

After portal branch ligation in the rat, cellular proliferation is associated with selective induction of c-Ha-ras, p53, cyclin E, and Cdk2

P Stärkel, L Lambotte, C Sempoux, C De Saeger, A Saliez, D Maiter, Y Horsmans

Abstract

Background—In liver regeneration after portal branch ligation we previously showed that early cellular changes are observed in both the proliferating and atrophying liver lobes. They are therefore not indicative of future proliferative response. In this study we attempted to define precisely, in the same model, the time at which the cellular processes diverge between the lobes by measuring various parameters associated with cellular proliferation. We also investigated the possible role of inhibitors of cell proliferation in the absence of progression towards the S phase in the atrophying lobes.

Aims—Expression of p53, c-Ha-ras, cyclin E, cyclin dependent kinase (Cdk2), transforming growth factor (TGF)- β , and interleukin (IL)-1 α and IL-1 β were assessed in relation to their potential role in proliferating and atrophying cellular phenomena.

Methods—Immunohistochemistry, northern blotting, western blotting, and reverse transcription-polymerase chain reaction were performed, mainly at time points corresponding to mid-G1/S phase progression (8–24 hours after surgery).

Results—The common and thus most likely non-specific response was still evident 5–8 hours after surgery and included an increase in IL-1 mRNA as well as p53 and cyclin E proteins. From 12 hours onwards, p53, c-Ha-ras, cyclin E, and Cdk2 were selectively induced in proliferating lobes whereas IL-1 β was predominantly activated in atrophying lobes. No changes in TGF- β or IL-1 α expression were observed at the same time points in any of the liver lobes.

Conclusions—The initial response to portal branch ligation and thus probably to partial hepatectomy seems to be non-specific for at least eight hours. Thereafter, p53, c-Ha-ras, cyclin E, and Cdk2 seem to drive cellular proliferation while IL-1 β is associated with cellular atrophy. In contrast, TGF- β and IL-1 α do not seem to play a role in determining the commitment of cells towards atrophy or proliferation.

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Keywords: portal branch ligation; liver regeneration; delayed early proto-oncogenes; cytokines; cyclin dependent kinase; rat

Partial hepatectomy (PH) induces many changes during the hours preceding DNA synthesis and cellular proliferation, including activation of proto-oncogenes, transcription factors, cytokines, and growth factors (for review see Fausto and Mead,¹ Taub,² Simpson and colleagues,³ Fausto and colleagues,⁴ and Michalopoulos and DeFrances⁵). However, it is not clear if all of these changes result from reduction in liver mass, and cause or are required for the proliferating phase. To clarify the role of these various changes, we have previously used different experimental models—namely, temporary partial hepatectomy and portal branch ligation (PBL).^{6–7} We have shown that early changes, including induction of immediate early proto-oncogenes and interleukin (IL)-6, as well as activation of nuclear factor κ B and Stat3, are observed in both the proliferating and atrophying lobes.⁷ Therefore, they are not indicative of the future proliferative response and are most likely produced in response to surgical stress. We have also shown that this initial response is independent of the reduction in liver mass.⁸

The PBL model also offers an unique opportunity to study the relationship of the changes observed at a later time after PH with stimulation or inhibition of liver growth. It was therefore intriguing to examine cellular expression of potential growth inhibitors and promoters from the mid-G1 to the G1/S phase in the regenerating and atrophying parts of the liver.

In liver regeneration, passage from G0 to G1 has been defined in hepatocytes by sequential activation of proto-oncogenes, including c-Ha-ras and p53 expression in mid-late G1 phase.⁹ The p53 gene plays a critical role in mediating G1 cell cycle arrest after DNA damage and loss of p53 function can result in loss of the G1 checkpoint arrest.¹⁰ However, there is some variability in induction of p53 regulated responses and it is important to consider the tissue when evaluating the role of p53 in suppressing cell growth.¹⁰ Important elements controlling progression of cells through the cell cycle are cyclins and cyclin dependent kinases (Cdks).¹¹ These form active kinase complexes during critical periods of the cell cycle (checkpoints) allowing transition from one stage of

Laboratories of Gastroenterology, Université Catholique de Louvain, 1200 Brussels, Belgium
P Stärkel
C De Saeger
Y Horsmans

Laboratories of Experimental Surgery, Université Catholique de Louvain, 1200 Brussels, Belgium
L Lambotte
A Saliez

Laboratories of Pathology, Université Catholique de Louvain, 1200 Brussels, Belgium
C Sempoux

Laboratories of Diabetology, Université Catholique de Louvain, 1200 Brussels, Belgium
D Maiter

Correspondence to: Dr Y Horsmans, Department of Gastroenterology, St Luc University Hospital, Av. Hippocrate 10, 1200 Brussels, Belgium.
Horsmans@gaen.ucl.ac.be

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Abbreviations used in this paper: IL, interleukin; PBL, portal branch ligation; PH, partial hepatectomy; RT-PCR, reverse transcription-polymerase chain reaction; TBS, Tris buffered saline; TGF, transforming growth factor; TNF, tumour necrosis factor; Cdk, cyclin dependent kinase; dTT, dithiothreitol; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

the cell cycle to the next. Cyclin E is required to catalyse the G1/S transition in normal cells^{12, 13} as well as Cdk2 activity to stimulate cell proliferation.¹⁴ Cdk2 preferentially assembles with cyclin E¹¹ and cyclin E/Cdk2 complexes become active during the late G1 phase contributing to the irreversible commitment of cells to enter the S phase.¹⁴

Hepatocyte growth seems to be modulated by several mechanisms which may result in increased effects of hepatocyte growth promoters such as tumour necrosis factor α (TNF- α), or in a reduced effect of hepatocyte growth inhibitors, as suggested by studies with Kupffer cell depleted rats.^{15, 16} Transforming growth factor (TGF)- β was shown to act as a growth inhibitor *in vitro* and *in vivo* involving paracrine and probably also autocrine mechanisms.^{17, 18} IL-1, which shows biphasic upregulation after partial hepatectomy, was recently proposed as a potential downregulator of hepatocyte proliferation.¹⁹ However, the exact role of these molecules in counterbalancing the stimulatory effects of mitogens in liver regeneration remains unclear.

In this study, we examined the role of p53, TGF- β , and IL-1, three potential negative growth regulators, as well as the role of the putative cell cycle promoters c-Ha-ras, cyclin E, and Cdk2, in relation to cellular atrophy and proliferation using the PBL model.

Materials and methods

ANIMALS

Male Wistar rats (220–270 g body weight) were obtained from the Rat Breeding Facilities of the Catholic University of Louvain Medical School, Brussels, Belgium. All animals were kept in a temperature and humidity controlled environment in a 12 hour light-dark cycle. At all times they were allowed free access to water and standard food pellet diet (Usine d'Alimentation Rationnelle, Villemoisson-sur-Orge, France). The animals were handled according to the guidelines established by the Catholic University of Louvain.

SURGICAL PROCEDURES AND EXPERIMENTAL DESIGN

All operations were carried out under light ether anaesthesia at room air between 9.00 am and 12.00 am using a clean but not sterile technique. In PBL of 70% liver mass, a median laparotomy was performed and the branch of the portal vein feeding the anterior and lateral lobes was carefully dissected under an operating microscope and completely ligated with a 7-0 suture. Care was taken not to injure the hepatic artery or bile duct and to avoid haemorrhage. In controls (0 hours), livers were removed without any prior surgical procedure. Postoperatively, animals had free access to water and food. Rats were sacrificed by exsanguination after puncture of the abdominal aorta and transection of the inferior vena cava in the thoracic cavity at one, two, five, eight, 12, 16, 24, and 30 hours after PBL. The livers were removed, the ligated and non-ligated lobes were rapidly weighted, snap frozen in liquid nitrogen, and stored at -80°C . Three rats were

killed at each of the indicated time points in all control groups as well as in the proto-oncogene, IL-1, cyclin E, and Cdk2 PBL groups. Six rats per indicated time points were used for the TGF- β 1, TGF- β 2, and TGF- β 3 experiments. The following time schedule for PBL was chosen for the different experiments: IL-1: one, two, five, eight, 12, and 24 hours; TGF- β : eight, 12, and 24 hours; delayed early proto-oncogenes: 12, and 24 hours; cyclin E and Cdk2: one, two, five, eight, 12, 16, and 24 hours.

PREPARATION OF HOMOGENATES, AND NUCLEAR AND CYTOPLASMIC EXTRACTS

Homogenates were obtained by homogenising whole liver tissue from the ligated and non-ligated lobes with an Ultra-turrax T25 device (IKA Labortechnik, Staufen, Germany) in a buffer containing 250 mM sucrose, 50 mM Tris HCl, pH 7.4, 5 mM MgSO_4 , 1 mM PMSF, and 2 $\mu\text{g}/\text{ml}$ of aprotinin and leupeptin at 4°C . Homogenates were filtered and stored at -80°C . Nuclear and cytoplasmic extracts were prepared separately from the anterior (ligated) and posterior (non-ligated) lobes, as described by Hattori *et al* with slight modifications.²⁰ All solutions, tubes, and centrifuges were maintained at 4°C . All buffers were supplemented with protease and phosphatase inhibitors as follows: 2 $\mu\text{g}/\text{ml}$ each of antipain, aprotinin, bestatin, and leupeptin, 0.5 mM NaF, and 1 mM Na_3VO_4 (all protease and phosphatase inhibitors were purchased from Sigma Chemical Co., Bornem, Belgium). The nuclear extracts were resuspended in the nuclear extract dialysis buffer (25 mM HEPES, 40 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol (dTT), 0.5 mM PMSF, 0.5 mM NaF, and 1 mM Na_3VO_4) and dialysed for four hours against 250 ml of nuclear extract dialysis buffer with one change of dialysis solution. After dialysis, the extracts were centrifuged at 14 000 rpm for five minutes at 4°C in a micro-centrifuge (Sorvall RMC 14; DuPont, Newtown, USA) to remove insoluble material, frozen in aliquots in liquid nitrogen, and stored at -80°C until use.

After pelleting of the nuclei, the supernatants were carefully aspirated and diluted with one volume of a buffer containing 10% glycerol, 25 mM HEPES, 40 mM KCl, 0.1 mM EDTA, 1 mM dTT, 0.5 mM PMSF, protease, and phosphatase inhibitors. This crude cytoplasmic extract was transferred to 28 ml ultracentrifuge tubes and spun at 24 000 rpm for two hours at 4°C in a SW28 ultracentrifuge rotor. The resultant supernatant was aspirated, frozen in aliquots in liquid nitrogen, and stored at -80°C until use as a cytoplasmic extract. Protein contents were determined using a BCA protein assay with serum albumin as a standard (Pierce Chemical, Rockford, Illinois, USA).

RNA ISOLATION

Total RNA was prepared from frozen liver tissue using the guanidine thiocyanate and cesium chloride method.²¹

NORTHERN BLOTTING

RNA (20 µg) was resolved on 1% agarose gels containing 1% formaldehyde and transferred to Hybond-N membranes (Amersham, Little Chalfont, UK). cDNA probes were ³²P labelled by the method of "random priming" using a Megaprime DNA labelling system (Amersham). Oligonucleotide probes were ³²P end labelled using a T₄ polynucleotide kinase (Amersham). After ultraviolet fixation, filters were hybridised with the ³²P labelled oligonucleotide or cDNA probes.²² The blots were exposed to a Hyperfilm MP (Amersham) for four hours to seven days. Densitometry using a Ultrascan XL device (Pharmacia LKB, Uppsala, Sweden) was performed. The results are expressed in arbitrary densitometric units adjusted for the respective 28S ribosomal signal. The p53 cDNAs were kindly provided by T Soussi (Inserm U301, Paris, France).²³ The c-Ha-ras cDNAs were purchased from Oncor (Gaithersburg, Maryland, USA). The 28S rRNA oligonucleotide was obtained from Oncogene Sciences (Uniondale, New York, USA).

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

Total liver RNA (40 µg) was treated with 10 U of Rnasein (Promega) and 1 U of DNASE (Promega) for 15 minutes at 37°C in an incubation buffer (Tris HCl 10 mM, NaCl 50 mM, MgCl₂ 25 mM) to avoid DNA contamination during PCR. The absence of genomic DNA contamination was assured by submitting samples, where reverse transcriptase had been omitted from the reaction, to PCR amplification. After incubation, the purified RNA was extracted using phenol-chloroform-isoamyl alcohol (25:24:1) (Sigma) and chloroform (Sigma), and precipitated by adding sodium acetate 3 M and 100% ethanol. Purified RNA (5 µg) was preincubated with random hexamer and water for 10 minutes at 70°C. Moloney murine leukaemia virus reverse transcriptase 400 U (Gibco BRL, Merelbeke, Belgium) was added, together with dTT and deoxynucleoside triphosphate, and the reaction was continued for a further 60 minutes at 37°C. The RT products were amplified in a two step PCR using Taq DNA polymerase (Boehringer Mannheim, Brussels, Belgium) and specific cDNA primers under reaction conditions listed in table 1. Ribosomal protein L19 (RPL19) RNA was co-amplified as an internal standard.

The first PCR step of five, five, and three cycles was performed using only the specific primers for cyclin E, TGF-β2, and TGF-β3, respectively, followed by the second PCR step of 25 cycles during which the RPL19 primers were added. The PCRs for IL-1α and TGF-β1 were first initiated with RPL19 primers for five and four cycles, respectively, followed by the second step PCR containing primers for IL-1α (25 cycles) and TGF-β1 (26 cycles). Amplification of IL-1β cDNA was carried out in a single step 24 cycle PCR using IL-1β specific primers, and the internal RPL 19 standard was amplified in a separate PCR of 30 cycles. Digoxigenin-UTP was included to allow detection of PCR products later on.

In parallel, RNA samples obtained from lipopolysaccharide treated rats (positive control for IL-1α, IL-1β, TGF-β1, TGF-β2, and TGF-β3), and cyclin E and RPL19 RNA samples were serially diluted and co-amplified in the same PCR procedure. Standard dilution curves for IL-1α, IL-1β, TGF-β1, TGF-β2, TGF-β3, cyclin E, and RPL19 were thus obtained. PCR products were separated by electrophoresis through an agarose gel (1.5%) and transferred to a nylon membrane. Bands were revealed using the Boehringer Dig Luminescent (Boehringer Mannheim) detection kit following the manufacturer's instructions. The bands were subjected to densitometric analysis, and semiquantitative analysis of signal strength was obtained by comparing measured values with their standard dilution curves. IL-1α, IL-1β, TGF-β1, TGF-β2, TGF-β3, and cyclin E values were finally adjusted for the respective RPL19 values.

WESTERN BLOTTING

A total of 25–40 µg (Cdk2, cyclin E), 125 µg (TGF-β), and 300 µg (IL-1β) of homogenate, nuclear, or cytosolic proteins were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (Hybond C-extra; Amersham). The blotted membrane was blocked (one hour, room temperature) in Tris buffered saline (TBS) containing 5% membrane blocking reagent (Amersham). All of the following incubations were carried out at room temperature in TBS containing 1% membrane blocking reagent. The incubation steps were followed by five washing steps of three minutes with TBS containing 0.1%

Table 1 Primers and reaction conditions used in polymerase chain reaction amplifications

Gene	Oligonucleotide sequences	Product size (bp)	Cycles	Reaction conditions
IL-1α	Sense: acaggtagttagaccacct Antisense: tagtgagcaactatagggt	389	30	94°C 5 min, 94°C 1 min, 58°C 1 min, 72°C 2 min, 72°C 10 min
IL-1β	Sense: gtggcagctaccatgtctt Antisense: gagaggctgctgatgtaccag	506	24	94°C 5 min, 94°C 1 min, 60°C 1 min 30, 72°C 2 min, 72°C 10 min
TGF-β1	Sense: ggactctccactgcaagac Antisense: cagagctgcgctgcagag	392	30	94°C 5 min, 94°C 1 min, 62°C 1 min, 72°C 2 min, 72°C 10 min
TGF-β2	Sense: ttgcgaggtatcgatggcac Antisense: gcataattgctgccttcgcc	214	30	94°C 5 min, 94°C 1 min, 62°C 1 min 30, 72°C 2 min, 72°C 10 min
TGF-β3	Sense: ccagctccaagcgacag Antisense: gtgcgcgagtggtgttg	164	28	94°C 5 min, 94°C 1 min, 64°C 1 min 30, 72°C 2 min, 72°C 10 min
Cyclin E	Sense: ctgaccattgtctcctggct Antisense: cagcttgactgtctggaca	385	30	94°C 5 min, 94°C 1 min, 60°C 1 min 30, 72°C 2 min, 72°C 10 min
RPL19	Sense: agtatgctcaggcttcagaa Antisense: gcaggtcttaagaccaggaa	501	30	94°C 5 min, 94°C 1 min, 56°C 1 min 30, 72°C 2 min, 72°C 10 min

Tween 20. The following primary and secondary antibodies were used: cyclin E rabbit polyclonal (Santa Cruz Biotechnology, Santa Cruz, California, USA; 1:000; one hour); Cdk2 rabbit polyclonal (Santa Cruz; 1:000; one hour); TGF- β 1, TGF- β 2, and TGF- β 3 mouse monoclonal (Genzyme, Cambridge, Massachusetts, USA; 1:3000; two hours); IL-1 β goat polyclonal (Santa Cruz; 1:250; one hour 30 minutes); peroxidase conjugated goat anti-mouse and mouse anti-rabbit IgGs (Jackson ImmunoResearch, West Grove, Pennsylvania, USA; 1:20000; one hour); anti-sheep/goat biotinylated Ig (Amersham; 1:4000; 45 minutes); and a streptavidin-horseradish peroxidase conjugate (Amersham; 1:4000; 15 minutes). The antigen-antibody reaction was visualised using the Amersham chemiluminescent detection system followed by exposure of the membranes to Hyperfilm-ECL (Amersham) for 1–3 minutes. Equal protein load on membranes and complete transfer were checked by staining gels and membranes with Coomassie blue. All blots were produced in triplicate and the amount of immunoreactive nuclear protein on each membrane was quantified using a UltroScan XL device (Pharmacia LKB). The area under the curve values were adjusted for total protein load. The final result of each sample was defined as the mean of three immunoblots performed on an identical cytoplasmic extract sample.

IMMUNOPRECIPITATION AND Cdk2 KINASE ACTIVITY

Immunoprecipitations and kinase assays were performed as described by Jaumot and colleagues²⁴ with slight modifications. Briefly, 500 μ g of cytosolic or nuclear proteins were incubated with 1 μ g of anti-Cdk2 antibody (Santa Cruz) for four hours at 4°C in a buffer containing 50 mM Tris, pH 7.4, Triton X100 0.1%, 5 mM EDTA, 250 mM NaCl, 50 mM NaF, 0.1 mM Na₃VO₄, 1 mM PMSF, and 2 μ g/ml leupeptin, aprotinin, antipain, and bestatin. Protein A/G Plus Agarose 20 μ l (Santa Cruz) was then added and incubation was carried on at 4°C for an additional two hours. Immunoprecipitates were harvested by centrifugation at 2500 rpm at 4°C for five minutes and washed four times in washing buffer A (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM dTT, 0.1 mM PMSF, 0.1 mM Na₃VO₄, and 0.1% Tween 20) and twice in washing buffer B (50 mM HEPES, 1 mM dTT). To determine kinase activity, Cdk2 immunoprecipitates were incubated at 30°C for 20 minutes in a kinase buffer (20 mM HEPES, 10 mM Mg acetate, and 1 mM dTT) containing 3 μ g of Histone H1 (Santa Cruz), 20 μ M ATP, and 10 μ Ci ATP ³²P (Amersham). The reaction was terminated by adding Laemmli sample buffer followed by 12.5% SDS-PAGE. The gels were dried and the phosphorylated proteins were visualised by autoradiography.

IMMUNOHISTOCHEMISTRY

Immunohistochemical staining of hepatic paraffin sections was performed as described previously with slight modifications.²⁵ Thin slices of liver tissue were immediately fixed in phosphate buffered 4% formaldehyde for a period of 24 hours. Before immunostaining, the slides were placed in a citrate buffer (pH 5.7) and treated with one cycle of three minutes at 750 W and four cycles of 3.5 minutes at 350 W in a microwave oven (Whirlpool, Göteborg, Sweden) to ensure optimal retrieval of the antigens.²⁶ The following primary antibody was used: an anti-p53 rabbit polyclonal antibody (Novocastra Laboratories, Newcastle, UK) at a dilution of 1:400 (overnight, 4°C). The specificity of the staining was ensured by omitting the primary antibody from the reaction and by replacing the primary antibody by a non-relevant rabbit polyclonal antibody. The overall surface of the sections was measured and all p53 labelled nuclei were counted. The results of nuclei counts were adjusted for the section surface.

STATISTICAL ANALYSIS

Results are expressed as mean (SEM). Statistical differences between groups were tested using one way analysis of variance (ANOVA). Statistical significance was assumed for p values <0.05.

Results

EXPRESSION OF p53 AND C-Ha-ras mRNA

Low p53 (fig 1A, C) and c-Ha-ras (fig 1B, C) transcript levels were found in all control animals. Following PBL, a significant increase in both transcripts was observed only in regenerating lobes from 12 hours onwards after PBL. Peak expression of p53 (150% increase) occurred at 12 hours ($p < 0.01$) followed by a decline to baseline values at 24 hours (fig 1A, C) whereas c-Ha-ras progressively increased until 24 hours (100% increase; $p < 0.001$) (fig 1B, C). No increase over baseline levels was noted in the anterior lobes.

IMMUNOHISTOCHEMISTRY OF p53 PROTEIN

No major histological differences between the lobes, in particular no foci of necroses, were observed until 30 hours after PBL. p53 expressing nuclei were homogeneously distributed over the whole liver and did not show preferential periportal or centrilobular distribution at any of the investigated time points (fig 2A–F).

Only a few hepatocyte nuclei expressing p53 protein were observed in the control groups (fig 2G). No elevation over baseline levels in p53 positive nuclei was present in both lobes one hour after PBL whereas a slight but significant increase in nuclear staining was found at five hours (posterior lobes $118 (42) \times 10^{-2}/\text{mm}^2$ and anterior lobes $127 (22) \times 10^{-2}/\text{mm}^2$; $p < 0.01$ v 0 hours). However, there was no statistically significant difference between both lobes at this time point (fig 2G). In the posterior lobes, a further elevation in p53 positive nuclei was observed at 30 hours after PBL while the

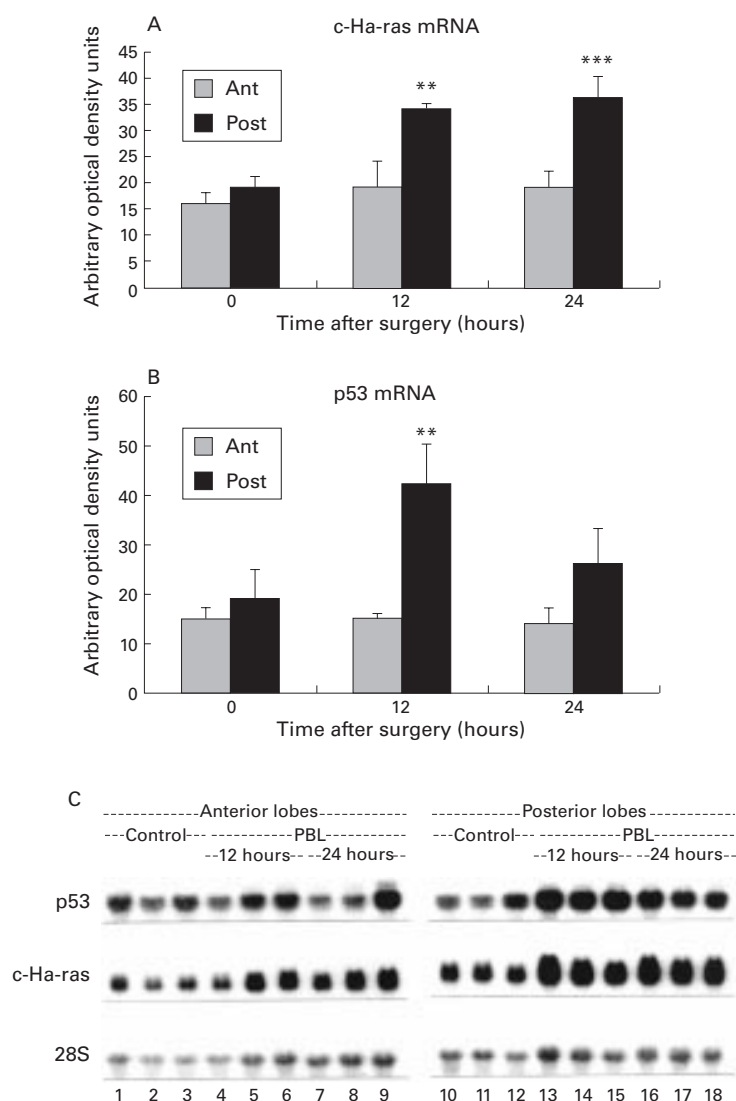


Figure 1 Northern blot analysis of p53 and c-Ha-ras mRNA. (A) Quantification of c-Ha-ras mRNA by densitometric analysis of the blots. The adjusted values did not reveal any significant rise in c-Ha-ras mRNA in the anterior lobes. In the posterior lobes, a significant increase in c-Ha-ras level was noticed from 12 hours after portal branch ligation (PBL). ** $p < 0.01$, *** $p < 0.001$. (B) Quantification of p53 mRNA by densitometric analysis of the blots. After adjustment for the respective 28S ribosomal signals, no elevation above baseline levels was observed in atrophying (Ant) lobes whereas p53 mRNA was significantly upregulated (** $p < 0.01$) in regenerating (Post) lobes at 12 hours after PBL. (C) Northern blots of p53, c-Ha-ras, and 28S mRNAs extracted from control animals (lanes 1–3 and 10–12) as well as from the anterior and posterior lobes of PBL animals (lanes 4–9 and 13–18, respectively). Total liver mRNA (20 μ g) was separated by electrophoresis on agarose gels, hybridised with their respective 32 P labelled cDNAs, and blotted. Signals were analysed by densitometry and adjusted for their 28S signals.

number of nuclei staining positive for p53 significantly declined in the anterior lobes (fig 2G). At this time point, a significant difference between both lobes became obvious (anterior lobes $62 (14) \times 10^{-2}/\text{mm}^2$ v posterior lobes $347 (123) \times 10^{-2}/\text{mm}^2$; $p < 0.05$).

EXPRESSION OF CYCLIN E

Cyclin E mRNA

Low amounts of cyclin E transcripts were detected in untreated animals. After PBL, no changes in cyclin mRNA expression were noticed in the atrophying lobes until 24 hours. In the regenerating lobes however mRNA levels started to rise from 12 hours onwards and a significant increase ($p < 0.01$) was clearly seen at 24 hours after surgery (fig 3)

Cyclin E protein

In the cytosol. Western blot analysis revealed two distinct bands of approximately 52 and 54 kDa, which most likely correspond to the previously described active cyclin E molecules originating from alternatively spliced mRNAs.^{27,28} Very low levels of cyclin E were present in the cytosol of control and sham operated animals. No increase was seen after sham operation but cyclin E was equally upregulated in the anterior and posterior lobes during the first five hours after PBL (fig 4A). A significant reduction in cyclin E protein expression occurred at 12 and 24 hours in the atrophying lobes. Nevertheless, protein levels remained above control values at these time points in the anterior lobes (fig 4B). In contrast, in the regenerating lobes, a further significant increase in overall cyclin E expression ($p < 0.001$) was observed from eight hours onwards after PBL which was mainly due to upregulation of faster migrating (52 kDa) cyclin E band (fig 4A, B). Cyclin E protein expression reached a peak in the posterior lobes at 12 hours and remained at significantly higher levels in these lobes compared with the atrophying lobes until 24 hours after surgery (fig 4B).

In the nuclear fraction. Cyclin E was only barely detectable in nuclear extracts from control and PBL animals before 12 hours after surgery. Thereafter, cyclin E was detected as two distinct bands of about 52 and 54 kDa in the regenerating liver lobes. At 12 hours after PBL, the 52 kDa band was predominately found whereas the 54 kDa band was only weakly expressed. Interestingly, at 24 hours the ratio of the slow migrating (54 kDa) to the faster migrating (52 kDa) band changed. Strong upregulation of the first was observed whereas the latter did not increase further (fig 5).

EXPRESSION OF Cdk2

In the cytosol

Low amounts of Cdk2 protein expression were found in quiescent livers of control animals (fig 5A). As early as one hour after PBL, a similar increase in Cdk2 levels was observed in the atrophying and regenerating lobes. Thereafter, Cdk2 levels decreased steadily in both parts of the liver to reach control values at eight hours after surgery. From 12 hours onwards, a divergent evolution became obvious between both parts of the liver. Cdk2 levels remained similar to controls in the atrophying lobes whereas significant ($p < 0.01$) upregulation was noted in the regenerating lobes (fig 5B). Only in the posterior lobes were high amounts of Cdk2 continuously detected until 24 hours after PBL.

In the nuclear fraction

Low signals of Cdk2 were also detected in liver nuclear extracts prepared from untreated control animals (fig 6A). In the atrophying lobes, no increase in Cdk2 expression was seen during the whole experiment. Indeed, immunquantification revealed levels of Cdk2 expression slightly below control values in the anterior lobes (fig 6B). In the regenerating lobes however Cdk2 levels increased progressively from 12

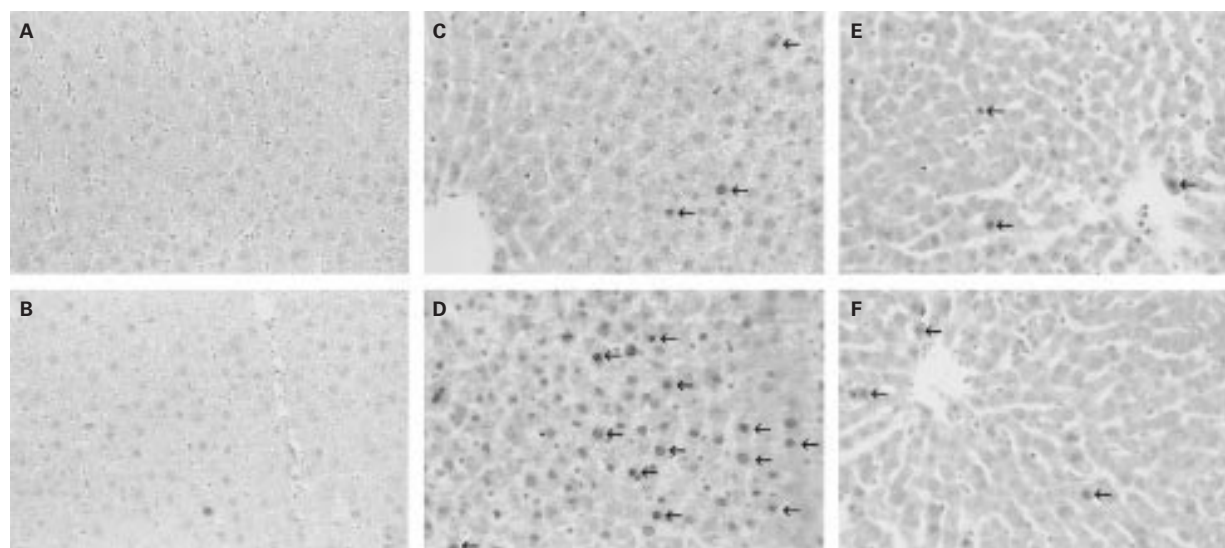


Figure 2 Immunohistochemistry of p53 protein expression after portal branch ligation (PBL). (A–F) Histology and p53 nuclear staining. Slides were stained immunohistochemically with a polyclonal anti-p53 antibody. No staining is observed in the anterior and posterior lobes of control animals (A, B). At five hours, nuclear staining (indicated by arrows) is slightly enhanced in both parts of the liver (E, F). At 30 hours, no further increase in p53 staining is noticed in the anterior lobes (C) whereas marked upregulation in positive staining nuclei is seen in the posterior lobes (D). (G) Quantification of p53 labelled nuclei. The labelled nuclei were counted in the anterior (Ant) and posterior (Post) lobes of control (0 hours) and PBL animals at one, five, and 30 hours. The final result was adjusted for the surface (mm^2) of the section. A similar and significant increase in p53 positive nuclei was noticed in the regenerating (Post) and atrophying (Ant) lobes at five hours after PBL ($**p < 0.01$ and $***p < 0.001$, respectively) whereas at 30 hours a further rise in p53 positive nuclei was only shown in the regenerating lobes. At this time point a significant difference between the lobes was observed ($*p < 0.05$).

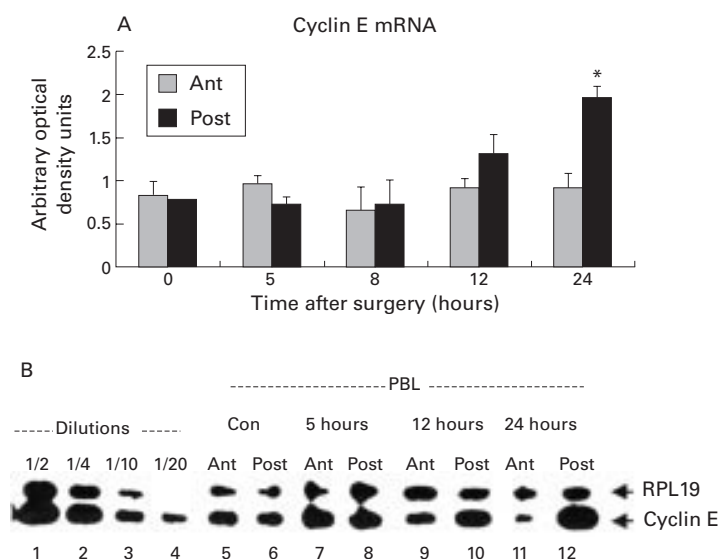


Figure 3 Expression of cyclin E mRNA in the atrophying and regenerating lobes after portal branch ligation (PBL). (A) Quantification of cyclin E mRNA by densitometric analysis. Total liver RNA obtained from the atrophying (Ant) and regenerating (Post) lobes was subjected to reverse transcription-polymerase chain reaction (RT-PCR), as described in materials and methods. The results were adjusted for their respective RPL19 levels and then compared with a standard dilution curve obtained by amplification of a cyclin E mRNA sample in the same PCR procedure. A significant induction of cyclin E mRNA was only observed in the regenerating lobes at 24 hours after PBL ($*p < 0.05$). (B) RT-PCR of cyclin E mRNA. Representation of RT-PCR of RPL19 and cyclin E mRNA in control animals (lanes 5 and 6) and in the atrophying (Ant) and regenerating (Post) lobes at various time points after PBL (lanes 7–12). Total liver RNA was subjected to RT-PCR as described in materials and methods. The results were adjusted for their respective RPL19 levels and compared with a standard dilution curve obtained by amplification of liver mRNA from lipopolysaccharide treated rats in the same PCR procedure (lanes 1–4).

hours onwards after PBL. At 24 hours, highly significant upregulation ($p < 0.001$) of Cdk2 was observed in the nuclear fractions prepared from posterior lobes (fig 6B).

HISTONE H1 KINASE ACTIVITY

Cdk2 was immunoprecipitated and a histone H1 kinase assay was performed on immunoprecipitates harvested from cytosolic and nuclear fractions. Cdk2 kinase activity was detectable in the cytosolic fraction from either part of the liver during the 24 hour experiment without major variations over the examined time period (fig 7A). No Cdk2 associated activity was found in nuclear fractions from the atrophying lobes at any of the investigated time points. In contrast, Cdk2 kinase activity was clearly and selectively present in the nuclear fractions obtained from the regenerating lobes at 24 hours after PBL (fig 7B). Detectable Cdk2 activity in the nucleus correlated with strong upregulation of nuclear Cdk2 protein and corresponded to the peak of DNA synthesis.

EXPRESSION OF TGF- β

TGF- β 1, TGF- β 2, and TGF- β 3 mRNAs

No significant changes in TGF- β 1, TGF- β 2, or TGF- β 3 mRNA levels were observed in both lobes during the first 24 hours after PBL (fig 8).

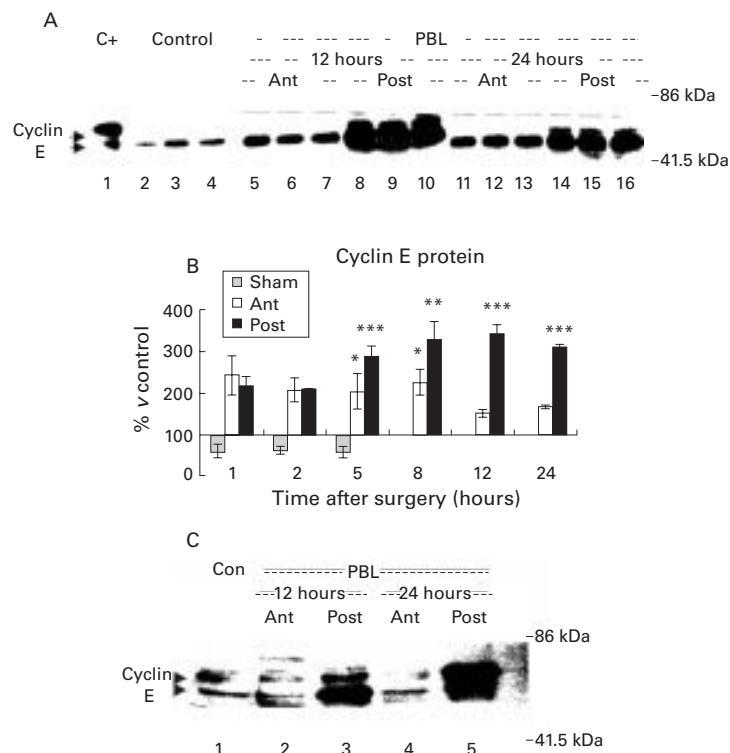


Figure 4 Western blot of cyclin E protein. (A) Cytoplasmic proteins (40 μ g) were resolved using 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and blotted with an anti-cyclin E polyclonal antibody. Samples from control animals (0 hours) and from the atrophying (Ant) and regenerating (Post) lobes of animals at various times after portal branch ligation (PBL) were analysed. The antibody recognised two bands of approximately 52 and 54 kDa in control and PBL animals. A representative blot of three control (lanes 2–4) and three PBL cytosol extracts at 12 hours (lanes 5–10) and 24 hours (lanes 11–16) after surgery is shown. KNKR nuclear extracts were used as a positive control (C+, lane 1). (B) Quantification of cyclin E protein in the cytoplasm by densitometry. Cyclin E protein was rapidly upregulated in both lobes within the first hours after PBL. A further significant increase was observed in the regenerating lobes between 8 and 24 hours after PBL ($^{**}p < 0.01$, $^{***}p < 0.001$, respectively). In contrast, cyclin E protein expression decreased in the atrophying lobes during the same time without, however, reaching control levels. (C) Nuclear proteins (40 μ g) were subjected to western blot analysis. Very low amounts of cyclin E protein were detected in the nuclear fraction of control livers (Con). A faint band of approximately 52 kDa was found in nuclear fractions of both lobes at all investigated time points which increased selectively in the regenerating lobes (Post) from 12 hours onwards (lane 3). A slower migrating approximately 54 kDa band, initially weakly present in both parts, was strongly upregulated in the nuclear fractions from the regenerating (Post) lobes at 24 hours (lane 5).

TGF- β proteins

Use of an antibody recognising all three TGF- β isoforms did not show any difference in TGF- β expression up to 24 hours after PBL (not shown).

EXPRESSION OF IL-1

IL-1 α

No significant changes in IL-1 α mRNA were seen, in atrophying or regenerating lobes throughout the entire experiment (not shown).

IL-1 β

IL-1 β mRNA. IL-1 β mRNA levels increased similarly in the anterior and posterior lobes until five hours after PBL. From eight hours onwards, a difference became apparent between both lobes. In atrophying lobes, IL-1 β decreased slightly thereafter but remained significantly higher than control levels until 24 hours after PBL (fig 9A, B). In contrast, in the regenerating lobes, IL-1 β mRNA expression had returned to control values eight hours after PBL (fig 9A, B).

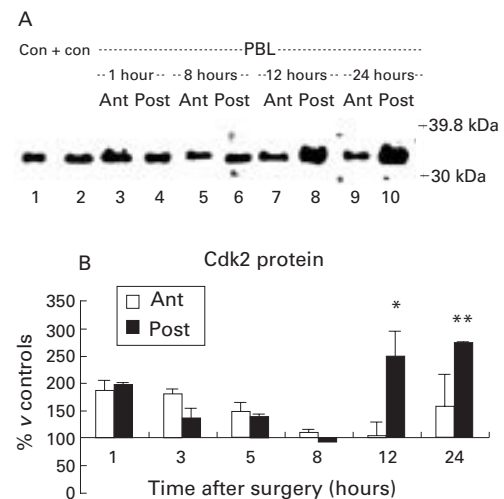


Figure 5 Western blot of cyclin dependent kinase (Cdk2) protein in the cytoplasm. (A) Cytoplasmic proteins (30 μ g) were resolved using 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and blotted with an anti-Cdk2 polyclonal antibody. Samples from control animals (0 hours) and from the atrophying (Ant) and regenerating (Post) lobes of animals at various times after portal branch ligation (PBL) were analysed. An approximately 34 kDa band was found in control livers (Con, lane 2) and in both lobes after PBL. Increased expression was observed in the regenerating (Post) lobes mainly at 12 and 24 hours after PBL (lanes 8 and 10, respectively). KNKR nuclear extracts were used as a positive control (Con+, lane 1). (B) Quantification of Cdk2 protein in the cytoplasm by densitometry. Cdk2 was similarly increased in both lobes during the very first hours after PBL but returned to control levels at eight hours. Thereafter, Cdk2 increased again significantly ($^{*}p < 0.05$; $^{**}p < 0.01$) only in the regenerating (Post) lobes until 24 hours after PBL.

IL-1 β protein. To investigate if persistent IL-1 β mRNA expression in atrophying lobes correlated with IL-1 β protein expression, western blot analysis of liver homogenates derived from the atrophying and regenerating lobes at eight, 12, and 24 hours after PBL was carried out. At high protein loads, an approximately 31 kDa band was detected by the antibody raised against the 31 kDa precursor of the IL-1 β protein. This band was observed only in the atrophying and not in the regenerating lobes at eight, 12, and 24 hours after PBL (fig 9C). Maximal levels of IL-1 β immunoreactive protein were reached in the atrophying lobes at approximately 12 hours after PBL.

Discussion

Portal branch ligation constitutes an ideal model to study the specificity and role of several factors in the development of liver atrophy or regeneration as both processes are concomitantly observed within the same liver. In this model of liver regeneration, opposite responses were induced eight hours or later after surgery in the anterior and posterior lobes related to cellular atrophy and cellular proliferation, respectively. Persistent expression of IL-1 β was observed in the atrophying lobes whereas p53, c-Ha-ras, cyclin E, and Cdk2 were selectively and persistently activated in the regenerating lobes. We have previously shown that the early cellular responses in the PBL model, including activation of nuclear factor κ B, Stat3, IL-6, c-fos, c-myc, and c-jun

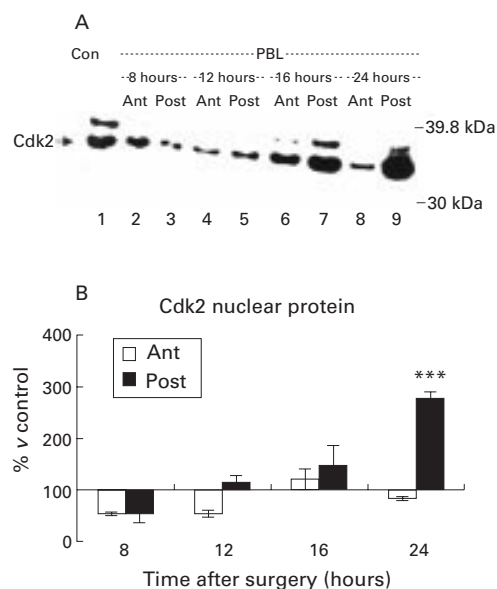


Figure 6 Western blot of cyclin dependent kinase (Cdk2) protein in the nuclear fraction. (A) Cytoplasmic proteins (30 µg) were resolved using 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and blotted with an anti-Cdk2 polyclonal antibody. Samples from control animals (Con) and from the atrophying (Ant) and regenerating (Post) lobes of animals at various times after portal branch ligation (PBL) were analysed. Low levels of Cdk2 were found in control livers (Con, lane 1) and in the atrophying (Ant) lobes after PBL. Increased expression was observed in the regenerating (Post) lobes from 16 hours onwards, reaching a peak at 24 hours after PBL (lanes 7 and 9, respectively). (B) Quantification of Cdk2 protein in the nuclear fraction by densitometry. No significant increase in Cdk2 expression compared with control levels was seen in the atrophying (Ant) lobes after PBL. In the regenerating (Post) lobes however Cdk2 levels started to rise from 12 hours after PBL and reached a significant peak (***) at 24 hours after PBL.

were similarly obtained in the anterior and posterior lobes.⁷ The present observations of similar activation of p53 protein and IL-1 in both parts of the liver during the first 5–8 hours after PBL also favours the absence of specificity of these early events. However, the methods used in this study were semiquantitative and do not allow exclusion of an eventual, although unlikely, threshold phenomenon during the early phase. As cross talk and cooperation between proteins and transcription factors exists, we cannot formally exclude the possibility that specific interplay of these factors at early time points may influence future cell fate. At present it is difficult to investigate these mechanisms. However, and more importantly, our results provide strong evidence that the cellular response in the atrophying and regenerating lobes follows a divergent specific pathway at approximately 12 hours after PBL. The results also suggest that hepatocytes in the atrophying lobes do not progress beyond early G1 phase. In contrast, sustained activation of p53 beyond the early phase and late activation of c-Ha-ras followed by cyclin E/Cdk2 induction in the regenerating lobes drives hepatocytes to progress further towards late G1 phase and finally into S phase of the cell cycle.

At 12 and 24 hours after PBL, no elevation in p53 mRNA over baseline levels was found in the atrophying lobes whereas the regenerating lobes showed a marked increase in p53 mRNA

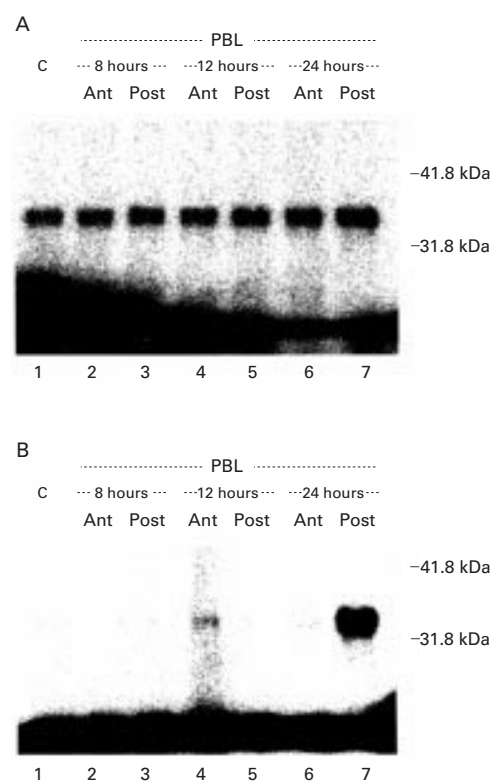


Figure 7 Cyclin dependent kinase (Cdk2) associated activity in cytoplasmic and nuclear fractions. Cdk2 protein was immunoprecipitated and a histone H1 kinase assay was performed followed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. (A) Low constitutive activity was detected in the cytosol of control livers (C) and in the atrophying (Ant) and regenerating (Post) lobes after portal branch ligation (PBL) (lanes 2–7) which did not significantly vary during the experiment. (B) In contrast, no Cdk2 activity was observed in the nuclear fractions of control (C, lane 1) and PBL livers (lanes 2–6) until 24 hours. At 24 hours after PBL a sharp increase in Cdk2 activity was selectively found in the regenerating lobes (lane 7).

levels, peaking at 12 hours. Although an early non-specific rise in p53 protein was noted in both lobes five hours after PBL, p53 protein expression did not increase further in the anterior lobes thereafter while highly significant upregulation of p53 expression was observed in the posterior lobes 30 hours after PBL. These results are in agreement with previous data showing p53 protein peaks at six and 30 hours after PH.²⁹ However, p53 mRNA and protein levels were not tightly coupled, suggesting post-transcriptional regulation of p53 expression. Taken together, our findings suggest a link between p53 and processes leading to cellular proliferation.

Although the precise role of p53 in the network of cellular regulation in normal liver is largely unknown, there is increasing evidence that p53 may be required for G1 phase progression, as suggested by upregulation of its mRNA in many cycling cells, including primary cultured hepatocytes.^{30–33} During the cell cycle in vitro, the abundance of p53 increases from G1 to S^{30–33} and a shift of p53 protein into the nucleus is observed at the G1/S transition.³⁴ In the regenerating liver, p53 expression is increased in terms of mRNA^{1–9–35–36} and protein levels, peaking at six and 30 hours after PH.^{29–37}

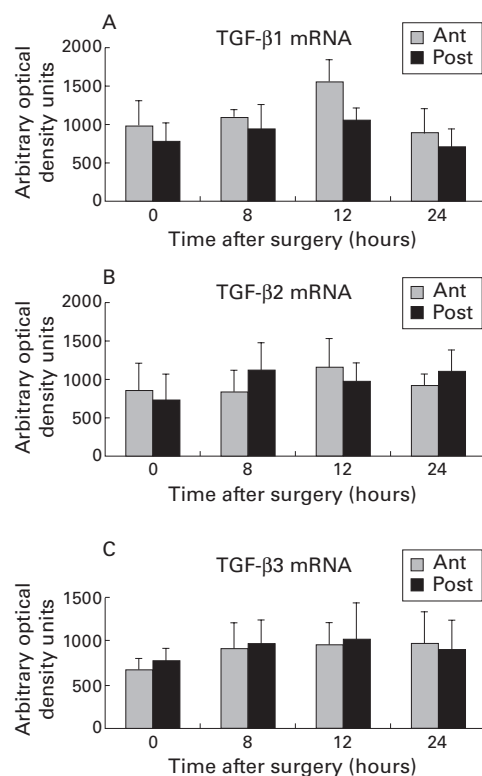


Figure 8 Quantification of transforming growth factor (TGF)- β 1, - β 2, and - β 3 mRNA by densitometric analysis. Total liver RNA was subjected to reverse transcription-polymerase chain reaction, as described in materials and methods using TGF- β 1 (A), TGF- β 2 (B), and TGF- β 3 (C) specific primers. The results were adjusted for their respective RPL19 levels and compared with a standard dilution curve obtained by amplification of liver mRNA from lipopolysaccharide treated rats in the same polymerase chain reaction procedure. Compared with control levels (0 hours), no significant differences in TGF- β mRNA levels were noted at any of the investigated time points after portal branch ligation (eight, 12, or 24 hours).

The requirement of p53 for cell cycle progression *in vivo* is further supported by the observation that in ethanol fed rats, which show a strong reduction in hepatocyte replication and recovery of liver mass after PH, no increase in p53 mRNA expression was found during late G1 despite activation of TNF- α , IL-6, and Stat3.³⁸ In addition to its potential role in the regenerative phenomena, p53 has also been shown to inhibit cell division by blocking cell cycle progression in G1 phase.^{39–42} Therefore, it could act as a negative modulator of liver regeneration in the regenerating lobes, as indicated by its sustained activation (30 hours) beyond the peak of DNA synthesis (24 hours). Alternatively, p53 could contribute to cell cycle block in early G1 of the cells in the atrophying lobes. Nevertheless, absence of p53 activation in our study once the early non-specific phase had been passed suggests that p53 may not be involved in the atrophic process of the anterior lobes.

Selective and progressive induction of c-Ha-ras in the regenerating part of the liver from 12 hours onwards after PBL as well as upregulation of ras oncogenes after PH⁹ suggests a strong link between this proto-oncogene group and cellular proliferation. Ras acts as a

transducer of the mitogenic signal and its function is required in G1 for passage through the restriction point of the cell cycle.^{43–44} c-Ha-ras may thus be one of the elements which contributes to the transformation of an initially non-specific cellular response into a specific one—that is, regeneration. However, the exact mechanisms through which ras proteins promote cell cycle progression remain unclear.

Progression through the cell cycle is governed by intermittent activation of cyclins and their associated kinase partners at key points of the cell cycle.¹¹ There is growing evidence that the cyclin E/Cdk2 complexes are the principal players contributing to the irreversibility of G1/S transition^{14–15} and their presence and activation are required to pass this cell cycle checkpoint.^{28–46} Our data on p53 and c-Ha-ras activation in the posterior lobes suggest that hepatocytes in the regenerating lobes progress to late G1 phase whereas those in the atrophying lobes are arrested in early-mid G1 phase. To further sustain this hypothesis and provide a potential link between p53/c-Ha-ras activation and the cell cycle, expression of cyclin E as well as expression and activity of its preferred kinase partner, Cdk2, were investigated in both parts of the liver. Cyclin E and Cdk2 were selectively upregulated in the cytoplasm of regenerating hepatocytes near the G1/S transition followed by transfer of cyclin E and Cdk2 into the nucleus. Activation of Cdk2 then takes place in the nucleus itself, as suggested by the selective and marked increase in Cdk2 associated kinase activity predominately in the nuclear fraction of the regenerating hepatocytes at 24 hours after surgery. Several recent studies have reported an increase in cyclin E and Cdk2 expression and activity close to the G1/S boundary after PH^{47–48} and a mechanism involving nuclear translocation and activation of Cdk2 has also been suggested.²⁴ Interestingly, in our study, Cdk2 kinase activity was accompanied by the predominant appearance of the slower migrating cyclin E band of approximately 54 kDa in the nuclear fraction on western blot analysis. A similar observation has recently been made by Albrecht and colleagues⁴⁸ in nuclear extracts from rats who underwent PH. One explanation may be that the transition from inactive into active cyclin E is associated with changes in its phosphorylation status. It has been demonstrated that cyclin E undergoes phosphorylation changes near the G1/S transition in the cycling cell, including primary hepatocytes in culture.^{49–50} Alternatively, the 54 kDa form of cyclin E may represent the formerly reported longer splice variant of cyclin E²⁷ and it is this variant which is mainly found and required in active cyclin E/Cdk2 complexes. Our observations emphasise the important role of cyclin E and Cdk2 in determining the commitment of the cell to undergo proliferation. The requirement of cyclin E/Cdk2 in processes leading finally to cellular proliferation and subsequently liver regeneration is further supported by observations in 2-AAF treated rats or in C/EBP beta knockout mice where lack of cyclin E expression at the G1/S phase restriction point is associated with

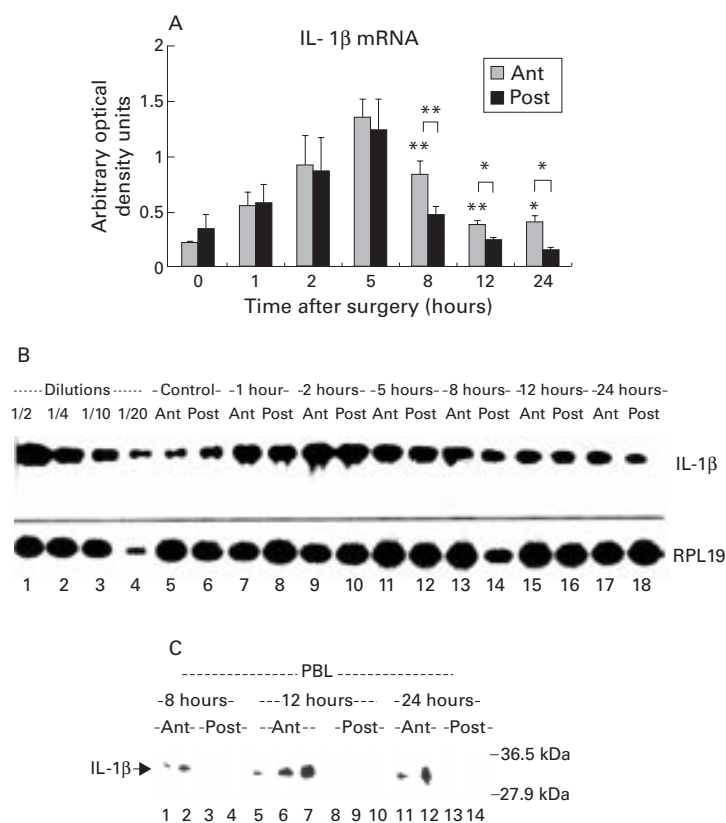


Figure 9 Expression of interleukin (IL)-1 β in atrophying and regenerating lobes after portal branch ligation (PBL). (A) Quantification of IL-1 β mRNA by densitometric analysis. IL-1 β mRNA was significantly induced in both lobes during the first hours (1–5) after PBL. From eight hours after PBL, IL-1 β mRNA returned to control levels in the regenerating lobes (Post) whereas significant activation of IL-1 persisted in the atrophying lobes (Ant) (* $p < 0.05$, ** $p < 0.01$). (B) Reverse transcription-polymerase chain reaction (RT-PCR) of IL-1 β mRNA. Representation of a RT-PCR of RPL19 and IL-1 β mRNA in control animals (lanes 5–6) and in the atrophying (Ant) and regenerating (Post) lobes at various time points after portal branch ligation (lanes 7–18). Total liver RNA was subjected to RT-PCR, as described in materials and methods. The results were adjusted for their respective RPL19 levels and compared with a standard dilution curve obtained by amplification of liver mRNA from lipopolysaccharide treated rats in the same PCR procedure (lanes 1–4). (C) Western blot of IL-1 β protein in liver homogenates. At high protein load (up to 300 μ g), an approximately 31 kDa band (IL-1 β precursor) was observed in the atrophying lobes at eight (lanes 1, 2), 12 (lanes 5–7), and 24 hours (lanes 11, 12) after PBL. No signal was detected in the proliferating lobes at the same time points (lanes 3, 4, 8–10, 13, 14, respectively).

disturbed liver regeneration after PH.^{51 52} In addition, our data provide a potential link between ras induction and activation of cyclin E/Cdk2 which are selectively seen in the regenerating lobes. Interestingly, peak expression of both is seen 24 hours after PBL which correlates with peak DNA synthesis in this model of liver regeneration.^{7 53} Ras activity is required for phosphorylation of pRb in response to mitogenic signalling.^{54 55} Enforced pRb phosphorylation may be achieved, at least in part, by ras induced derepression and/or enhancement of cyclin E/Cdk2 activity.⁵⁶ However, it has not yet been clearly established if ras directly targets cyclin E/Cdk2 complexes or if indirect mechanisms are mainly involved, such as degradation of cyclin/Cdk inhibitory proteins and/or induction of other cyclins sequestering free cyclin/Cdk inhibitory proteins.^{14 57 58}

Hepatocytes as well as non-parenchymal cells can express TGF- β ^{18 59 60} and TGF- β has been shown to inhibit hepatocyte proliferation in vivo and in vitro.^{17 61–63} It has been reported that TGF- β exerts its inhibitory effect in the

late G1 phase of the cell cycle near the G1/S boundary and may be involved in blocking cyclin E expression in hepatocytes.^{64 65} We therefore examined expression of TGF- β mRNA and protein expression in both lobes, in particular during mid-late G1 phase (8–24 hours). No increase in TGF- β mRNA or TGF- β protein levels was noticed between eight and 24 hours after PBL in any part of the liver. Therefore, our results do not suggest a role for TGF- β as a negative regulator of hepatocyte proliferation, at least during this critical period after PBL. Consequently, mechanisms other than TGF- β seem to be important in regulating hepatocyte proliferation in vivo before the first wave of DNA synthesis. Nevertheless, TGF- β may interfere to some extent with repression of hepatocyte proliferation or the final adjustment of liver size after the major wave of cell growth as peak TGF- β induction has been reported after 24 hours following PH or CCl₄ administration, time points which have not been assessed in our studies.^{17 18 66}

Given the low probability of TGF- β being a major inhibitor of hepatocyte cell cycle progression in late G1, we assessed expression of IL-1, a negative regulator of hepatocyte proliferation in vitro,^{67 68} in both parts of the liver after PBL. During the last years, a potential suppressor effect of IL-1 on hepatocyte proliferation in vivo has also been suggested based on studies examining IL-1 expression after PH and in Kupffer cell depleted rats subjected to PH.^{16 19} In addition, administration of an IL-1 receptor antagonist prior to PH resulted in a marked increase in hepatocyte proliferation in these animals.⁶⁹ In our studies, IL-1 α levels did not change in any of the lobes during the first 24 hours after PBL hence excluding a major role of this cytokine in regulating liver cell proliferation. However, and interestingly, IL-1 β expression showed sustained activation in the atrophying lobes from eight hours onwards after PBL whereas in the regenerating lobes IL-1 β returned to control levels. This persistent activation of IL-1 β in the atrophying part of the liver constitutes a further argument for IL-1 β being involved in downregulation of cell cycle progression in the liver. However, the mechanisms by which IL-1 β influences the cell cycle are still unknown. Nevertheless, although IL-1 β was downregulated in the regenerating lobes between eight and 24 hours after PBL, we cannot exclude a regulatory influence of this cytokine at later time points.

In conclusion, the present results show that the cellular response, initially similar in the atrophying and regenerating lobes, follows a divergent specific pathway from 12 hours onwards after PBL. Our findings underline the importance of events occurring between eight and 12 hours (mid G1 phase) to determine the final outcome—that is, atrophy or proliferation—of each hepatocyte. p53, c-Ha-ras, cyclin E, and Cdk2 seem to drive the commitment of cells to proliferate. IL-1 β but not TGF- β is associated with cellular atrophy and could play a role as a negative regulatory factor

of hepatocellular proliferation in the atrophying lobes at a critical point in mid G1 phase of the cell cycle.

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