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Janeen H. Trembley
University of Minnesota

Clifford J. Steer
University of Minnesota

PERSPECTIVES ON LIVER REGENERATION

JANEEN H. TREMBLEY[1] AND CLIFFORD J. STEER[2]
Department of Medicine
University of Minnesota Medical School

Biological regeneration is a topic which has fascinated scientists for centuries. In the 18th century, scientists observed the ability of various animals to replace amputated limbs and entire body segments following bisection.[1] Dramatic examples of regeneration such as these are mainly restricted to invertebrate animals. One notable exception is the ability of the urodele amphibians, such as the newt, to regenerate tail, limbs, jaws and ocular tissues. The replacement of a limb complete with the various cell types such as cartilage, muscle and bone is an example of regeneration defined in the classic sense. The liver has the remarkable ability to fully replace lost tissue mass and cells after acute injury. The regrowth of the liver following tissue removal or other injury is traditionally termed regeneration but is technically compensatory hyperplasia. One key difference lies in the fact that while cell number, cell type and organ mass are faithfully replaced, the original gross morphology is not duplicated. For example, two-thirds partial hepatectomy in the mouse or rat entails the removal of the left lateral and median liver lobes. The regrowth of the remnant liver results in essentially tripling the mass and cell number of the remaining lobes.

The liver is an epithelial organ composed of parenchymal cells, or hepatocytes, and nonparenchymal cell types including Kupffer, Ito, bile duct epithelial (cholangiocytes) and fenestrated endothelial cells. The liver performs many essential functions such as glucose regulation, synthesis of blood proteins, secretion of bile and drug metabolism. The optimal mass of the liver required to perform these functions is determined by body size and the liver is remarkably adaptable to changes in demand. For example, transplanted livers grow or shrink in size in response to the body mass and demands of the recipient. Once liver mass falls below the optimal threshold level due to physical, chemical or biological injury, the liver responds by initiating growth until the threshold level is again achieved. Liver growth which is initiated by a mitogenic stimulus without coincident liver cell loss is neither a regenerative nor compensatory growth response, but rather is termed direct hyperplasia and is the subject of another review.[2] This review will address the regenerative growth response.

AN OVERVIEW OF THE REGENERATIVE PROCESS

Adult hepatocytes are normally quiescent, highly differentiated cells exhibiting little mitosis (only 1 in 10,000 to 20,000 cells). A variety of injuries can result in liver cell loss and regrowth. These include chemical injuries such as carbon tetrachloride administration and viral infection. A well characterized model for liver regeneration is the 70% partial hepatectomy (PH), which will be the basis for much of the information presented in this review.

After resection of the left lateral and median liver lobes, an ordered sequence of events occurs. The multitude of events which occur post-PH can be characterized by three major phases (fig 1). The first phase involves priming the remnant liver cells for growth in which the remaining hepatocytes and nonparenchymal cells synchronously exit their resting G_0 state and enter the G_1 phase of the cell cycle. The second phase includes progression through one or more cell division cycles. The final phase involves organization of the newly replicated cells and extracellular matrix for normal liver function.

The priming events of the first phase include immediate changes in membrane potential, rapid influx of sodium, increased intracellular pH, calcium release from intracellular stores and an increase in urokinase receptor and urokinase activity within 5 minutes. Furthermore, cytokine and growth factor signaling occur within 30 minutes post-PH and are the catalyst for subsequent events. Latent transcription factors within the liver cells are activated and, in turn, induce immediate-early genes. In phase two, delayed-early genes are activated followed by DNA synthesis and progression through the cell cycle. Two waves of cell division typically follow PH. The first major wave of cell proliferation involves primarily hepatocytes while subsequent proliferation also includes nonparenchymal cells. Peaks in DNA synthesis for the rat occur predictably around 18-24 hours and 42-48 hours post-PH, with the first peak representing a more dramatic and synchronous period of DNA replication. The time course of liver regeneration in the mouse is typically delayed by approximately 12 hours compared to the rat. Mitosis proceeds 6 to 8 hours after DNA synthesis. In young adult rats, as many as 95% of the hepatocytes undergo at least a single cycle of replication and within 4 days the majority of liver cells has been replaced. Finally, in phase three, remodeling of the liver cells occurs. Within 7 to 10 days the extracellular matrix microarchitecture and liver mass are restored and the normal complement of liver specific gene expression is present.

THE POTENTIAL FOR HEPATOCYTE PROLIFERATION

There is abundant evidence that existing hepatocytes are the source of new hepatocytes in both adult liver regeneration and in postnatal liver development. Hepatocyte proliferation and the origin of newly replicated cells can be examined in vivo by at least two distinct processes. The first is that of liver regeneration induced by PH. The second occurs in developing animals where, in rodents, the liver mass increases approximately 10-fold between 1 and 4 weeks of age. Taking advantage of this developmental period of liver growth, cell replication was

[1] Present address: St. Jude Children's Research Hospital, Department of Tumor Cell Biology, 332 North Lauderdale, Memphis, TN 38105.
[2] Correspondence address: Clifford J. Steer, M.D., Department of Medicine, University of Minnesota Medical School, 420 Delaware St. S.E., Minneapolis, MN 55455. Tel: (612) 624-6648; e-mail: steer001@tc.umn.edu

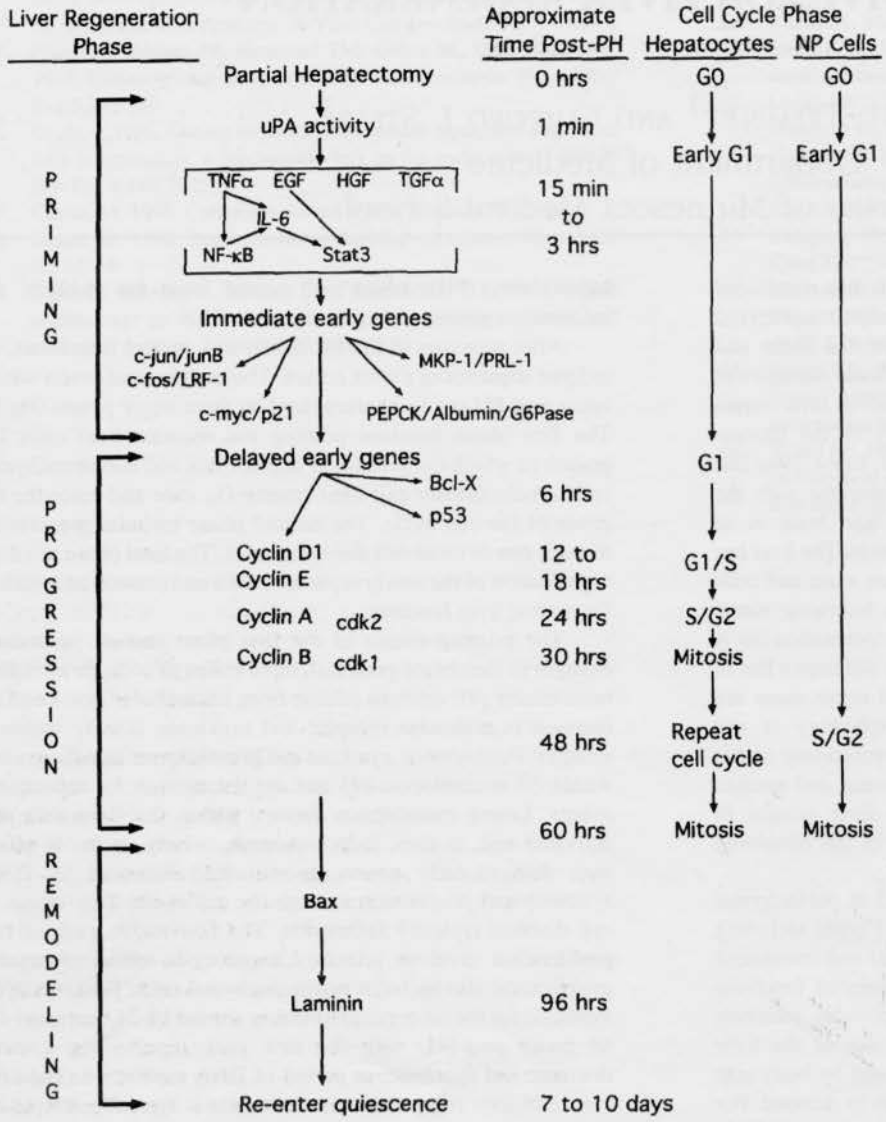

Figure 1. Sequential events after partial hepatectomy in the rat can be subdivided into three major phases. The diagram illustrates the types of regulatory events involved, including increased expression of immediate- and delayed-early genes, active participation of members of the cyclin family and modulation of certain apoptosis-associated gene products. Cell cycle progression for nonparenchymal (NP) cells is typically delayed approximately 24 hours compared to hepatocytes. The complexity of the entire process is as profound as the simplicity of this diagram. Other details are described in the text.
Abbreviations: cdk, cyclin-dependent kinase; EGF, epidermal growth factor; G6Pase, glucose-6-phosphatase; HGF, hepatocyte growth factor; IL-6, interleukin-6; LRF, liver regeneration factor; MKP, map kinase phosphatase; NF-κB, nuclear factor-κB; PEPCK, phosphoenolpyruvate carboxykinase; PH, partial hepatectomy; PRL, phosphatase of regenerating liver; Stat3, signal transducer and activator of transcription-3; TGFα, transforming growth factor alpha; TNFα, tumor necrosis factor alpha; uPA, urokinase plasminogen activator.

monitored in mice transgenic for the human α_1-antitrypsin/β-galactosidase expression construct. Blue transgenic cells which began as singlets or doublets randomly scattered in neonatal livers were later present in large clusters in young adult rats.[3] These results indicate that the blue-marked cells arose from preexisting hepatocytes and did not migrate from a stem cell compartment.

Hepatocytes have a remarkable capacity for repeated proliferation and are long-lived cells which, in rodents, remain viable for more than 2 years. Similar to the experiment described above, the use of transgenic mice provided useful information on the clonogenic potential of hepatocytes. First, transgenic mice expressing the urokinase plasminogen activator (uPA) coding sequence were constructed. Expression of this gene produced elevated plasma uPA and fatal hemorrhaging in newborn mice.[4] Interestingly, two lines of uPA transgenic mice were established from surviving founder mice in which uPA was expressed at low levels. Within these transgenic lines, half of the offspring died due to hemorrhage but the remaining offspring appeared normal and survived. In those mice, a spontaneous genetic rearrangement had occurred which shut down expression from the uPA gene. The hepatocytes containing the rearrangement displayed a marked proliferative advantage and repopulated the livers.[5] Second, the toxic, chronically regenerative effect of uPA expression in these transgenic mice was used to determine whether adult liver cells rather than neonatal cells could repopulate an impaired liver. Genetically differentiable donor hepatocytes were transferred into transgenic uPA mice by splenic injection and were able to proliferate and replace up to 80% of the recipient parenchyma.[6] Furthermore, these livers retained the ability to regenerate following PH. Finally, rat hepatocytes were transplanted into immunosuppressed uPA transgenic mice where most of the recipient mouse liver was replaced by rat hepatocytes.[7] Overall, rat liver can regenerate at least 12 times, and a single hepatocyte has the theoretical clonogenic capacity to undergo at least 34 divisions and generate 50 rat livers.[8] These results have exciting potential for gene therapy applications, as discussed in a later section.

One of the striking features of liver regeneration is that these highly differentiated cells simultaneously proliferate and

continue to perform the functions necessary for viability of the organism. Moreover, the regrowth of the liver following PH does not involve a significant stem cell component. There are many definitions of stem cells in the literature. One definition is that stem cells are pluripotent and can self renew. Thus, a liver stem cell should be capable of producing different hepatic cell types as well as replicating into other stem cells. Data presented to date strongly suggest the presence in the liver of a bipotent stem cell called the oval cell. These cells can differentiate in culture into either hepatocytes, bile ductule cells and possibly other cell types.[9] Taken one step further, the hepatocyte can be viewed as a unipotential stem cell since this fully, but not terminally, differentiated cell can respond to regenerative signals and reproduce itself. Following 60-70% cell loss due to PH, the liver is still able to carry out the necessary functions for organ viability without activation of its stem cells. However, if damage to the liver is severe enough that liver function is compromised and the hepatocytes cannot proliferate, oval cells are activated and replicate. This is illustrated in an experimental model of liver regeneration in which treatment with 2-acetylaminofluorene (AAF) is followed by PH. The AAF treatment inhibits hepatocyte mitosis thus provoking an oval cell-mediated regeneration response post-PH.[9]

Growth Factor and Cytokine Signaling

The role of growth stimulatory and, to a lesser extent, growth inhibitory factors in the liver's regenerative response have been extensively investigated in the hopes of identifying the key 'start' and 'stop' signals. However, the precise role of various positive and negative growth factors still remains unclear. A complete mitogen is a substance which is capable of stimulating DNA synthesis and mitosis of cultured cells in serum-free media. Transforming growth factor alpha (TGFα), epidermal growth factor (EGF) and hepatocyte growth factor (HGF), or scatter factor, are each complete mitogens for liver cell growth. Hepatic comitogens alone do not cause proliferation of liver cells in culture but act to enhance the stimulatory effect of complete mitogens. These include insulin, glucagon, insulin-like growth factors, norepinephrine, various hormones, calcium, vitamin D and certain nutrients. Finally, several liver growth inhibitors have been identified including the transforming growth factor beta (TGFβ) family and interleukin 10 (IL-10).

Research into the role of growth factors in liver cell proliferation has been performed in vitro using primary hepatocytes due, in part, to the improved ability to manipulate the cellular environment. Hepatocytes are isolated for primary culture by collagenase perfusion. The process causes these cells to leave G_0 and enter G_1. Following isolation and plating, several genes which traditionally mark the early to middle G_1 phase of the cell cycle are expressed. These genes include c-*fos*, c-*jun*, c-*myc*, and p53. However, when grown in serum-free media, these primary hepatocytes cannot progress through G_1 into S phase (DNA synthesis) without the addition of growth factors. Thus, primary hepatocytes are primed for proliferation and are responsive to growth factors in contrast to resting or quiescent hepatocytes in vivo which are not responsive to growth factors. Loyer and colleagues determined that the mitogen-dependent restriction point in adult rat hepatocytes occurred in mid to late G_1, or 42-48 hours after seeding.[10] The addition of either EGF or TGFα in conjunction with the cofactor pyruvate was sufficient to allow these cells to progress through DNA synthesis.

Removal of 30% of the liver creates a situation similar to collagenase isolation of primary hepatocytes. The cells in the remnant liver exit G_0 and are primed for DNA synthesis, but require the addition of growth factor(s) to enter S phase. Fausto and colleagues infused growth factors into the mesenteric veins of rats for 24 hours using an osmotic pump placed in the peritoneal cavity. They found that quiescent hepatocytes exhibited little response to HGF, EGF, and TGFα; but if the liver cells were first primed by 30% PH, cell cycle progression occurred.[11]

Epidermal growth factor and transforming growth factor-α

Basal levels of EGF RNA are detectable in rat liver using the reverse transcription polymerase chain reaction technique.[12] EGF RNA abundance increases by 10-fold within 15 minutes post-PH then diminishes below basal levels around 4 hours post-PH. Fractionation of liver cells prior to RNA isolation revealed that EGF RNA was expressed by hepatocytes and Ito cells, whereas the EGF receptor RNA was identified in hepatocytes, endothelial, Kupffer and Ito cells. Typically, EGF is synthesized as a 140 kilodalton (kDa) transmembrane precursor protein that is processed and released from the cell surface as a 6 kDa signaling peptide. However, in regenerating liver, EGF protein accumulates in rat and mouse hepatocytes as a 60 kDa polypeptide.[12] This work established that an autocrine mechanism exists for EGF signaling during liver regeneration.

Both EGF and TGFα bind the EGF receptor. Binding of the receptor leads to autophosphorylation which exposes a recognition site for SH2 domain-containing proteins and causes phosphorylation of cellular substrates which, in turn, affect transcription. In the rat, the number of EGF receptors increases by approximately 2-fold in the first 3 hours post-PH then decreases again until day 4.[13] The majority of EGF in mouse and rat is produced in the salivary glands. Removal of the salivary glands in the mouse 2 weeks prior to PH resulted in a 50% decrease in plasma EGF concentration, abolished the increase in EGF levels post-PH, and greatly delayed peak DNA synthesis.[14] Injection of EGF restored the time course of the regenerative response. In the rat, sialoadenectomy at the time of or 3 hours post-PH decreased DNA synthesis and mitosis by 50% but did not affect the expression of the early response genes c-*jun*, c-*fos* and c-*myc* .[15] If salivary gland removal was performed 6 hours or more post-PH, no reduction in DNA synthesis was observed. Furthermore, administration of EGF from 3 to 9 hours post-PH in sialoadenectomized rats was sufficient to restore normal regenerative activity. These authors observed that overall, diminished EGF levels delayed the regeneration response to PH in the first 24 hours, but liver mass recovery in sialoadenectomized versus control rats was similar 7 days post-PH. Thus, EGF appears to affect early G_1 events in liver regeneration which occur after the priming phase from 0 to 3 hours post-PH. Jones and colleagues reported a more severe effect of salivary gland removal 24 hours pre-PH in rats.[16] They reported that DNA synthesis was inhibited by 90% 24 hours after 33% PH. Thus, it is apparent that EGF signaling during liver regeneration occurs through both autocrine and endocrine mechanisms.

TGFα mRNA levels increase 4 hours following PH and remain elevated for at least 48 hours. TGFα peptide levels increased 2-fold between 24 and 48 hours via an autocrine loop mechanism.[11] Hepatocytes produce TGFα and bind the growth factor through the EGF receptor in the plasma membrane.

Transgenic mice overexpressing TGFα demonstrate an increased rate of hepatocyte replication leading to increased liver size. This is compensated for after 3 to 5 months of life by increased cellular turnover.[11] Furthermore, by 15 months of TGFα overexpression, 85% of these mice exhibit hepatic tumors. In contrast, TGFα knockout mice develop normally except for abnormal hair growth.[17]

Hepatocyte growth factor

HGF is produced by mesenchymal cells which, in the liver, are represented by Ito, Kupffer and endothelial cells. Blood levels of HGF increase 20-fold by 1 hour post-PH.[8] However, proteolytic processing of the inactive single-chain form of HGF to the active form may occur as early as 1 minute post-PH. Active uPA is responsible for this cleavage post-PH and this protein is detected 1 minute post-PH.[18]

The importance of this growth factor is highlighted from HGF knockout mice which die during embryonic development between days 13 and 16. In these embryos, the liver is reduced in size, there is extensive loss of hepatocytes and placental development is impaired.[19,20] Homozygous deletion of the HGF receptor, c-Met, also results in embryonic lethality.[21] Overexpression of HGF in transgenic mice produced a phenotype in which liver regrowth following PH occurred 2 to 3 times faster than wild type. The resulting liver was larger than normal and contained smaller hepatocytes with diploid DNA content.[22,23] Moreover, the proliferative stimulus of prolonged HGF expression caused formation of hepatocellular adenomas and carcinomas in most transgenic mice beyond 1.5 years of age.[23] Finally, overexpression of a truncated c-Met receptor containing the regulatory and catalytic cytoplasmic domains caused a block to apoptosis and permited immortalization of these transgenic hepatocytes.[24] These results indicate that HGF may play an essential role in liver morphogenesis and that deregulated HGF signaling is oncogenic for hepatocytes.

Tumor necrosis factor-α and interleukin-6

The cytokines tumor necrosis factor (TNF)α, IL-6, and IL-10 are also expressed by nonparenchymal cells of the liver and expression for these factors increases within 3 hours post-PH.[25] Recent data strongly support a signal pathway(s) in which TNFα, IL-6 and IL-10 interact in regulating each other. EGF and IL-6 activate the transcription factor signal transducer and activator of transcription-3 (Stat3) and TNFα induces nuclear factor (NF)-κB transcription factor activation.[26-28] Mice homozygous for an IL-6 gene deletion are developmentally normal but exhibit a dramatic decrease in the number of S phase hepatocytes during liver regeneration.[29] In conjunction with decreased response in DNA synthesis, these mice demonstrate no Stat3 activation and decreased c-*fos*, *jun*B, c-*myc* and cyclin D1 expression. In contrast to the effect of IL-6 depletion on hepatocyte proliferation during liver regeneration, nonparenchymal cells exhibited DNA synthesis and gene expression patterns similar to wild type mice. One further effect of IL-6 deficiency was the development of necrotic areas in the liver. Liver regeneration does occur eventually in these IL-6 negative mice. Injection of IL-6 prior to PH restores Stat3 activation and nearly normal hepatocyte proliferation. IL-6 knockout mice exhibited increased death following PH. Specifically, 40% of knockout mice died post-PH versus 10% mortality in wild-type and 8% death in knockout mice which received an IL-6 injection pre-PH.

Treatment of rats with antibodies to TNFα prior to surgery inhibited liver regeneration after PH. The effects of antibody injection included decreased DNA synthesis 24 hours post-PH, significantly decreased serum IL-6 and diminished induction of activator protein-1 (AP-1) transcription factor activity.[30] In addition, mice deficient in type 1 TNFα receptor (TNFR-1) displayed severely impaired DNA synthesis, no detectable activation of Stat3 or NF-κB, decreased AP-1 activity and lower IL-6 levels.[31] Furthermore, 50% of these animals died between 3 and 5 days post-PH with the surviving mice demonstrating slower liver regeneration. The loss of TNFα receptors can be compensated for by injection of IL-6 30 minutes before PH. This restored DNA synthesis and the Stat3 pathway, but not NF-κB DNA binding. Normally, TNFα levels are downregulated posttranscriptionally by IL-10 and TGFβ1. Treatment of rats with gadolineum chloride (GdCl) depletes the liver of active Kupffer cells and increases TNFα and TNFα-inducible cytokines such as IL-6. After PH in GdCl treated rats, induction of IL-10 was greatly decreased, TNFα was transiently overexpressed and the regenerative response was greater than normal.[25] Since TGFβ1 expression is not significantly affected by GdCl treatment prior to PH, TGFβ1 is thought to affect TNFα downregulation in these animals.

The results of TNFα and IL-6 on liver regeneration suggest a signaling pathway in which PH induces expression of TNFα followed by activation of NF-κB which induces IL-6. IL-6 causes activation of Stat3. Activation of Stat3 and NF-κB together initiates immediate-early gene expression. Expression of both IL-10 and TGFβ1 acts to prevent TNFα overexpression. Furthermore, increased stress-activated protein kinase activity following PH might play a positive role in proliferative signaling during regeneration, possibly by stimulating IL-6 production.[32]

Growth inhibitors

Expression of the growth inhibiting cytokines TGFβ1, 2 and 3 is increased following PH.[33] TGFβ2 and TGFβ3 expression peak early post-PH, whereas TGFβ1 expression peaks around 48 hours. TGFβ isoforms are transcribed by both nonparenchymal and parenchymal cells following PH, however, hepatocytes release latent TGFβ whereas nonparenchymal cells release active TGFβ.[33] TGFβ1 is the best characterized of the three TGFβ isoforms and receptors for this molecule are present on virtually all cells. Transgenic mice in which a mature form of porcine TFGβ1, under control of the mouse albumin promoter was expressed, exhibited increased hepatocyte mitotic and apoptotic activity and hepatic fibrosis.[34] Furthermore, high plasma expression levels of the transgene negatively affected other organs, most notably the kidneys. Finally, inhibin-beta C is a recently identified TGFβ family member whose gene expression is downregulated by at least 8-fold following PH in the mouse.[35]

The Immediate-Early Growth Response

Similar to other mitogenic conditions, the regenerating liver uses multiple signal transduction pathways. Most signaling pathways begin with ligand binding to specific receptors. The event transduces a signal by mechanisms such as receptor autophosphorylation and subsequent binding of protein complexes, kinase activity of the receptor, or coupling of the receptor to other signaling systems such as G-proteins. For example, binding of receptor tyrosine kinases by the growth factors EGF, TFGα and HGF following PH are most likely key events initiating early

growth response pathways. Much of the information about signal transduction pathways has been defined in other systems and in isolated hepatocytes and will not be reviewed here. The early growth response is dependent upon immediate-early gene expression, has been extensively investigated in regenerating liver and provides the basis for the following discussion.

The primary growth response after PH consists of the transcriptional activation of immediate-early genes, which is initially accomplished through activation of latent transcription factors. By definition, immediate-early genes do not require protein synthesis for their activation. Preexisting factors within liver cells function to activate genes normally quiescent in these mature, differentiated cells. It is generally accepted that increased circulation of growth factors like those described above triggers this response. Two key transcriptional activators are Stat3 and partial hepatectomy factor (PHF)/NF-κB, a liver specific form of NF-κB.[36] Both of these DNA-binding factors are activated by phosphorylation events. Stat3 activation and nuclear translocation occur by phosphorylation of a tyrosine residue, and phosphorylation of the inhibitor protein IkBα or RL/IF-1 results in release of PHF/NF-κB. PHF/NF-κB DNA-binding activity peaks at 30 to 60 minutes post-PH, whereas Stat3 activity is induced by 30 minutes but peaks at 2 to 3 hours post-PH.

Many transcription factors are induced as immediate-early genes, resulting in a transcriptional cascade during the G_1 phase of the initial liver regeneration cell cycle. Leucine zipper transcription factors which dimerize to form AP-1 type transcriptional complexes are important participants in this induction. High levels of AP-1 DNA-binding complexes containing the proteins c-Jun, JunB, c-Fos, and the liver specific partner protein liver regeneration factor-1 (LRF-1) are detected for several hours after the G_0 to G_1 transition.[36]

Two other immediate-early genes involved in proliferative signaling include EGF and c-*myc*. The functions of EGF in liver regeneration were discussed above. The transcriptional activator protein c-Myc is a protooncogene which plays a role in both cell proliferation and cell death. The roles of two immediate-early genes, map kinase phosphatase (MKP)-1 and phosphatase of regenerating liver (PRL)-1, which encode protein tyrosine phosphatases, have yet to be delineated.[36] The discovery of novel phosphatase gene activation during liver regeneration is intriguing because of the role played by the cdc25 phosphatase family in activating cyclin/cyclin dependent kinase (cdk) complexes during cell proliferation. The parallel induction of most immediate-early genes in hepatocytes as well as nonparenchymal cells provides evidence that the exit from G_0 is simultaneous for all cells in the remnant liver.[37] Moreover, because DNA synthesis in nonparenchymal cells occurs in the second wave of cell proliferation, these data suggest a prolonged G_1 phase in nonparenchymal cells. Interestingly, activation of NF-κB and AP-1 and expression of TNFα are specific to the compensatory hyperplastic response since these effects are not observed in direct hyperplasia induced by nafenopin or cyproterone acetate.[38]

Changes in gene activity post-PH also act to maintain the liver's functions. For example, several immediate-early genes encode proteins important for glucose regulation and metabolism, thus compensating for the loss in liver mass. These genes include glucose-6-phosphatase, insulin-like growth factor binding protein-1, and phosphoenolpyruvate carboxykinase.[36] Albumin is also expressed as an immediate-early gene. Thus, the combined upregulation of immediate-early transcription factor and liver function genes allows the remnant liver to both grow and perform its myriad differentiated functions.

Progression Through Liver Regeneration

Following the transcriptionally regulated immediate-early gene response occurring from 0 to 3 hours post-PH, gene expression patterns and regulation become more complex. Work performed in Taub's laboratory demonstrated that genes are induced in three expression patterns following PH.[37] The patterns include growth regulated expression beginning at surgery through 60-72 hours post-PH, cell cycle related expression, and liver-specific gene expression after the growth phase from 60 hours post-PH and beyond. Work in the Steer laboratory has investigated mechanisms for gene regulation from all three categories. One of the main conclusions from this work is that genes induced beyond the immediate-early phase of liver regeneration are predominantly regulated at the post-transcriptional level by several different and potentially additive mechanisms. The following discussion will cover specific examples of gene regulation post-PH for genes involved in various aspects of cell proliferation and hepatocyte function.

An interesting example of alternate gene regulation following PH involves the genes for ornithine decarboxylase (ODC) and connexin 32 (Cx32). ODC is an RNA helicase belonging to the DEAD box family and is the rate limiting enzyme for polyamine biosynthesis required for maximal DNA synthetic activity.[39] ODC transcript levels increased by 3 hours post-PH and continued to increase steadily until they reached peak expression of 37-fold over nonregenerating levels at 24 hours. ODC transcript expression then steadily declined until baseline levels were again achieved by 96 hours.[40] ODC transcription rate did not change post-PH; however, the in vivo chemical half-life increased from 2.5 hours in nonregenerating liver to greater than 12 hours at 24 hours post-PH. Furthermore, at times of increased ODC mRNA stability and expression, the gene exhibited demethylation at *Hin*P1 I restriction sites and the transcript was located on heavier polysomes suggesting increased translation.[40,41] Moreover, the rate of poly(A) tail removal for

	Quiescent Liver		Regenerating Liver	
	ODC	Cx32	ODC	Cx32
Transcript Expression	↓	↑	↑	↓
Transcription Rate	—	—	No change	No change
mRNA Half-Life	2.5 h	10.9 h	>12 h 24 h post-PH	3.8 h 12 h post-PH
Relative Translation	↓	↑	↑	↓
Poly(A) Tail Shortening Rate	↑	↓	↓	↑
Genomic Methylation	↑	N.D.	↓	N.D.

Figure 2. Inverse modulation of ODC and Cx32 gene expression in the regenerating liver after 70% partial hepatectomy. "No change" in transcription rate is relative to that in the quiescent liver. In addition to ODC, increases in mRNA half-lives for *p53*, c-*myc* and H-*ras* were also associated with decreased genomic methylation suggesting a potential role for DNA methylation in posttranscriptional gene regulation. See text for details. ODC, ornithine decarboxylase; Cx32, connexin 32; N.D., not determined.

ODC transcripts was greatly increased in nonregenerating liver when the mRNA half-life was shortest compared to a time post-PH when half-life was increased.

Cx32 transcripts from the β_1 gap junction gene represent an example of complimentary regulation to that of ODC. Gap junction proteins form intercellular channels which allow cell-to-cell communication. Cx32 transcripts are abundant in nonregenerating liver and cycle in their pattern of expression post-PH showing very low levels at 12 and 48 hours but abundant expression at intervening times.[42] This pattern is consistent with those for other gap junction proteins during liver regeneration. Fladmark and colleagues proposed that the advantage to downregulating gap junctions during liver regeneration-induced cell cycling may be in maintaining separate pools of metabolites and signaling molecules between hepatocytes that are replicating and those maintaining liver-specific functions.[43] No change in Cx32 gene transcription rate was seen during liver regeneration but the mRNA half-life decreased from 10.9 to 3.8 hours concordant with decreased transcript abundance at 12 hours post-PH.[41] Furthermore, Cx32 transcripts were associated with heavier polysome fractions in nonregenerating liver suggesting greater mRNA stability. In contrast, the rate of poly(A) tail shortening increased at 12 hours post-PH consistent with decreased mRNA stability. Cx32 protein levels paralleled transcript levels but was slightly delayed.[42] These data, summarized in figure 2, illustrate that many posttranscriptional mechanisms of gene regulation are used during liver regeneration to affect the dramatic fluctuations observed in gene expression. Thus, the liver has many levels of gene regulation available for manipulation.

Cyclins and cyclin-dependent kinases

Several laboratories have investigated the cell cycle regulatory family of cyclins and cdks during liver regeneration. Cyclins form complexes with members of another family of genes, the cdks. The cyclin subunit plays a regulatory activating role and the serine/threonine protein kinase catalyzes final phosphorylation. It is apparent that most, if not all, transitions and checkpoints in the eukaryotic cell cycle involve cyclin/cdk activity. Cyclins and their partner cdks form complexes which are active at specific times during the cell cycle. Targets for cyclin/cdk kinase activity include retinoblastoma tumor suppressor protein, histone H1, E2F-1 and RNA polymerase II.[44]

At the transcript level, most of the cyclins and cdks exhibit cell cycle-dependent expression which peak at cell cycle time points in which they are known to be active.[45-47] However, the cycling pattern of transcript expression exhibited by these genes is not due to changes in transcription rate as detectable by nuclear run-on assays; and changes in mRNA stability only partially account for the transcript fluctuations observed.[45,48] Furthermore, cyclin steady-state protein levels do not consistently correlate with mRNA expression patterns.[46] For example, in contrast to transcript expression, cyclin B1 protein is readily detectable in nonregenerating liver and total liver expression level does not change appreciably during regeneration.[47] However, the steady-state expression pattern for cdk1 does occur in a cell cycle-related manner and cyclin B/cdk1 kinase activity is detected at the appropriate mitotic phase during the first wave of regeneration.[46,47] Cyclin A also is detected in resting liver and a unique tyrosine-phosphorylated form was detected during the G_2 phase of the first wave of cell proliferation following PH.[49]

p53

The *p53* tumor suppressor gene product is a critical component in cellular pathways for DNA damage-induced G_1 and G_2 arrest as well as for apoptosis. p53 is a transcription factor which binds to and activates, in a signal specific manner, various response genes including the cdk inhibitor *p21* and the apoptotis gene *bax* .[50] Mice in which the *p53* gene has been knocked out are viable but exhibit pronounced tumor formation. Steady-state mRNA and protein levels are loosely coupled for p53 during liver regeneration. The *p53* gene is induced in a delayed-early manner and transcript expression peaks at 6 hours post-PH exhibiting a 35-fold increase over nonregenerating liver.[40] *p53* transcript levels then exhibit two further peaks in expression (15- to 20-fold over baseline) 24 and 42 hours post-PH. Protein levels for p53 peak 5-fold over baseline 6 to 12 hours post-PH and 40-fold over baseline 30 hours post-PH in the rat.[51] Thus, maximal p53 protein levels in regenerating liver correlate with G_1 and mitosis in the first wave of cell division.

p53 plays a role in sensitizing hepatocytes to both growth and death signals. Quiescent hepatocytes from *p53* null mice are phenotypically normal. However, once these cells are released from G_0 by isolation for culture, a greater proportion of *p53* null cells enter DNA synthesis than wild-type hepatocytes.[52] Induction of liver regeneration by carbon tetrachloride in *p53* knockout mice also resulted in greater DNA synthetic activity. Furthermore, isolated *p53* null cells are less responsive than wild-type cells to the addition of the mitogens EGF, insulin and fetal bovine serum as well as to mitosuppressive agents such as TGFβ. In contrast, the livers of *p53* null mice were more responsive in vivo to a nongenotoxic mitogen.

p53 plays an integral role in G_1 and G_2 checkpoint arrest in gamma-irradiated hepatocytes. Irradiation of mice 48 hours after carbon tetrachloride-induced liver regeneration (late G_1) resulted in reduced hepatocyte G_1/S arrest in *p53* null versus wild-type mice.[52] This was followed by a striking rise in the mitotic index 24 hours after irradiation, indicating no significant G_2 arrest in response to DNA damage. However, many of the mitotic figures were abnormal, suggesting that the mitotic peak resulted from damaged hepatocytes proceeding to and arresting in mitosis. p53 appears to be important in the regulation of proliferating, but not quiescent, hepatocytes.

p21

p21 is a member of the cdk inhibitor (CKI) family of genes. The CKI proteins can inhibit cyclin/cdk kinase activity by binding to either the complex or the cdk alone. As mentioned previously, the *p21* gene contains p53 binding sites and the p21 protein product is hypothesized to be a key downstream mediator of p53 regulatory pathways. In correspondence with this notion, *p21* is induced by many of the same cellular signals which induce *p53*. Alternately, *p21* also is induced during cellular senescence[44]. Albrecht and colleagues observed that *p21* transcripts were barely detectable in mouse and rat liver pre-PH but the abundance increased by 1 hour post-PH.[53] Transcript levels decreased at the onset of DNA synthesis in both rodents then increased again. Dietary protein deprivation also resulted in increased *p21* mRNA expression. Moreover, the same pattern of *p21* induction was exhibited in both *p53* null and wild-type mice post-PH. However, following protein deprivation, the increase in *p21* transcript expression after PH was p53-dependent. *p21* expression was induced in cycloheximide pre-treated mice subjected to PH, defining *p21* as an immediate-early growth

response gene. *p21* transcript expression post-PH is predominantly regulated at the posttranscriptional level, which suggests why the *p21* gene response in PH-stimulated regenerating liver is independent of p53.[53]

A liver specific minigene construct was used to create transgenic mice overexpressing p21 in the liver.[54] Endogenous p21 was undetectable in the liver by two methods whereas the transgenic livers expressed readily detectable p21 in the hepatocytes. Transgenic mice exhibited decreased liver mass by 49 to 62%, overall stunted growth and a shorter lifespan. Furthermore, there were fewer hepatocytes, more nonparenchymal cells than normally observed and an abundance of oval cells. This is consistent with the notion, stated earlier, that if hepatocytes are impaired from proliferating, oval cell replication will increase. The majority of cyclin D1 and cdk4 proteins in hepatocytes were found complexed with p21 which indicates that phosphotransfer activity is inhibited for this kinase. No detectable increase in apoptosis was observed. Following PH in the p21 overexpressing mice, DNA synthesis was less than 15% of normal values and occurred mainly in oval cells. Furthermore, no mitoses were observed indicating a possible G_2 block as well. Thus, p21 overexpression results in a dominant negative effect in the normal regenerative response to PH. Additionally, mice lacking p21 develop normally but exhibit defective G_1 arrest in response to DNA damage or nucleotide pool perturbation.[55]

Apoptosis in the Liver

Similar to other cells in culture, hepatocytes demonstrate signal-specific responses to apoptotic stimuli. Several gene products have been identified which have an antiapoptotic effect. These proteins include the retinoblastoma (Rb) tumor supressor gene product and the apoptosis-associated gene products, Bcl-2 and Bcl-X_L. Rb is a key regulatory protein for the progression from G_1 to S phase of the cell cycle and also plays a role in development.[56] The functions of Rb are controlled through fluctuations in the protein's phosphorylation status. Hypophosphorylation inhibits, whereas hyperphosphorylation allows, entrance into S-phase and cell cycle progression. Loss of functional Rb in the cell results in deregulation of transcriptional activity and either tumorigenesis or apoptosis. Mice lacking the *Rb* gene die during embryogenesis and demonstrate increased cell division and apoptotic cell death in the hematopoietic and nervous systems, liver, and skeletal muscle precursor cells.

Rb appears to play a protective role against apoptosis in hepatocytes. Rb protein expression peaks at 12, 30, and 72 hours post-PH in the rat, with expression at 30 hours representing a greater than 100-fold increase over nonregenerating Rb abundance.[51] *Rb* transcript expression is uncoupled from that of the protein.[57] TGFβ1 treatment inhibited *Rb* gene expression and protein phosphorylation in culture, while Rb protein expression was inhibited in regenerating liver.[51,58] In vitro, this TGFβ1 induced reduction in Rb protein abundance and phosphorylation resulted in apoptosis in both primary rat hepatocytes and in HuH-7 human hepatoma cells.[58] Furthermore, depletion of Rb protein expression by antisense oligonucleotide also resulted in hepatocyte death. Overexpression of *Rb* inhibited the apoptosis of hepatic cells induced by TGFβ1, *Rb* antisense and REC2 (DNA recombinase).[51,58,59]

The *bcl*-2 gene family members *bcl*-2 and *bcl*-X_L also act to protect cells from apoptosis. In fact, overexpression of either of these genes can block apoptosis from numerous stimuli.[60] *Bcl*-2 and *bcl*-X_L gene expression occurs early during regeneration with maximal expression 6 hours following PH.[61] *Bcl*-2 transcripts are expressed by nonparenchymal cells and exhibit less than 2-fold induction whereas *bcl*-X_L transcripts are expressed by hepatocytes and exhibit greater than 20-fold induction. Bcl-2 and Bcl-X_L protein levels do not fluctuate significantly during regeneration. Coupled *bcl*-X_L transcript and protein expression occur in a delayed-early response pattern following PH in the mouse.[62] Most likely, the functions performed by Bcl-2 in other cell types are fulfilled by Bcl-X_L in hepatocytes. HGF is reported to suppress epithelial cell apoptosis, and interestingly, BAG-1 (an anti-apoptotic protein) associates with the c-Met receptor for HGF, thus linking HGF antiapoptotic effects with the survival branch of the *bcl*-2 family.[63]

Other members of the *bcl*-2 family are proapoptotic in function. These gene products include Bax, Bad, and Bak. Bax homodimers promote cell death, while bax heterodimers formed with Bcl-2 or Bcl-X_L do not. Bad and Bak can also bind to Bcl-2 and Bcl-X_L, thus promoting the formation of Bax homodimers and cell death. *Bax* transcript and protein abundance increase following PH in the rat in a stepwise fashion.[61] The Bax protein is most abundant in regenerating liver during the period when proliferation has predominantly ceased and apoptosis-mediated reorganization of the liver is occurring. Similarly, Bax protein abundance increases following withdrawal of the drug clofibrate, an inducer of direct hyperplasia, and is associated with the increased apoptosis of extraneous hepatocytes.[61]

p53 is a key cellular status sensor which can halt the cell proliferation machinery in order to effect repairs, but can also induce apoptosis. Cultured *p53* null hepatocytes are able to survive and proliferate under conditions in which wild-type cells cease to proliferate and undergo apoptosis.[52] However, these cells were competent to enter into p53-independent apoptosis following ultraviolet irradiation. Liver cells can also undergo Fas ligand/ receptor mediated cell death. Hep 3B human hepatoma cells, which lack *p53*, can still undergo apoptosis in response to apoptotic stimuli.[58,59]

Remodeling of Regenerated Liver and Extracellular Matrix

Most studies on liver regeneration focus on proliferation. However, restoration of liver mass and function also requires regulation of apoptosis and extracellular matrix (ECM) to effect the remodeling of parenchymal and nonparenchymal cells into functional units. Remodeling occurs during development, tissue repair and regeneration. It requires coordination of matrix deposition, matrix degradation, cell proliferation and apoptosis. The hepatocyte is the only epithelial cell in the body not separated from the vascular space by two continuous basement membranes, which may allow rapid exchange of components between the plasma and hepatocytes.[64] Actually, hepatic failure due to cirrhosis results, in great part, from formation of basement membrane between hepatocytes and the vascular space.

The processes of cell division and subsequent hepatic remodeling require using preexisting ECM. It is intuitive that some degradation would take place to facilitate movement. Activation of uPA occurs within 5 minutes post-PH and this, in turn, is proposed to initiate a proteolytic cascade resulting in hepatic matrix degradation and release of active HGF.[8] Four days post-PH, mitotic activity has ceased and hepatocytes exist as clusters of 10 to 14 cells which lack sinusoids and ECM.[64]

Furthermore, by this point the cell/ECM ratio has greatly increased. At this time, laminin-positive Ito cells become detectable and appear to extend processes to invade the hepatocyte clusters. Fenestrated endothelial cells then penetrate the clusters and separate the hepatocytes into cell plates, thus restoring normal hepatic vascular structure. Once this is accomplished, laminin production ceases. Along with hepatic plates, the biliary tree must also regenerate in the hepatectomized liver. Intrahepatic bile duct epithelial cells, or cholangiocytes, line the intrahepatic biliary tree and function to modify bile. In these cells, DNA synthesis increased by 1 day, peaked on day 3 and returned to control values by 28 days post-PH.[65] By 10 days post-PH, the normal distribution of ECM and regrowth of the biliary tree are complete.

Unanswered Questions and Perspectives

There are several key questions which remain unanswered about liver regeneration. First, what are the critical signals occurring within minutes of surgery which catalyze the entire regenerative process? It is unlikely that a single master signal exists and we now know that even a change in membrane potential is not a critical event.[66] Following PH, blood flow to the remnant liver increases 3-fold and is probably responsible for delivering necessary signaling agents for regeneration. In this regard, studies performed almost half a century ago revealed that when two rats are joined parabiotically, PH of one rat causes proliferation in the intact liver of the other. Furthermore, regeneration proceeds in a periportal to pericentral pattern in the direction of portal blood flow within the remnant lobes. Second, how do liver cells maintain their differentiated functions and yet exit quiescence to begin proliferating? Third, how does the liver sense when the optimal mass has been achieved and stop hepatocyte proliferation? Many genes, such as protooncogenes and tumor suppressor genes, appear to be involved in multiple cellular processes, including cell proliferation, development and apoptosis. While this seems antithetical, it also reflects an economy of function. A change in the intricate balance between signals for proliferation and apoptosis can be swiftly acted upon by key proteins capable of functioning in either pathway.

The ultimate goal of regeneration research is to develop therapeutics directed to replacing damaged tissues in vivo. In most vertebrates, the capacity for regeneration is limited to a few tissues, such as bone, skeletal muscle and, of course, liver. For bone and muscle, regeneration in some ways recapitulates embryonic differentiation from stem cells. In contrast, it is widely accepted that the liver is able to regenerate without activation of stem cells. But, we also know that hepatocytes can undergo partial dedifferentiation, allowing them to reenter the cell cycle while maintaining critical differentiated functions. The liver is not a salamander or a newt, but it certainly exhibits some remarkable regenerative properties. The approach of regenerative biology is to identify the cellular and molecular differences that distinguish tissue embryogenesis from wound repair and then to recreate an embryonic regenerative environment in an injured tissue. In fact, why do tissues scar rather than regenerate? They may even contain cells competent to undergo replication but lack stimulatory signals to effect regeneration. On the other hand, they may be receptive to signals that suppress regeneration and promote scarring. It seems that most tissues appear to lack stem cells required for regeneration. However, is it conceivable that they simply lie dormant with all the machinery necessary for regeneration—waiting for just the right combination of stimuli? Perhaps we should take a closer look at livers and urodeles and learn from them how to induce regeneration by dedifferentiation. In the future, we may ultimately develop the technology to regenerate vital organs. The fun and excitement will be in getting there!

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