Impaired hepatocyte survival and liver regeneration in Atm-deficient mice

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Atm is a stress-induced DNA damage checkpoint protein kinase with multiple roles in cell-cycle progression. Recent evidence indicates that Atm also plays a role in stem cell maintenance and self-renewal. It is not known whether Atm has a role during tissue regeneration. Using liver regeneration as a model system, we examined the role of Atm in this process. Here, we show that the expression levels of Atm protein were gradually increased during liver regeneration and this was correlated with the onset of DNA replication. The induction of Stat3 and JNK signaling, which are essential processes in normal regeneration response, was attenuated during the early phases of liver regeneration in Atm-deficient mice. P53 was transiently phosphorylated at serine 23 during liver regeneration in an Atm-dependent manner. In addition, we found that cyclin A induction was delayed and p21 was over-expressed, both of these processes were correlated with reduced and delayed DNA replication in Atm−/− mice during liver regeneration. Finally, we show that increased apoptosis was observed in Atm−/− mice in response to partial hepatectomy, indicating that Atm is required for the survival of hepatocytes. Collectively, these data indicate that liver regeneration is impaired in Atm-deficient mice. Given that liver is the first line of defense against environmental toxins, the elucidation of the function of Atm and Atm-mediated signaling pathways in liver metabolism and in response to environmental toxins is of fundamental interest.

INTRODUCTION

Mammals are constantly exposed to environmental toxic chemicals, mostly through dietary intake. One of the most important functions of liver is to detoxify the ingested toxin. Environmental toxins may damage liver after ingestion and accordingly animals have evolved with the ability to repair damaged liver through regeneration. Liver regeneration is an evolutionary adaptive response to the constant exposure of toxic compounds, viral infection and ischemia (1). Experimentally, liver regeneration can be induced in animal models by removing the large and median lobes of liver [two-third partial hepatectomy (PH)] (2). The remaining liver grows rapidly and restores the original mass within a few days (3).

Liver regeneration involves a complex interplay of signaling events, which are precise and highly regulated to ensure the functional restoration of damaged liver (1). In response to PH, over 95% of the hepatocytes exit the G0-phase and enter the cell cycle synchronously. The DNA synthesis begins in 24 h and peaks at 36–40 h post-PH in mouse models. The molecular mechanisms that regulate these events include early signaling events, leading to increased production of growth factors such as HGF, TNFα and IL-6, followed by induction of immediate early genes such as AP-1, Stat3 and NFκB (1). Mice deficient for TNFα or IL-6 signaling have exhibited impaired liver regeneration and defective induction of immediate early genes (4,5). Importantly, during liver regeneration, proliferating hepatocytes continue to perform crucial metabolic and detoxification functions (1). However, little is known about how proliferating hepatocytes respond to genotoxin during liver regeneration and it is also not clear whether a DNA damage response pathway is active in proliferating hepatocytes in vivo.

In response to genotoxic insults, a DNA damage response pathway is activated and is largely mediated by ATM, a DNA damage sensing sensor and transducer with protein
kinase activity (6,7). ATM is mutated in patients with the genomic instability syndrome, ataxia telangiectasia (A-T) (8). A-T patients are characterized by progressive cerebellar degeneration, immune deficiencies, growth retardation, premature aging and predisposition to tumorigenesis (6,7). Interestingly, most A-T patients have high alpha-fetal protein (AFP) expression in the serum that has been used as one of the diagnostic markers for A-T syndromes (9). The origin of the abnormal AFP in A-T patients is not clear. AFP is normally produced in the yolk sac and in fetal liver during development. However, expression of AFP is switched off in mature adult hepatocytes (10,11). Under pathological conditions, AFP is re-expressed and accumulated in serum in patients with hepatoblastoma, hepatocellular carcinoma and germ cell tumors (10,11). Interestingly, AFP expression can also be induced in experimental animal model systems by PH and treatment with hepatotoxin CCl4 (12). Given that hepatocellular carcinoma and hepatoblastoma are not frequently observed in A-T patients (13), it is possible that increased AFP expression in A-T patients is due to the constant regenerative response of the liver cells to genotoxic insults. This suggests frequent turnover of hepatocytes in A-T patients, although such clinical data are not available at present.

To characterize the role of Atm in response to genotoxic insults in hepatocytes and a role in liver regeneration response, we have examined the function of Atm in a liver regeneration model system. Here, we show that Atm is required for survival of hepatocytes and that liver regeneration is impaired in Atm-deficient mice.

RESULTS
Expression of Atm during liver regeneration

To determine whether Atm plays a role in hepatocyte proliferation and survival in vivo, the expression of Atm was examined by immunoblot during liver regeneration. It was found that Atm was only weakly expressed in normal liver; its expression was slightly enhanced at 3 h post-PH and greatly increased at 36–72 h post-PH (Fig. 1A). In contrast, Chk2, a downstream target of Atm kinase (14), expression appears to be constant during the early stages (0–24 h) of liver regeneration and increased from 36 to 72 h post-PH (Fig. 1A). Therefore, increased Atm and Chk2 expression appears to correlate with the peak cellular activity (e.g. DNA synthesis) during liver regeneration. This result contrasts with the general view that ATM levels are invariant in different phases of cell cycle (15). Although it has been shown previously that the majority of the ATM protein resides in the nucleus (16–19), a small fraction of this protein does exist in the microsomal fraction and in the cytoplasm of Purkinje neurons (19–23). To determine the subcellular localization of Atm protein in hepatocytes during liver regeneration, immunohistochemical analysis was performed on the regenerating liver. Atm was localized to the nucleus of both regenerating hepatocytes and non-parenchymal cells between 48 and 72 h (Fig. 1B and C). The labeling intensity varied in the regenerating hepatocytes, possibly reflecting the different stages of individual cells in the cell cycle.

DNA synthesis during liver regeneration is delayed and attenuated in Atm−/− liver

The observed correlation of Atm expression with active phases of liver regeneration suggests a potential role of Atm in liver regeneration. To test this hypothesis, liver regeneration was examined utilizing an Atm knockout mouse model system. The requirement for Atm during cell-cycle progression of hepatocytes was determined by monitoring DNA synthesis at various times after PH, utilizing BrdU incorporation in both wild-type and Atm−/− livers. A peak of DNA synthesis was observed in proliferating hepatocytes at 36 h post-PH in wild-type livers (Fig. 2A). In contrast, DNA synthesis in hepatocytes of the Atm−/− liver was drastically reduced (Fig. 2B; P < 0.001) and delayed, appearing only 48 h post-PH in Atm−/− livers (Fig. 2C). Therefore, these results suggest that Atm is required for the induction of DNA synthesis after PH and are consistent with the observation that Atm was induced to the highest level during the peak DNA synthesis phase (Fig. 1).

Atm is required for early signaling events during liver regeneration

To further characterize the role of Atm in liver regeneration, the early events (priming phase) of this process were examined in both wild-type and Atm−/− livers. Consistent with the previous observation that Stat3 was phosphorylated and activated in early hours after PH (24), it was observed that the expression of phosphorylated Stat3 was increased in wild-type liver 3 h post-PH. In contrast, the induction of phosphorylated Stat3 was clearly attenuated in Atm−/− livers (Fig. 3A). In addition, c-Jun was phosphorylated at Ser63 in a biphasic pattern at 1 and 6 h post-PH in wild-type liver, whereas the intensity of the phosphorylated c-Jun was diminished in Atm−/− livers (Fig. 3A). Although the expression of the phosphorylated c-Jun was completely turned off by 12 h post-PH (Fig. 3A, WT), the expression of the phosphorylated
Defective induction of cyclin A and overexpression of p21 in Atm<sup>−/−</sup> livers

Induction of immediate early genes during liver regeneration is followed by the expression of growth promoting genes required for cell-cycle progression (1). As Atm<sup>−/−</sup> livers exhibited defective DNA synthesis during liver regeneration, the expression of key proteins involved in cell-cycle progression is of interest. In wild-type liver, we found that cyclin A expression was induced and peaked between 36 and 48 h, coincident with the S-phase (Fig. 4A). In contrast, maximum cyclin A expression was delayed, appearing only after 48–72 h post-PH in Atm<sup>−/−</sup> livers (Fig. 4A). Therefore, the delayed expression of cyclin A correlated with the observed differential DNA synthesis between wild-type and the mutant mice (Fig. 2C). The change in cyclin A expression appears to be specific, because other cell-cycle regulators, such as cyclin E, cyclin B1, Cdk1, Cdk4, Cdc25A, and cyclin D1, were not altered during liver regeneration in wild-type and mutant mice (Fig. 4A) (data not shown).

It has been previously shown that Atm-deficient cells have increased p21 expression, which is responsible for the slow proliferation of Atm-deficient cells (26–28). We observed that p21 protein levels were increased at 24 h post-PH and maintained through 72 h in wild-type liver (Fig. 4B). In contrast, p21 protein levels in Atm<sup>−/−</sup> livers were further increased when compared with wild-type regenerating liver and the higher expression levels were maintained throughout this process (Fig. 4B). The expression of p27 and p57, two related CDK inhibitors, was similar between wild-type and mutant mice during liver regeneration (Fig. 4B). Taken together, these data suggest that defective cell-cycle progression in Atm<sup>−/−</sup> liver after PH is due to an increased p21 expression and delayed induction of cyclin A.

Impaired liver regeneration and hepatocyte survival in Atm-deficient mice

Previous investigations have shown that overexpression of p21 in hepatocytes impairs mouse liver regeneration (29). In addition, the increased p21 and delayed BrdU incorporation in Atm<sup>−/−</sup> liver suggest that Atm<sup>−/−</sup> mice may be unable to regenerate liver tissue. Indeed, between 24 and 72 h post-PH,
was used for time 0. (mice for each time point were used for analysis except one mouse, which was used for time 0. Increased p21 expression in regenerating Atm−/− liver, compared with wild-type liver after PH. Liver samples were identical as in (A). The expression of CDK inhibitors of the Cip/Kip family was examined by immunoblot. Samples were loaded equally in all time points as judged by the expression levels of control proteins β-actin and β2-microglobulin.

Atm null mice displayed prolonged lethargy and weakness, which was not evident in wild-type mice. A cohort of 27 wild-type and 28 Atm−/− mice were observed after PH. It was found that Atm−/− mice had a reduced rate of survival when compared with wild-type mice (Fig. 5; P = 0.011). These results suggest that Atm−/− mice have impaired liver regeneration.

One of the hallmarks of A-T patients is hypersensitivity to radiation (7). At the cellular level, sensitivity to radiation leads to diminished clonogenic survival. In addition, spontaneous apoptosis was seen in Atm-deficient lymphocytes, suggesting a role of Atm in cell survival (30). Paradoxically, Atm is also required for ionizing radiation-induced apoptosis in developing CNS (31). To determine whether Atm has a role in survival of hepatocytes after PH, a TUNEL assay was performed in liver tissues before and after PH. Few TUNEL positive nuclei could be detected in both wild-type and Atm−/− livers before PH and 24 h after PH (data not shown). At 48 h post-PH, Atm−/− livers exhibited increased TUNEL positive nuclei in both hepatocytes and nonparenchymal cells when compared with wild-type controls (Fig. 6; P < 0.001). These results indicate that Atm is required for survival of proliferating hepatocytes and nonparenchymal cells.

DISCUSSION

Tissue regeneration in mammals is restricted to a few organs such as liver, finger tips (at a young age) and peripheral nerves (32). It is not clear whether liver regeneration involves stem cells within the liver or whether hepatocytes have retained certain stem cell properties (1,33). A role of Atm in stem cell maintenance and/or self-renewal was recently demonstrated in hematopoietic stem cells, in which Atm-deficiency progressively confers reduced ability to reconstitute in irradiated bone marrow (34). Consistent with the role of Atm in stem cell self-renewal, it has been demonstrated that Atm-deficient neural stem cells possessed poor proliferative capacity and reduced survival when compared with wild-type stem cells in vivo and a reduced ability to form neurospheres, a property of neural progenitor cells, in vitro (20,35). During liver regeneration, the remnant hepatocytes enter the cell cycle synchronously. Therefore, the liver regeneration model provides an excellent in vivo system to study the role of Atm in tissue regeneration and in cell proliferation. In this study, for the first time, we demonstrated that increased Atm protein levels correlated directly with peak DNA synthesis in liver tissues. The importance of Atm in hepatocyte proliferation and regeneration is demonstrated by the observation that Atm deficiency impaired the ability of hepatocytes to proliferate. This impairment of proliferation is correlated with p21 overexpression and the delayed induction of cyclin A. During liver regeneration, the increased p21 expression was observed starting around 24 h post-PH. Consistent with this observation is the previously documented induction of p21 expression in the regeneration of CCl4 damaged mouse liver (37). The significance of increased p21 expression during liver regeneration is not clear, although it could be related to hepatocyte proliferation because the amount of p21 expression is directly related to proliferative capacity (36). It was shown previously that p21 interacts with Cdk2/cyclin D complex and is required for Cdk kinase activity at physiological relevant levels (36). However, increased p21 expression blocks multiple CDKs and inhibits cell-cycle progression (37). It has also been shown that in transgenic animals, targeted overexpression of p21 driven by the transthyretin promoter in hepatocytes impaired liver regeneration by the decreased proliferation of these cells (29). Therefore, defective liver regeneration in Atm-deficient mice could be due to the overexpression of p21 in hepatocytes.
Liver regeneration is characterized by rapid induction and mobilization of transcription factors such as Stat3 and AP-1 proteins (1). Phosphorylated (thus activated) Stat3 was detected in regenerative liver 3 h post-PH, whereas induction of phosphorylated Stat3 was defective in Atm null mice. Therefore, impaired liver regeneration may be due to the impaired induction of Stat3. Stat3 signaling has been linked to hepatoprotection by induction of anti-caspase regulators and by reduction of oxidative injury (38). Therefore, defective induction of Stat3 might play a role in impaired liver regeneration and in decreased hepatocyte survival observed in Atm-deficient mice.

Enhanced oxidative stress during liver regeneration in Atm-deficient mice could decrease overall cellular survival. Reactive oxygen species (ROS) could be conceivably generated during liver regeneration as a result of metabolic overload and/or overactive mitochondrial oxidation. The induction of gene expression during liver regeneration shares many similarities with the cellular response to oxidative stress (39).

ROS, produced during normal metabolism or produced by metabolic products of environmental toxins, can induce DNA damage and is one of the major causes of genome instability in the liver. Recent studies have clearly implicated a role of ATM in the oxidative stress response (40) and shown that antioxidants could significantly ameliorate tumorigenesis and neurodegeneration in Atm-deficient mice (41–44). Therefore, the impaired liver regeneration in Atm-deficient mice may be due to the inability of hepatocytes to deal with increased oxidative stress during liver regeneration. In support of this concept, we found that JNK activation is sustained in Atm-deficient livers during regeneration. ROS may play a role in sustained JNK activation in Atm-deficient cells, because it was previously shown that A-T cells treated with CdCl$_2$, a potent pro-oxidant, showed marked increase of JNK activation and heme oxygenase-1 induction (45). The sustained JNK activation in Atm-deficient livers may be due to the inhibition of MAP kinase phosphatases by ROS (46). Furthermore, it was recently demonstrated that the ability of Atm-deficient hematopoietic cells to reconstitute bone marrow was improved greatly with the administration of antioxidants (34). These observations suggest that treatment with antioxidants would improve liver regeneration and enhance survival in Atm-deficient mice.

A-T patients have high levels of AFP in their serum, although the significance of this diagnostic parameter is not clear (9). High levels of AFP expression are associated with liver cancer and germ cell cancers, which are not frequently observed in A-T patients. Interestingly, it has been reported that increased expression of AFP mRNA correlates with liver regeneration (47,48). Nevertheless, we did not observe AFP expression during liver regeneration in both wild-type and Atm-deficient livers by immunoblot or immunohistochemical analysis (Lu and Wang, unpublished data). Absence of the expression of AFP protein after PH could be due to post-transcriptional regulation of AFP gene, the difference in strain of mouse used or a variance between mouse and human hepatocytes (11). Alternatively, in comparison with Atm-deficient mice which are housed in controlled environment and under identical food consumption, A-T patients likely encounter greater variations in environment and diet, which may damage the liver in these patients but not in the normal human population. Such increased sensitivity to environmental toxins experienced by A-T patients would result in increased turnover of hepatocytes. Consistent with this hypothesis, we found that Atm is required for the survival of proliferating hepatocytes. Atm-deficiency will lead to constant turnover of proliferating hepatocytes damaged by environmental toxins, which might contribute to the increased AFP expression in A-T patients.

This study provides a framework to further analyze the role of Atm and Atm-mediated signaling pathways in liver regeneration. Despite the tremendous advance in the mechanistic understanding of DNA damage signaling and repair in general, very little attention has been given to the liver, an important organ involved in metabolism, detoxification and homeostasis and the first line of defense against xenobiotics. Our data clearly implicate the significant role of Atm and Atm-mediated signaling pathways in liver metabolism and regeneration.
MATERIALS AND METHODS

Mice
Atm knockout mice were obtained by breeding of Atm heterozygotes as described (30). Mice were fed ad libitum and housed in a barrier facility with 12-h light cycle. Six- to eight-week-old healthy animals were used in this study.

Liver regeneration
Liver regeneration was carried out as described (49) under avertin anesthesia. In brief, the abdominal cavity was opened with longitudinal incision and the left and medial lobes were ligated, followed by excision to remove the two lobes. After closing the abdominal wall by suture, animals were recovered on a warm plate and later returned to cages and fed ad libitum. At the indicated time, mice were sacrificed and liver tissues were harvested.

BrdU incorporation and TUNEL assay
Mice were injected with 0.2 ml of 10 mM BrdU (Sigma) intraperitoneally 1 h before tissue harvesting. Cell proliferation studies were performed using BrdU labeling kit (Zymed) and incorporation of BrdU was determined from three wild-type and three Atm−/− mice. Over 2000 nuclei were scored for each liver sample. TUNEL assay was performed using the DeadEnd Fluorometric TUNEL system as described by the manufacturer (Promega). Quantitation of TUNEL assay was performed in three optical fields of liver sections for each genotype. Over a thousand nuclei were counted, and mean and standard deviation of the data were determined. P-values were determined by two-tailed Student’s t-test assuming equal variance.

Histological and immunohistochemistry
Liver tissues were fixed in 10% neutral formalin (Sigma) overnight. The tissues were dehydrated in a series of ascending concentration of alcohol, followed by two changes in xylene, and then embedded in paraffin. Five to seven micron sections were cut and stained with H&E, according to standard procedure. For immunohistochemistry, the liver sections were rehydrated in a series of descending alcohol concentrations, following deparaffinization in xylene. Endogenous peroxidase activity was quenched by incubating tissue sections in 0.1% H2O2 for 30 min. After a brief rinse in PBS, ATM antigen was retrieved by incubation in 10 mM citrate buffer (pH 6.0), followed by heating in a microwave oven for 10 min at full power. Mouse monoclonal anti-ATM antibody (MAT-2, kindly provided by Dr Y. Shiloh) was used for IHC. Expression of ATM was detected using ABC kit (Vector), followed by counterstaining with Fast green.

Immunoblot assay
Liver tissues were harvested at the indicated time post-PH and stored at −80°C. Liver tissues were rinsed in cold PBS, followed by homogenization in modified RIPA buffer (50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 1% NP-40, 0.25% Na-deoxycholate and 150 mM NaCl) containing 1× cocktail of proteinase inhibitors (Roche). Cleared lysates were resolved on SDS–PAGE gel and transferred to PVDF membrane. Protein expression was detected by enhanced chemiluminescence. The following antibodies were used in this study: anti-ATM (MAT 3 from Y. Shiloh); anti-Chk2 (Upstate); anti-β-actin (Sigma); anti-Stat3pS727, anti-c-Jun pS63, anti-p53 pS20, anti-p53 pS392 and anti-p53 S15 (Cell Signaling); anti-cyclin A, anti-cyclin B1, anti-cyclin D1, anti-cyclin E, anti-CDK1, anti-CDK4, anti-p21, anti-p27, anti-p57 and anti-β2microglobulin (Santa Cruz).

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Conflict of Interest statement: Yaolin Wang holds stock in Schering-Plough Corp., the maker of Clarinex, Vytorin and Temodar and is currently working at Schering.

REFERENCES


