The Cdk Inhibitor p21 Is Required for Necrosis, but It Inhibits Apoptosis following Toxin-induced Liver Injury*

Received for publication, January 29, 2003, and in revised form, May 13, 2003
Published, JBC Papers in Press, May 20, 2003, DOI 10.1074/jbc.M300996200

Young Hye Kwon, Aleksandra Jovanovic, Michael S. Serfas, and Angela L. Tyner‡

From the Department of Molecular Genetics, University of Illinois, Chicago, Illinois 60607

Lever injury and repair were examined in wild type, p21\textsuperscript{Waf1/Cip1}\textsuperscript{-/-}, and p27\textsuperscript{Kip1}\textsuperscript{-/-} deficient mice following carbon tetrachloride (CCl\textsubscript{4}) administration. In wild type liver, p21 expression is induced in a biphasic manner following injection of CCl\textsubscript{4}, with an early peak of p21 expression occurring in pericentral hepatocytes at 6 h, prior to evidence of injury, and a second peak succeeding regenerative proliferation. In contrast, p27 is present throughout the quiescent liver, but its expression decreases following CCl\textsubscript{4} injection. Surprisingly, p21-deficient animals were resistant to CCl\textsubscript{4}-induced necrotic injury, indicating that rapid induction of p21 in pericentral hepatocytes following CCl\textsubscript{4} injection contributes to subsequent necrosis. Expression of cytochrome P450 2E1, which plays an essential role in CCl\textsubscript{4}-induced necrotic injury, was not affected in p21-deficient mice. Although they had the least injury, p21-deficient mice had the highest levels of hepatic proliferation that correlated with increases in hyper-phosphorylated retinoblastoma protein and Cyclin A expression. Increased replication in p21-deficient livers was counteracted by an increase in hepatocyte apoptosis as detected by caspase-3 activation. p21 plays distinct and opposing roles regulating hepatocyte survival during injury and subsequent repair, with early induction of p21 contributing to necrotic injury and later expression to cessation of proliferation and hepatocyte survival.

Cyclin kinase inhibitors (CKIs)\textsuperscript{1} play key roles in negatively regulating cell proliferation (reviewed in Refs. 1 and 2). p21\textsuperscript{Waf1/Cip1}\textsuperscript{-/-} is the founding member of the Cip/Kip family of CKIs, which also includes p27\textsuperscript{Kip1} and p57\textsuperscript{Kip2} (reviewed in Refs. 3 and 4). The Cip/Kip CKIs bind to a broad range of cyclin Cdk complexes and inhibit the activity of the cyclin E and cyclin A-dependent kinase Cdk2 (1, 5, 6). In the adult mouse, p27 is expressed throughout the liver lobule, whereas expression of p21 protein cannot be detected. However, in the absence of p27, p21 protein accumulates, and at least one of these CKIs is needed for proper maintenance of hepatocyte quiescence (7).

The liver provides an outstanding model for studying the physiological roles of CKIs in tissue repair and remodeling. It has the unique ability to regenerate following tissue loss because of chemical or physical injury (reviewed in Refs. 8 and 9). Regulation of the regenerative process must include signals that stop cell proliferation, once the liver has been restored to the appropriate size. Carbon tetrachloride (CCl\textsubscript{4}) has been widely used to study mechanisms of hepatic injury and repair following toxin-induced injury (10). It is metabolized in the centrilobular zone of the liver where the production of highly reactive trichloromethyl radicals leads to the necrotic death of pericentral hepatocytes (11, 12). One to 2 days following CCl\textsubscript{4} injection, liver cells within the perportal and intermediate zones divide in response to the severe damage and cell death that occurs in the pericentral region.

Previously we detected a dramatic increase in expression of mRNA encoding the Cki p21\textsuperscript{Waf1/Cip1} in mouse pericentral hepatocytes between 4 and 8 h after CCl\textsubscript{4} injection, long before any clear evidence of liver injury was apparent. A second peak of p21 mRNA expression was observed between 1 and 2 days post-injection in periportal hepatocytes that had divided to replace the dead cells. The two peaks of p21 mRNA expression observed following CCl\textsubscript{4}-induced injury were detected in both wild type and p53-deficient mice, indicating that p21 gene expression was not p53-dependent (13). In the current study, we have addressed the roles of p21 and the related Cki p27 during the course of CCl\textsubscript{4}-induced liver injury and regeneration using mice deficient for p21 or p27. These studies provide a better understanding of the molecular mechanisms regulating liver injury and repair following chemical injury, as toxins and liver cell necrosis play significant roles in a number of clinical liver diseases (reviewed in Ref. 14).

EXPERIMENTAL PROCEDURES

Animals—Conventionally raised viral antigen free wild type, p21\textsuperscript{-/-} (15), and p27\textsuperscript{-/-} (16) male mice at 8–10 weeks of age were used in all experiments, unless otherwise indicated. At least three mice per genotype were examined per time point. Mice were fed a commercial diet and water ad libitum. To induce injury 10 \textmu g body weight of a 10\% solution of carbon tetrachloride in corn oil or corn oil alone was injected intraperitoneally, and mice were sacrificed at the times indicated. The left lobe of the liver was fixed in 4\% paraformaldehyde and paraffin-embedded for sectioning. Other portions of liver were frozen in liquid nitrogen and kept at -70 °C for preparation of protein lysates and total RNA.

Immunoblotting—In general, 50 \mu g of total liver protein was separated on 12 or 15\% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). For pRb and p107 proteins, 150 \mu g of protein was loaded and separated on an 8\% gel. All membranes were stained with Ponceau S to confirm equal loading and transfer of protein. Immunoblotting was performed using the following primary antibodies obtained from Santa Cruz Biotechnology, Inc., Santa Cruz, CA: p21 (SC-397), p27 (SC-528), Cdk2 (SC-163), Cdk4 (SC-280), cyclin A (SC-596), cyclin E (SC-481), p107 (SC-518), and PCNA (SC-56). Other antibodies used included cyclin D1 (MS-210), NeoMarkers, Fremont, CAI, phospho-Rb (9308, Cell Signaling Technology, Beverly, MA), and \beta-actin (A-5441, Sigma). Immunoreactive
bands were detected using the SuperSignal Substrate (Pierce). Immunoprecipitations were performed using anti-p21 antibody (PC55, Oncogene Research Products, San Diego). Rabbit IgG was used as a control for antibody specificity.

**Semiquantitative and Quantitative PCR—**Semiquantitative RT-PCR was performed to examine expression of mouse p21, p27, and cytochrome P450 2E1 (CYP 2E1) as described (7). The following primer pairs and conditions were used for detecting expression of p21 (upstream primer, AGTGTGGCTGTGTCCTGGC; downstream primer, ACACCAAGTGCAAGACAGC; annealing temperature 62 °C, 30 cycles; product 311 bp), p27 (upstream primer, TCGCGAAGCTCGAGG; downstream primer, TGAATGCTCTTCCATATTCC; annealing temperature 62 °C, 30 cycles; product 300 bp), and CYP 2E1 (upstream primer, AGTTTCACACTGCGTGACTCG; downstream primer, CCTGGGACACAGGAATGTCC; annealing temperature 57 °C, 30 cycles; product 125 bp). Expression of mouse S16 ribosomal protein was examined as an internal control as described previously (13). For each combination of primers, the kinetics of PCR amplification were studied; the number of cycles corresponding to plateau was determined, and PCR efficiencies were calculated.

Quantitative real-time PCR was performed to measure cyclin A gene expression (upstream primer, AGTACCTGCCTTCACTCATTGCTG; downstream primer, TCTGGTGAAGGTCCACAAGACAAG) using the ABI PRISM 7700 (Applied Biosystems, Foster City, CA) and SYBR Green technology. Mouse S18 expression was examined as a control using the primers described (17). 96-Well optical plates with reaction mixtures were heated for 2 min at 50 °C and 4 min at 95 °C, followed by 40 cycles of PCR consisting of 15 s at 95 °C and 20 s at 63 °C. At the end of the run, samples were heated to 95 °C with a ramp time of 10 s to confirm dissociation curves to check that single PCR products were obtained. PCRs were also analyzed by gel electrophoresis to confirm that a single product of the expected size was amplified. Validation experiments were performed to demonstrate that efficiencies of target and reference amplifications were approximately equal. The comparative C_1_ method for relative quantitation of gene expression described by Livak and Schmittgen was used to determine cyclin A expression levels. Experiments were carried out in triplicate for each data point. Sequence Detection Systems 1.7 software (Applied Biosystems, Foster City, CA) was used for analysis.

**Assessment of Liver Injury—**Levels of serum alanine aminotransferase (ALT) were measured to assess the degree of hepatic necrosis. Serum was collected from animals and analyzed for ALT activity using an in vitro diagnostic kit from Sigma.

The DeadEnd™ Fluorometric TUNEL System (Promega Corp., Madison, WI) was used to examine injury in the liver. Fluorescein-12-UTP-labeled DNA in liver sections was visualized using fluorescence microscopy. To examine caspase-3 activation in situ, liver sections were pretreated for antigen recovery and accessibility by microwave heating in 0.01 M citrate (pH 6.0), 4 °C for 5 min. The Tyramide Signal Amplification kit (PerkinElmer Life Sciences) and cleaved caspase-3 antibody (Amp-175, Cell Signaling Technology, Beverly, MA) were used for immunohistochemistry.

**Autoradiographic Analyses—**For determination of hepatocyte proliferation, 2 μCi of methyl-[3H]thymidine (Amersham Biosciences)/g body weight was injected intraperitoneally into three mice of each genotype 1 h prior to sacrifice. Tissue sections were dipped into NTB-2 emulsion and exposed for 17 days. Developed slides were counterstained with hematoxylin-eosin, and the number of hepatocytes incorporating [3H]thymidine was determined by counting H-labeled hepatocyte nuclei in 3 grids per field. At least 5 fields were counted on each slide, and 3 slides were analyzed for each sample.

**RESULTS**

**Differential Regulation of p21 and p27 following Liver Injury—**Expression of p21 and p27 protein was examined in the liver during the course of CCl_4-induced liver injury and regeneration in wild type mice and mice lacking p21 or p27. Wild type, p21−/−, and p27−/− mice injected with CCl_4 or corn oil alone were sacrificed at 0, 3, 6, 9, 12, 24, 36, 48, 72, and 168 h after injection. Total proteins were isolated and subjected to immunoblotting with antibodies against p21, p27, or β-actin (Fig. 1A). We were unable to detect p21 protein in wild type liver lysates, but its expression was induced by 3 h after CCl_4 injection. Two peaks of p21 protein were detected at 6 and 48 h in wild type mice. p53-independent transcriptional regulation plays a key role in the induction of p21 following CCl_4 injection (13), and the two peaks of p21 protein expression followed peak mRNA expression detected using RT-PCR (Fig. 1B).

Significant p27 protein expression was detected in wild type quiescent liver, but shortly after CCl_4 administration p27 levels decreased. Decreases in p27 protein levels after injury appear to be post-transcriptionally regulated, because p27 mRNA levels remain fairly constant during the course of regeneration (Fig. 1B). Interestingly, significant levels of p21 protein were detected in p27-deficient mice at the zero time point (Figs. 1A and 5B), although no increase in p21 RNA expression was detected in p27-deficient mice (Fig. 1B). Our earlier studies indicate that p21 accumulates in the livers of p27-deficient mice through a post-transcriptional mechanism and compensates for the absence of p27 in maintaining hepatocyte quiescence (7).

Differences in p21 expression were also detected at later time points in p27-deficient mice when compared with wild type
mice (Figs. 1A and 5B). The early peak of p21 protein expression detected in wild type animals was muted, and the later peak of p21 expression occurred at 36 h in p27-deficient mice versus 48 h in the wild type mice. These data indicate that hepatocytes deficient for p27 differentially regulate expression of p21 at several time points. In p27-deficient mice the pattern of p21 RNA expression was very similar to that of wild type mice, suggesting that the differences in p21 expression are regulated post-transcriptionally in p27-deficient hepatocytes.

We found previously (7) that p21 protein expression was regulated at the level of protein stability in cultured primary hepatocytes.

Decreased Necrotic Injury in p21-deficient Mice—CCl₄ is a classical inducer of centrilobular necrosis. When hepatocytes undergo necrosis, ALT is released from hepatocytes into the bloodstream, resulting in abnormally high serum levels of this enzyme. To assess the level of hepatocyte necrosis, we measured serum ALT levels throughout the course of liver injury and repair (Fig. 2). Peak levels of serum ALT were detected at 18 h post-CCl₄ injection in both wild type and p27−/− mice. At this time point serum levels of ALT in p21−/− mice were ~5-fold less than in the other two genotypes. Release of ALT was delayed in p21−/− mice, with peak levels appearing at 24 h. However, cumulative serum ALT in p21−/− animals never reached the levels detected in wild type and p27 null animals. p27-deficient mice had higher levels of serum ALT at

**Fig. 2.** p21-deficient mice have reduced serum ALT levels following injection of CCl₄. Blood samples were drawn by cardiac puncture; plasma was separated by centrifugation, and ALT enzyme levels were measured as described. ALT levels are shown as units/liter ± S.E. Reduced levels of serum ALT in p21-deficient mice are indicative of reduced necrotic liver injury.

**Fig. 3.** Assessment of liver injury using the TUNEL assay and immunohistochemical localization of cleaved caspase-3. A, the TUNEL assay was performed with sections of liver tissue from wild type (WT), p21−/−, and p27−/− mice at 12, 18, and 24 h after CCl₄ injection. Injury occurs in pericentral hepatocytes surrounding the central veins. Examples of central veins (CV) and portal triads (PT) are labeled. Strong TUNEL labeling of pericentral hepatocytes was detected in wild type and p27−/− liver sections at 18 and 24 h but not in p21−/− sections. B, tissue sections from livers at 24 h post-CCl₄ injection were incubated with an antibody specific for cleaved caspase-3. Few cells in the wild type tissue sections were positive for cleaved caspase-3 expression, indicating that the strong TUNEL labeling shown for wild type sections in A represents necrosis and not apoptosis. Highest levels of apoptotic cells (cleaved caspase-3-positive) were detected in the p21−/− liver sections (arrows). Antibody binding was visualized using fluorescein isothiocyanate (green), and 4,6-diamidino-2-phenylindole was used to stain nuclei (blue). Size bar represents 100 μm.
Functions of p21 and p27 following CCl4-induced Injury

Increased Regenerative Proliferation and Expression of Cyclin A in Mice Lacking p21 after CCl4-induced Liver Injury—Expression of Cdk2, Cdk4, Cyclin A, Cyclin D1, Cyclin E, and PCNA was examined during the course of CCl4-induced regeneration in wild type, p21−/−, and p27−/− mice (Fig. 5A). Expression of most of these genes was increased during the proliferative phase of liver regeneration (36–72 h) in these mice. A notable prolonged increase in Cyclin A expression was detected in the livers of p21−/− mice when compared with wild type controls and p27−/− mice.

In Fig. 5A relative amounts of protein expressed at different time points in each genotype are displayed. However, differences in experimental conditions preclude making comparisons regarding absolute levels of protein expressed at each time point between livers of mice of each genotype. To compare directly protein expression levels in the different genotypes, 50 μg of liver protein from wild type and p21- and p27-deficient mice at 0, 6, 24, 36, and 48 h post-CCl4 injection were loaded on the same gel, and immunoblotting was performed (Fig. 5B). Increased p21 protein levels were detected in the liver at the zero time point and 24 and 36 h post-CCl4 injection in mice lacking p27, whereas reduced levels of p21 are expressed at 6 h in the p27-deficient animals. Increased Cdk and PCNA expression was also present at the zero time point in p27 null mice (see also Ref. 7). A substantial increase in Cyclin A levels was detected in p21−/− liver samples at 48 h post-CCl4 injection.

To determine whether p21 or p27 are necessary for regulation of hepatocyte proliferation following CCl4-induced injury, [3H]thymidine was injected into wild type, p21−/−, and p27−/− mice 1 h prior to the time of sacrifice at 24, 36, 48, 72, and 168 h following CCl4 injection, and [3H]thymidine incorporation was examined in liver sections (Fig. 6). Significant regenerative proliferation was not observed at the 24- and 168-h time points in any of the genotypes. In all genotypes of mice, peak proliferation was detected at 36 h post-CCl4 injection. Mice deficient for p21 had the highest level of proliferation, with ~30% more hepatocytes incorporating [3H]thymidine in the 1-h period in p21−/− mice than in wild type mice. Higher levels of cell proliferation were maintained in p21 null mice at 48 and 72 h post-CCl4 injection.

The p27-deficient animals had lower levels of cell proliferation at all time points. This may be due in part to the growth inhibitory effect of increased expression of p21 in the livers of these mice (see Fig. 5B). Immunoprecipitations were performed with p21 antibodies and wild type and p27-deficient liver lysates, and immunoblotting was performed with antibodies against different Cdks and cyclins. We easily detected association of p21 with Cdk2, Cdk4, and cyclin D1 in p27-deficient liver lysates (Fig. 7). Because lower levels of p21 are expressed in wild type liver, longer exposure times were required to detect the same co-immunoprecipitated proteins leading to increased background levels. At 6 h post-CCl4 injection, p21 was complexed with Cdk2, and the p21-Cdk2 complex was more abundant in wild type mice. However, no increase in proliferation was detected in p21-deficient mice at the 6-h time point (data not shown).

Prolonged Hepatocyte Proliferation Correlates with Increased Phosphorylation of pRb and Induction of Genes Required for S-phase Progression in p21-deficient Mice—Hyperphosphorylation of members of the retinoblastoma protein (pRb) family by Cdks leads to inhibition of the growth-suppressive functions of members of the pRb family, activation of the E2F family of transcription factors, and gene expression required for cell cycle progression. p21 is an inhibitor of pRb phosphorylation raising the possibility that p21-deficient mice might have increased
phosphorylation of members of the pRb family. We examined phosphorylation of pRb in the livers of p21−/− and wild type mice following CCl₄ administration (Fig. 8A). Hyperphosphorylated pRb levels were highest at the 48-h time point post-CCl₄ injection in wild type mice. Peak p21 protein induction is also detected at 48 h in wild type mice, which prevents the sustained proliferation detected in livers of p21-deficient mice. In p21-deficient mice, prolonged hyperphosphorylation of pRb was detected from 24 to 72 h post-CCl₄ injection in the liver, indicating that p21 activity is important for inhibiting pRb hyperphosphorylation during liver regeneration. Increases in levels of the pRb-related protein p107 were also detected in the p21−/− mouse livers between 36 and 72 h after CCl₄ injection.

Increased phosphorylation of members of the pRb family leads to the alleviation of repression and induction of genes required for S-phase progression such as Cyclin A (21, 22). Because Cyclin A promoter activity is regulated by pRb (23), we examined Cyclin A mRNA expression levels using quantitative real time PCR. In p21-deficient animals the increased phosphorylation and inhibition of pRb repressive functions corresponded with increased Cyclin A mRNA (Fig. 8B) and protein expression (Fig. 5). The increased levels of Cyclin A may contribute to the prolonged proliferation observed in the p21−/− liver during regeneration.
Increased Apoptosis Counteracts Increased Proliferation in p21-deficient Mice

Although the lowest levels of necrosis were detected in p21-deficient mice, these animals had highest levels of hepatocyte proliferation during regeneration. In many different systems, expression of p21 has been correlated with an inhibition of apoptosis (reviewed in Ref. 24). To determine whether apoptosis plays a role in regulating liver mass in p21-deficient mice, we used antibodies specific for activated caspase-3 to examine apoptosis in the livers of wild type and p21-deficient mice. Many hepatocytes were positive for activated caspase-3 in p21-deficient livers, but not in wild type livers, at 36 h post-CCl4 administration, when proliferation is at its peak (Fig. 9). Diffuse green staining in wild type liver sections represents background that is specific for the injured region of the liver lobule. Results obtained using three different wild type and p21-deficient mice from three independent experiments are presented in Fig. 9. We do detect some variability in the numbers of cleaved caspase-3-positive cells in the p21-deficient animals, which may be due in part to differences in metabolic activity in the liver arising from variations in feeding, because the animals have free access to food. Still, in all cases the p21-deficient mice have a significant increase in apoptotic cell number. Thus, in the absence of p21 there is more proliferation at 36 h post-CCl4 injection, but this proliferation is counteracted by an increase in programmed cell death.

DISCUSSION

The liver is the largest gland in the body and the site where drugs and chemicals are metabolized. Toxins produced during metabolism may give rise to injury, but the healthy liver is...
capable of repairing itself. We explored the roles of p21 and p27 throughout the course of liver injury and repair following CCl₄-induced liver injury, and we have determined the functions of the early and late peaks of p21 expression following CCl₄ injection. The early peak of p21 expression promotes necrotic injury, and this is one of the first reports that suggests a role for p21 in necrosis. The later peak of p21 induction serves as a cell cycle checkpoint and inhibitor of cell proliferation during regeneration. For the most part, p27-deficient mice responded like wild type mice, although increased p21 expression in p27-deficient mice led to a slight increase in necrotic injury at 24 h (Fig. 2) and decreased proliferation at later time points (Fig. 6).

Although animals of all three genotypes recovered from the liver injury, it appears that p21 plays a major role in regulating proliferation and cell viability following toxin-induced injury and subsequent regeneration.

Although p21 expression was not detected in the uninjured liver, high levels were expressed within 3–6 h specifically in pericentral hepatocytes after CCl₄ injection (13). In p21-deficient mice no significant aberrant proliferation was detected during the first 24 h post-CCl₄ injection. However, p21-deficient animals displayed a marked decrease in the level of necrotic injury, indicating that p21 expression promotes necrosis. Disruption of the p21 gene did not lead to altered expression of CYP2E1, which plays an essential role in CCl₄-induced necrosis.

The mechanisms underlying the dependence of necrotic injury on induction of p21 remain unclear. The immediate early genes c-fos and c-myc are rapidly induced in the pericentral region before p21 is expressed following CCl₄ injection (13). In p21-deficient mice no significant aberrant proliferation was detected during the first 24 h post-CCl₄ injection. However, p21-deficient animals displayed a marked decrease in the level of necrotic injury, indicating that p21 expression promotes necrosis. Disruption of the p21 gene did not lead to altered expression of CYP2E1, which plays an essential role in CCl₄-induced necrosis.

The mechanisms underlying the dependence of necrotic injury on induction of p21 remain unclear. The immediate early genes c-fos and c-myc are rapidly induced in the pericentral region before p21 is expressed following CCl₄ injection (13). In p21-deficient mice no significant aberrant proliferation was detected during the first 24 h post-CCl₄ injection. However, p21-deficient animals displayed a marked decrease in the level of necrotic injury, indicating that p21 expression promotes necrosis. Disruption of the p21 gene did not lead to altered expression of CYP2E1, which plays an essential role in CCl₄-induced necrosis.

The mechanisms underlying the dependence of necrotic injury on induction of p21 remain unclear. The immediate early genes c-fos and c-myc are rapidly induced in the pericentral region before p21 is expressed following CCl₄ injection (13). In p21-deficient mice no significant aberrant proliferation was detected during the first 24 h post-CCl₄ injection. However, p21-deficient animals displayed a marked decrease in the level of necrotic injury, indicating that p21 expression promotes necrosis. Disruption of the p21 gene did not lead to altered expression of CYP2E1, which plays an essential role in CCl₄-induced necrosis.

The mechanisms underlying the dependence of necrotic injury on induction of p21 remain unclear. The immediate early genes c-fos and c-myc are rapidly induced in the pericentral region before p21 is expressed following CCl₄ injection (13). In p21-deficient mice no significant aberrant proliferation was detected during the first 24 h post-CCl₄ injection. However, p21-deficient animals displayed a marked decrease in the level of necrotic injury, indicating that p21 expression promotes necrosis. Disruption of the p21 gene did not lead to altered expression of CYP2E1, which plays an essential role in CCl₄-induced necrosis.

Fig. 8. Hyperphosphorylation of pRb and p107 and increased expression of cyclin A mRNA in p21-deficient mice correlate with prolonged proliferation. A, immunoblotting experiments demonstrate prolonged hyperphosphorylation of pRb in the regenerating livers of mice deficient for p21. In addition, increased levels of p107 are detected in p21–/– mouse livers after CCl₄ injection. B, quantitative real-time PCR was used to examine Cyclin A mRNA expression in wild type (WT) and p21-deficient mice. Cyclin A mRNA expression levels were normalized to S18 RNA expression levels using the comparative Ct method. Increased Cyclin A mRNA levels were detected in p21-deficient mice from 24 to 48 h after CCl₄ injection.

Fig. 9. Increased apoptosis in the livers of p21-deficient mice during the replicative phase of liver regeneration counteracts excessive proliferation. Paraffin-embedded liver sections from wild type (WT) and p21–/– mice at 36 h post-CCl₄ injection were stained with antibodies against activated caspase-3, and antibody binding was visualized using fluorescein isothiocyanate, and 4,6-diamidino-2-phenylindole was used to stain nuclei. Data from three wild type and three p21–/– mice are shown. Increased apoptosis of hepatocytes surrounding the central veins is observed in p21-deficient livers. Examples of central veins (CV) and portal triads (PT) are marked. Size bars represent 100 μm.
was expressed at low levels until it was induced again from 36 to 72 h, coincident with the major phase of regenerative proliferation that occurs following toxin-induced injury. The second peak of p21 expression is consistent with a role for p21 as a cell cycle checkpoint protein and an inhibitor of cell proliferation following the initial wave of hepatocyte replication. We examined cell proliferation in wild type, p21−/−, and p27−/− mice during the course of CCl4-induced injury and repair. In all cases peak proliferation as measured by tritiated thymidine incorporation was detected at 36 h post-CCl4 injection. The highest numbers of proliferating cells were detected in p21−/− mice at all time points examined (Fig. 6). The extended time course of hepatocyte proliferation in animals lacking p21 at 48 and 72 h post-CCl4 injection was not observed following partial hepatectomy, after which accelerated early peak proliferation, but no prolonged proliferation, was observed in p21−/− female mice (25). p21 expression was higher in p27−/− mice compared with wild type livers at several time points examined, and p27-deficient animals had lowest levels of proliferation. We showed previously that p21 protein accumulates in the p27-deficient liver where it can inhibit hepatocyte proliferation (7).

pRB and p107 have been shown to have overlapping functions in the developing liver (26). We observed increased levels of hyperphosphorylated pRB and increased expression of p107 post-CCl4 injection in the livers of p21 null mice. Hyperphosphorylation of pRB abrogates its growth-suppressive functions and disrupts its ability to bind members of the E2F family, leading to activation of a number of genes required for DNA synthesis (reviewed in Ref. 27). Increased expression of Cyclin A would support the longer duration of DNA replication detected in p21−/− animals following CCl4 injury. Recently it was reported that expression of another E2F target gene, the Cdk-activating phosphatase Cdc25A, is higher in p27-deficient livers, and this may also contribute to the increased cell proliferation detected in p21−/− mice (28).

We asked how liver mass is properly maintained in p21-deficient mice, which had the lowest levels of liver injury but highest levels of cell proliferation. By performing immunohistochemistry with antibodies specific for activated caspase-3, we found that livers from the p21-deficient mice had significantly higher levels of apoptosis, particularly during the peak of proliferation. Thus apoptosis is important for control of liver size in the face of too much cell proliferation during the course of liver regeneration.

Many reports have indicated that p21 induction may inhibit apoptosis by a variety of mechanisms (reviewed in 24), and our data suggest an apoptosis inhibiting role for p21 in the liver. Increased E2F activity, which may occur as a consequence of disruption of the p21 gene, could contribute to increased apoptosis by a number of p53-dependent and independent mechanisms (reviewed in Refs. 29–31). It was reported recently (32) that deregulation of E2F activity may directly contribute to the regulation of caspase expression. Increased apoptosis of hepatocytes during the proliferative phase of liver regeneration may also be regulated by environmental signals, as the liver is remodeled and there is an increased number of cells in an organ with restricted size as a consequence of p21 disruption.

We have shown here that p21 plays a central role in regulating both proliferation and cell viability in the toxin-injured liver. The importance of countering excessive proliferation with increased cell death has been recognized as a necessary mechanism for preventing the development of cancer (33). We are currently investigating the susceptibility of p21-deficient mice to developing liver tumors after treatment with specific carcinogens. Both decreased and increased p21 expression have been correlated with the development of hepatocellular carcinoma (34, 35), perhaps attesting to the dual functions of p21 as a regulator of injury and proliferation. Because of its abilities to regulate both proliferation and cell death, modulation of p21 activity following liver injury may have therapeutic benefits in patients with liver disease.

Acknowledgments—We are indebted to Andrei Gautel, Nissim Hay, Jason Poole, and Robert Streit for helpful discussions and comments about the manuscript.

REFERENCES

1. Sherr, C. J., and Roberts, J. M. (1999) Genes Dev. 13, 1501–1512
2. Sherr, C. J. (2000) Cancer Cell 7, 5–7
3. Dotto, G. P. (2000) Biochem. Biophys. Acta 1471, M43–M56
4. Hart, A. L., Serfas, M. S., and Tyner, A. L. (1996) Proc. Soc. Exp. Biol. Med. 213, 137–148
5. Ekhholm, S. Y., and Reed, S. I. (2000) Curr. Opin. Cell Biol. 12, 676–684
6. Tyner, A. L., and Hart, A. L. (2000) in Gi Phase Progression (Boonstra, J., ed) Landes Bioscience, in press, early publication at http://www.eurekah.com.
7. Kwon, Y. H., Jouaville, A., Serfas, M. S., Kiyokawa, H., and Tyner, A. L. (2002) J. Biol. Chem. 277, 41417–41422
8. Fausto, N. (2000) J. Hepatol. 32, 19–31
9. Michaliszyn, G. K., and DeGregori, J. (1997) Science 276, 60–68
10. Sell, S. (2001) Hepatology 33, 738–750
11. Slater, T. F. (1984) Biochem. J. 222, 1–15
12. Inoue, M. (1994) in The Liver: Biology and Pathobiology (Arias, I., Boyer, J., and Fausto, N., ed.) 3rd ed., pp. 435–459, Raven Press, Ltd., New York
13. Serfas, M. S., Gouman, E., Feuerman, M. H., Hart, A. L., and Tyner, A. L. (1995) Cell Growth Diff. 6, 951–961
14. Rosser, B. G., and Goris, G. J. (1995) Gastroenterology 108, 252–275
15. Brugaeras, J., Chandrasekaran, C., Gordon, J. I., Beach, D., Jacks, T., and Hannon, G. J. (1995) Nat Rev 377, 552–557
16. Kiyokawa, H., Kineman, R. D., Manova-Todorova, K. O., Soares, V. C., Hoffman, E. S., Ono, M., Khanam, D., Hayday, A. C., Frohmam, L. A., and Koff, A. (1996) Cell 85, 721–732
17. DeRyckere, D., and DeGregori, J. (2002) Methods 26, 57–57
18. Fausto, N., and Campbell, J. S. (2003) Mech. Dev. 120, 117–130
19. Grasl-Kraupp, B., Ruttikay-Needycky, B., Kozela, H., Bukowska, K., Bursch, W., and Schulte-Her台账, R. (1990) Hepatology 21, 1405–1418
20. Wong, F. W., Chan, W. Y., and Lee, S. S. (1998) Toxicol. Appl. Pharmacol. 153, 109–118
21. DeGregori, J., Kovalik, T., and Nevin, J. R. (1995) Mol. Cell. Biol. 15, 4215–4224
22. Nevin, J. R., Leong, G., DeGregori, J., and Jakoi, L. (1997) J. Cell. Physiol. 173, 233–238
23. Knudsen, K. E., Fribourg, A. F., Strobeck, M. W., Blanchard, J. M., and Knudsen, E. S. (1999) J. Biol. Chem. 274, 27632–27641
24. Gautel, A. L., and Tyner, A. L. (2002) Mol. Cancer Ther 1, 639–649
25. Albeck, J. H., Poon, R. Y., Ahonen, C. L., Rieland, B. M., Deng, C., and Crary, G. S. (1998) Oncogene 16, 2141–2150
26. Lee, M. H., Williams, B. O., Mulligan, G., Mavilk, S., Bronson, R. T., Dyson, N., Harlow, E., and Jacks, T. (1996) Genes Dev. 10, 1621–1632
27. Kaelin, W. G., Jr. (1999) Biosciws 21, 950–958
28. Jaime, M., Pujol, M. J., Serratosa, J., Pantoja, C., Canela, N., Casanovas, O., Serrano, M., Agell, N., and Bachs, O. (2002) Hepatology 35, 1063–1071
29. Phillips, A. C., and Vousden, K. H. (2001) Apoptosis 6, 173–182
30. Girob, D. (2002) FEBS Lett. 529, 122–125
31. Sears, R. C., and Nevin, J. R. (2002) J. Biol. Chem. 277, 11617–11620
32. Nabh, K., Polkoff, J., Davuluri, R. V., McCurrah, M. E., Jacobin, M. D., Marita, M., Zhang, M. Q., Lazebnik, Y., Bar-Sagi, D., and Lowe, S. W. (2002) Nat. Cell Biol. 4, 859–864
33. Evans, G. I., and Vousden, K. H. (2001) Nature 411, 342–348
34. Hui, A. M., Kanai, Y., Sakamoto, M., Tsuda, H., and Hirohashi, S. (1997) Hepatology 25, 575–579
35. Weyama, H., Shiraki, K., Sugimoto, K., Ito, T., Fujikawa, K., Yamanaka, T., Takase, K., and Nakano, T. (2002) Hum. Pathol. 33, 429–434
The Cdk Inhibitor p21 Is Required for Necrosis, but It Inhibits Apoptosis following Toxin-induced Liver Injury

Young Hye Kwon, Aleksandra Jovanovic, Michael S. Serfas and Angela L. Tyner

J. Biol. Chem. 2003, 278:30348-30355.
doi: 10.1074/jbc.M300996200 originally published online May 20, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M300996200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 33 references, 11 of which can be accessed free at
http://www.jbc.org/content/278/32/30348.full.html#ref-list-1