Conditional Deletion of c-myc Does Not Impair Liver Regeneration

Feng Li, Yan Xiang, James Potter, Ramani Dinavahi, Chi V. Dang, and Linda A. Lee

Department of Medicine, The Johns Hopkins University School of Medicine, Baltimore, Maryland

Abstract

The oncogene c-myc encodes a transcription factor that has long been considered essential to liver regeneration, the process by which fully differentiated hepatocytes proliferate in an attempt to maintain a normal functional mass in response to hepatic injury. Experimental liver regeneration can be induced upon 70% partial hepatectomy and is accompanied by an increase in c-myc expression accompanying the synchronous entry of remaining hepatocytes into the cell cycle. Because liver regeneration is an essential process for achieving liver homeostasis, therapies directed at reducing MYC expression in hepatocellular carcinoma are fraught with the theoretical possibility of injuring adjacent noncancerous liver cells, thereby restricting the liver’s normal regenerative response to injury. To determine if intact c-myc is required for liver regeneration, we reduced hepatic c-myc in c-mycfl/fl mice using an adenoviral vector that expresses Cre recombinase. Despite a 90% decrease in hepatic expression of c-myc, restoration of liver mass 7 days later was not compromised. Reconstituted liver retained the same decrease in hepatic c-myc, indicating that hepatocytes deficient in c-myc were able to proliferate in response to partial hepatectomy. Although c-myc is required for embryonic development, our findings indicate that it is not required for the maintenance of the adult liver. (Cancer Res 2006; 66(11): 5608-12)

Introduction

Over 70% of the normal adult liver is comprised of hepatocytes that are in the G0 phase of the cell cycle. Despite terminal differentiation, hepatocytes retain the remarkable ability to proliferate and maintain the functional mass of the liver when liver function is threatened by acute or chronic injury. The molecular mechanisms by which liver regeneration occur remain poorly understood, as do the mechanisms that govern cessation of the regenerative process once the original liver mass has been finally restored. The rodent two-thirds partial hepatectomy is the classic experimental model to study the control of liver regeneration and the cell cycle in vivo (1). Surgical resection of two thirds of the liver triggers the synchronous entry of 90% of hepatocytes in the remnant liver into G1. Peak DNA synthesis occurs by 24 hours after partial hepatectomy (2), and the original liver mass, with no significant change in liver architecture, is restored within 7 days, reflecting an average 1.6 cycles of replication. Dramatic changes in hepatic gene expression accompany all stages of liver regeneration. The gene c-myc, whose expression is induced immediately after partial hepatectomy, has garnered significant attention as a major regulatory node in a complex transcriptional network triggered during the G1 phase of liver regeneration (3). c-myc encodes a basic helix-loop-helix leucine zipper transcription factor known to regulate the expression of cell proliferation and metabolism genes in diverse cell types. In the liver, it may participate in the “priming” of hepatocytes (4), a necessary state that permits hepatocytes to respond to cytokines released by partial hepatectomy. Despite the extensive body of literature implicating c-Myc as a primary effector of liver regeneration, it has yet to be proven that c-Myc is absolutely required. Determining whether c-myc is truly important in liver regeneration would further our understanding of the biological function of c-myc, an oncogene overexpressed in many human malignancies, including hepatocellular carcinoma. Moreover, this knowledge could aid proposed molecular or chemical strategies directed at reducing c-Myc expression or function in hepatocellular carcinoma, as they are fraught with the theoretical possibility of injuring adjacent noncancerous liver cells, thereby restricting the liver’s normal regenerative response to injury (5, 6). We, thus, sought to biologically test whether c-myc is truly essential for restoration of liver mass following partial hepatectomy. We reduced allelic c-myc in the livers of transgenic mice possessing floxed c-myc alleles (c-mycR/R, ref. 7) and discovered no significant delay in the restoration of normal liver mass following two-thirds partial hepatectomy.

Materials and Methods

Transgenic mice. All animals received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the NIH (NIH publication 86-23 revised 1985). c-mycR/R mice were gifts of F. Alt (Harvard University, Boston, MA). Transgenic c-mycR/R mice were backcrossed to the parental strain, C57/BL6 and F1 progeny crossed to generate the transgenic strain c-mycR/R. Male mice ~6 weeks of age were used for all experiments. Adenoviral vectors. AdCre (F. Bunz, The Johns Hopkins University) was propagated using the human embryonic kidney cell line 293 and purified in cesium-chloride gradients. AdLacZ was created by subcloning the β-galactosidase gene into the EcoRI site of the replication-defective, cytomegalovirus promoter–driven AdLox vector (J. Rade, The Johns Hopkins University). Animals were injected through the tail vein with 8 × 109 plaque-forming units (pfu) 4 days before partial hepatectomy.

Partial hepatectomy. Animal protocols were approved by the Johns Hopkins University Animal Control and Use Committee. Mice anesthetized with isoflurane underwent 70% partial hepatectomy according to methods described by Higgins and Anderson (1).

PCR. Regular PCR conditions and primer sequences to assay both floxed c-myc (c-myc) and the deleted c-myc allele (Δc-myc) are as described (7). Δc-myc was also detected by Taqman probe and normalized to the nuclear gene GAPDH, which was amplified from the same aliquot using Sybr Green core reagent and used as an internal DNA control. The probe and primer sequences for Δc-myc are as follows: probe, 5′-FAM-TCCCTCAGCCTAATAAGTCTCTCAAATGACCCTTTAATACTTTAC-3′; forward primer, 5′-GGGAAGAGCCCTGTCCAGACCA-3′; reverse primer, 5′-GATGCTAGAAGGTTCTCCTGAGACAA-3′. The primers for GAPDH are as follows:

Requests for reprints: Linda A. Lee, 720 Rutland Avenue, Ross 1029, Baltimore, MD 21205. Phone: 410-502-5058; Fax: 410-955-0185; E-mail: llee12@jhmi.edu.

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forward, 5′-CTAAAGATCTCGAAGCAAGG-3′; reverse, 5′-AAGTGGCACAGATAAACG-3′. We determined the gel intensity of the c-myc band of the Cre animal to be only 10% of the LacZ animal in Fig. 1, indicating that the Cre animal must have 90% c-myc deletion. Because this percent deletion correlates with the normalized real-time value of Δc-myc/GFAP = 1.03 in this same Cre animal, we defined a value above 1.03 in any animal as representative of a 90% deletion of c-myc. Any value below 1.03 was calculated as (value / 1.03) × 90% to be the percent of deletion. The expression of c-myc, L-myc, N-myc, and c-fos were detected with total RNA by Taqman probes. Primer sequences are indicated in the box above.

**Primary hepatocyte preparation and adenoviral infection.** Primary murine hepatocytes were isolated by the collagenase perfusion method and cultured on collagen-coated 35-mm plates as described for rat hepatocytes (8). Hepatocytes isolated from untreated c-mycfl/fl mice were plated and 1 day later transduced with AdLacZ or AdCre at 2 × 10^9 pfu per plate. Cell number was assayed at days 2, 7, and 9 after viral transduction. Cell number and viral units were proportionally increased for flow cytometry assay. Propidium iodide staining was done at day 5 after viral infection.

**Results**

c-myc knockout in the mouse is embryonic lethal (9); thus, we reduced hepatic c-myc expression in adult male transgenic mice engineered with c-myc d/d (7) by injecting into the tail vein adenovirus expressing Cre-recombinase (AdCre). Adenoviral vector injection into the tail vein is an efficient method for transgene delivery to the liver. This i.v. delivery method results in >90% uptake of the vector genome by the liver, with only modest expression in any other organ (10, 11). Control animals were injected with adenovirus expressing the bacterial β-galactosidase gene (AdLacZ). The AdCre virus carries a green fluorescent protein (GFP) cassette, allowing the detection of transduced cells. Diffuse GFP expression in the Cre-infected livers was observed 4 days after injection (Fig. 1A). Regular and real-time PCR were used to quantify c-myc allelic deletion in each animal liver (Fig. 1B and Fig. 2B). When compared with control AdLacZ livers, 90% of the floxed c-myc allele had been deleted in AdCre livers.

Four days after injection, all mice underwent 70% partial hepatectomy. Three or four mice in each group were sacrificed 2, 5, and 7 days after partial hepatectomy. No statistical difference in the liver/body mass ratio between AdCre and AdLacZ mice occurred 2 and 7 days after resection (Fig. 2A). There was, however, a significant difference (P < 0.05) at day 5, when the liver/body mass ratio in AdCre mice transiently lagged behind that of AdLacZ animals. To determine if liver in which c-myc was conditionally deleted would regenerate, we quantified and compared the percentage of deleted c-myc allele in the regenerated liver 7 days after partial hepatectomy and the earlier hepatotomized liver for each animal. We found that fully regenerated livers remained as deficient in the c-myc allele as the hepatotomized livers removed earlier from the same animal (Fig. 2B). The persistent loss of the c-myc allele in the regenerated liver indicates that the reconstituted liver mass indeed arose from liver deficient in c-myc. Although AdCre animals seemed healthy and active, the architecture of c-myc-deficient livers was disorganized, and many hepatocytes and their nuclei seemed enlarged compared with those of AdLacZ livers (Fig. 2C). We used the number of cells per high power field (cells/hpf) to estimate average hepatocyte size and found on day 7 that AdLacZ livers possessed 496 ± 94 cells/hpf and AdCre livers 279 ± 41 cells/hpf. Because the area/cell can be used to estimate cell volume using the formula, 4/3πr³ (assuming a cell is spherical), the difference in cell number indicates average hepatocyte size was increased in AdCre livers compared with AdLacZ livers. Restoration of liver mass results in part from the increase in cell size, but on day 2, we calculated the mitotic index in both AdCre and AdLacZ livers to be 1%. We also determined that there was no significant difference in cells/hpf on day 2 compared with day 7 AdCre livers. Had no proliferation occurred and liver mass attributable only to increases...
in cell size, cells/hpf on day 7 AdCre liver should have been significantly fewer than on day 2. Thus, the restoration of liver mass results from both increased cell size and cell proliferation.

The finding that hepatocytes lacking c-myc could still regenerate was supported by proliferation studies conducted in vitro. Hepatocytes isolated from untreated animals and then transduced with AdCre proliferated at a similar rate as AdLacZ-treated cells (Fig. 3A). Flow cytometry of AdCre and AdLacZ ex vivo transduced hepatocytes shows that the percentage in S phase was the same in both populations (Fig. 3B). Interestingly, there was an increase in the 4N population in the AdCre hepatocytes.

Deletion of floxed c-myc using Cre recombinase could affect the expression of other early response factors, such as the transcription factor c-Fos, which also has been implicated along with c-Myc as being a critical transcriptional instigator of hepatic regeneration (3). We found that disruption of c-myc expression did not interfere with the expression of c-fos, in both groups of animals 2 days after resection (Fig. 4). We also detected no statistical difference in L-myc or N-myc expression in AdCre- and AdLacZ-regenerated livers, suggesting that the expression of these Myc family members did not change to compensate for c-myc deletion (Fig. 4).

Discussion

The oncogene c-MYC encodes a transcription factor that has long been considered essential to liver regeneration, the process by which fully differentiated hepatocytes proliferate in an attempt to maintain a normal functional mass in response to hepatic injury. c-Myc activity is often coupled to cell proliferation, giving rise to the expectation that c-MYC would be critical for normal liver regeneration, a model used to study the cell cycle in vivo. Our study shows, however, that restoration of the liver/body mass ratio still occurs within 7 days following partial hepatectomy despite a deletion of c-myc. Although liver mass was restored in the usual time frame following partial hepatectomy, we found that liver

Figure 2. Liver-deficient in c-myc regenerates in response to partial hepatectomy. A, liver/body mass ratio at days 2, 5, or 7 after partial hepatectomy. B, average % c-myc allele deletion from both AdCre and AdLacZ mice before and after partial hepatectomy as measured by real-time PCR. C, liver H&E staining micrographs from AdCre or AdLacZ mice before or 2, 5, or 7 days after partial hepatectomy.

Figure 3. Hepatocytes deficient in c-myc proliferate ex vivo. A, growth curves of AdCre- or AdLacZ-treated hepatocytes isolated from c-myc<sup>fl/fl</sup> mice. Points, average of four experiments comparing AdCre and AdLacZ; bars, SE. B, propidium iodine stained AdCre or AdLacZ hepatocytes.
architecture was not entirely normal. We observed that the hepatic plate of the c-myc-deficient regenerated liver was disorganized, with some enlarged hepatocytes and nuclear enlargement persisting through day 7, suggesting that the restoration of liver mass could be partially due to increased hepatocyte growth independent of proliferation. The enlarged hepatocytes correspond to the increased population of 4N hepatocytes that we detected by flow cytometry (Fig. 3B). Nevertheless, it is clear that cell proliferation also occurred, as ascertained by the stable mitotic index at day 2 in AdCre and AdLacZ livers and maintenance of a constant number of cells/hpf in c-myc-deficient livers between days 2 and 7.

Baena et al. also observed that c-myc deletion did not alter the rate of hepatocyte proliferation (12). Despite our agreement with those findings, Baena et al. found deletion of c-myc compromised liver regeneration. We assayed liver regeneration by comparing the liver/body mass ratios on day 7. Baena et al. measured liver regeneration indirectly by examining the expression of the proliferation-associated gene PCNA, a c-Myc responsive gene whose expression might in fact be reduced in the absence of MYC. Furthermore, it is uncertain whether or not restoration of liver mass would occur by day 7 in their model because they ended their study on day 2 following partial hepatectomy.

Baena et al. found that c-myc deletion in newborn mice results in a decrease in both hepatocyte size and polyploidization as the livers mature, but we found that c-myc deletion in the adult liver produces the opposite. It is entirely possible that these contrasting results arise from inherent differences in our animal models; that is, we used adult mice whose livers contain a higher percentage of polyploid cells, as opposed to the newborn mice they used whose livers are comprised of diploid hepatocytes that are known to be more highly replicative (13, 14). Our experimental models also differ in that we eliminated floxed c-myc alleles in liver using an adenoviral vector to express Cre recombinase, whereas Baena et al. deleted c-myc alleles by injecting newborn homozygous (c-myc^lox^lox;mx-cre^-) mice with plpC to activate the mx promoter and express Cre recombinase. It is not readily apparent why a deficiency of c-myc would lead to increased hepatocyte ploidy. Although Beer et al. have linked c-myc overexpression to endoreduplication, a process characterized by enforced DNA replication and disruption of mitosis (15), it is not apparent that the increase in 4N hepatocytes in our c-myc-deficient model results from the same mechanism of endoreduplication. A possible interpretation of our data is that c-myc is not required for hepatocyte endoreduplication.

Our findings in primary hepatocytes confirmed our histologic data that c-myc is dispensable for hepatocyte proliferation in the adult liver, despite the fact that c-myc is required for embryonic development (9). The functional effect of c-Myc in developing tissues, where it may play a larger role in cell proliferation, may differ from its role in the homeostasis of some adult tissues as we observed in our study. We previously observed that transient overexpression of c-myc in the adult mouse liver increases cell size and protein synthesis without a significant increase in cell proliferation (16). Because c-Myc function in the mature liver may not center around cell proliferation, it is possible that c-Myc plays a greater role in other cellular processes essential to cell growth, such as glucose metabolism and ribosomal biogenesis (17). Even so, the liver still was able to recover its mass in the usual amount of time whatever the effect on these other c-myc affected pathways. We have yet to determine how c-myc deletion in these regenerated livers affects hepatic physiology because c-Myc has been shown to regulate many genes involved in metabolism (18), and its overexpression improves glucose homeostasis in diabetic mice (19). Further study is necessary to understand how changes in these physiologic properties may affect liver regeneration and function.

Other studies done in cells or tissues of mature animals support the notion that c-Myc function is context dependent. For example, primary keratinocytes retain the ability to proliferate when c-myc expression is diminished (20). Moreover, recovery and maintenance of normal small intestinal crypt epithelium is possible even in the absence of c-myc (21). Not all adult tissues can proliferate in the absence of c-myc, however. Conditional elimination of c-myc in the bone marrow leads to accumulation of hematopoietic stem cells that fail to differentiate, indicating that in some adult tissues, c-myc is necessary for normal homeostasis (22). Because the function of c-Myc seems to change based on the cell or tissue context, it seems likely that genes regulated by c-Myc in vivo will vary substantially depending on cell type, adding another layer of complexity to understanding the role of c-Myc in cell proliferation, growth, differentiation, and tumorigenesis (23).

Deregulated expression of c-Myc is the hallmark of many types of human malignancies. Experimental models exist in which Myc-induced tumors of pancreatic B islets, liver, and skin regress once Myc activation is inhibited (reviewed in ref. 24). These models imply that interference with c-Myc expression or activity is a viable approach to treat certain human malignancies. Antisense oligonucleotides, triple helix forming oligonucleotides, and RNA interference are but a few approaches already undertaken to reduce the effect of deregulated c-Myc expression in tumor cells. The finding that liver regeneration can still occur in our mouse model despite a deficiency in c-myc raises the notion that oncogene knockdown strategies targeting c-MYC in hepatoma or metastatic lesion will not automatically compromise the regenerative capacity of surrounding normal hepatocytes. However, it is difficult to predict from our data how hepatocytes of chronically diseased livers, in which human liver tumors often arise, would behave if expression of c-MYC were abrogated.

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