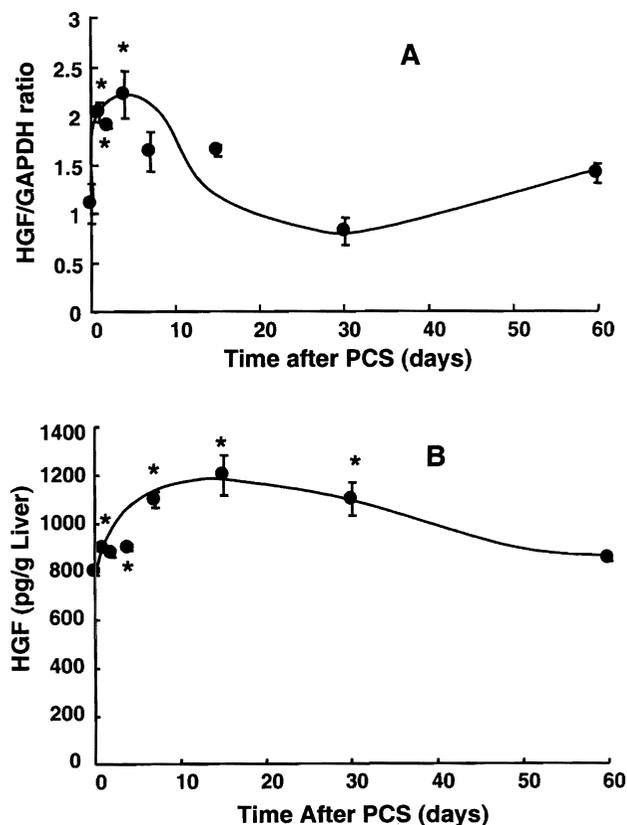


Fig. 5. Effect of PCS on hepatic HGF mRNA (A) and protein (B). The mRNA expression and the protein levels of HGF were determined in the liver samples from three rats for each time point. For each rat duplicate determinations were made. Values are averages of such determinations \pm SEM. * $P < 0.05$ vs. '0' time.

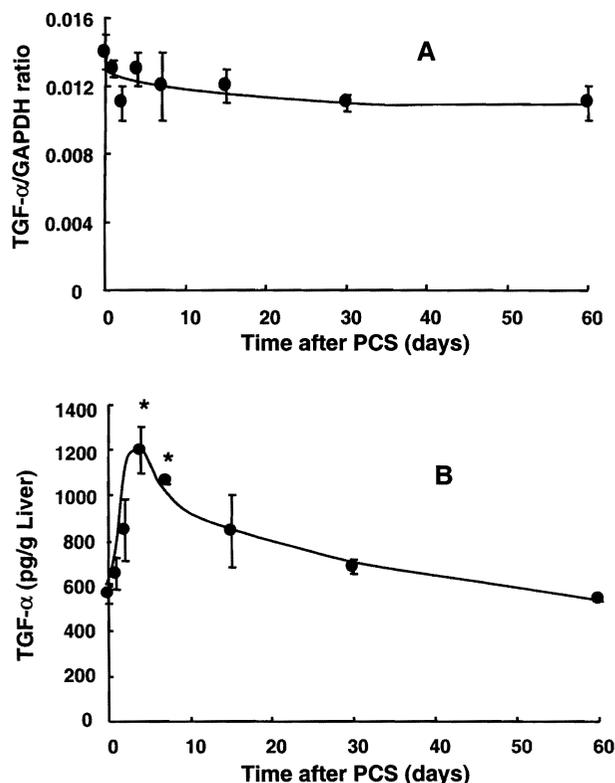


Fig. 6. Effect of PCS on hepatic TGF- α (A) mRNA and (B) protein. The mRNA expression and the protein levels of TGF- α were determined in the liver samples from three rats for each time point. For each rat duplicate determinations were made. Values are averages of such determinations \pm SEM. * $P < 0.005$ vs. '0' time.

been unequivocally established in in vivo models [7,8,19] including PCS [11,13]. Moreover, although HGF has been reported to protect several cell types including hepatocytes from apoptotic death [37–41], it does not protect cultured fetal hepatocytes from TGF- β -induced apoptosis [42], and it was found to be proapoptotic for certain cells such as Math A mouse sarcoma cells [43], aflatoxin-transformed rat liver epithelial cells [44] and hepatoma HepG2 cells [45]. There is also considerable in vitro evidence that HGF-stimulated DNA synthesis in hepatocytes is completely inhibited by TGF- β both in the presence and absence of insulin [27] (Table 1). Coinfusion of TGF- β with a variety of exogenous growth factors in previous studies of PCS could override the hepatotrophic effects only of TGF- α [11,13]. However, the weight of collective evidence including the studies of PCS reported here suggests that the apoptotic effect of TGF- β can overwhelm otherwise physiologically significant increases in hepatic tissue concentrations of TGF- α as well as HGF and ALR.

Recently, it was reported that the IL-1 β mRNA concentration increases 24 h after PCS in rat [46]. It was suggested that the antiproliferative property of IL-1 β [47] may be primarily responsible for the liver atrophy since TGF- β mRNA did not change at this time. However, the protein concentrations of these molecules were not determined, and,

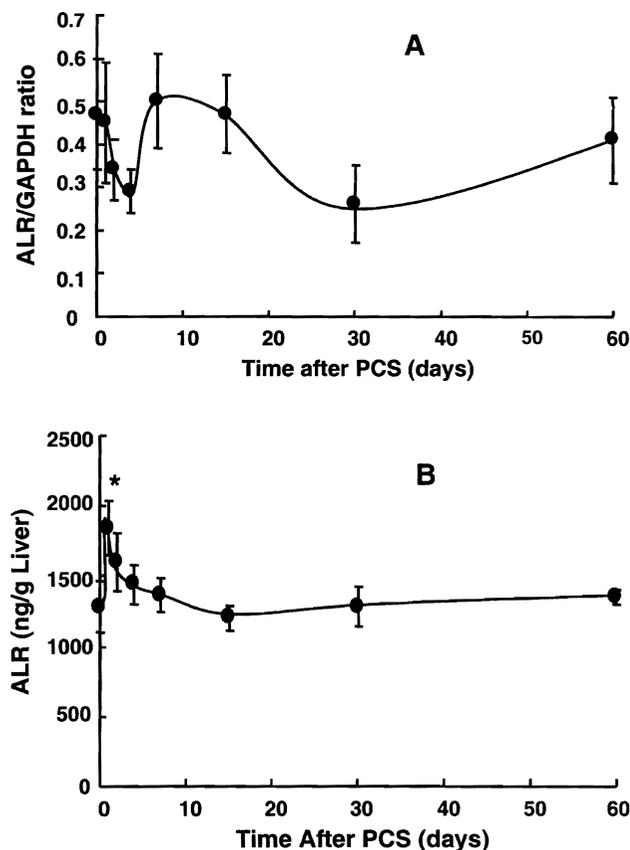


Fig. 7. Effect of PCS on hepatic ALR (A) mRNA and (B) protein. The mRNA expression and the protein levels of ALR were determined in the liver samples from three rats for each time point. For each rat duplicate determinations were made. Values are averages of such determinations \pm SEM. * $P < 0.05$ vs. '0' time.

therefore, it is difficult to speculate on the role of IL-1 β in PCS. In this regard, it should be noted that IL-1 β has also been found to exert no effect on hepatocyte proliferation [48], and in yet other studies it was found to be antiapoptotic [49,50]. Thus a careful analysis of IL-1 β (mRNA and protein) with respect to the changes of PCS, warrants further investigation.

Table 1
Effect of TGF- β on HGF-induced [3 H]thymidine incorporation in hepatocytes^a

	– Insulin	+ Insulin
Vehicle	318 \pm 34	494 \pm 53
HGF (25 ng/ml)	983 \pm 73	1331 \pm 128
TGF- β (5 ng/ml)	140 \pm 24	89 \pm 5
HGF + TGF- β	113 \pm 6	109 \pm 2
Serum (5%)	940 \pm 14	932 \pm 15

^a After an overnight culture, cells were placed in serum-free medium with or without 10^{-6} M insulin. Growth mediators were added at the concentration indicated and incubation was continued for 24 h. [3 H]thymidine (1 μ Ci/ml) was added to all wells and the reaction was terminated after 4 h. Results are expressed as CPM/well \pm SEM of triplicate determinations of an experiment performed twice.

The actual loss of hepatocytes due to apoptosis probably is much higher than could be demonstrated decisively in our study. At the 7-day time point, some apoptotic hepatocytes could be seen in the sinusoidal lumen presumably just before their removal from the liver by a phenomenon known as 'dropout' that has been demonstrated in human patients undergoing liver allograft rejection [51–54]. With anti-Fas antibody it appeared that the dead hepatocytes 'leaked out' rather than being phagocytosed by neighboring macrophages and granulocytes [55]. Because we did not observe any evidence of phagocytosis of apoptotic hepatocytes by macrophages, these cells would have been excluded from the apoptotic counts. In addition, apoptotic bodies that could not be identified, but followed similar pattern as for apoptotic hepatocytes were also noted in the liver after PCS. The precise identification of these bodies could not be made because of the rapidity of the apoptotic process and the limitations of TdT-mediated dUTP nick end labeling (TUNEL) assay and other procedures [56].

In conclusion, the results of this study demonstrate that the hepatic atrophy that results from PCS occurs despite the upregulation of endogenously produced growth promoters whose effect appeared to be interdicted by growth inhibitory mechanisms of which the physiologic action of TGF- β is the most obvious candidate. TGF- β -induced apoptosis of liver cells appears to be an integral part of the complex mechanism that constrains liver cell and non-parenchymal cell replication, allowing a stable equilibrium to be established after PCS of low-grade hyperplasia despite global organ atrophy. The results may have direct clinical implications in liver diseases involving portal vein obliteration. Ischemia caused by this condition has been shown to cause hepatocellular apoptosis and liver atrophy in the absence of chronic inflammation [57]

Acknowledgements

This work was supported by DK29961, DK54411, A140329 and A38899 and a VA merit award. We thank John Prelich, Lori Perez and Jennifer Costentine for excellent technical assistance, and Terry Mangan for outstanding secretarial support.

References

- [1] Eck NVK. voprosu o perevyazkie vorotnois veni: Predvaritelnoye soobshchjenye (concerning ligation of the vena porta). *Voen Med J* 1877;130:1–2 (Translation by Child CG III: Eck's Fistula. *Surg Gynecol Obstet* 1953; 96:375–6).
- [2] Hahn M, Massen O, Nencki M, Pavlov J. Die Eck'sche Fistel zwischen der unteren Hohlvene und der Pfortader und ihre Folgen fur den Organismus. *Arch Exp Pathol Pharm* 1893;32:161–210.
- [3] Starzl TE, Porter KA, Francavilla A. The Eck fistula in animals and humans. *Curr Probl Surg* 1983;20:687–752.
- [4] Putman CW, Porter KA, Starzl TE. Hepatic encephalopathy and light electron microscopic changes of the baboon liver after portal diversion. *Ann Surg* 1976;184:155–161.
- [5] Fisher ER, Fisher B. Ultrastructural hepatic changes following partial hepatectomy and portacaval shunt in rat. *Lab Invest* 1963;12:929–942.
- [6] Fisher B, Lee SH, Fisher ER, Saffar E. Liver regeneration following portacaval shunt. *Surgery* 1962;52:88–102.
- [7] Fausto N, Webber EM. Liver regeneration. In: Arias IM, Boyer JL, Fausto N, Jakoby WB, Schachter DA, Shafritz DA, editors. *The liver: biology and pathobiology*, 3rd ed.. New York, NY: Raven Press, 1994. pp. 1059–1084.
- [8] Michalopoulos GK, DeFrances MC. Liver regeneration. *Science* 1997;276:60–66.
- [9] Higgins GM, Anderson RM. Experimental pathology of liver. *Arch Pathol* 1931;12:186–202.
- [10] Francavilla A, Hagiya M, Porter KA, Polimeno L, Ihara I, Starzl TE. Augmenter of liver regeneration (ALR): its place in the universe of hepatic growth factors. *Hepatology* 1994;20:747–757.
- [11] Francavilla A, Starzl TE, Porter K, Foglieni CS, Michalopoulos GK, Carrieri G, et al. Screening for candidate hepatic growth factors by selective portal infusion after canine Eck's fistula. *Hepatology* 1991;14:665–670.
- [12] Starzl TE, Porter KA, Putnam CW. Intraportal insulin protects from the liver injury of portacaval shunt in dogs. *Lancet* 1975;2:1241–1246.
- [13] Francavilla A, Azzarone A, Carrieri G, Foglieni CS, Zeng QH, Cillo U, et al. Effect on canine Eck fistula liver of intraportal TGF- β alone or with hepatic growth factors. *Hepatology* 1992;16:1267–1270.
- [14] Mitsue S, Hamanoue M, Tanabe G, Ogura Y, Yoshidome S, Aikou T, et al. Expression of HGF and TGF- β 1 mRNA after partial hepatectomy in rats with liver cirrhosis. *Surg Today* 1995;25:237–243.
- [15] Tomiya T, Ogata I, Kenji F. Transforming growth factor α levels in liver and blood correlate better than hepatocyte growth factor with hepatocyte proliferation during liver regeneration. *Am J Pathol* 1998;153:955–961.
- [16] Webber EW, Fitzgerald MJ, Brown PI, Bartlett MH, Fausto N. Transforming growth factor- α expression during liver regeneration after partial hepatectomy and toxic injury, and potential interactions between transforming growth factor- α and hepatocyte growth factor. *Hepatology* 1993;18:1422–1431.
- [17] Russell WE, Dempsey PJ, Sitaric S, Peck AJ, Coffee Jr RJ. Transforming growth factor- α (TGF α) concentrations increase in regenerating rat liver: evidence for a delayed accumulation of mature TGF α . *Endocrinology* 1993;133:1731–1738.
- [18] Gandhi CR, Kuddus RH, Subbotin VM, Prelich J, Murase N, Rao AS, et al. A fresh look at augmenter of liver regeneration (ALR) in rats. *Hepatology* 1999;29:1435–1445.
- [19] Fausto N, Laird AD, Webber EM. Role of growth factors and cytokines in hepatic regeneration. *FASEB J* 1995;9:1527–1536.
- [20] Oberhammer F, Bursch W, Parzefall W, Breit P, Erber E, Stadler M, et al. Effect of transforming growth factor β on cell death of cultured hepatocytes. *Cancer Res* 1991;51:2478–2485.
- [21] Oberhammer F, Pavelka M, Sharma S, Tiefenbacher R, Purchio AF, Bursch W, et al. Induction of apoptosis in cultured hepatocytes and in regressing liver by transforming growth factor β 1. *Proc Natl Acad Sci USA* 1992;89:5408–5412.
- [22] Bursch W, Oberhammer F, Jirtle RL, Asakari M, Sedivy R, Grasl-Kraupp B, et al. Transforming growth factor- β 1 as a signal for induction of cell death by apoptosis. *Br J Cancer* 1993;67:531–536.
- [23] Hully JR, Chang L, Schwall RH, Widmer HR, Terrell TG, Gillett NA. Induction of apoptosis in the murine liver with recombinant human activin A. *Hepatology* 1994;20:854–861.
- [24] Lee SH, Fisher B. Portacaval shunt in the rat. *Surgery* 1961;50:668–672.
- [25] Kuddus RH, Nalesnik MA, Subbotin VM, Rao AS, Gandhi CR. Enhanced synthesis and reduced metabolism of endothelin-1 (ET-1)

- by hepatocytes- an important mechanism of increased endogenous levels of ET-1 in liver cirrhosis. *J Hepatol* 2000;33:725–732.
- [26] Bioulac-Sage P, Saric J, Boussarie L, Balabaud C. Congenital portacaval shunt in rats: liver adaptation to lack of portal vein – a light and electron microscopic study. *Hepatology* 1985;5:1183–1189.
- [27] Nakamura T, Tomita Y, Hirai R, Yamaoka K, Kaji K, Ichihara A. Inhibitory effect of transforming growth factor- β on DNA synthesis of adult rat hepatocytes in primary culture. *Biochem Biophys Res Commun* 1985;133:1042–1050.
- [28] Weinbren K. The portal blood supply and regeneration of the rat liver. *Br J Exp Pathol* 1955;36:583–591.
- [29] Starzl TE, Porter KA, Kashiwagi N, Putnam CW. Portal hepatotropic factors, diabetes mellitus and acute liver atrophy, hypertrophy and regeneration. *Surg Gynecol Obstet* 1975;141:843–858.
- [30] Weinbren K, Washington SLA, Smith CY. The response of the rat liver to alterations in total portal blood flow. *Br J Exp Pathol* 1975;56:148–156.
- [31] Hawkins PA, DeJoseph MR, Hawkins RA. Eliminating metabolic abnormalities of portacaval shunting by restoring normal liver blood flow. *Am J Physiol* 1996;270:E1037–E1042.
- [32] Starzl TE, Porter KA, Watanabe K, Putnam CW. The effects of insulin, glucagon, and insulin/glucagon infusions upon liver morphology and cell division after complete portacaval shunt in dogs. *Lancet* 1976;1:821–825.
- [33] Child CG, Barr D, Holswade GR, Harrison CS. Liver regeneration following portacaval transposition in dogs. *Ann Surg* 1953;138:600–608.
- [34] Starzl TE, Francavilla A, Porter KA, Benichou J, Jones AF. The effect of splanchnic viscera removal upon canine liver regeneration. *Surg Gynecol Obstet* 1978;147:193–207.
- [35] Francavilla A, Starzl TE, Scotti C, Carrieri C, Azzarone S, Zeng QH, et al. Inhibition of liver, kidney, and intestine regeneration by rapamycin. *Transplantation* 1991;53:496–498.
- [36] Saile B, Matthes N, Knittel T, Ramadori G. Transforming growth factor β and tumor necrosis factor α inhibit both apoptosis and proliferation of activated rat hepatic stellate cells. *Hepatology* 1999;30:196–202.
- [37] Tanaka T, Mizuno K, Umesaki N, Ogita S. Suppressed apoptotic susceptibility in human endometrial epithelial cells pretreated with hepatocyte growth factor. *Clin Exp Obstet Gynecol* 1998;25:125–128.
- [38] Kosai K, Matsumoto K, Nagata S, Tsujimoto Y, Nakamura T. Abrogation of Fas-induced hepatic failure in mice by hepatocyte growth factor. *Biochem Biophys Res Commun* 1998;244:683–690.
- [39] Ueki T, Kaneda Y, Tsutsui H, Nakanishi K, Sawa Y, Morishita Y, et al. Hepatocyte growth factor gene therapy of liver cirrhosis in rats. *Nat Med* 1999;5:226–230.
- [40] Kopp JB. Hepatocyte growth factor: mesenchymal signal for epithelial homeostasis. *Kidney Int* 1998;54:1392–1393.
- [41] Liu Y, Sun AM, Dworkin LD. Hepatocyte growth factor protects renal epithelial cells from apoptotic cell death. *Biochem Biophys Res Commun* 1998;246:821–826.
- [42] Fabregat I, Sanchez A, Alvarez AM, Nakamura T, Benito M. Epidermal growth factor but not hepatocyte growth factor, suppresses apoptosis induced by transforming growth factor-beta in fetal hepatocytes in primary culture. *FEBS Lett* 1996;384:14–18.
- [43] Gohda E, Okayuchi H, Iwao M, Yamamoto I. Induction of apoptosis by hepatocyte growth factor/scatter factor and its augmentation by phorbol esters in Meth A cells. *Biochem Biophys Res Commun* 1998;245:278–283.
- [44] Conner EA, Teramoto T, Wirth PJ, Kiss A, Garfield S, Thorgeirsson SS. HGF-mediated apoptosis via p53/bax-independent pathway activating JNK1. *Carcinogenesis* 1999;20:583–590.
- [45] Yanagawa K, Yamashita T, Yada K, Ohira M, Ishikawa T, Yano Y, et al. The antiproliferative effect of HGF on hepatoma cells involves induction of apoptosis with increase in intracellular polyamine concentration levels. *Oncol Rep* 1998;5:185–190.
- [46] Laurent S, Otsuka M, De Saeger C, Maiter D, lambotte L, Horsmans Y. Expression of presumed specific early and late factors associated with liver regeneration in different rat surgical models. *Lab Invest* 2001;81:1299–1307.
- [47] Boulton R, Woodman A, Calman D, Selden C, Tam F, Hodgson H. Nonparenchymal cells from regenerating rat liver generate interleukin-1a and -1b: a mechanism of negative regulation of hepatocyte proliferation. *Hepatology* 1997;26:49–58.
- [48] Morita M, Watanabe Y, Akaike T. Protective effect of hepatocyte growth factor on interferon gamma-induced cytotoxicity in mouse hepatocytes. *Hepatology* 1995;21:1585–1593.
- [49] Bohlinger I, Leist M, Barsig J, Uhlig S, Tiegs G, Wendel A. Interleukin-1 and nitric oxide protect against tumor necrosis factor α -induced liver injury through distinct pathway. *Hepatology* 1995;22:1829–1837.
- [50] Takehara T, Hayashi N, Tatsumi T, Kanto T, Mita E, Sasaki Y, et al. Interleukin 1 β protects mice from Fas-mediated hepatocyte apoptosis and death. *Gastroenterology* 1999;117:661–668.
- [51] Tsamandas AC, Jain AB, Felekouras ES, Fung JJ, Demetris AJ, Lee RG. Central venulitis in the allograft liver: a clinicopathologic study. *Transplantation* 1997;64:252–257.
- [52] Afford SC, Hubscher S, Strain AJ, Adams DH, Neuberger JM. Apoptosis in the human liver during allograft rejection and end-stage liver disease. *J Pathol* 1995;176:373–380.
- [53] Krams SM, Egawa H, Quinn MB, Villanueva JC, Garcia-Kennedy R, Martinez OM. Apoptosis as a mechanism of cell death in liver allograft rejection. *Transplantation* 1995;59:621–625.
- [54] Afford SC, Randhawa S, Eliopoulos AG, Hubscher SG, Young LS, Adams DH. CD40 activation induces apoptosis in cultured human hepatocytes via induction of cell surface fas ligand expression and amplifies fas-mediated hepatocyte death during allograft rejection. *J Exp Med* 1999;189:441–446.
- [55] Ogasawara J, Watanabe-Fukunaga R, Adachi M, Matsuzawa A, Kasugai T, Kitamura Y, et al. Lethal effect of the anti-Fas antibody in mice. *Nature* 1993;364:806–809.
- [56] Sakamoto T, Liu Z, Murase N, Ezure T, Yokomoto S, Poli V, et al. Mitosis and apoptosis in the liver of interleukin-6-deficient mice after partial hepatectomy. *Hepatology* 1999;29:403–411.
- [57] Shimamatsu K, Wanless IR. Role of ischemia in causing apoptosis, atrophy, and nodular hyperplasia in human liver. *Hepatology* 1997;26:343–350.