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BASIC RESEARCH

Expression patterns of cytokine, growth factor and cell cycle-related genes after partial hepatectomy in rats with thioacetamide-induced cirrhosis

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Abstract

AIM: To examine the differences in the responses of normal and cirrhotic livers to partial hepatectomy in relation to the factors influencing liver regeneration.

METHODS: Cirrhosis was induced in rats by administration of thioacetamide. Untreated rats were used as controls. The control rats as well as the cirrhotic rats were subjected to 70% partial hepatectomy. At different time points after hepatectomy, the livers were collected and the levels of cytokines, growth factors and cell cycle proteins were analyzed.

RESULTS: After hepatectomy, the cirrhotic remnant expressed significantly lower levels of cyclin D1, its kinase partner, cdk4, and cyclin E as compared to the controls up to 72 h post hepatectomy. Significantly lower levels of cyclin A and cdk2 were also observed while the cdk inhibitor, p27 was significantly higher. In addition, the cirrhotic group had lower IL-6 levels than the control group at all time points up to 72 h following resection.

CONCLUSION: The data from our study shows that impaired liver regeneration in cirrhotic remnants is associated with low expression of cyclins and cdks. This might be the consequence of the low IL-6 levels in cirrhotic liver remnant which would in turn influence the actions of transcription factors that regulate genes involved in cell proliferation and metabolic homeostasis during the regeneration process.

Key words: Cirrhosis; Partial hepatectomy; Cytokines; Growth factors; Cell cycle

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INTRODUCTION

A healthy liver has the capacity to regenerate itself after injury. This results in the restoration of liver mass and function[1,2]. In the healthy state, hepatocytes are in the resting G0 state and rarely divide. However, following injury or hepatectomy, the process of regeneration is activated. Hepatocytes then undergo transition into the G1 phase, followed by the S phase, G2 phase and mitosis. The passage through the cell cycle is modulated by the interplay between cyclins, cyclin-dependent kinases (cdks) and inhibitors of cdks[3-6].

Liver regeneration is influenced by a multitude of factors. These include cytokines and growth factors which are necessary to make quiescent hepatocytes replicate[1,2,7]. It has now been established that a set of priming events is necessary before the normally quiescent hepatocytes can respond to the growth factors. Both tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) have been shown to be the factors that facilitate the priming events in regeneration[8,9]. Following the priming events, growth factors then play an important role as mitogens generating a cascade of signals leading to DNA synthesis and cell division[10].

In humans, surgical resection is often carried out on a diseased liver and is the main therapeutic intervention for hepatocellular carcinoma[11]. The remnant liver is usually cirrhotic with impaired regenerative capacity. In this study, we examine the differences in the responses of normal and cirrhotic livers to partial hepatectomy in relation to the factors influencing hepatocyte proliferation and division. The thioacetamide-induced rodent model of liver cirrhosis was employed as induction of cirrhosis with thioacetamide is highly reproducible, irreversible and produces morphological features similar to that observed in human cirrhosis[12].

MATERIALS AND METHODS

Reagents

Mouse monoclonal antibodies against cyclin D1 and cdk2, and rabbit polyclonal antibodies against cyclin A, cyclin D3, cyclin E, cdk4 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Acrylamide, N, N'-methylene-bis-acrylamide, glycine, sodium dodecyl sulfate, 2-mercaptoethanol, isopropyl alcohol and mineral oil were from Sigma (St. Louis, MO, USA). Tween 20 was from Duchefa (Haarlem, Netherlands). Enhanced chemiluminescence western blotting detection kit, high performance film and nitrocellulose membrane were from Amersham Biosciences (Little Chalfont, Buckinghamshire, England). Sucrose, Bio-Rad protein assay dye, N, N, N', N'-tetra-methylethylenediamine, ammonium persulfate and prestained SDS-PAGE Standards Broad Range were from Bio-Rad (Hercules, CA, USA). Trizol reagent was from GIBCO BRL. Chloroform and sodium chloride were obtained from Merck (Darmsdadt, Germany). Access reverse transcription-PCR system was from Promega (Madison, WI, USA). Rat TNF-α and IL-6 ELISA kits were from Bender MedSystems (Vienna, Austria).

Induction of cirrhosis and partial hepatectomy

The induction of cirrhosis, partial hepatectomy (PH) and harvesting of the livers has been previously described[13,14]. In brief, cirrhosis was induced in ten-week-old male Wistar-Furth rats by intraperitoneal injection of 300 mg thioacetamide/kg thrice weekly for 10 wk. Healthy, control animals were kept under the same conditions without any treatment. After one week acclimation to allow for the washout of thioacetamide, all animals underwent 70% PH as described by Higgins and Anderson[15]. Two experimental groups were studied. Healthy rats in the control groups as well as cirrhotic rats in the cirrhotic group were submitted to PH. Within each group, six rats were sacrificed at 3, 6, 24, 48 and 72 h after surgery. The excised and the resected livers were harvested and used for further analysis.

Preparation of rat liver lysates

The liver was chopped into small pieces and homogenized in 5 volumes of buffer containing 0.25 mol/L sucrose, 5 mmol/L HEPES and 0.5 mmol/L EGTA, pH 7.5. The homogenate was centrifuged at 12 000 × *g* at 4 °C for 10 min. The supernatant (the liver lysate) was then stored in small aliquots at -80 °C till required. The protein concentration was determined using the Bio-Rad protein assay dye reagent with bovine serum albumin as the standard. For each time point, the lysates (containing equal amount of protein from each liver, $n = 6$) were pooled and then used for Western blot and ELISA analysis.

Western blotting

25 μg of the pooled sample was resolved on SDS-PAGE and transferred onto nitrocellulose membrane. Mouse monoclonal antibodies against cyclin D1 and cdk2, and rabbit polyclonal antibodies against cyclin A, cyclin D3, cyclin E, cdk4 were used to detect the respective proteins. The bound antibodies were visualized by using enhanced chemiluminescence. The bands were then quantified using the Analytical Imaging Station software (Imaging Research Inc, St. Catharines, Ontario, Canada).

Table 1 Primers for reverse-transcription-PCR of HGF, TGF-α, p21, p27 and p53 mRNAs and 28S rRNA.

Target sequence[1]	Primer	Sequence (5′- 3′)
28S	28S Forward	GGC CAA GCG TTC ATA GCG AC
	28S Reverse	GAG GCG TTC AGT CAT AAT CC
HGF[38]	HGF Forward	CCC GGT GCT GCA GCA TGT CCT
	HGF Reverse	TCC CCT CGA TTT CGA CAG
TGF-α[38]	TGF-α Forward	GAC AAG TTG AAC AAG AAC CTC
	TGF-α Reverse	CGT CAT CCA CCT AAT ACA TAA G
p21[52]	p21 Forward	ATG TCC GAT CCT GGT GAT GTC
	p21 Reverse	CAC TTC AGG GCT TTC TCT TGC
p27[52]	p27 Forward	GCA GCT TGC CCG AGT TCT AC
	p27 Reverse	TTC TTG GGC GTC TGC TCC AC
p53[53]	p53 Forward	TCC TCC CCA ACA TCT TAT CC
	p53 Reverse	GCA CAA ACA CGA ACC TCA AA

[1]The 28 S primers were kindly provided by Assoc. Prof. Chang Chan Fong (Department of Biochemistry, National University of Singapore). The other primers were as previously reported[38,52,53].

Quantitative assay for TNF-α and IL-6

The concentrations of TNF-α and IL-6 present in the liver lysates were determined using the Rat TNF-α and IL-6 ELISA kits. The assays were carried out according to the protocol described in the manufacturer's manual.

RNA extraction, reverse transcription, and PCR

Total RNA was extracted from the liver samples using the Trizol reagent. The extraction was carried out according to the manufacturer's instructions. For each time point, the total RNA (containing equal amount of RNA from each liver as determined by absorbance at 260 nm, $n = 6$) was pooled and then used for reverse transcription-PCR.

Six sets of primers were used for PCR and the sequences are shown in Table 1. The isolated RNA was subjected to reverse transcription-PCR using the Access reverse transcription-PCR system (Promega, USA). A typical 25 μL reverse transcription-PCR reaction contained 0.4 μg of RNA, 0.1 mmol/L dNTPs, 0.5 μmol/L of the forward and corresponding reverse primers, 0.5 mmol/L of $MgSO_4$, and 0.05 U/μL of AMV reverse transcriptase and 0.05 U/μL of T*fl* DNA polymerase. The reverse transcription was carried out at 48 °C for 45 min followed by denaturation at 94 °C for 2 min. Following reverse transcription, the cDNA was subjected directly to PCR. Each cycle consists of denaturation at 94 °C for 30 s, annealing at 60 °C for 1 min and extension at 68 °C for 2 min. At the end of the last cycle, a final extension at 68 °C for 7 min was carried out. The number of PCR cycles used was the lowest needed to produce a product which could be visualized on the gel. The product was separated by agarose-gel electrophoresis and visualized by ethidium-bromide staining. The gel image was captured and the bands were then quantified using the Analytical Imaging Station software (Imaging Research Inc, St. Catharines, Ontario, Canada).

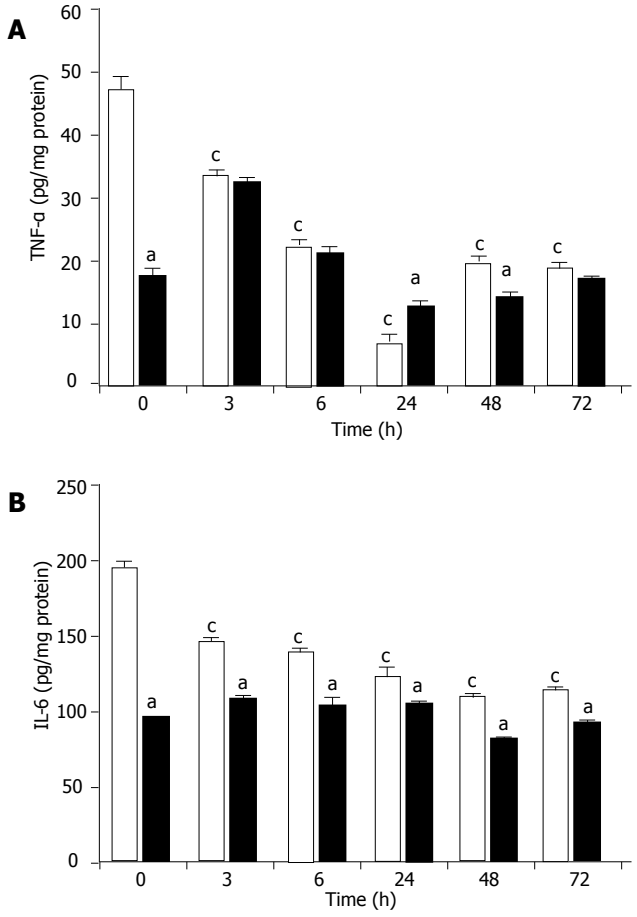

Figure 1 Hepatic **A**: TNF-α and **B**: IL-6 concentrations after PH on healthy rats (white bars) and cirrhotic rats (black bars). At different time points after the operation, the remnant liver was harvested, homogenized and the supernatant was used for analysis. For each time point, the supernatant from 6 animals was pooled and used for ELISA analysis. All analyses were carried out in triplicates and the results are expressed as mean + SD. $^{c}P < 0.05$, ANOVA analysis, for comparison between control rats before (at 0 h) and after PH. $^{a}P < 0.05$, ANOVA analysis, for comparison between the cirrhotic group and the corresponding control group at each time point.

RESULTS

Two groups of animals were used in the study. The control group and the cirrhotic group were subjected to 70% PH. All data generated in this study were first examined to determine the changes that occur after PH in the controls. This was followed by comparisons to determine if there were significant differences between the control group and the cirrhotic group both before and after 70% PH.

Hepatic TNF-α and IL-6

Prior to PH, TNF-α and IL-6 were both detected in the liver extracts from the control group and the cirrhotic group. The levels in cirrhotic livers were significantly lower than that in healthy livers. Following resection, the hepatic levels of both TNF-α and IL-6 were significantly lowered from 3 h till 72 h post-resection in the control group. Significantly different TNF-α levels between the controls and cirrhotics were only observed at 24 h and 48 h post-PH. Reduced IL-6 levels were observed in the cirrhotic group before and up to 72 h following PH (Figure 1A and B).

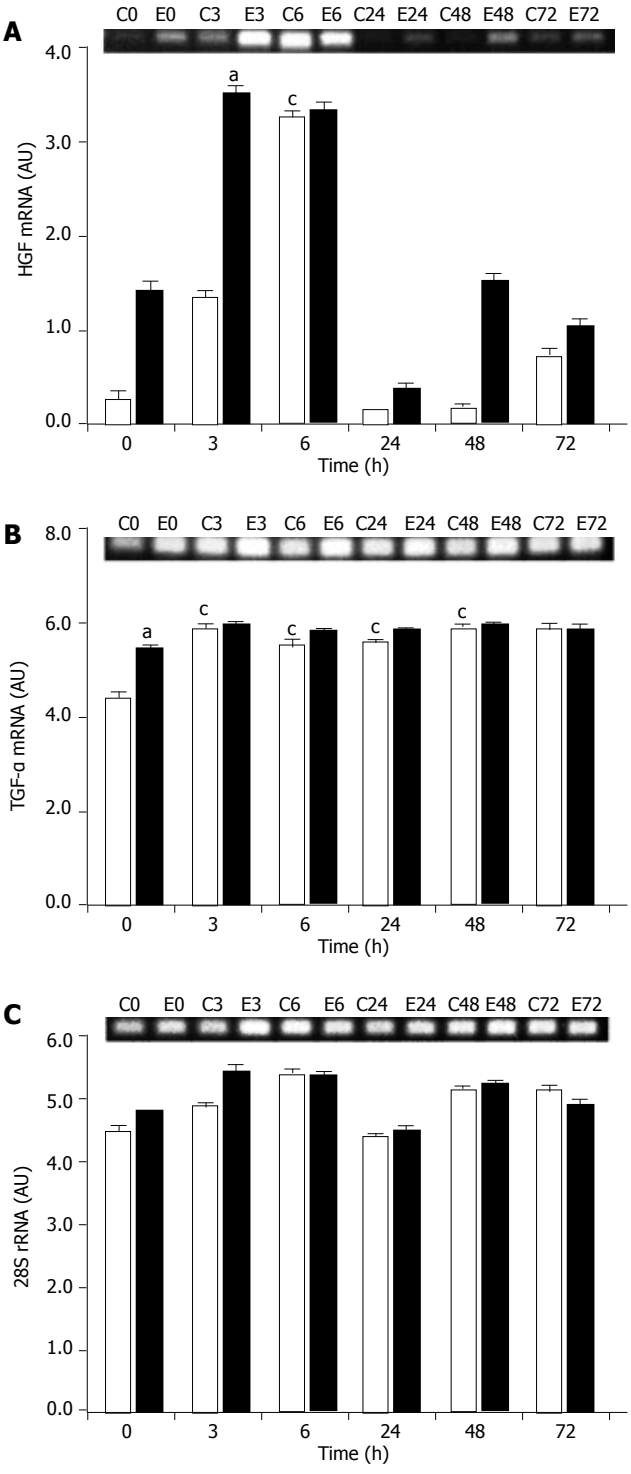

Figure 2 Hepatic **A**: HGF, **B:** TGF-α and **C:** 28S rRNA expressions after PH on healthy rats (white bars) and cirrhotic rats (black bars). At different time points after the operation, the remnant liver was harvested, and RNA was extracted. For each time point, the RNA from 6 animals was pooled and used for reverse-transcription followed by PCR. The PCR product was separated by agarose-gel electrophoresis and visualized by ethidium-bromide staining. The gel image was captured and the bands were quantified using the Analytical Imaging Station software. All analyses were carried out in triplicates. A representative gel (C, control, healthy rats; E, cirrhotic rats; the number indicates the time post-PH in h) and the quantification analysis expressed as mean + SE (AU = arbitrary units) are shown. $^{c}P<0.05$, ANOVA analysis, for comparison between control rats before (at 0 h) and after PH. $^{a}P < 0.05$, ANOVA analysis, for comparison between the cirrhotic group and the corresponding control group at each time point.

Expression of growth factors

Total RNA was extracted from the liver samples and sub-

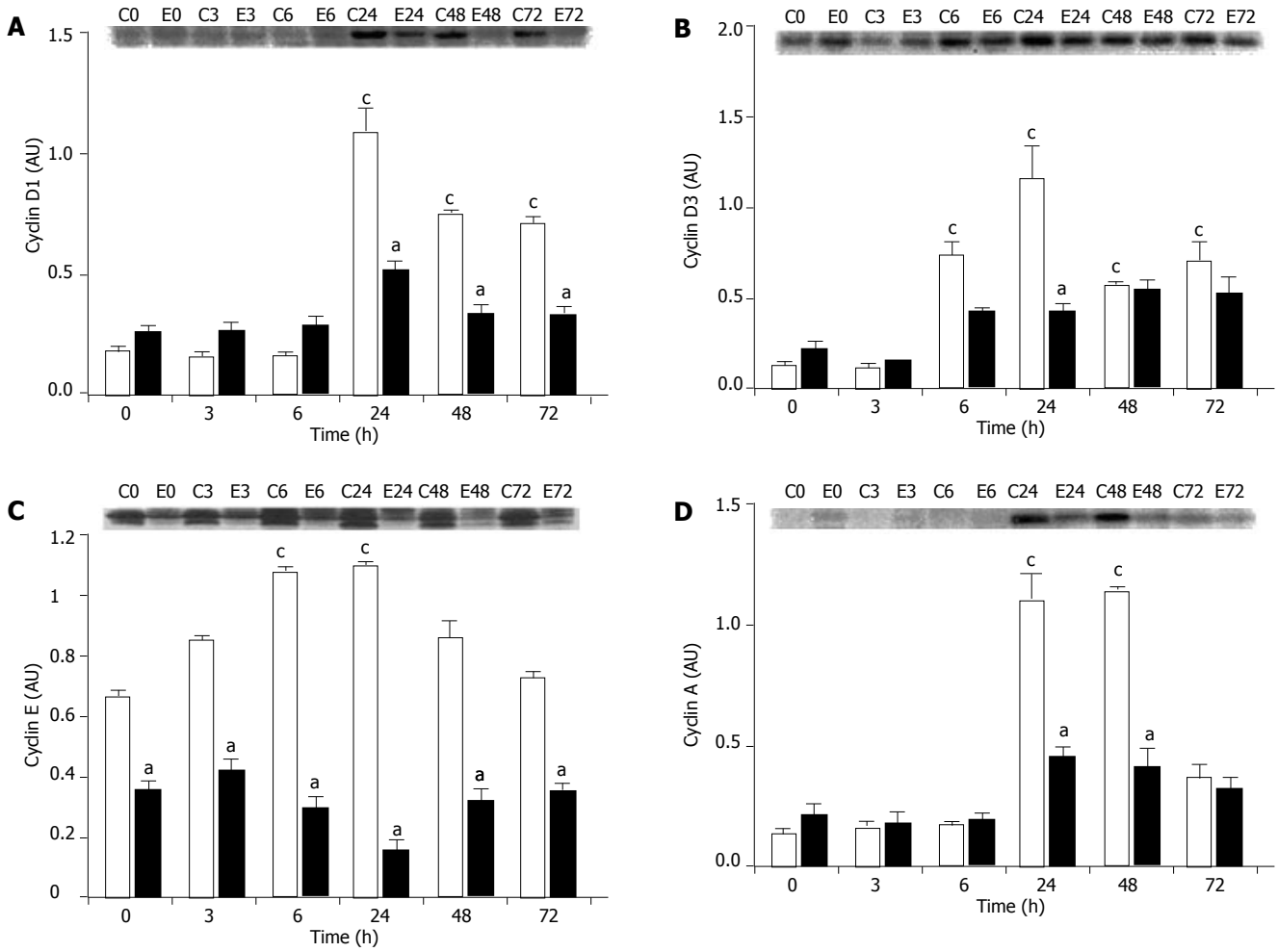

Figure 3 Effect of PH on cyclin expressions in healthy rats (white bars) and cirrhotic rats (black bars). At different time points after the operation, the remnant liver was harvested, homogenized and the supernatant was used for analysis. For each time point, the supernatant from 6 animals was pooled and used for Western blot analysis. The blot image was captured and the bands were quantified using the Analytical Imaging Station software. All analyses were carried out in triplicates. A representative blot (C, control, healthy rats; E, cirrhotic rats; the number indicates the time post-PH in h) and the quantification analysis expressed as mean+SE (AU = arbitrary units) are shown. **A:** Cyclin D1 expression. **B:** Cyclin D3 expression. **C:** Cyclin E expression. **D:** Cyclin A expression. $^{c}P < 0.05$, ANOVA analysis, for comparison between control rats before (at 0 h) and after PH. $^{a}P < 0.05$, ANOVA analysis, for comparison between the cirrhotic group and the corresponding control group at each time point.

jected to reverse transcription-PCR analysis for transcripts encoding for the hepatocyte growth factor (HGF) and the transforming growth factor alpha (TGF-α). A transient increase in HGF was observed at 6 h post-PH for the control group. The trend was also similar for the cirrhotic group, although a significant increase was also observed at 3 h post-PH (Figure 2A).

In contrast, TGF-α was significantly higher at all time points up till 48 h post-resection in the control group. TGF-α level in the cirrhotic livers was significantly higher than controls before PH. After PH, the levels remained high and there were no differences between the control group and the cirrhotic group (Figure 2B). The 28S rRNA was used as a control and no significant differences in the expression were observed (Figure 2C).

Markers of cell cycle progression, cdk inhibitors and p53 expression

Western blot analysis was carried out to examine the changes in cyclins and cdks. In the control group, cyclin D1 levels were elevated from 24 to 72 h post-PH (Figure 3A) while cyclin D3 levels peaked at 24 h post-PH and remained significantly elevated up till 72 h post-PH (Figure 3B). In addition, cdk4 was also significantly increased at the 6 h time point (Figure 4A). Comparison of the cirrhotic group with the corresponding controls showed that cyclin D1 levels were significantly lower at 24 to 72 h post-PH and the peak in cyclin D3 expression was not observed. The cdk4 levels were also lower than that of the corresponding controls from 6 to 72 h post-resection (Figure 3A, 3B and 4A).

As previously described, the antibody against cyclin E recognized multiple isoforms[16,17]. These represent alternative spliced variants of the gene. Cyclin E levels in the controls increased significantly within 24 h following resection. However, in the cirrhotic group, this increase was not evident and cyclin E levels were lower than the controls at all time points (Figure 3C). For the control animals, the highest levels of cyclin A and cdk2 expression were at 24 and 48 h after resection. At these time points, the levels of cyclin A and cdk2 in the cirrhotic animals were significantly lower than that in the corresponding controls (Figure 3D and 4B).

Reverse transcription-PCR analysis showed that for

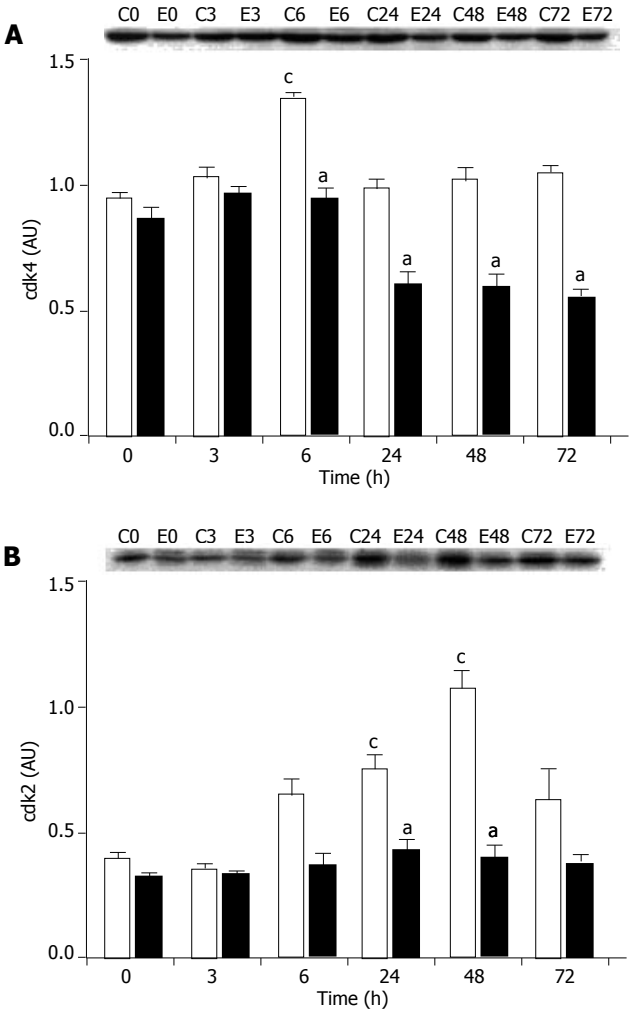

Figure 4 Effect of PH on cyclin-dependent kinase expressions in healthy rats (white bars) and cirrhotic rats (black bars). At different time points after the operation, the remnant liver was harvested, homogenized and the supernatant was used for analysis. For each time point, the supernatant from 6 animals was pooled and used for Western blot analysis. The blot image was captured and the bands were quantified using the Analytical Imaging Station software. All analyses were carried out in triplicates. A representative blot (C, control, healthy rats; E, cirrhotic rats; the number indicates the time post-PH in h) and the quantification analysis expressed as mean+SE (AU = arbitrary units) are shown. **A:** Expression of cdk4. **B:** Expression of cdk2. $^{c}P < 0.05$, ANOVA analysis, for comparison between control rats before (at 0 h) and after PH. $^{a}P < 0.05$, ANOVA analysis, for comparison between the cirrhotic group and the corresponding control group at each time point.

both p21 and p53, the increase in expression was before the 24 h time point for the control group and the cirrhotic group (Figure 5A and C). However, the expression of p27 in the cirrhotic group was significantly higher than the controls before as well as after resection (Figure 5B).

DISCUSSION

In this study, the thioacetamide-induced rodent model of liver cirrhosis was used to examine the factors that may contribute to impaired regeneration of cirrhotic livers after PH. Changes in the expression patterns of cytokines, growth factors and cell cycle proteins were examined over a period of 72 h.

Cyclins, cdks and cdk inhibitors were all detected in healthy livers, similar to previous observations[16]. Cirrhotic livers also expressed comparable levels of D-type cyclins, cyclin A, cdks, p21 and p53 as the healthy livers. However, cyclin E was significantly lowered while p27 was significantly raised.

Following PH, quiescent hepatocytes are induced to proliferate. Cyclins and their kinase partners play key roles in regulating the progress through the different phases of the cell cycle[18,19]. The D-type cyclins together with their kinase partners play a key role in regulating G1 progression[20]. The cyclinE/cdk2 complex regulates G1 to S phase transition[21], while the cyclinA/cdk2 complex is necessary for initiation of DNA replication in the S phase[22-24]. As expected, the expression of cyclin D1, cyclin E and cyclin A increased significantly in the controls after resection as the liver undergoes regeneration. This is congruent with other studies which showed that after PH, there is firstly an induction of G1 cyclins preceding S phase followed by subsequent induction of cyclin A and cyclin B[4,25,26].

Transient increases in the mRNA levels of cdk inhibitors p21 and p27, as well as p53 were also observed at 3-6 h after PH in the controls. Similar observations have been previously described in various other studies using rodent models[6, 27-30]. It is thought that in the regenerating liver, p53 plays a dual role[30]. The first is as a cell cycle check resulting in a delay in the G1 phase to allow damage repair[29]. The second is the induction of the expression of growth factors or their receptors. Indeed, p53 responsive elements have been found on the promoters of HGF and TGF-α, both of which are involved in the proliferative response[31,32]. The expression of the cdk inhibitor, p21 is also induced post PH, probably in part as a response to p53 induction[33]. This may serve to prevent premature progression through G1 phase[34] or to stabilize the cyclinD-cdk4 complex and promote its translocation to the nucleus[35]. p27 acts as a brake on cyclinE-cdk2 activity and has to be sequestered by cyclin D1 for cdk2 to be active[36].

Comparison between the control and cirrhotic groups showed that the changes in cyclin/cdk expressions after PH were not mirrored in the cirrhotic livers. Cyclin D1 and its kinase partner, cdk4 were expressed at much lower levels at 24-72 h post PH, cyclin A and cdk2 were decreased at 24-48 h post PH while cyclin E was persistently lowered up to 72 h post PH. In addition, the expression of the cdk inhibitory protein, p27 was also elevated at 6-48 h post PH. Thus, unlike healthy livers, cirrhotic livers expresses significantly lower levels of cyclins [this study and those by Zhao *et al*[37] and Masson *et al*[38]]. Given the persistent low levels of G1 cyclins, it is likely that the block to cell cycle progression occurs at the G1 phase.

The key factors that influence liver regeneration include the cytokines TNF-α and IL-6 and the growth factors HGF and TGF-α. The cytokines TNF-α and IL6, are necessary to prime and initiate liver regeneration after PH[2,8,9]. Signals to initiate liver regeneration for TNF-α are mediated by TNF receptor 1. Knockout mice lacking this receptor have diminished ability to restore liver mass after PH leading to increased mortality[39]. High postoperative mortality and impaired response to PH were also observed in IL-6 deficient mice[40]. Besides the cytokines, growth factors also play important roles in liver regeneration. TGF-α and HGF are potent mitogens for hepatocytes[10]. The re-

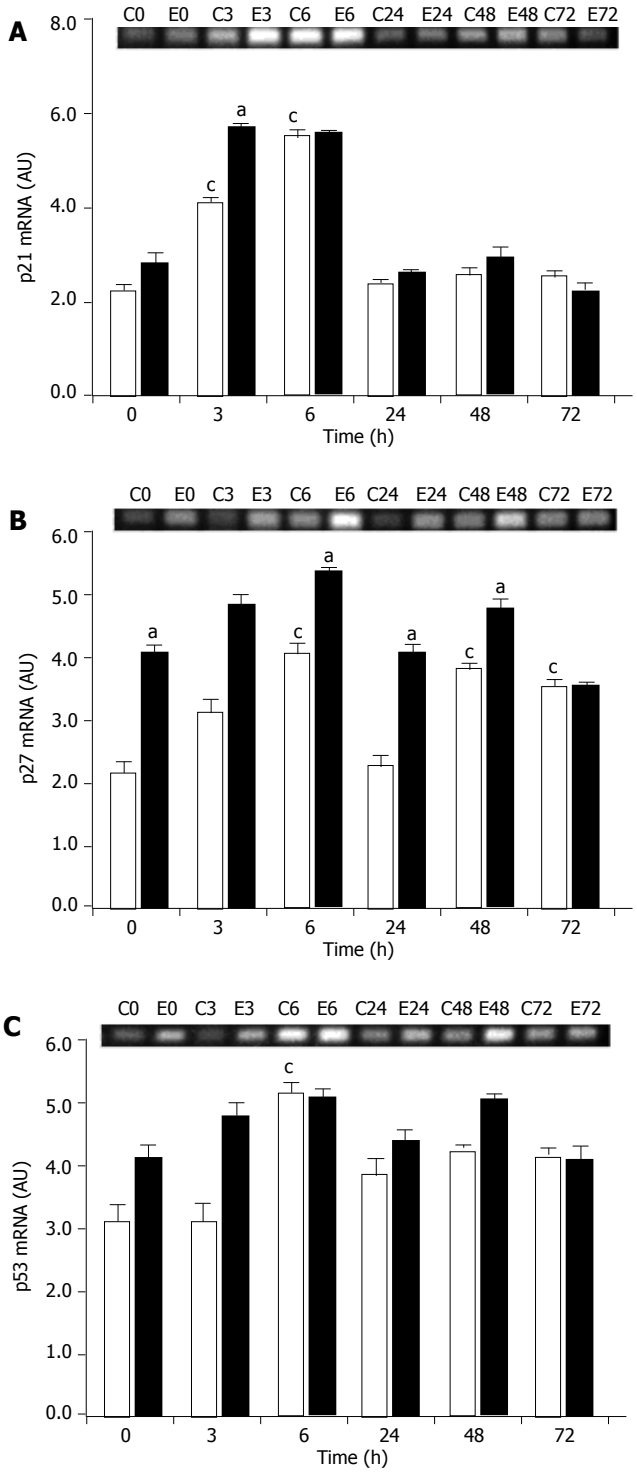

Figure 5 Hepatic **A:** p21, **B:** p27 and **C:** p53 expressions after PH on healthy rats (white bars) and cirrhotic rats (black bars). At different time points after the operation, the remnant liver was harvested, and RNA was extracted. For each time point, the RNA from 6 animals was pooled and used for reverse-transcription followed by PCR. The PCR product was separated by agarose-gel electrophoresis and visualized by ethidium-bromide staining. The gel image was captured and the bands were quantified using the Analytical Imaging Station software. All analyses were carried out in triplicates. A representative gel (C, control, healthy rats; E, cirrhotic rats; the number indicates the time post-PH in h) and the quantification analysis expressed as mean + SE (AU = arbitrary units) are shown. $^{c}P < 0.05$, ANOVA analysis, for comparison between control rats before (at 0 h) and after PH. $^{a}P < 0.05$, ANOVA analysis, for comparison between the cirrhotic group and the corresponding control group at each time point.

sponse to HGF is mediated by its interaction with c-met, a protooncogene which encodes a receptor tyrosine kinase. In conditional met mutant mice, impaired regeneration occurs after PH and this is due to a defective exit from quiescence and diminished entry into S phase[41]. In addition, HGF also upregulates the expression of TGF-α and the presence of anti-TGFα antibodies can block DNA synthesis in regenerating livers[42].

Cirrhotic livers have significantly lower TNF-α and IL-6 levels compared to healthy livers prior to PH. It is unclear why this should be so. However, similar reductions have been observed in patients with primary biliary cirrhosis and fulminant hepatitis[43,44]. This is not simply due to a general depression in RNA synthesis in the diseased livers as the expression of other genes such as IL-1 and interferons were unaffected[44]. In addition, unchecked fibrosis will lead to impaired diffusion of nutrient and hepatotropic factors and this may also be a contributing factor to the reduced levels of TNF-α and IL-6. In contrast, HGF expression was unchanged in cirrhotic livers. TGF-α expression was significantly increased in cirrhotic livers and this is consistent with the role of TGF-α in the chronic regeneration that occurs in the cirrhotic process[45].

Following resection of healthy controls, it was observed that both TNF-α and IL-6 levels in the liver decreased progressively over a period of 72 h. This is to be expected as TNF-α and IL-6 are necessary mainly for the initial priming events. In addition, TNF-α is a pro-inflammatory cytokine and high levels are not desirable once the priming signals have been initiated. In this study, a peak in both cytokines was not observed after PH. This is probably due to the fact that the increase in both cytokines was expected to be at an early time point after resection[39,46] and the first time point in this study may be too late for this observation. A peak in the expression of HGF was observed at 3-6 h post PH while that of TGF-α was elevated up to 48 h post PH. Similar to that observed by Zhao *et al*, hepatic expression of the growth factors HGF and TGF-α was not significantly different in both normal and cirrhotic livers after PH[37].

In contrast to the growth factors, it was observed that following partial hepatectomy, the IL-6 levels in cirrhotic livers are significantly lower than that of the healthy controls. The role of IL-6 in liver regeneration has been extensively examined. To date, the roles of IL-6 in liver regeneration include the activation of acute phase response, induction of proliferation and hepatoprotection. The binding of IL-6 to the gp130-IL-6 receptor complex results in the activation of Janus kinase (JAK). This in turn activates the mitogen-activated protein kinase (MAPK) and STAT3 signaling pathways[7,9]. In IL-6 deficient mice, liver failure and defective hepatocyte regeneration is observed following PH. Subcutaneous injection of IL-6 which results in sustained action of IL-6 was able to completely reverse the high post-operative mortality of IL-6 deficient mice[47]. It is interesting to note that both IL-6 and cyclin D1 levels were reduced in the regenerating cirrhotic liver. AP1 activity was also markedly inhibited in regenerating cirrhotic livers with reduced expression levels of c-Jun and JunD[37]. Taken together, these observations are comparable to that in IL-6 deficient mice which shows marked reduction in STAT3 activation and depressed AP-1, myc and cyclin-D1 expressions[40].

Il-6 also mediates the induction of C/EBP expression[48]. Impaired C/EBPβ activities have been observed in cirrhotic livers following resection[37] and this could account for the reduction in cyclin E and cyclin A. In C/EBPβ -/- mice, cyclins A, B and D were reduced near the peak of DNA synthesis during liver regeneration[49]. Although it is still uncertain as to whether C/EBPβ regulates cyclin expression via transcriptional or post-transcriptional mechanisms, it is clear that in C/EBPβ -/- hepatocytes, cell cycle progression is blocked close to or at the G1/S phase transition[49].

The data from our study thus shows that impaired liver regeneration in cirrhotic remnants is associated with low expression of cyclins including cyclin D1. In rats with carbon tetrachloride-induced cirrhosis, similar changes in the expression of cyclins and IL-6 were also observed[38]. Markedly reduced IL-6 levels could influence the action of transcriptions factors (such as STAT3, AP1, C/EBPβ and HNF-1α) which are necessary for the regulation of genes that coordinate the balance between cell proliferation and metabolic homeostatsis during liver repair and growth. It addition, liver regeneration is also critically dependent on ATP stores and the reduction in liver proliferation in cirrhotic livers may be due in part to inadequate mitochondrial respiratory function. Cirrhotic livers indeed have reduced complex I activity and lower respiratory control ratios following PH as shown by us and others[13,50,51].

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