Hepatoblast-like Cells Populate the Adult p53 Knockout Mouse Liver: Evidence for a Hyperproliferative, Maturation-arrested Stem Cell Compartment

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Abstract

Although p53 regulates the cell cycle and apoptosis, gross embryonic development is normal in the p53 knockout (−/−) mouse. In this study, we comprehensively assessed liver development in p53 −/− mice (from embryonic day 15 to adult) for evidence of a cell cycle-induced perturbation in differentiation. Liver cell proliferation in the embryo and newborn is similar in p53 −/− and +/+ mice; in contrast, −/− adult hepatocytes divide at twice the rate of wild types. Developmental expression patterns of liver-specific markers that are up-regulated (e.g., phosphoenolpyruvate carboxykinase and aldolase B) and down-regulated (e.g., α-fetoprotein) are similar. Therefore, embryonic and perinatal liver development is normal in the absence of p53. However, the p53 −/− adult liver displays small blast-like cells, the majority being hepatic and some lymphoid. These cells appear in periportal regions and can infiltrate the parenchyma. The hepatic blast-like cells express both mature and immature liver markers, suggesting that differentiation of the liver stem cell compartment is blocked.

Introduction

The liver arises from the primitive gut between 8.5 and 9 days gestation (E8.5–9) 3 in the mouse. Cell proliferation and functional differentiation result in the initiation of expression of liver-specific genes during liver development. These marker genes can be grouped according to the developmental timing of their appearance into fetal, neonatal, and late suckling clusters (1). The earliest marker of liver cell commitment is AFP; its mRNA is first detected at E8.5 in immature hepatoblasts and diminishes to undetectable levels in the adult (2). The primitive liver begins to produce other serum proteins, Alb and TN, around E9.5 and E13, respectively (3, 4). These proteins are expressed by the liver throughout adult life and are reliable markers of a parenchymal hepatic cell. A group of metabolic enzymes is expressed at the late fetal/neonatal period to prepare the liver for its adult physiological role; these include PAH, PEPCK, and G-6-Pase (5, 6). The late suckling period, around 3 weeks postnatal (3W) is accompanied by the expression of enzymes that include tryptophan oxygenase and serine dehydratase (7, 8).

Early fetal liver contains haematopoietic cells, as well as immature hepatoblasts that are purported to be bipotential progenitor cells, being capable of differentiating into hepatocytes and biliary epithelium (9–11). The progenitor cells express a range of markers that are expressed by embryonic hepatocytes including, AFP, Alb, the M 2 isoform of pyruvate kinase (M 2-PK), and CK19 (12). Differentiation of progenitor cells to mature hepatocytes is accompanied by the down-regulation of AFP and CK19 and the switching of expression from M 2-PK to the adult L-isofrom of pyruvate kinase (L-PK; Ref. 13). If the progenitor cell differentiates into biliary epithelium, it retains expression of CK19 but not of hepatic markers (AFP, Alb, and M 2-PK; Ref. 14). Embryonic liver progenitor cells and the facultative liver stem cell (oval cells) have been reported to express similar markers and to differentiate into both hepatocytes and bile duct cells (13). Oval cells proliferate during experimental carcinogenesis or when chronic and severe liver trauma is associated with the inhibition of hepatocyte division and thus their ability to replace lost parenchyma.

Maturation of all tissues requires a balance between cell proliferation (both to attain cell numbers and to acquire the differentiated phenotype) and apoptosis (to maintain tissue homeostasis). The two events of proliferation and differentiation are often mutually exclusive; a proliferating cell usually becomes quiescent (G 0) prior to differentiation, and a correct balance of these processes is crucial for normal tissue development (15). For these reasons, regulation of the cell cycle has been implicated in cell differentiation. Proteins controlling the balance between proliferation and differentiation act by regulating various checkpoints throughout the cell cycle. p53 is one such protein reported to control different stages of the cell cycle, because it acts to protect the genome after DNA damage or cellular stress. A rapid rise in the levels of activated p53 results in either growth arrest or apoptosis (16). p53 can promote G 1 or G 2 arrest, block mitotic spindle formation, and assist in DNA repair by transcriptionally acti-
vating genes such as p21\(^{WAF1}\) (17), Gadd45 (18, 19), and cyclin G (20, 21). p53 signals cell apoptosis under certain conditions, by enhancing transcription of genes such as BAX (22, 23), APO 1 (24), and IGF-BP3 (25). Thus, p53 has a pivotal role in maintaining genome stability. In its absence, a cell will divide without checkpoint control and consequently accumulate DNA mutations.

We chose the p53 knockout mouse to study the relationship between proliferation and differentiation in the liver, anticipating that dysregulated cell proliferation would result in aberrant differentiation. Three lines of p53 null mice produced independently were reported to develop normally (26–28). This was contrary to expectation, given the role of p53 in cell cycle control and apoptosis. Predictably, the lack of p53 rendered the null mice susceptible to broad-spectrum tumor formation within (on average) 6 months. The most common form of tumor was lymphoma (28). This was contrary to expectation, given the role of p53 in anticipating that dysregulated cell proliferation would result in aberrant differentiation. Three lines of p53 null mice produced independently were reported to develop normally (26–28).

This study reports that the proliferation and differentiation of embryonic and postnatal p53 \(-/-\) liver is normal, suggesting that p53 is not essential for embryonic and perinatal liver development. However, the adult p53 \(-/-\) liver shows increased hepatocyte proliferation and the presence of hepatoblast-like cells. The hepatoblast-like cells actively proliferate and accumulate without differentiating, suggesting that blocked ontogeny occurs in the stem cell compartment of the adult p53 null liver.

**Results**

**Proliferation of Liver Cells during Development Is Unaltered by the Absence of p53.** PCNA-positive cell numbers in the livers of p53 \(-/-\), \(+/-\), and \(+/+\) animals during embryonic (E15 and E19) and postnatal (NB, 1W, 2W, and 3W) development steadily decline with liver maturity from 50% at E15 to 6.5% at 3W postnatal (Fig. 1A). There is no significant difference (\(P > 0.05\)) in the number of proliferating cells in p53 \(-/-\), \(+/-\), and \(+/+\) livers at all developmental time points studied (Fig. 1). NB liver from p53 \(-/-\) (Fig. 1B) and \(+/+\) (Fig. 1C) liver is chosen to represent the pattern of PCNA staining in developing liver; positive cells stain with a range of intensities. In embryonic liver, there are two apparent cell populations expressing PCNA, hepatocytes and smaller cells with less cytoplasm, which are mainly hematopoietic cells. Furthermore, postnatal hepatocyte PCNA expression reflects that of the total cell population, showing no significant difference between the p53 null and wild type (result not shown).

**Differentiation of Liver Cells Is Unaltered by the Absence of p53.** Northern blots of total RNA derived from p53 \(-/-\), \(+/-\), and \(+/+\) livers were hybridized to cDNAs of the developmental liver markers Alb, AFP, PEPCK, aldolase B, and PAH (Fig. 2). Alb, aldolase B, and PAH mRNA is present in the E15 p53 \(-/-\), \(+/-\), and \(+/+\) mouse and steadily increases throughout development to reach the adult level. AFP mRNA is highly expressed at E15 and until 1W postnatal, after which it is down-regulated to undetectable levels. PEPCK is a postnatal cluster enzyme, and accordingly, it first appears in the NB and increases postnatally to adult levels. Quantification of the Northern blots revealed no significant difference (\(P > 0.1\)) between p53 \(-/-\), \(+/-\), and \(+/+\) animals in terms of the pattern of expression of all developmental mRNAs during liver development (Table 1).

**p53 \(-/-\) Adult Livers Contain Small Basophilic Blast-like Cells.** Histological examination of 27 p53 \(-/-\) adult livers revealed that all contained small basophilic cells, which we have described as blast-like cells, in varying numbers.
The livers were classified into three categories according to the number of blast-like cells and the extent of disruption to normal liver architecture. The three categories are clearly distinguishable when we compare adult wild-type (Fig. 3A) and adult p53−/− with mild (Fig. 3B), moderate (Fig. 3C), and severe (Fig. 3D) lobular disruption by blast cells. Wild-type liver shows a portal and central area, with neat cords of hepatocytes connecting the two zones, p53−/− liver, mildly affected by blast-like cells, appears normal, with the exception of a small number of blast-like cells around all portal areas. The moderately affected p53−/− liver has increased numbers of blast-like cells around all portal areas and small clusters located centrally within the lobule. At high resolution (Fig. 3C, inset), the blast-like cells are seen to have very little cytoplasm, and their nuclei vary slightly in size. Blast-like cells in severely affected p53−/− adult liver occupy 70–95% of the liver parenchyma. Normal liver architecture is disrupted, with portal and central areas difficult to identify.

There is no correlation between the age of the animal and the number of blast-like cells in the liver. Of 27 mice studied, 15 were mild, 5 were moderate, and 7 were severe, i.e., 55.6, 18.5, and 25.9%, respectively. The p53−/− adult mice that were mildly affected by blast cells ranged in age from 12 to 33 weeks. Mice with livers moderately affected ranged from 11 to 30 weeks and with severely affected livers, from 7 to 33 weeks.

**Proliferative Index of Adult p53−/− Hepatocytes Is 2–2.5 Times That of Wild Type.** Immunolocalization of PCNA in p53−/− (mild), +/+ and +/− adult liver shows a greater number of positive cells in p53−/− and +/+ compared with wild type. Two distinct cell populations in the p53−/− and +/+ livers are PCNA positive, hepatocytes (cells with large round nuclei) and smaller blast-like cells (small with a scant cytoplasm). p53−/− liver with a mild blast-like cell phenotype has 2.5 times the number of PCNA-positive hepatocytes than their p53 +/+ counterparts, and the p53 +/+ liver has 1.3 times (Fig. 4).

Expression of PCNA by blast-like cells in mild, moderate, and severely affected p53 null adult livers demonstrates an increase in proliferative index with increased numbers of blast-like cells. Approximately one-tenth of blast-like cells (8.8% ± 1.1) present in mild liver express PCNA. In moderate and severe livers, the proliferative index of blast-like cells approaches 100% (result not shown). Interestingly, 30–40% of the few remaining hepatocytes in the severe livers are positive for PCNA expression.

Primary cultures of p53−/− and +/+ hepatocytes incorporate [3H]thymidine at levels of 1.55 ± 0.04 dpm/μg DNA and 0.81 ± 0.03 dpm/μg DNA, respectively. PCNA and [3H]thymidine studies taken together show that 2–2.5 more adult p53 null hepatocytes are proliferating compared with wild type.

One-third partial hepatectomies were performed on adult p53−/− liver with mild disruption because of blast-like cells and their wild-type counterparts. Four days after hepatectomy, there was no significant increase in the number of blast-like cells (identified by H&E morphology and M2-PK staining) nor any significant change in their PCNA expression (9.3 ± 0.8%). There was an increase in PCNA expression in hepatocytes as a result of the procedure; 0.12% of total wild-type hepatocytes were proliferating versus 3.34% of hepatocytes in p53−/− liver. Only 0.03% of hepatocytes in sham-operated animals express PCNA. This equates to a 29-fold increase in PCNA positive in the p53−/− compared with the wild-type liver.

**Blast-like Cells Are Predominantly Hepatic and Express Both Mature and Immature Liver Markers.** A typical portal area of moderately affected p53−/− liver contains both small basophilic blast-like cells and larger hepatocytes (Fig. 5A). Staining of serial sections revealed that nearly one-fifth (17.6% ± 2.1) of the blast cells stained positively for B- and T-cell markers (Fig. 5B). The remainder of blast-like cells express Alb (Fig. 5C), TN (Fig. 5D), L-PK (Fig. 5E), and M2-PK (Fig. 5F). All blast cell colonies contained some cells expressing B and T markers as well as Alb-, TN-, L-PK-, and M2-PK-expressing cells. In half of the blast cell clusters, there was a small fraction (9.8% ± 3.1) of cells that stain positively for CK19 (Fig. 5G). The blast-like cells do not express tyrosine aminotransferase and AFP (result not shown). These hepatic markers were not expressed in various nonhepatic tissues (lymph node, spleen, lung, and thymus) tested. The blast-like cells are a mixed cell population consisting mainly of immature hepatic cells and a small population of lymphoid cells. Expression of Alb and L-PK in mature hepatocytes surrounding areas of blast-like cells is patchy, comprising cells staining with a range of intensities. In general, there is a decrease in hepatocyte expression of Alb and L-PK with increasing numbers of blast-like cells (data not shown).

**Apoptosis Does Not Balance Increased Proliferation in the p53−/− Adult Mouse.** We did not observe a significant increase in liver size until there was severe liver disruption caused by blast cells, in which case the liver was ~2-fold normal size. p53 +/+ and −/− mild and moderately affected livers were totally negative for caspase-3 throughout the liver lobule (result not shown). Four p53−/− livers severely disrupted by blast cells (91.1 ± 2.1% blast cells) all show strong caspase-3 staining in the islands of hepatocytes surrounded by completely negative blast-like cells (Fig. 6). The number of
Characterization of p53 Null Liver

Table 1  mRNA expression of liver-specific proteins during development

<table>
<thead>
<tr>
<th>Age</th>
<th>AFP</th>
<th>Albumin</th>
<th>PEPCK</th>
<th>Aldolase B</th>
<th>PAH</th>
</tr>
</thead>
<tbody>
<tr>
<td>E15 −/−</td>
<td>1.24 ± 0.07</td>
<td>0.50 ± 0.03</td>
<td>ND</td>
<td>0.55 ± 0.02</td>
<td>0.34 ± 0.03</td>
</tr>
<tr>
<td>E15 +/+</td>
<td>1.45 ± 0.12</td>
<td>0.49 ± 0.06</td>
<td>ND</td>
<td>0.56 ± 0.03</td>
<td>0.23 ± 0.05</td>
</tr>
<tr>
<td>E19 −/−</td>
<td>1.34 ± 0.12</td>
<td>0.52 ± 0.04</td>
<td>ND</td>
<td>0.65 ± 0.03</td>
<td>0.32 ± 0.06</td>
</tr>
<tr>
<td>NB −/−</td>
<td>1.44 ± 0.07</td>
<td>0.77 ± 0.09</td>
<td>0.96 ± 0.05</td>
<td>1.03 ± 0.06</td>
<td>0.47 ± 0.09</td>
</tr>
<tr>
<td>NB +/+</td>
<td>0.86 ± 0.07</td>
<td>1.02 ± 0.14</td>
<td>1.06 ± 0.07</td>
<td>0.54 ± 0.07</td>
<td>0.32 ± 0.06</td>
</tr>
</tbody>
</table>

* ND, not detected.

hepatocytes staining per cluster varies between the animals from 42 ± 8.0 to 79 ± 5.7%.

Discussion

Given the established relationship between cell proliferation and differentiation, it is surprising that p53 knockout mice were both viable and developed normally (26–28). However, studies analyzing the p53 knockout mouse have not documented the development of specific tissues. This study reports liver development in the absence of p53. The liver is a useful model for studying development because stages in hepatocyte differentiation are characterized by the expression of specific clusters of liver enzymes (1). We hypothesized that because p53 controls the cell cycle as well as apoptosis, its absence may increase the rate of liver cell proliferation and retard liver differentiation.

We show that liver proliferation during development is not affected by the absence of p53, and accordingly, neither is the developmental expression pattern of Alb, AFP, PAH, PEPCK, or G-6-Pase. Thus, our results are consistent with previous findings, which suggest that p53 is not required for normal embryonic development and specifically, liver development. We conclude that either p53 has no major role in liver cell proliferation, or that other cell cycle regulators are capable of maintaining normal cell proliferation in the developing liver in the absence of p53. Because p53 has been shown recently to be a member of a family of proteins, other members, i.e., p63 and p73, may compensate for its absence (reviewed in Ref. 29).

We report that p53 null adult hepatocytes have proliferative indices between 2 and 2.5 times that of wild type. In addition, we document the presence of highly proliferative, small, basophilic blast-like cells in all p53 null adult livers examined. Their numbers are variable and not age related, which rules out age-dependent changes as the trigger for blast-like cell appearance. We classify the degree of lobular disruption by these blast-like cells as mild, moderate, and severe.

The blast-like cells may be responsible for the increased proliferation of hepatocytes in p53 −/− adult liver in a number of ways: (a) they could achieve this by creating an environment favoring increased hepatic cell proliferation via the production of promitotic cytokines; (b) the hepatic blast-like cells may differentiate into hepatocytes while retaining their proliferative ability; and (c) the increased proliferation may be an inherent property of a mature p53-deficient cell, and the presence of blast-like cells may be the result of aberrant differentiation in the absence of p53. The first two possibilities are unlikely, given the increase in adult hepatocyte proliferation that is observed in vitro in the absence of blast-like cells, as well as in vivo with blast-like cells present. This implies that a lack of p53-mediated checkpoint control has both increased the number of hepatocytes in the proliferative phase and induced the appearance of a cell population with blocked differentiation.

Partial hepatectomies were performed on the adult p53 null mice to assess the contribution of the blast-like cells during liver regeneration. There were two possibilities: either the blast-like cells would not proliferate after surgical resection, or they possess a regenerative capacity and divide to replace lost tissue. The partial hepatectomy data support the former, and there was no increase in proliferation of the
blast-like cells. They are not involved in liver regeneration; this lack of contribution to facilitate liver regeneration is similar to the behavior of the liver progenitor (oval) cell (30).

Because it has been reported (26–28) that malignant lymphomas are the most common (>70%) tumor to affect the p53 null mouse, it was necessary to exclude the possibility that the blast-like cells in the liver are metastasizing lymphomas. We show that only one-fifth of the blast-like cells are of lymphoid origin, and the remainder are predominately hepatic, expressing both immature and mature liver markers. The hepatic blast-like cells in the liver of the adult p53 null mouse express the definitive liver marker Alb, the mature hepatocyte marker L-PK, and the immature oval cell and hepatoblast markers M2-PK and a small fraction, CK19. We conclude that they are a heterogeneous population, with the majority similar to the hepatoblast in E16–18 liver because they express Alb, M2-PK, and L-PK, as reported for the hepatoblast (3, 31). It is of interest that a small percentage of CK19-positive hepatic blast-like cells are either more immature than CK19-negative blast-like cells, or they are differentiating along the biliary lineage.

We report a gradual decrease in expression of Alb and L-PK by hepatocytes in livers affected by increasing numbers of blast-like cells. This is similar to the reduced expression of Alb and L-PK by hepatocytes reported in various models of experimental hepatocarcinogenesis in which oval cells proliferate (33–35).

The increase in hepatocyte proliferation and the presence of highly proliferative blast-like cells in the livers of all p53 null mice should result in increased tissue mass unless apoptosis is increased to maintain tissue size (15). We found increased proliferation in hepatocytes and blast-like cells in p53 null mice, but no significant increase in tissue mass and no expression of caspase-3, a marker of apoptosis. In contrast, severe p53 null liver con-
tained 90% blast-like cells and exhibited a massive increase in tissue size and caspase-3 expression in the small populations of hepatocytes. There is an increase in hepatocyte proliferation, concomitant with this apoptotic response. It is possible that the presence of blast-like cells in the mild and moderate p53 −/− animals is not sufficient to cause a significant increase in tissue mass. However, when highly proliferative blast-like cells are occupying the majority of the liver (as in the severe p53 −/− adult), this results in increased tissue size, and apoptosis is increased in an attempt to regulate organ mass. Alternatively, the cycling hepatocytes may themselves signal apoptosis.

Fig. 5. Characterization of blast-like cells in adult p53 −/− liver. Photomicrographs showing serial sections (4 μm) of moderate adult p53 −/− liver stained with H&E (A), B and T mixture (B), Alb (C), TN (D), L-PK (E), M2-PK (F), and CK19 (G). Positive blast-like cells (closed arrows) are seen in all panels; hepatocytes expressed Alb, TN, and L-PK (open arrows). Scale bar, 100 μm.
There are two possible explanations for the cellular origin of the hepatic blast-like cells in the livers of p53−/− adult mice: dedifferentiation of mature cells, or maturation arrest of immature cells. We favor the latter because all blast cell populations were phenotypically similar, and cells at intermediate stages of differentiation, between hepatocytes and blast-like cells, were not seen. The possibility exists that these blast-like cells are the progeny of liver stem cells that are found in fetal liver and decrease to low numbers in the adult (10). Activation of the liver stem cell compartment in the adult occurs after chronic liver injury, including experimental carcinogenesis. Possibly the p53 null liver recruits stem cells in response to early stage preneoplasia, after which maturation is blocked. The appearance of hepatoblast-like cells indicates that liver stem cell differentiation is arrested.

Materials and Methods

Mice. The p53+/− mice were a generous gift from Dr. Tyler Jacks (USA; Ref. 27) and were housed in a specific pathogen-free animal facility. Female p53+/− mice were crossed with male p53−/− mice to generate p53−/− and +/- offspring. The p53 gene status was individually assessed using PCR (27). Specific pathogen-free, wild-type C57BL/6J mice were obtained from the Animal Resource Center (Murdoch, Western Australia).

Liver Preparation for Immunohistochemistry. Livers from p53−/−, +/−, and +/- mice were collected for various developmental ages: E15, E19, NB, 1W, 2W, 3W, and adult (>6 weeks). Livers were fixed in Carnoy’s solution (60% ethanol, 30% chloroform, and 10% acetic acid) for 2 h before being processed through graded ethanol and embedded in paraffin wax. Liver sections of 6 μm (or 4 μm for serial sections) were cut and attached to poly-L-lysine-coated slides and dried at 37°C. The sections were dewaxed using Histo-Clear and hydrated using decreasing concentrations of ethanol. Liver sections were stained with H&E to verify morphology and for cell counts.

PCNA Immunohistochemistry. The indirect immunoperoxidase method for detection of proteins (36) was used to localize PCNA. Endogenous peroxidases were blocked with treatment with 2.5% aqueous periodic acid (Sigma) for 5 min and then with 0.02% sodium borohydride (Ajax Chemicals) for 2 min. Samples were blocked for 1 h with 10% fetal bovine serum (Life Technologies, Inc.) in PBS (pH 7.4) containing 0.2% saponin (Sigma). Liver sections were then incubated for 4 h at room temperature in biotinylated rat antimouse PCNA (Leinco Technologies) antibody. After three washes with PBS/0.2% saponin, a peroxidase-coupled streptavidin (Dako) was added to the sections and incubated overnight at 4°C in a humidified chamber. The following day, the washing procedure was repeated, and the sections were reacted with 3,3′-diaminobenzidine (Sigma) for 5 min.

B and T Cell and Caspase-3 Immunohistochemistry. To identify cells of the lymphoid lineage, a mixture of rat monoclonal antibodies (B- and T-cell mixture) reactive with CD3, CD4, CD8 Thy 1.2 (T-cell antigens), and JID and 6B2 (B-cell antigens) was used as a primary antibody. Apoptosing cells were localized using antibody to the COOH terminus of the human caspase-3 p20 subunit (Santa Cruz Biotechnology), a protease expressed during the early stages of apoptosis (37, 38). Endogenous peroxidases were blocked by incubation in 3% H2O2, and then sections were incubated in primary antibody overnight at 4°C in a humidified chamber. The following day, sections were washed and then incubated for 1 h at room temperature in the appropriate secondary antibody, antirat IgG conjugated to HRP (Amersham) for B and T cocktail, or antigoat IgG HRP (Rockland) for caspase-3. The sections were washed and then reacted with 3,3′-diaminobenzidine for 5 min.

Blast Cell Characterization by Immunohistochemistry. To characterize hepatic blast cells, sections were reacted with primary antibodies directed against Alb (39), TN (polyclonal rabbit), M2-PK (Rockland), L-PK (40), and CK19 (Amersham). Endogenous peroxidases were blocked with 2.5% periodic acid, followed by 0.02% sodium borohydride, and sections were incubated with primary antibody for 1 h at room temperature. The sections were washed then incubated for 1 h at room temperature with the appropriate secondary antibody, antirat IgG conjugated to HRP (Amersham) for Alb and L-PK, antigoat IgG HRP for M2-PK, and antiamoig IgG HRP (Sanofi Diagnostis Pasteur) for CK19. Following this, sections were washed and then reacted with DAB for 5 min. All sections following staining were mounted in glycerol/gelatin and coverslipped.

Quantification of Positive Cells following Immunohistochemistry. Cells positive for various antigens were scored over 5–10 fields and expressed as a percentage of total cells in the field. Scores for all animals were expressed as mean ± SE. Where appropriate, results from different groups of animals were compared for variability using one-way ANOVA.

[3H]Thymidine Labeling of Cells. Primary hepatocyte cultures were established from adult p53−/− and wild-type mice. Whole liver was perfused via the portal vein with three consecutive solutions: 50 ml of perfusion buffer [0.05 m KCl (BDH), 1.16 m NaCl (BDH), 0.02 m HEPES (BDH), 0.25 m NaHCO3 (Ajax Chemicals), 0.06 m glucose (BDH)] plus 0.61 m M EDTA, 12.5 ml of perfusion buffer, and finally 62.5 ml of...
perfusion buffer plus 1.3 mM CaCl₂ and 1.11 × 10⁻⁴ units of type I collagenase. The liver was removed from the animal and dissociated in Leibowitz media (Life Technologies, Inc.) with 200 μg/ml bovine serum albumin. The cells were filtered through large-gauge gauze, washed twice with Leibowitz medium before being resuspended in an appropriate volume of Minimum Essential Medium, 10% FBS, and 10⁻⁸m insulin. Cells were plated onto collagen-coated plates at a density of 4.5 × 10⁴/30-mm diameter dish. Once adhered, cells were incubated in 1 μCi of [³H]thymidine (Amersham, Buckinghamshire, United Kingdom) per ml of culture medium for 2 h (incorporation tested linear over 1, 2, and 3 h; result not shown). The cells were thoroughly washed and then scraped from the dish, pelleted, resuspended in 5% trichloroacetic acid, and then heated at 90°C for 1 h. Samples were taken for scintillation counting and DNA assay. The hot acid hydrolysis method (41) was used to assay DNA. Cytosine arabinoside was used to inhibit cell proliferation to serve as a control for [³H]thymidine incorporation as a result of DNA repair or nonspecific binding (result not shown).

Partial Hepatectomy. The mice were anesthetised, a small incision was made in the abdomen, and the frontal lobe of the liver was pushed out. The lobe was ligated near the hilus, and the liver tissue was excised, completing a one-third partial hepatectomy. The excised liver was fixed for 2 h in Carnoy’s fixative. The remainder of the liver was placed inside the abdominal cavity, and the incision was sutured closed. Control, sham-operated mice were opened, and the inside the abdominal cavity, and the incision was sutured in Carnoy’s fixative. The remainder of the liver was placed of the liver was pushed out. The lobe was ligated near the hilus, and the liver tissue was excised, completing a one-third partial hepatectomy. The excised liver was fixed for 2 h in Carnoy’s fixative. The remainder of the liver was placed inside the abdominal cavity, and the incision was sutured closed. Control, sham-operated mice were opened, and the liver was exposed and sutured closed. Four days after the surgery, the animals were sacrificed by cervical dislocation, and the livers were removed and fixed for 2 h in Carnoy’s fixative. The livers, before and after partial hepatectomy, were stained with H&E, M2-PK, and PCNA.

RNA Isolation and mRNA Detection. Whole livers from p53+/−, +/+ and +/+ mice over various developmental ages, E15, E19, NB, 1W, 2W, 3W, and adult, were collected. For each time point, three mice per genotype were used, and their RNA was analysed individually. Total RNA was isolated using Trizol reagent (Life Technologies, Inc.) according to the manufacturer’s instructions. Samples of total RNA (15 μg) were electrophoresed through a 1.0% agarose gel containing 2.2 μm formaldehyde (42) and then transferred to Gene-screen Plus (NEN) membranes by capillary blotting. Alb, AFP, G-6-Pase, PAH, PEPC, and GAPDH mRNAs were detected by hybridization with their respective cDNAs: Albumin 3116 (43), prAF 87 AF (44), PAH (45), pmcPEPCK2.4 (46), pBRAIolase B (47), and prGAPDH-13 (48). All cDNA probes were labeled with [α-³²P]dCTP (Amersham) using a Mega-Prime kit (Amersham) and hybridized at 42°C for 24 h. The hybridized membranes were washed according to the manufacturer’s instructions before being exposed to a BAS IIIs phosphorimaging plate (Fuji, Japan). The phosphorimaging screen was scanned using a Fuji Bas 2500 phosphorimager (Fuji, Japan). Quantitative analysis of the images was performed using NIH Image software (Bethesda, MD); mRNA levels were expressed relative to GAPDH mRNA. Quantitative values were compared for variance using a one-way ANOVA.

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We acknowledge Dr. Tyler Jacks for permission to use of the p53 knockout mouse and thank Drs. Jerry Adams and Alan Harris for providing heterozygote p53 knockout mice.

References


