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LIVER REGENERATION

Anna Mae Diehl

Johns Hopkins University, Baltimore, MD

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1. ABSTRACT

Unlike other vital organs, the liver typically regenerates after injury. Indeed, the very factors that cause liver injury initiate a reparative process in the residual liver that includes the induction of cytoprotective mechanisms, deletion of mortally wounded cells, repair of less damaged survivors, liver cell proliferation to replace the cells that died, the deposition of new matrix, and tissue remodeling to restore normal hepatic mass and architecture. During liver regeneration, the liver normally continues to perform vital, liver-specific functions. Unfortunately, the hepatic regenerative response sometimes becomes disrupted - either failing to occur or occurring in a disordered, or incomplete fashion. Abnormal regeneration contributes to the pathogenesis of fulminate liver failure, cirrhosis, and primary liver cancers. Research in the field of regenerative biology has identified several events that are required for liver regeneration. These include injury-induced changes in the hepatic microenvironment, the ability of surviving liver cells and/or their progenitors to proliferate, and a temporary suspension of homeostatic mechanisms that

normally couple cell proliferation to programmed cell death. The signals that mediate these complex biologic responses are being detailed. A better understanding of the extra- and intracellular signals that prompt the injured liver to regenerate should suggest treatments to promote liver regeneration in patients with otherwise fatal liver diseases.

2. INTRODUCTION

Liver regeneration is a remarkable process that allows complete restoration of hepatic architecture and tissue specific function after many different types of liver injury (1). Liver regeneration occurs when liver cells proliferate to replace other liver cells that have died. The enormous regenerative capacity of the adult liver distinguishes the liver from other vital organs, which have a much more limited ability to regenerate. In addition to its role during recovery from injury, liver regeneration is required to maintain the day-to-day survival of the organ because, even in healthy adults, some liver cells undergo programmed cell death, i.e., apoptosis, every day. If these cells were not replaced, the liver would gradually atrophy. Liver size remains constant because dead cells are replaced by proliferation of some of the remaining cells. Ultimately, liver mass is determined by the relative rates of cell proliferation and cell death. When the liver is injured, the rate of liver cell death increases as liver cells are killed by necrosis and/or apoptosis. This injury triggers a proliferative response in the surviving cells, permitting the organ to regenerate and allowing eventual recovery to normal health. During regeneration, restoration of tissue integrity requires the balanced reconstitution of several different liver cell populations (e.g., hepatocytes, biliary epithelial cells, endothelial cells, stellate cells, lymphocytes and macrophages), as well as the hepatic matrix that provides their scaffolding. Over the past three decades, much has been learned about the mechanisms that regulate liver regeneration. This knowledge has suggested new therapeutic strategies that may benefit patients with a wide variety of liver diseases.

3. GENERAL MECHANISMS THAT REGULATE HEPATOCYTE MASS

3.1. In Healthy Adult Livers

3.1.1. Continuous rejuvenation by hepatic progenitors

The role of hepatic progenitor (i.e., stem) cells in maintaining healthy liver mass has been the subject of much debate. Controversy rages because unique markers for liver stem cells have not been defined yet (2), limiting conclusive identification of this compartment, which is generally believed to include small, oval cells that exist along the canals of Herring (3). This oval cell population is probably heterogeneous, containing a relatively large number of cells that are committed to the liver lineage but which can differentiate into either hepatocytes or biliary epithelial cells, and a much smaller sub-population of other cells that are more pluripotent, including a tiny number of true stem cells that are capable of differentiating along either an epithelial (e.g., hepatic or pancreatic) or hematopoietic lineage (4). Because healthy adult livers have a very low proliferative index (i.e., < 1:1000 hepatocytes are replicating their DNA at any point in time) (5), it is believed that relatively few progenitor cells reside there. However, recent evidence that stem cells derived from adult bone marrows localize within the livers of adult recipients (6) and differentiate into hepatocytes (7) demonstrates that hepatic progenitor cells persist in adulthood These cells arise in, or circulate through, the bone marrow (8), suggesting that the tiny population of stem cells that exists in adult livers is probably replenished continuously from a larger boned marrow reservoir of stem cells. However, the signals that recruit stem cells to the adult liver and the factors that instruct their expansion and subsequent differentiation are poorly understood.

3.1.2. Replication of mature hepatocytes

The low proliferative index of mature hepatocytes matches the low prevalence of hepatocyte apoptosis in healthy adult livers. During health, there is a gradient of hepatocyte DNA synthesis across the liver lobule, with the highest proliferative index occurring in peri-portal hepatocytes and the lowest index occurring in hepatocytes near terminal hepatic venules (1, 5). Some investigators propose that the progressive decline of proliferative activity in aging progeny from peri-portal stem cells drives the portal-to-central gradient of hepatocyte DNA synthesis (9-12). Others argue that the portal-to-central gradient of soluble, gut-derived hepatotrophic factors is predominately responsible for the zonal distribution of proliferation in mature hepatocytes (13, 14). Ongoing debate is fueled by recent evidence that hepatic progenitors may be distributed throughout the liver lobule, rather than merely clustered peri-portally (15, 16). In addition, the marrow-like environment of the spleen may provide a reservoir for hepatic progenitors (13), providing a steady supply of precursor cells that can be carried into the liver from the spleen with the portal blood flow. In any case, the importance of portal venous factors (soluble or cellular) as regulators of day-to-day hepatocyte proliferative activity is supported by evidence that the liver atrophies when deprived of portal blood flow (14, 17, 18). In addition, hepatocyte proliferation normally fluctuates over the course of the day and is highest post-prandially, when portal venous blood flow to the liver increases (19, 20).

3.2. During Recovery from Injury

3.2.1. Hyperplasia of mature hepatocytes

Because DNA synthesis and mitoses increase dramatically in mature-appearing (i.e., differentiated) adult hepatocytes after the liver has been injured by 70% partial hepatectomy (PH) or various hepatotoxins, it is thought that adult hepatocytes can proliferate when they are "challenged" to do so (1). However, despite decades of study, the ultimate proliferative capacity of mature hepatocytes remains unclear. As mentioned earlier, most mature hepatocytes in healthy livers are not actively synthesizing DNA. Indeed, extensive analyses of growth-related gene expression in adult livers demonstrates that most adult hepatocytes have exited the proliferative phases of the cell cycle and spend most of their lives in a growth-arrested (G-0) state. At issue is whether or not these cells can re-enter the cell cycle and replicate.

Various approaches, including the isolation and culture of primary adult hepatocytes, have been used to

answer this question. When cultured in the presence of growth factors, adult hepatocytes up-regulate pre-replicative genes, such as c-jun, and synthesize DNA, but proliferate poorly, if at all (21). However, it is possible that the poor replicative activity of hepatocytes in vitro is merely an artifact of arduous isolation protocols or sub-optimal culture conditions (22-26). Indeed, other experimental approaches, such as transplantation experiments with rat hepatocytes, have generated support for the concept that mature hepatocytes retain a considerable capacity for proliferation in vivo. In one study, labeled hepatocytes isolated from adult donor livers were transplanted directly into one lobe of a recipient rat's liver after ligating portal vein branches to the other lobes [27]

Deprived of portal blood flow, those segments involuted but eventually, the entire recipient liver mass was repopulated by donor hepatocytes. Based on the number of hepatocytes that were transplanted, the authors concluded that a single hepatocyte can divide at least 34 times, giving rise to 1.7 x 10(10) daughter cells. This implies that a single rat hepatocyte has the clonogenic potential to generate 50 adult rat livers. However, it is difficult to exclude the possibility that the donor liver cells used in these studies might have included one or two pluripotent progenitor (i.e., stem) cells.

Perhaps the oldest and most widely cited argument for the proliferative capability of mature hepatocytes is the adult liver's ability to regenerate completely after PH (28). Indeed, the ancient Greek myth about Prometheus revolves around the liver's ability to regenerate after pieces of liver have been removed. Post-PH regeneration has immediate clinical relevance to the recovery from hepatic resection and, hence, to liver trauma, cancer surgery, split-liver transplantation and living donor hepatic transplantation. By studying the process of liver regeneration after PH in rodents, much has been learned about the mechanisms that are likely to regulate normal liver regeneration in humans. Thus, this information will be discussed in detail subsequently.

3.2.2. Stem cell hyperplasia

Within the last 5 years, several studies have shown that hepatic progenitors persist in adults and some have demonstrated that these cells regenerate the injured liver under certain circumstances. For example, components of the normally-tiny oval cell compartment (i.e., the resident hepatic progenitor cells) expand and re-populate the remnant liver when animals are subjected to PH following pre-treatment with drugs that inhibit the proliferation of more mature hepatocytes (29-31). This finding suggests that the regenerative capacity of hepatic progenitor cells is enormous, even in adults. More definitive proof for this concept was recently published by Grompe's group who were able to completely regenerate the lethally damaged livers of FAH knockout mice (a model for tyrosinemia) by transplanting bone marrow-derived stem cells from healthy wild type donors (7). These results have revolutionized thinking among liver regeneration researchers and opened new areas for investigation. Important issues that must be addressed include the role of endogenous stem cells in chronic liver diseases and the utility of stem cell transplantation as a therapy for various acute and chronic forms of liver injury.

3.2.3. Hepatocyte Hypertrophy

Hepatocytes enlarge (i.e., hypertrophy) in many liver diseases. A recent report from Thorgeirsson's group suggests that hepatic hypertrophy may occur as a compensatory mechanism to restore normal liver mass when hepatocyte proliferation is inhibited. Rats that underwent PH after being treated with dexamethasone or 5-fluorouracil to inhibit hepatocyte proliferation were able to recover liver mass without inducing hepatic DNA synthesis. The restoration of liver mass was accomplished by the preferential enlargement/hypertrophy of the peri-portal hepatocytes. The hypertrophied state was not stable, because once the DNA synthesis inhibitors were withdrawn, the enlarged hepatocytes entered the cell cycle and normal liver structure and DNA content were re-established (32).

4. ANIMAL MODELS OF LIVER REGENERATION

Regeneration is important for recovery from a wide variety of liver injuries. To design therapies to optimize hepatic regeneration, it is necessary to clarify the mechanisms that regulate this complex process. Work in cell culture systems has been helpful in delineating the signal transduction pathways of several hepatocyte mitogens. However, because, as mentioned earlier, primary cultured hepatocytes have limited replicative ability and precisely coordinated responses among various liver cell populations are necessary for liver regeneration, in vitro studies must be complemented by work in animal models. Because cellular proliferation occurs very infrequently in the healthy liver, these animal models injure the liver to provoke a regenerative response that can be studied. Two general strategies (partial liver resection or toxin-induced liver injury) have been used to induced hepatic regeneration.

4.1. Partial hepatectomy

Two -thirds partial hepatectomy (PH) in mice and rats has been a particularly useful model for defining events that regulate the proliferation of mature hepatocytes because it provides a unique opportunity to evaluate mammalian cell cycle progression in vivo. Because virtually all hepatocytes are in G-0 at the time of PH and PH is a temporally circumscribed "insult", remaining hepatocytes enter the pre-replicative phase of the cell cycle (G-1) relatively synchronously. To monitor the kinetics of DNA synthesis following PH, rats were partially hepatectomized and killed at various times during the first day and after 1, 2, 3, 7, and 14 days post-PH. One hour before killing, each rat was injected intraperitoneally with tritiated thymidine or bromo-deoxyuridine to label newly synthesized DNA and autoradiography or immunohistochemistry was done to calculate the hepatocyte labelling index (33, 34). These studies demonstrate that DNA synthesis (S phase) in mature hepatocytes begins at around 24 hours and the labeling index reaches its peak value of 3-4% on day 3 post-PH.

This represents a 26-fold (2600 %) increase in the hepatocyte labeling index over basal, pre-PH values. During the same time hepatocytes double their ploidy, suggesting that half of the observed labeling index increase is directed to DNA accumulation and half to cell division. If this interpretation is valid, then the hepatocyte production rate increases 13 fold during the initial 72 h after PH. However, careful morphometry of regenerating livers suggests that hepatocyte proliferation may not be this robust because the hepatic acini increase in size by only about 15% during this time period. This is not sufficient to accommodate the number of hepatocytes that would be generated by an acute 1300% increase in proliferative activity (33). This finding suggests that much of the acute post-PH increase in hepatocyte labeling index occurs because damaged hepatocyte DNA is being repaired. Never-the-less, the initial 72 hours after PH offers a "window" to scrutinize events that regulate G-0 to G-1 and G-1 to S phase transition in adult hepatocytes. Indeed, it may be the only system available to study G-0 to G-1 phase transition in normal hepatocytes, because virtually all freshly isolated and cultured primary or neoplastic hepatocytes have already entered G-1 (1). Traditionally, the PH model is considered to be a "pure" model of regeneration because the remnant liver lobes are not directly manipulated (i.e., injured) at the time of PH (28). Indeed, the dearth of overt injury has prompted some concern that the PH model may not reliably mimic events that regulate hepatocyte proliferation after toxic or infectious liver injury (1). However, serum aminotransferase levels usually increase in healthy rats and mice after PH. Also, careful inspection of liver sections in these animals typically reveals rare scattered foci of swollen hepatocytes that are variably associated with hemorrhage and small cell infiltrates (35-37). Moreover, sections that have been stained to detect apoptosis demonstrate increased apoptotic bodies during the first hour following PH (38). Despite early post-PH liver damage, the liver goes on to regenerate the resected mass within 10-14 days (1).

4.2. Toxin-induced liver injury

Regeneration can also be studied after liver injury has been caused by various hepatotoxins (e.g., d-galactosamine, carbon tetrachloride, thioacetamide, acetaminophen) (39). This strategy mimics a common clinical problem (i.e., toxic liver injury). Thus, results obtained in this model system are likely to be clinically relevant to large groups of patients. However, ongoing liver cell injury may make it difficult to differentiate mechanisms that promote hepatocyte proliferation from those that lead to heaptocyte death. In addition, because toxicity generally occurs over hours to days, the compensatory hepatocyte proliferative response is not synchronous. Hence at any given point in time, proliferating hepatocytes are likely to be in different stages of the cell cycle, confounding data interpretation. Finally, in many types of toxic liver injury, inflammatory cells infiltrate the liver, and it may be difficult to distinguish responses occurring in these cells from those that are occurring in resident liver cell populations (40). Given the complexity of toxin-induced models of hepatocyte proliferation, this review focuses on information that has been obtained by studying the more simplistic PH model.

5. REGULATION OF REGENERATION AFTER PH

5.1. Initiation of regeneration (G-1 phase of the cell cycle)

5.1.1. Injurious Factor

All types of liver injury challenge surviving hepatocytes to proliferate. In general terms, regeneration from liver injury begins because some injury related-factors initiate a proliferative response in the surviving cells. Ironically, this “initiator” function is performed by specific molecules that can also mediate cell death. For example, the injury related cytokine, tumor necrosis factor alpha (TNFα) and some of its intracellular products, reactive oxygen species (ROS), are necessary to push quiescent (G-0) hepatocytes into the early pre-replicative phase of the cell cycle (i.e., G-1) after PH. Proof of the fact that these potentially toxic molecules initiate the regenerative response has been provided by studies of experimental animals that were pre-treated with neutralizing antibodies to TNFα or which were genetically manipulated so that they did not express type 1 TNF receptors, which contain cytosolic "death" domains. PH is not followed by the usual burst of ROS production by hepatic mitochondria and liver regeneration fails to occur in such animals (38, 41, 42). However, disruption of type 2 TNF receptors, which lack these "death" domains, has no effect on liver regeneration (43).

5.1.2. Oxidant Generation and Protective Adaptations

It remains poorly understood why activation of type 1 TNF receptors (which are capable of initiating apoptosis) and increased mitochondrial oxidant release (which can damage cellular DNA and other vital components) (44) culminate in regeneration, rather than death, after PH. TNF α kills cultured adult hepatocytes only when protein synthesis has been inhibited, suggesting that these cells typically induce various protective mechanisms that block death signals that are initiated by TNF-TNF receptor 1 interaction (45). Apparently, activation of similar cytoprotective mechanisms occurs in regenerating hepatocytes and this is sufficient to prevent massive hepatocyte lethality after PH. In support of this concept, the activity of the anti-apoptotic transcription factor NF-kB increases in a TNF-dependent manner within 15 minutes after PH (42, 46). Pre-treatment of mice with adenoviral vectors for IKappaB prevents NF-kB activation and causes massive liver cell apoptosis after PH (47). Presumably, NF-kB induction protects hepatocytes by activating the transcription of a number of cytoprotective genes. To date, one of these, inducible nitric oxide synthase (iNOS), has been identified. Nitric oxide, the product of iNOS, inhibits TNF-dependent activation of caspases and is known to protect hepatocytes from TNF-mediated apoptosis (48-50). In normal mice, iNOS is induced in hepatocytes during the middle of the pre-replicative period following PH (51). Although iNOS-deficient mice do not have obviously abnormal livers before PH, PH induces massive liver cell apoptosis and liver failure, rather than hepatocyte proliferation, in these

animals (52). Together with evidence that several other stress-activated genes, including manganese superoxide dismutase and bcl-xL, are induced during the pre-replicative period following PH (53), this finding suggests that regeneration succeeds when hepatocytes survive oxidative stress and escape apoptosis. This finding is particularly intriguing in light of evidence that hepatogenesis requires similar protection during embryonic development.

5.1.3 Preservation of Differentated Functions

As hepatocytes enter G-1 in response to TNF and TNF-regulated cytokines, such as interleukin-6 (IL-6), that are induced by PH (41), hepatocyte gene expression is re-programmed to meet the unique demands imposed by the stress of liver injury. In addition to up-regulating genes that protect hepatocytes themselves from oxidants and apoptosis, hepatocytes modify their expression of other genes that are involved in the generalized acute phase response (54-56). This occurs because TNF and TNF-inducible cytokines regulate transcription factors, such as CCAAT/enhancer binding protein (C/EBP) isoforms, that modulate the expression of many liver-specific genes (57-62). These C/EBP isoforms also regulate cell proliferation. C/EBP α, a growth arrest gene (63, 64), is constitutively expressed in mature hepatocytes (65). TNF inhibits C/EBP α (66). C/EBP β (also known as liver activating protein (LAP) or nuclear factor IL-6 (NF-IL-6)), a growth-permissive C/EBP isoform, is weakly expressed by hepatocytes in the healthy liver but is one of the major transcription factors that regulate acute-phase gene expression by hepatocytes during inflammation (67). C/EBP β is induced by IL-6. During inflammation, a third C/EBP isoform, C/EBP delta, is also induced in hepatocytes (59).

5.1.4. Release from Replicative Quiescence

Reciprocal changes in C/EBP isoform expression and activity are thought to regulate cell cycle progression, although the mechanisms involved are not completely understood. Shortly after PH, the DNA binding activity of C/EBPα transiently declines, whereas that of C/EBPβ and C/EBP delta transiently increases. Pretreatment with anti-TNF antibodies before PH inhibits these differential variations in the C/EBPs (68), indicating that TNF plays a critical role in regulating these changes during liver regeneration. Other studies show that TNF modulates C/EBP function indirectly by inducing IL-6 (41) and also directly by interacting with its receptors on hepatocytes to rapidly modulate the DNA-binding activity of various C/EBPs (69). These cytokine-induced changes in C/EBP activity help to rapidly reprogram hepatocyte gene expression to meet regenerative demands. Both C/EBPα and C/EBPβ also operate at post-transcriptional levels to modulate hepatocyte progression through the pre-replicative (G1) period. For example, C/EBPα physically interacts with p21 protein. This interaction stabilizes p21 which functions as a potent cell cycle inhibitor in hepatocytes (70, 71). Therefore, the down regulation of constitutively expressed growth-arrest C/EBP isoforms, such as C/EPBα, permits liver cell proliferation (72-74). Meanwhile the induction of LIP, a truncated translation products of the C/EBPβ gene, further inhibits interactions between C/EBPα and p21, thereby promoting p21 degradation and cell cycle progression (71). The up regulation of "growth-permissive" C/EBPβ isoforms that can substitute for C/EBPα to trans-activate C/EBP-regulated genes also preserves differentiated, liver-specific gene expression as the liver regenerates (73). This response is essential, as demonstrated by evidence that liver regeneration and survival after PH are inhibited significantly in C/EBPβ knockout mice (75). This discussion illustrates the complex molecular mechanisms that balance requirements for increased cellular proliferation with the necessity of maintaining liver-specific functions as the adult liver regenerates.

5.2. Replication of Genetic Material (S phase)

Cells that enter into the pre-replicative phase of the cell cycle do not always progress into S phase, where DNA replication occurs. In fact, a number of mechanisms exist to arrest cell cycle progression at the boundary between G-1 and S phase in cells that have suffered serious oxidative damage (76). Even in non-damaged cells, progression into replicative phases of the cells cycle (i.e., S phase) requires signals in addition to those that are directly generated by the injury-related cytokines that initiate entry into G-1.

5.2.1. Mitogenic Factors

Factors that help G-1 hepatocytes to progress into S phase have been isolated from the serum of animals that have undergone PH. Some of these (e.g., EGF, transforming growth factor alpha (TGF α)), hepatocyte growth factor (HGF)) are considered complete mitogens because they stimulate DNA synthesis when added to serum-free primary hepatocyte cultures. Others (e.g., insulin, glucagon, norepinephrine, hepatotrophic stimulator substance (HSS)), termed comitogens, can only enhance mitogen-induced DNA synthesis under these conditions but are incapable of promoting synthesis in the absence of mitogens. Still other factors (members of the TGF β/activan superfamily) inhibit mitogen-induced DNA synthesis in cultured hepatocytes (39).

Although all these factors were isolated after PH, it has been difficult to prioritize their importance as mediators of liver regeneration. For example, in one study, pretreatment of rats with neutralizing antibodies to EGF failed to inhibit liver regeneration after PH (77). Other experiments showed that transgenic mice with targeted over expression of TGF alpha in the liver did not have supra-maximal liver regeneration after PH (78, 79) (44-45). Subsequent work demonstrated that the livers of TGF alpha-null mice regenerate after PH (80). One possible explanation for these results is that there are redundant mechanisms to ensure hepatocyte proliferation after PH. Indeed, both EGF and TGF α bind to the same hepatocyte receptor, and so, these mitogens can probably substitute for each other (80). It has not yet been possible to clarify the role of HGF after PH because knockout mice with homozygous deletions of genes that encode either HGF or its receptor, c-Met, show arrested hepatic development and die as embryos (81, 82). Direct intraportal injections of

large doses of HGF caused only a slight increase in hepatocyte DNA synthesis in healthy rats (83). However, tail vein injection of large volumes (1 ml) of HGF expressing plasmid DNA into healthy mice (which normally have blood volumes of < 3 ml) resulted in sustained induction of hepatic HGF expression, increased hepatocyte DNA synthesis and hepatomegaly (84). The acute volume overload induced by this experimental approach causes temporary vascular congestion within the liver and the associated liver injury might have contributed to HGF's proliferative actions. In support of this possibility, when infusion of other mitogens is preceded by even a minor hepatic insult (e.g., infusion of collagenase, one-third hepatectomy), dramatic increases in hepatocyte DNA synthesis ensue (85). Presumably, injury-related signals also modulate the actions of co-mitogens, such as insulin and adrenergic hormones, because these factors do not cause healthy hepatocytes to proliferate (39). However, liver regeneration after PH is inhibited in insulin-deficient rats (86) and in rats treated with inhibitors of alpha- or beta-adrenergic receptors (87). Thus, hormones that regulate metabolic and stress responses do play some role in regulating the regenerative response to PH. Taken together, the previous results demonstrate that mitogens and co-mitogens are most effective when introduced to hepatocytes that have been "primed" (i.e., sensitized) to accept proliferative signals because of antecedant, injury-related events.

5.2.2. Mitogenic Signaling

Given the difficulty of dissecting the hepatotrophic effects of various mitogens and comitogens in intact animal models of liver regeneration, most of the knowledge about their signaling pathways has been obtained by working in vitro. By studying cultured cells in tightly controlled conditions, much has been learned about the cellular mechanisms that promote cellular proliferation in response to mitogens and comitogens. In various types of cells, mitogenic factors promote proliferation by interacting with specific receptors on the plasma membrane. Most mitogens and comitogens activate receptors with intrinsic tyrosine kinase activity (i.e., receptor tyrosine kinases (RTKs)). Autophosphorylation of tyrosine residues in the receptor's cytosolic domain recruits cytosolic adaptor proteins that couple the receptor to various enzymes that generate different effector molecules. Receptors for some comitogens (e.g., glucagon, norepineprine) lack intrinsic tyrosine kinase activity but can couple to RTKs or to cytosolic tyrosine kinases that permit activation of some of the same effector cascades. These effector cascades generally involve a series of phosphorylation reactions that are mediated by protein kinases, which are, themselves, activated by phosphorylation. Tyrosine kinase activation of Ras, Raf, and downstream serine-threonine kinases, including the mitogen-activated kinases (MAPKs), has been shown to be particularly important in promoting proliferation because mutations that permit constitutive activation of this cascade generally result in neoplastic transformation in hepatocytes (reviewed in (88)).

Although several cytosolic and nuclear targets of MAPKs have been identified, transcription factors that are phosphorylated by MAPKs are probably the critical downstream, growth-regulatory molecules because mutations that constitutively activate these target nuclear proteins are also transforming (89). MAPK-induced transcription factors promote cellular proliferation by increasing the expression of various genes, including those that encode transcription factors and other proteins that regulate progression through later stages of the cell cycle. Somewhat paradoxical is recent evidence that sustained (i.e., "hyper") phosphorylation of MAPKs inhibits cellular proliferation. At least one mechanism involved has been identified: prolonged activation of MAPK leads to prolonged activation of C/EBPβ, a MAPK target, which then causes excessive transcription of the growth inhibitor, p21 (90-92).

Mitogens also activate many other kinases, including the stress-activated kinase cascade (SAPK), protein kinase C and protein kinase B (Akt) that modulate cellular sensitivity to apoptosis (93-95). Interestingly, all of these mitogen-regulated kinase cascades are also regulated by the injury-related cytokine TNF α. Mitogens and TNF also regulate the inhibitor kappaB kinases (IKK) which promote the disassociation of NF-kB from its cytosolic binding proteins, permitting NF-kB to localize in the nucleus where it activates the transcription of target genes that prevent apoptosis (96, 97). Interestingly, IKK activity is required for the MAPK cascade to induce neoplastic transformation in cultured liver cells because neoplasia is blocked by agents that specifically inhibit the redistribution of NF-kB to the nucleus (94). These results provide a mechanism that may explain why NF-kB-regulated anti-apoptotic responses are necessary for the expansion of liver cell number that is required for both liver development (98, 99) and regeneration (47).

5.3. Cell Division (G2-Mitosis (M) phase)

After cellular DNA has been replicated, additional mechanisms are activated to assure that the cell is ready to divide into two daughter cells. Damaged cells that escaped the G-1-S checkpoint are often arrested at this stage of the cell cycle (100). Because cells in G2-M have completed S phase, they contain extra copies of DNAs and are designated as polyploid (i.e., tetraploid, octaploid, etc, according to the number of extra sets of chromosomes that they contain). PH increases the number of polyploid hepatocytes (33). The proportion of polyploid hepatocytes increases with age (101, 102) and exposure to oxidative stress or other DNA damaging agents (24, 103). Experiments with fibroblasts from mice in which the mismatch repair gene, Msh 2, had been inactivated suggest that DNA base excision repair mechanisms may regulate DNA ploidy (104). Interestingly, certain types of cells that are vulnerable to TNF killing die during the G2-M period after being exposed to TNFα during late G-1 (105). This information suggest that hepatocyte ploidy may increase post-PH because TNF-damaged hepatocytes become arrested in G2 so that they can be repaired or deleted. If true, then this might help to explain why massive hepatocyte apoptosis occurs after the period of hepatocyte DNA synthesis in mice that were pre-treated with NK-kB inhibitors and then subjected to PH (47).

6. DOWN-REGULATION OF THE PROLIFERATIVE RESPONSE

A major unanswered question in liver regeneration is how the liver "knows" when regeneration has been completed and reverts to its normal state of low proliferative activity. TGF β and other factors that inhibit cellular proliferation have been identified in the liver, both before and after PH (106). TGF β expression increases transiently in the regenerating liver, suggesting that it may provide important growth-arrest signals. Consistent with this possibility, treatment with TGF b delays liver regeneration following PH (107). However, transgenic mice that over-express TGF β are able to regenerate their livers after PH (108). Thus, additional mechanisms must be involved in terminating the proliferative response that follows PH. It is conceivable that replicative quiescence (G-0) is the "default" pathway in hepatocytes and that proliferation requires the persistence of factors that initiate entry into G-1 (e.g., cytokines) as well as factors that promote progression through the replicative stages of the cell cycle (e.g., mitogens and co-mitogens). In any case, a better understanding of these events has important implications for the pathogenesis of hepatic malignancies that typically arise in the context of diseases that cause chronic liver injury and repair.

7. SUMMARY OF MECHANISMS THAT REGULATE HEPATOCYTE PROLIFERATION AFTER PH

During liver regeneration after PH, injury-related cytokines, mitogens and comitogens interact with hepatocyte receptors that activate kinases that transiently modify the activity of pre-formed transcription factors such that these factors enhance the transcription of a wide array of target genes. All of this happens rapidly (within minutes) and does not require de novo protein synthesis. This immediate response is terminated when the kinase targets are returned to their normal, inactive state by phosphatases. However, by then, a more sustained response has already been initiated because the synthesis of various proteins that are necessary for cellular repair and proliferation has been induced. Some of these proteins are transcription factors that, in turn, increase the expression of other genes. Others are enzymes that are necessary to protect hepatocytes from apoptosis and to maintain hepatocyte-specific functions during increased proliferative activity. Still others are structural proteins that must be duplicated during cellular replication. During hepatocyte proliferation, all of these different types of proteins are not induced simultaneously. Rather, mechanisms coordinate the sequential induction of proteins that are necessary for the cell to progress through a series of "check points" that ensure that it is healthy and prepared to replicate. After PH, the number of proliferating hepatocytes increases transiently and proliferative activity declines to basal, pre-PH levels once the liver mass has been restored.

A more detailed review of growth-regulatory signal transduction is beyond the scope of this chapter. Suffice it to say, the work of many groups is leading to a relatively detailed understanding of several proliferative signaling cascades that can be activated by factors that regulate hepatocyte progression through the cell cycle. However, much remains to be learned about the mechanisms that coordinate hepatocyte replication with the proliferation of other types of liver cells (e.g., biliary epithelial cells, endothelial cells, stellate cells, and components of the innate immune system) and with the reconstitution of the connective tissue matrix of the liver. Similar to hepatocyte proliferation, the latter responses are also necessary to regenerate this complex tissue.

8. TISSUE REPAIR AND REMODELING AFTER PH

Although extra-hepatic tissues are major sources of certain mitogens (e.g., EGF is produced by salivary and duodenal Brunner's glands) and co-mitogens (e.g., insulin is produced by pancreatic beta cells) (86, 109), most of the growth-modulatory factors that regulate hepatic regeneration are derived within the hepatic microenvironment. This is well illustrated by the PH model, which normally provokes a cascade of cytokines within the liver.

8.1. Cytokine Microenvironment

TNF α is an early participant in the cytokine cascade that is induced within minutes of PH (110). TNF α, in turn, induces IL-6, which increases later in the pre-replicative period (41). Studies of IL-6-null mice demonstrate that this cytokine plays a key role in the regenerative response. Hepatocyte proliferation after PH is severely inhibited and post-PH liver injury and mortality are increased in mice that are genetically-deficient in IL-6 (111). Pre-treatment of TNF receptor-1-deficient mice with IL-6 restores normal regeneration after PH, demonstrating that IL-6 is a key effector of the TNF-initiated proliferative response to PH (42). However, IL-6 does not promote DNA synthesis in cultured hepatocytes (112). This suggests that, similar to TNF α, the proliferative effects of IL-6 require cooperation of other growth-modulatory molecules that are produced in the injured liver. Evidence that TNF and IL-6 stimulate hepatic stellate cells to produce HGF supports this concept (113). HGF, in turn, activates hepatocyte production of TGF α, which functions as an autocrine growth factor. Hepatic expression of many other cytokines also increases transiently after PH. Some of these (e.g., IL-12 and interferon γ) promote the biologic activity of TNF α. Others (e.g., IL-10 and TGF β) inhibit it (114). Thus, experimental manipulations or naturally occurring events that inhibit the injury-related cytokines, TNF α and IL-6, after PH create a ripple of effects that profoundly influence the endogenous production of numerous other growth regulators.

The cellular source(s) of the cytokines that are produced after PH are being identified. More than a decade ago, Cornell demonstrated that liver regeneration was inhibited by various strategies that limited the intestinal production bacterial lipopolysaccharide (LPS) endotoxin (115, 116). This observation suggests that intestine-derived bacterial products, such as LPS, which are potent-inducers of macrophage TNF α production, mediate TNF α

induction after PH. However, the discordance between the kinetics of TNF α induction by LPS in Kupffer cells (which requires about an hour) (117) and the kinetics of TNF α induction after PH (which occurs within minutes) (118) suggest that this explanation cannot be entirely correct. The rapidity of these TNF-dependent responses suggests that PH initially either increases the delivery of TNF α to the liver or activates a latent pool of pre-formed TNF α protein that exists in some liver cell population (s), perhaps even hepatocytes themselves. Shortly thereafter, the hepatic expression of TNF mRNA increases transiently. While this delayed TNF induction might result from the activation of hepatic macrophages, this possibility is not supported by experiments that used gadolinium chloride to deplete hepatic Kupffer cells before PH. In rats that underwent PH after gadolinium chloride treatment, increases in hepatic TNF α mRNA levels were amplified (rather than abolished), suggesting that cells other than macrophages are the predominant producers of TNF α after PH (110, 119). Consistent with this concept, in situ reverse transcription polymerase chain reaction studies to localize TNFα gene expression demonstrated no detectable expression of TNF α in sinusoidal lining cells of normal or gadolinium chloride-pretreated rats before PH. During the initial hour after PH, clusters of small TNF-positive cells were observed in peri-portal areas around portal venules and bile ductules of normal rats. These foci were more prominent in gadolinium chloride-pretreated rats. Small TNF (+) cells were also detected near rare terminal hepatic venules. When these studies were done, the cellular sources of TNFα could not be specified with certainty (120). However, the data merit reconsideration given very recent evidence that proliferating oval cells express TNF α during certain rodent models of hepatocarcinogenesis (121). During later time points after PH, cytokine gene expression increases in extra-hepatic sites, such as white adipose tissue (122), contributing to the generalized (i.e., systemic) cytokine response that follows this liver injury. Interestingly, recent bone marrow transplantation experiments in IL-6 deficient recipients demonstrate that bone marrow-derived cells are another source of IL-6 during liver regeneration (123). Taken together, these results demonstrate that liver regeneration is orchestrated by a complex dialogue among various liver cell populations. These interactions ensure the orderly replacement of lost cells without significantly compromising liver-specific functions. Injury-related signals that originate within the liver also help to recruit extrahepatic tissues to release factors (e.g., growth-regulator hormones, neurotransmitters, mitogens, cytokines) that augment local repair efforts.

8.2. Matrix deposition and lobular reorganization

Given the importance of nonparenchymal cell activation and matrix production to the pathogenesis of chronic liver disease, it is clear that the regulation of these events during normal regeneration has important implications. After PH, liver nonparenchymal cells enter S phase about 24 hours after hepatocytes. As a result, within 2 to 3 days after PH liver histology is characterized by small clumps of liver cells that surround capillaries (34). Stellate cell processes then penetrate these clumps and begin to secret laminins and matrix-modeling molecules, changing the capillaries into true sinusoids that become lined by a fenestrated endothelium and Kupffer cells. During the process, the local matrix composition changes from one of high laminin content to that which is more typical of the healthy adult liver (i.e., primarily fibronectin, collagen types I and IV, and smaller amounts of other proteins and glycosaminoglycans). This reconstitutes the liver's lobular architecture and permits blood from portal veins to traverse through sinsoids before emptying into hepatic venules.

Because after PH hepatocytes in periportal zones of lobules proliferate 24 to 36 hours before hepatocytes in pericentral (zone 3) regions, restoration of the normal lobular architecture proceeds in a wavelike fashion from the portal to the central areas of the liver lobules during liver regeneration. By the end of the process, somewhat larger liver lobules have been formed in which the distance between the portal and hepatic venules is longer than it was before PH (33). It remains uncertain if and how these "mega" lobules are remodeled to restore the size and number of hepatic lobules that existed before PH.

9. FUTURE APPLICATIONS OF LIVER REGENERATION KNOWLEDGE

Manipulation of the liver's regenerative response could be used both as treatment modality for various acute and chronic liver diseases, as well as a strategy to expand the efficacy of liver transplantation.

9.1. Rescue from Fulminant Liver Failure

Most patients who die as a result of massive acute hepatic necrosis, die because the injured liver fails to perform many of its vital functions. Because hepatocyte proliferation is exuberant in the livers of such patients, one treatment option is to supplement liver function until the native liver becomes repopulated by a sufficient number of hepatocytes to resume its usual tasks. This strategy requires ready access to relatively large numbers of mature, well-differentiated hepatocytes. A couple of different approaches have been utilized to achieve this objective.

9.1.1. Auxiliary Heterotopic Liver Transplantation

The placement of a small liver graft in the upper abdomen, near the injured native liver (auxiliary heterotopic liver transplantation), has been successful as a "bridge" to recovery in patients with fulminant liver failure (124). Once regeneration of the native liver is complete, immunosuppressive therapy is discontinued, permitting the heterotopically placed allograft to be destroyed. Orthotopic liver transplantation using small for size graft from cadaveric donors is sometimes done to save patients with fulminate liver failure when a full-sized graft is unavailable.

In one study, recipient survival was improved by combining auxiliary partial orthotopic liver donor transplantation to assist the function of the small-for-size graft (125).

9.1.2. Hepatocyte Transplantation

Studies in animals with massive acute hepatic necrosis indicate that suspensions of hepatocytes that are injected into the spleen migrate into the liver, where they replicate and eventually repopulate the organ (27). Even more exciting are recent experiments that suggest bone marrow transplantation might eventually become a treatment for patients with certain types of acute liver failure. Grompe's group showed that the congenitally damaged livers of FAH-null mice (which have tyrosinemia) could be completely repopulated with healthy hepatic progenitors by injecting bone marrow from normal donor mice (7). The demonstration of Y chromosome positive hepatocytes in female bone marrow transplant recipients from male donors proves that bone marrow-derived hepatic progenitors also persist post-natally in humans (6). Therefore, at least theoretically, transplantation of bone marrow or bone marrow-derived hepatic stem cells from healthy donors may become a treatment for tyrosinemia and other congenital liver diseases, such as α1-anti-trypsin deficiency. The efficacy of hepatocyte (as opposed to bone marrow) transplantation has already been demonstrated in humans. For example, this was an effective strategy for transferring genes encoding the low-density lipoprotein (LDL) receptor into patients with congenital LDL receptor deficiency (126). In the latter study, patients with congenital LDL deficiency donated their own hepatocytes, which were manipulated ex vivo to express functional LDL receptors and the, reintroduced into the patient. The necessity of harvesting autologous hepatocytes will be obviated when sufficient numbers of hepatic progenitors can be purified from bone marrow. Assuming that the latter can be achieved, then stem cells from a patient's own bone marrow could be genetically manipulated ex vivo and then re-introduced into the patient. Alternatively, stem cells from healthy bone marrow donors could be transplanted into recipients with genetic liver disease. However, because the donor stem cells might generate progenitors that eventually replace the recipient's own bone marrow, this approach carries the risk of chronic graft-versus-host (or autoimmune) disease.

9.1.3. Artificial Liver Assist Devices

Bioartifical liver (BAL)-assist devices offer a less invasive means of temporarily augmenting liver function in patients with liver failure. Current devices use freshly isolated normal porcine hepatocytes or cultured human hepatoma cell lines. As such, the devices require a ready supply of hepatocytes that can survive ex vivo and perform excretory, synthetic, and/or biotransforming functions that cannot be accomplished by the failing native liver. The relative importance of various hepatocyte functions in promoting the recovery of patients with fulminant liver failure is unknown. Nonetheless, two bioartificial devices have demonstrated some efficacy in patients with fulminant liver failure: the BAL, in which pig hepatocytes are attached to microcarriers, and the extracorporeal liver assist device (ELAD), which contains a human liver-derived tumor cell line. Thus far, the benefits of both devices in humans appear to be less impressive than were reported in animal models of acute liver failure (127, 128). Perhaps this reflects present uncertainty about the quantity of hepatocytes, specific function(s), and duration of support that are required for clinical benefit. It is hoped that a better understanding of the effects that these devices exert on the metabolic functions of the damaged liver and on the recovery process will permit refinements in device design and improved outcomes.

9.2. Treatment of Decompensated Cirrhosis

9.2.1. Small-for-Size Donors (Split Livers and Living Donors)

Approximately 3,000 patients with end-stage liver disease die every year awaiting a suitable liver allograft. The demand for liver allograft is increasing faster than the number of viable donors, thus creating a burgeoning number of patients on waiting lists for liver transplantation. The number of organ donors has reached a plateau in the United States. Thus, it is likely that the number of patients who die while awaiting liver transplantation will continue to rise unless new approaches to expand the donor pool are developed. The liver's innate ability to regenerate permits the transplantation of small-for-size grafts. In healthy rodents and relatively well-compensated cirrhotic humans, the threshold mass of liver tissue that is necessary to preserve important liver-specific functions until regeneration occurs is about 30-40% of the original healthy liver mass. Thus, a single donor liver can be divided between two recipients, doubling the size of the donor pool. In addition, liver lobes can be procured from living donors by partial hepatectomy and then, transplanted into recipients after the diseased liver has been removed. Recipients of either split-liver grafts or small-for-size grafts from living donors will eventually regenerate to sizes that are appropriate for the recipient's body mass. The remnant liver in living donors also regenerates to replace the resected liver, protecting the donor from long-term deficits. In both situations, the regenerative response is typically accomplished within a month post-operatively. However, clinical experience demonstrates significantly increased perioperative mortality in recipients who were severely decompensated at the time of transplantation. The poor outcome in this group of patients suggests that some process either inhibits regeneration or exacerbates transplantation-related injury in their grafts. Until this phenomenon is better understood and treated, severely ill cirrhotic patients are not candidates for transplantation with reduced size grafts. However, there is great optimism that transplants from living donors will significantly decrease the spent on waiting lists for many, more stable patients with advanced liver disease (129).

10. SUMMARY

Unlike other vital organs, the liver typically regenerates after injury. Indeed, the very factors that cause liver injury initiate a reparative process in the residual liver that includes the induction of cytoprotective mechanisms, deletion of mortally wounded cells, repair of less damaged survivors, liver cell proliferation to replace the cells that died, the deposition of new matrix, and tissue remodeling to restore normal hepatic mass and architecture. During liver regeneration, the liver normally continues to perform vital, liver-specific functions. Unfortunately, the hepatic

regenerative response sometimes becomes disrupted - either failing to occur or occurring in a disordered, or incomplete fashion. Abnormal regeneration contributes to the pathogenesis of fulminate liver failure, cirrhosis, and primary liver cancers. Research in the field of regenerative biology has identified several events that are required for liver regeneration. These include injury-induced changes in the hepatic microenvironment, the ability of surviving liver cells and/or their progenitors to proliferate, and a temporary suspension of homeostatic mechanisms that normally couple cell proliferation to programmed cell death. The signals that mediate these complex biologic responses are being detailed. A better understanding of the extra- and intracellular signals that prompt the injured liver to regenerate should suggest treatments to promote liver regeneration in patients with otherwise fatal liver diseases.

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Send all correspondence to: Anna Mae Diehl MD, Professor, Johns Hopkins University, 720 Rutland Avenue Ross Research Building # 912, Baltimore, MD21205-2109, Tel: 410-955-7316, Fax: 410-955-9677, E-mail: amdiehl@welch.jhu.edu, amdiehl@jhmi.edu