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REVIEW

Liver Regeneration after Acetaminophen Hepatotoxicity

Mechanisms and Therapeutic Opportunities

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Acetaminophen (*N*-acetyl-para-aminophenol; APAP) overdose is the most common cause of acute liver failure in the Western world, with limited treatment opportunities. For years, research on APAP overdose has been focused on investigating the mechanisms of hepatotoxicity, with limited success in advancing therapeutic strategies. Acute liver injury after any insult, including APAP overdose, is followed by compensatory liver regeneration, which promotes recovery and is a crucial determinant of the final outcome. Liver regeneration after APAP-induced liver injury is dose dependent and impaired after severe APAP overdose. Although robust regenerative response is associated with spontaneous recovery and survival, impaired regeneration results in faster progression of injury and death after APAP overdose. APAP hepatotoxicity—induced liver regeneration involves a complex time- and dose-dependent interplay of several signaling mediators, including growth factors, cytokines, angiogenic factors, and other mitogenic pathways. Compared with the liver injury, which is established before most patients seek medical attention and has proved difficult to manipulate, liver regeneration can be potentially modulated even in late-stage APAP-induced acute liver failure. Despite recent efforts to study the mechanisms of liver regeneration after APAP-induced liver injury, more comprehensive research in this area is required, especially regarding factors that contribute to impaired regenerative response, to develop novel regenerative therapies for APAP-induced acute liver failure. (*Am J Pathol 2019, 189: 719—729; https://doi.org/10.1016/j.ajpath.2018.12.006*)

Acetaminophen (*N*-acetyl-para-aminophenol; APAP) is the most commonly used over-the-counter antipyretic and analgesic drug worldwide.[1] APAP is present in numerous medicines, either alone or in combination with other active ingredients, ranging from common cold formulations to combination products with opioids for severe painful conditions.

APAP Overdose and APAP-Induced Hepatotoxicity

APAP is considered well tolerated at therapeutic doses, with minimal side effects. However, overdose of APAP can cause severe liver damage that progresses to acute liver failure (ALF) and death. Toxicity due to APAP overdose may arise as a consequence of either an acute overdose or from repeated/staggered dosing over a short period of time.[2] At present, APAP overdose is the foremost cause of ALF in the Western world, accounting for nearly 50% of all of the ALF cases in the United States and 60% in the United Kingdom.[3,4] APAP overdose is associated with more than 78,000 emergency department visits, 33,000 hospitalizations, and around 500 deaths each year in the United States.[1,5] Although the majority of APAP overdose cases are intentional (around 70%), cases of therapeutic misadventures are also frequent.[1]

Extensive research has been performed since the 1970s to study the mechanisms of APAP-induced hepatotoxicity, and these mechanisms have been reviewed comprehensively.[6,7]

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Briefly, APAP hepatotoxicity can be divided into three phases (Figure 1). During the *initiation phase* after an overdose, APAP is rapidly metabolized to its reactive metabolite, *N*-acetyl-p-benzoquinone imine (NAPQI), which is removed by glutathione (GSH) conjugation, leading to rapid depletion of cellular GSH stores. The excess NAPQI forms cellular protein adducts, particularly in mitochondria, leading to mitochondrial dysfunction and the generation of reactive oxygen species (ROS). Initial mitochondrial ROS generation is exacerbated by a plethora of intracellular signaling events, including c-Jun N-terminal kinase activation, leading to an opening of mitochondrial permeability transition pores and the release of endonucleases that cause DNA damage.[7] All these events ultimately result in hepatocellular necrosis, specifically in the centrilobular region. During the *progression phase*, the initial acute liver injury (ALI) further progresses by extracellular mechanisms that are not completely clear. Necrotic cells release damage-associated molecular patterns such as high-mobility group box 1 protein and DNA fragments, which cause the recruitment of inflammatory cells (eg, neutrophils) and cytokine generation, eventually resulting in sterile inflammation.[6] Sterile inflammation is reported to be involved in both the progression of APAP-induced hepatotoxicity and its resolution. The release of proteolytic enzymes, such as calpains, from dying hepatocytes can also mediate the progression of APAP-induced liver injury by damaging neighboring hepatocytes.[8,9] Furthermore, the rate of replenishment of cellular GSH stores after initial depletion can affect the extent of oxidative damage and alter the course of APAP-induced liver injury. In addition, a recent study indicated that cellular O-linked β-*N*-acetylglucosamine (*O*-GlcNAcylation) can dysregulate hepatic GSH-replenishment response.[10] The injury phases of APAP hepatotoxicity are subsequently followed by a *recovery phase*, in which compensatory hepatocellular proliferation is initiated; dead cells are replaced by newly formed cells, leading to liver regeneration and recovery. In cases in which a robust liver-regeneration response is initiated, liver injury is resolved, and liver function is restored spontaneously. In cases in which liver regeneration fails, ALI can progress to ALF, with multiorgan failure and death.[11–14]

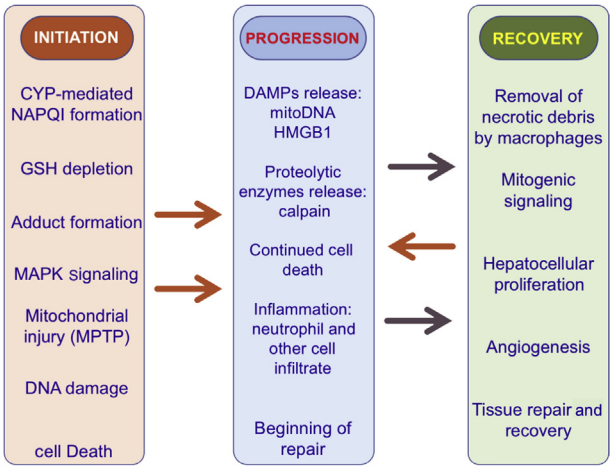

Figure 1 Three phases of acetaminophen (*N*-acetyl-para-aminophenol; APAP)-induced liver injury. Pathogenesis of APAP-induced liver injury, divided into three mechanistically interrelated but distinct phases: i) *initiation* (bioactivation of APAP and initial cell death), ii) injury *progression* (exacerbation of initial injury via extracellular mechanisms), and iii) *recovery* (compensatory liver regeneration and repair). CYP, cytochrome P-450; DAMP, damage-associated molecular pattern; GSH, glutathione; HMGB1, high-mobility group box protein 1; MAPK, mitogen-activated protein kinase; mitoDNA, mitochondrial DNA; MPTP, mitochondrial permeability transition pore; NAPQI, *N*-acetyl-p-benzoquinone imine.

Limitations of Current Treatment Strategy and the Potential of Regenerative Therapy

Despite decades of research, current treatment options after APAP overdose are extremely limited. Apart from symptomatic patient care, *N*-acetylcysteine (NAC) is the only clinically recognized pharmacologic intervention for APAP-overdose patients.[3] NAC provides cysteine precursor to restore the hepatic GSH levels. However, intervention with NAC is effective only when administered within 24 hours of APAP overdose, with early intervention leading to better prognosis.[15] Although NAC is given even to late-presenting patients, its effectiveness is questionable. In fact, the findings from recent studies suggest that prolonged treatment with NAC may be detrimental for recovery after APAP overdose.[16,17] Unfortunately, in patients who do not respond well to NAC therapy, liver transplantation is the only other option. However, liver transplantation is complicated by issues related to organ availability, graft rejection, lifelong immunosuppression, and exorbitant costs.[18] Another important point of consideration is that most patients seek medical attention late, such that injury is already established and difficult to manipulate.[19] A recent study demonstrated that high-volume plasmapheresis in combination with standard medical therapy (which includes NAC) can improve transplant-free survival in APAP-induced ALF patients and may be a promising therapeutic modality.[20] Additional approaches are required in the future to enhance standard NAC therapy. Recent studies have shown that patients with higher innate liver regeneration have higher transplant-free survival.[13,14] For years, the research to identify therapeutic targets for APAP overdose has been focused on investigating the mechanisms of APAP-induced liver injury. However, studies to determine the mechanisms of liver regeneration after APAP-induced ALF (ie, the recovery phase), are less common. Liver regeneration can be potentially modulated even at a late stage in the pathogenesis of APAP-induced ALF, and stimulating liver regeneration in patients with APAP-induced ALF might be an attractive therapeutic strategy.

Liver Regeneration and Its Role in Determining Final Outcomes after Drug-Induced ALI

Liver has an extraordinary capacity to regenerate on loss of liver tissue due to toxin-induced liver injury, surgical resection, infection, or trauma. Whereas experimentally, two-third partial hepatectomy (PH) is the most widely studied model of liver regeneration and has been extensively reviewed,[21,22] liver repair after chemical-induced injury has also been well documented. Liver regeneration as a compensatory response to liver injury has been well described for several toxicants, such as thioacetamide, carbon tetrachloride, chloroform, acetaminophen, and allyl alcohol.[23] The findings from acute studies using these hepatotoxicants suggest that liver regeneration follows the principles of dose response, and that liver regeneration after toxicant-induced ALI increases proportionately to the extent of liver injury but only up to a threshold dose; doses higher than the threshold dose actually inhibit liver regeneration.[23] Furthermore, these studies have demonstrated that liver regeneration plays a crucial role in determining the final outcome of toxicant-induced ALI, such that timely and proportionate stimulation of regeneration leads to regression of injury, but delayed or inhibited regeneration culminates in progression of injury and death.[12,23] The importance of liver regeneration in toxic injury has been especially highlighted by experiments in which liver regeneration was manipulated. For instance, inhibition of liver regeneration with antimitotic agents such as colchicine in a toxicant-induced ALI model resulted in an exacerbation of injury, leading to death. Similarly, stimulation of liver regeneration in these cases inhibited progression of injury, resulting in improved survival.[23]

Similar to other hepatotoxicants, APAP-induced liver injury is followed by compensatory liver regeneration, where the hepatocytes in closest proximity to the necrotic zones divide and replace dead cells.[12] Several lines of evidence indicate the important role of liver regeneration in determining outcome after APAP-induced liver injury. For instance, treatment with IL-6,[24] stem cell factor,[25] vascular endothelial growth factor (VEGF),[26] and GSH[27] enhanced regeneration, resulting in regression of injury or enhanced survival after APAP-induced toxicity in mice. Furthermore, mice with streptozotocin-induced diabetes were resistant to APAP-induced injury because of a higher regeneration capacity, and the inhibition of regeneration by an antimitotic agent (colchicine) in these mice resulted in increased mortality.[28] Finally, accumulating clinical evidence supports the association of liver regeneration after APAP overdose with enhanced survival. For instance, higher β-catenin activation was correlated with higher spontaneous liver regeneration, preventing the need for liver transplantation,[13] and increased serum α-fetoprotein, a marker of hepatocyte proliferation, was correlated with survival in patients with APAP-induced ALF.[14] Together, these data indicate that stimulating liver regeneration in APAP-induced ALF patients can potentially improve survival and recovery.

Mechanisms of Liver Regeneration after APAP-Induced Hepatotoxicity

Despite significant evidence that liver regeneration plays a crucial role in the resolution of APAP-induced ALF, the molecular mechanisms of liver regeneration after APAP toxicity are just beginning to be understood. It is important to note that these mechanisms may differ from most widely studied PH models because of the basic differences between APAP injury and PH (Table 1). A previous study utilizing incremental doses of APAP in mice showed that liver regeneration after APAP toxicity was dose dependent, similar to other chemical hepatotoxicants.[12] In this study, a lower moderate overdose of APAP in mice (300 mg/kg) caused extensive liver injury, but also significant compensatory regeneration, leading to regression of injury and spontaneous recovery.[12] However, after a severe overdose of APAP (600 mg/kg) liver regeneration was remarkably inhibited, resulting in sustained injury and decreased survival.[12] Of interest, the marked inhibition of regeneration at the higher dose was not due to a lack of critical liver mass, as >50% of hepatocytes were viable at this dose, even at peak injury. In fact, peak injury was not remarkably different between the two doses, whereas regeneration was significantly impaired only at the higher dose. Further comprehensive analysis of signaling pathways revealed that several pro- and anti-regenerative pathways were differentially affected in a dose-dependent manner.[12] The approach of using two doses, one that allows stimulation of regeneration and another that inhibits it, led to the identification of proliferative signaling pathways that drive liver regeneration and, more importantly, also revealed potential mechanisms that actively inhibit liver regeneration after APAP overdose. The high dose in this study simulated patients who cannot recover spontaneously after APAP overdose, who have

Table 1 Differences between Liver Regeneration after Partial Hepatectomy and Acute Liver Injury

Features	Partial hepatectomy	Acute liver injury
Starting point	A known starting point (time of surgery)	Extended process with undefined starting point
Location	All hepatocytes in the remaining lobes	Mostly areas surrounding the necrotic zones
Cell cycle	Synchronous	Unsynchronized
Inflammation	Not significant	Extensive
Injury	Moderate	Extensive (dose-dependent)

either delayed or completely inhibited liver regeneration, and who require liver transplantation. This and a few other studies (discussed in the following sections) have revealed several signaling pathways that mediate liver regeneration after APAP overdose.

Role of Growth Factors

Growth factors such as epidermal growth factor receptor (EGFR) ligands and hepatocyte growth factor (HGF) are considered primary mitogens (which can directly stimulate hepatocyte proliferation even in serum-free media or *in vivo* stand-alone) for hepatocytes, and are crucial for liver regeneration after PH and maintaining liver homeostasis.[22,29,30] In fact, EGFR ligands [eg, EGF and transforming growth factor (TGF)-α] and HGF are the only known primary mitogens for hepatocytes.[22] EGFR ligands and HGF act primarily through the activation of EGFR and c-Met receptor, respectively. Several studies in the PH model have shown that the elimination of a single extracellular signaling pathway can only delay or diminish liver regeneration, but cannot permanently abolish it.[22] However, a recent study demonstrated complete abolition of liver regeneration after PH by combined elimination of c-MET and EGFR signaling.[30] Similar complete abolition of hepatocellular proliferative response was observed after combined disruption of this signaling, even in a chemical mitogen−induced hepatomegaly model, which does not involve any tissue loss.[31] These studies highlight the indispensable role of signaling of these growth factors in hepatocyte proliferation and in maintaining a proliferative environment in liver. Although growth factor signaling plays a central role in liver regeneration and hepatocyte proliferation, its role in regeneration after APAP-induced liver injury is relatively unknown. After a moderately toxic dose of APAP in mice, both Egfr and c-Met were reported to be remarkably activated within 15 minutes and 3 hours, respectively.[11,12] Furthermore, EGFR activation was observed in primary human hepatocytes after treatment with APAP.[11] Of interest, the inhibition of EGFR alone in mice almost completely abolished compensatory hepatocyte proliferation, resulting in progression of injury and decreased survival after moderate APAP overdose, which normally results in robust liver regeneration and spontaneous recovery.[11] The inhibition of EGFR signaling alone in a PH model has been shown to only delay liver regeneration.[30,32] This finding indicates that the dynamics and mechanisms of liver regeneration are very different in the APAP-induced ALF model as compared with PH, and that liver regeneration after APAP overdose is more critically dependent on EGFR activation. However, the activation of both Egfr and c-Met was dose-dependently higher even after severe APAP overdose in mice, in which liver regeneration and recovery were impaired, leading to significant mortality.[12] A similar pattern of dose-dependent activation was also observed in downstream signaling mediators such as Erk1/2, Akt, c-Jun, and c-Fos.[12] This finding indicates that the activation of EGFR and c-MET signaling alone may not be sufficient to mount robust liver regeneration after severe APAP-induced liver injury. Similar inferences can be made based on the findings from some earlier clinical studies and investigations in nonrodent models.[33,34] In a study using Beagle dogs, the administration of hepatic stimulatory substance alone or in combination with TGF-α (an EGFR ligand), insulin-like growth factor II, and insulin did not affect survival or liver regeneration after a lethal dose of APAP.[33] In another study, plasma HGF levels were elevated in all APAP-induced ALF patients but were significantly higher in nonsurvivors compared with survivors.[34] Thus, the activation of growth-factor signaling is not always associated with the compensatory liver regeneration after APAP overdose, especially in severe cases, in which other factors may be more crucial.

Apart from mitogenic growth factors, some growth factors such as TGF-β are known to be mito-inhibitory in hepatocyte culture and during liver regeneration after PH.[21] A recent study demonstrated the expression of TGF-β1 and the activation of TGF-β signaling in perinecrotic areas after APAP overdose in mice and in APAP-induced ALF patients, which was associated with impaired liver regeneration and hepatocyte senescence.[35] The production of TGF-β1 in macrophages was found to be particularly important for inhibiting liver regeneration, as myeloid-specific *Tgfb1* knockout (KO) mice showed improved liver regeneration without alteration of liver injury after APAP overdose.[35] Importantly, delayed treatment with Tgf-β receptor 1 (Tgf-βr1) inhibitor, even 12 hours (when NAC is ineffective) after a sublethal dose of APAP, in mice resulted in improved liver regeneration. Furthermore, concurrent treatment with Tgf-βr1 inhibitor improved survival after lethal dosing of APAP in mice.[35]

Role of Cytokines

Cytokine-signaling pathways stimulated by factors such as IL-6 and TNF-α also contribute to liver regeneration. It has been postulated that cytokines act as priming factors for hepatocytes and improve their response to proliferative stimuli, but are not direct mitogens for hepatocytes.[22] Proliferative signaling of TNF-α in hepatocytes is majorly mediated by TNF receptor 1 (TNF-R1). Two independent studies have demonstrated that the deletion of *Tnfr1* lowered liver regeneration after APAP overdose in mice.[36,37] However, overall recovery was not altered in one of these studies[37] and *Tnfr1* KO mice were found to be more susceptible to initial APAP toxicity in the other study.[36] It is possible that the inhibition of liver regeneration after *Tnfr1* deletion in the latter study[36] could have been mediated secondary to exaggerated injury, which needs further exploration. TNF-α signaling ultimately results in stabilization and nuclear translocation of transcription factor NF-κB. Previous reports have shown increased hepatic

Tnf-α concentrations and NF-κB DNA binding after APAP treatment, which correlated with increased cyclin-D1 (a key regulator of cell cycle entry) protein expression and liver regeneration, in mice.[17,38,39] In contrast, decreased serum Tnf-α concentration, lower NF-κB DNA binding, and decreased expression of cyclin-D1 were reported after interventions that impaired liver regeneration after APAP-induced liver injury.[17,40] This association was further substantiated by a study of incremental doses of APAP, which revealed direct binding of p65-subunit of NF-κB to cyclin-D1 promoter after a moderately toxic and regenerating dose of APAP; binding was significantly lower after a severely toxic dose of APAP, in which liver regeneration was impaired.[12] Furthermore, the induction of Tnf-α gene expression, *trans*-activating phosphorylation of p65 at Ser536, and nuclear levels of p65 were lower after a severely toxic dose of APAP in mice.[12] These data suggest that a lack of activation of TNF-α/NF-κB signaling may be one mechanism, among others, of impaired liver regeneration after highly toxic dosing of APAP; further investigation is needed.

Similar evidence implicating a role of IL-6/STAT-3 signaling in regeneration after APAP-induced liver injury has been reported. Il-6 levels in liver and serum were found to be increased after APAP overdose in mice.[27,41,42] Furthermore, *Il6* KO mice displayed impaired liver regeneration after APAP toxicity, without any alteration of initial liver injury. Impaired liver regeneration in *Il6* KO was associated with prolonged elevation of aspartate aminotransferase (Ast) levels.[24] Pretreatment with Il-6 in these KO mice resulted in restoration of liver-regeneration parameters, along with a decrease in AST levels, indicating role of IL-6 in liver regeneration and recovery after APAP-induced hepatotoxicity.[24] IL-6 signaling culminates in STAT-3 activation, which in turn stimulates promitogenic gene expression. Impaired liver regeneration in *Tnfr1* KO mice after APAP overdose was associated with delayed phosphorylation of Stat-3, suggesting crosstalk of Tnf-α/NF-κB and Il-6/Stat-3 signaling in liver regeneration after APAP-induced liver injury, which is also observed in the PH model.[36] The *Il6* KO studies were conducted using a moderately toxic dose of APAP, which eventually resulted in spontaneous regeneration and recovery. However, the study using incremental doses of APAP demonstrated that, although Il-6 was induced and Stat-3 was activated after a moderately toxic dose and a regenerating dose of APAP, activation of Il-6/Stat-3 signaling was even higher and sustained after a severely toxic dose of APAP, in which liver regeneration was impaired.[12] This finding suggests that IL-6 signaling alone may not be sufficient to mount robust liver regeneration after severe APAP-induced liver injury. Furthermore, sustained and overactivation of IL-6/STAT-3 signaling has been implicated in impaired liver regeneration in a PH model,[43,44] an interesting facet of IL-6 signaling that remains to be investigated in an APAP model.

Role of Other Signaling Mediators

Wnt/β-catenin signaling is one of the few pathways that has been deeply investigated for its role in liver regeneration after APAP toxicity.[12,13,45,46] An incremental-dose study in mice showed activation of β-catenin signaling along with nuclear localization of β-catenin after a moderately toxic dose of APAP, in which compensatory liver regeneration was intact.[12] However, β-catenin signaling was inhibited after severe APAP overdose, in which regeneration was inhibited. Cell cycle regulator cyclin-D1 is a known target of β-catenin, and binding of β-catenin to cyclin-D1 promoter was also inhibited after severe APAP overdose.[12] In a different transgenic mouse model, integrin-linked kinase (*Ilk*) deletion in liver resulted in increased liver regeneration, which was also associated with activation of β-catenin signaling.[46] Liver-specific β-catenin KO mice had lower liver injury due to a lower level of APAP-metabolic activation enzymes hindering the investigation of a direct role of β-catenin in liver regeneration using this model.[13] However, β-catenin KO mice at an equitoxic dose of APAP (different doses that produce similar injury in different types of mice) compared with control mice showed lower liver regeneration.[13] More importantly, overexpression of a stable form of β-catenin in liver improved regeneration to a certain extent even after severe APAP overdose.[12] Also, treatment with pharmacologic inhibition of glycogen synthase kinase-3β (an upstream negative regulator of β-catenin), even very late after severe APAP overdose, in mice resulted in increased activation of β-catenin signaling and early initiation of liver regeneration without altering the peak regenerative response.[45] The findings from these studies indicate that inhibited β-catenin signaling is associated with impaired liver regeneration after a highly toxic dose of APAP, but that β-catenin activation signaling alone is not sufficient for liver regeneration. The role of specific Wnts in the activation of β-catenin signaling and their cellular source after APAP toxicity remain to be investigated.

Signaling via bile acids is another important pathway known to promote liver regeneration after PH, and few studies indicate its role in liver regeneration after APAP toxicity as well.[47,48] The administration of cholic acid in mice resulted in a remarkable increase in liver regeneration and early regression of injury after APAP overdose.[47] Downstream mediators of bile acids that play a role in liver regeneration after APAP toxicity are not clear. Clinically relevant, late intervention with engineered fibroblast growth factor 19 (a human analogue of murine fibroblast growth factor 15), which is an important downstream mediator of bile acid signaling, resulted in enhanced liver regeneration, survival, and decreased liver injury, even after severe APAP overdose, in mice.[48] However, liver regeneration was not altered in *Fgf15* KO mice utilizing an equitoxic dose of APAP compared with wild-type mice.[49] Nonetheless, stimulating bile acid signaling has potential

therapeutic benefit for APAP toxicity; further exploration is needed.

Apart from the lack of proregenerative signaling, other factors such as cell cycle inhibition (eg, via p53/p21 pathways) and hepatocellular senescence in areas surrounding necrotic zones may have a significant role in the impairment of liver regeneration observed after severe APAP-induced liver injury. Indeed, dose-dependent p53 activation and downstream p21 induction were observed after APAP overdose in mice, with greater activation after severe APAP overdose, which was associated with cell cycle arrest.[12,50] A recent study also demonstrated similar association of increased p21/p16 expression with severity of liver injury and impaired liver regeneration in APAP overdose patients. Furthermore, deletion of p21 in mice improved perinecrotic liver regeneration without altering liver injury after APAP overdose in this study. TGF-β signaling is important for increased p21 expression and impaired liver regeneration.[35] Systemic deletion of p53 in mice results in the induction of proliferative signaling, faster cell cycle progression and liver regeneration, and faster recovery after APAP overdose, despite causing higher peak liver injury.[51] Further investigations revealed massive and prolonged double-strand DNA damage as one of the underlying causes of activation of cell cycle arrest pathways after severe APAP overdose in mice.[50] Although transient DNA damage was also observed after moderate APAP overdose, it was followed by prompt activation of DNA repair pathways, which was missing after severe APAP overdose.[50] Similar hepatic DNA damage and cell cycle abnormalities were also reported in tissues from APAP-induced ALF patients, indicating the clinical relevance of these findings.[52] Thus, future development of therapeutic strategies for APAP-induced ALF should aim not only to stimulate proliferative signaling but also to promote DNA repair and inhibit cell cycle−arrest pathways. Perhaps concurrent activation of proliferative signaling along with blockage of antiproliferative pathways may prove to be more efficient in promoting liver regeneration and recovery after APAP-induced liver injury.

Role of Nonparenchymal Cells

Macrophages

Removal of necrotic cells after toxic injury is an important aspect of liver regeneration and imperative for replacement with newly formed hepatocytes. M2 macrophages are known to be recruited to necrotic area after APAP overdose in mice and play an important role in debris removal and tissue repair.[53,54] Recruitment of macrophages is mediated by monocyte chemoattractant protein (MCP) 1 and its receptor C-C chemokine receptor (CCR) 2 (expressed on monocytes), and the expression of both of these proteins increases in the necrotic regions after APAP overdose in mice.[53] Increased levels of MCP1 in serum and hepatic tissue and CCR2 in monocytes were reported even in APAP-induced ALF patients.[55] *Ccr2* KO mice had delayed resolution of injury after APAP overdose, accompanied by lower M2 macrophage accumulation, indicating an important role of M2 macrophage accumulation in tissue repair.[54] A recent study showed an accumulation of a specific resolution-type macrophage population (Mer tyrosine kinase positive) in necrotic areas of hepatic tissues of ALF patients and also demonstrated their role in the resolution of liver injury after APAP overdose in a mouse model.[56] Apart from debris removal and resolution of injury, macrophages might play an important and very early role in hepatocyte proliferation after APAP-induced liver injury, based on the facts that macrophages are known to produce mitogens such as HGF, TGF-α, platelet-derived growth factor, TNF-α, and IL-6 and that proliferation occurs first after APAP toxicity in a layer of hepatocytes surrounding the necrotic zones, where macrophages actively infiltrate and proliferate.[12,22] In contrast, macrophages can also produce mito-inhibitory factors such as TGF-β, which inhibits liver regeneration after APAP toxicity.[35] However, a direct role of macrophages in hepatocyte proliferation after APAP-induced liver injury remains to be investigated.

Endothelial Cells

Angiogenesis and restoration of microvasculature is also an important aspect of tissue repair after liver injury. VEGF is a mitogen for endothelial cells and is known to play an important role in angiogenesis during liver regeneration after PH.[22] Furthermore, VEGF-stimulated endothelial cells can produce HGF and can be directly involved in hepatocyte proliferation.[22] Several reports have demonstrated an important role of VEGF and its receptor VEGFR in liver regeneration and hepatocyte proliferation after APAP-induced hepatotoxicity. Hepatic Vegf levels and expression of its receptors Vegfr1, 2, and 3 were increased after APAP overdose in mice and rats.[57−59] Whereas treatment with VEGFR inhibitor in mice impaired hepatocyte proliferation,[57] the administration of human recombinant VEGF increased hepatocyte regeneration after APAP overdose in mice, without altering initial hepatotoxicity.[26] Furthermore, *Vegfr1* KO mice exhibited impaired restoration of microvasculature, diminished hepatocyte proliferation, and expression of growth factors such as HGF and fibroblast growth factor, associated with decreased survival after APAP overdose.[58]

Stellate Cells

Stellate cells are a predominant source of HGF in the liver; however, their role in liver regeneration after APAP hepatotoxicity remains to be explored. The findings from two studies from a single group suggest that stellate cells might play a role in liver regeneration after APAP hepatotoxicity. Although the depletion of activated stellate cells using gliotoxin resulted in impaired liver regeneration and increased mortality after APAP overdose in mice, treatment with stellate cell−derived conditioned medium enhanced liver regeneration and improved survival.[60,61] However, a

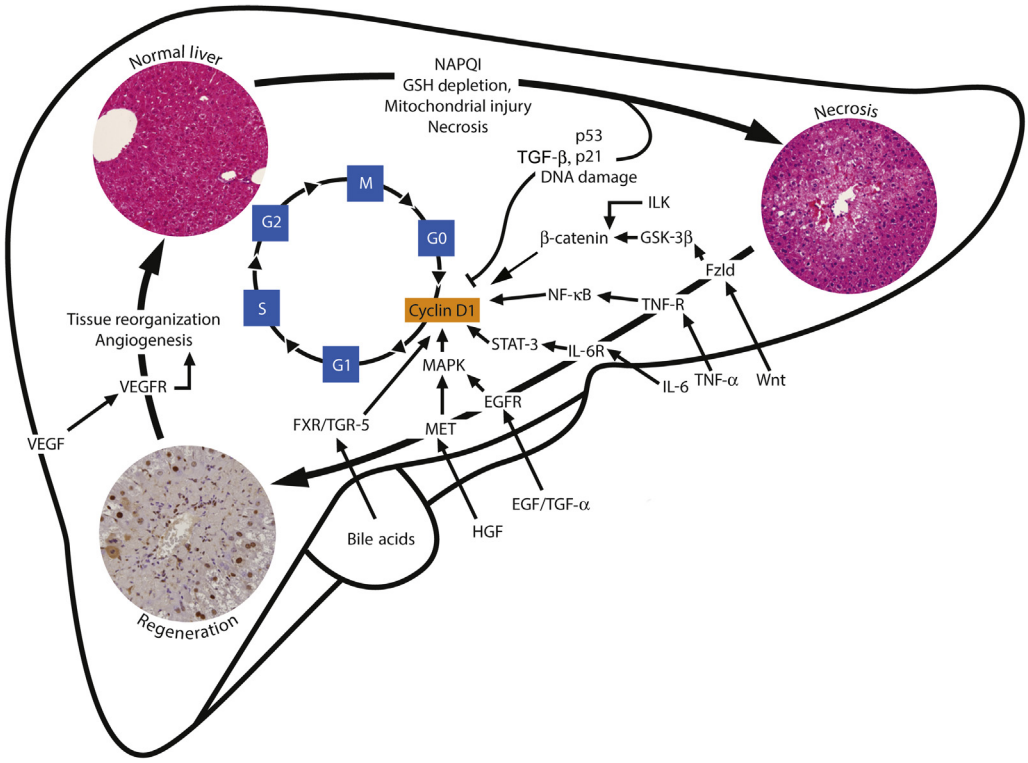

Figure 2 Molecular mechanisms of regeneration after acetaminophen (*N*-acetyl-para-aminophenol; APAP)-induced liver injury. Liver regeneration after APAP overdose involves a complex time- and dose-dependent interplay of several signaling mediators. Several proliferative signaling pathways that control cell cycle machinery, including growth factor signaling via epidermal growth factor receptor (EGFR) and c-MET [receptor for hepatocyte growth factor (HGF)], cytokine signaling [tumor necrosis factor (TNF)-α/NF-κB and IL-6/STAT-3], Wnt/β-catenin, and bile acid signaling are activated after APAP overdose, potentially contributing to liver regeneration. Some of these proliferative signaling pathways including Wnt/β-catenin and TNF-α/NF-κB signaling are inhibited after severe APAP overdose (others such as EGFR/c-MET and IL-6/STAT-3 signaling remain activated), which is accompanied by unchecked DNA damage and activation of antiproliferative pathways [transforming growth factor (TGF)—β and p53/p21] leading to cell cycle arrest and impaired liver regeneration. Angiogenesis and the restoration of microvasculature during normal liver regeneration involve the activation of vascular endothelial growth factor (VEGF)/VEGF receptor (VEGFR) signaling, which also indirectly contributes to hepatocyte proliferation via the stimulation of HGF release from endothelial cells. Top, hematoxylin and eosin—stained liver sections that are normal (left) and necrotic (right). Bottom, regenerating liver, shown as proliferating cell nuclear antigen (PCNA)-positive hepatocytes (brown nuclear staining). FXR, farnesoid X receptor; Fzld, frizzled protein; G0, gap 0 phase; G1, gap 1 phase; G2, gap 2 phase; GSH, glutathione; GSK, glycogen synthase kinase; ILK, integrin-linked protein kinase; M, mitosis phase; MAPK, mitogen-activated protein kinase; NAPQI, *N*-acetyl-p-benzoquinone imine; S, synthesis phase; TNFR, TNF receptor.

direct role of stellate cells on hepatocyte proliferation could not be inferred from these studies as peak liver injury was also altered in these studies. Further comprehensive studies with late interventions are required to confirm the role of stellate cells in liver regeneration after APAP hepatotoxicity.

Role of HPCs

The role of hepatic progenitor cells (HPCs) in liver regeneration has been a topic of intense debate. In most scenarios, proliferation of remnant hepatocytes to produce new hepatocytes is sufficient for liver regeneration. However, in cases of severe hepatic injury in which hepatocyte proliferation is inhibited, cells of biliary ductular origin (specifically, terminal bile ductules called the canals of Hering) can give rise to bipotent progenitor cells (known as oval cells), which differentiate into hepatocytes restoring liver regeneration.[21,22] A dose- and time-dependent biphasic oval cell or HPC reaction, largely restricted to the smallest portal tracts, has also been reported after the administration of sublethal doses of APAP in mice.[62] Similar HPC response has been reported in APAP-induced ALF patients with severe liver necrosis.[63] A few studies indicate that stellate cells, which recently emerged as a stem cell niche for hepatic progenitor cells, might play a role in HPC response after APAP overdose; further investigation is needed.[60,61] Of interest, the time course of HPC activation in mice was closely correlated with temporal changes in serum stem cell factor (SCF) after APAP-induced liver injury, suggesting a potential role of SCF in progenitor cell activation.[62,64] Another study reported increased expression of SCF and its receptor c-kit (progenitor cell marker) in liver after APAP overdose in mice.[25] Although mice treated with anti-Scf antibody and Scf-deficient mice exhibited increased mortality, treatment with exogenous SCF increased hepatocyte proliferation and improved survival after APAP-induced liver injury.[25,64] Furthermore, α-fetoprotein (a marker of fetal phenotype

Table 2 Important Mediators of Regeneration Following APAP-Induced Liver Injury

Mediators	Findings
Growth factors	
EGFR	Activated within 15 minutes after APAP overdose in mice in a dose-dependent manner[11]
	Activated in primary human hepatocytes after APAP treatment[11]
	EGFR inhibition (after peak injury development) impaired regeneration, exacerbated injury progression, and decreased survival[11]
HGF/MET	Activation of MET within 3 hours after APAP overdose in mice in a dose-dependent manner[12]
	Elevated HGF levels in ALF patients (higher in nonsurvivors)[34]
Cytokines	
TNF-α/NF-κB	TNF-α levels and DNA binding of NF-κB associated with cyclin D1 expression and improved regeneration in several studies[17,38,39]
	Activation of NF-κB signaling and binding to cyclin D1 promoter after moderately toxic (regenerative) dose; inhibited after severe APAP overdose in mice[12]
	TNF-R1 KO: lower regeneration; probably secondary to higher initial injury[36]
IL-6/STAT-3	Serum and liver levels of IL-6 increased after APAP overdose in mice[27,41,42]
	Dose-dependent activation of this pathway after APAP overdose in mice[12]
	IL-6 KO: impaired regeneration without altering initial injury but exacerbated injury progression; regeneration restored in KO mice by IL-6 pretreatment[24]
Paracrine mediators	
Wnt/GSK-3β/β-catenin	Activation of β-catenin and binding to cyclin D1 promoter after moderately toxic (regenerative) dose; inhibited after severe APAP overdose in mice[12]
	β-catenin KO: impaired regeneration[13]; overexpression: improved regeneration even after severe APAP overdose[12]
	Activation of β-catenin correlated with spontaneous liver regeneration in ALF patients[13]
	GSK-3 inhibition (late intervention) resulted in β-catenin activation and early initiation of liver regeneration after severe APAP overdose in mice[45]
Bile acids/FGF	Cholic acid treatment increased regeneration leading to early regression of APAP injury in mice[47]
	FGF15 KO: regeneration not altered at doses of comparable toxicity to WT mice[49]
	Engineered FGF19 treatment: enhanced regeneration, survival and decreased injury even after severe APAP overdose in mice[48]
VEGF/VEGFR	VEGF and VEGFRs increased after APAP overdose in mice and rats[57–59]
	VEGFR inhibition: impaired regeneration[57]; hrVEGF administration: improved regeneration without altering initial injury[26]
	VEGFR1 KO: lower HGF, impaired regeneration and restoration of microvasculature; decreased survival[58]
Mediators of cell cycle arrest	
TGF-β	Activated in both mice and APAP-induced ALF patients; role in p21 activation, senescence, and impaired regeneration[35]
	Myeloid-specific KO: improved regeneration without altering injury[35]
	TGF-βR1 inhibition: improved regeneration even after very delayed treatment; improved survival after concurrent treatment with APAP[35]
p53	Dose-dependent activation after APAP overdose in mice; correlated with extent of DNA damage and failed DNA repair[50]
	KO: faster cell cycle progression and regeneration despite higher peak injury[51]
p21	Dose-dependent activation in mice and APAP overdose patients[12,35]
	KO: improved regeneration without altering injury[35]

ALF, acute liver failure; APAP, acetaminophen; EGF, epidermal growth factor; EGFR, EGF receptor; FGF, fibroblast growth factor; GSK, glycogen synthase kinase; HGF, hepatocyte growth factor; hr, human recombinant; KO, knockout; TGF, transforming growth factor; TNF, tumor necrosis factor; TNF-R1, TNF receptor 1; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; WT, wild type.

and progenitor cells)—positive cells were found in APAP-induced ALF patients,[63] and serum AFP levels were correlated with a better prognosis and survival in APAP-induced ALF patients.[14] All of these studies suggest a potential role of progenitor cells in liver regeneration after APAP-induced ALF, which should be explored in future studies from the standpoint of regenerative therapy and biomarker development.

Conclusion

Liver injury and ALF inflicted by APAP overdose is a significant clinical issue with limited treatment options. Mechanisms of liver injury after APAP overdose have been extensively investigated to develop novel therapeutic strategies. However, most patients seek medical attention late, such that injury is already established and difficult to

manipulate. Liver injury after APAP overdose is subsequently followed by compensatory liver regeneration, which promotes recovery. Several recent studies have demonstrated a crucial role of timely liver regeneration in determining final outcome after APAP overdose. However, the development of novel therapies for ALF directed at stimulating liver regeneration is hampered because of the limited mechanistic understanding of liver regeneration after APAP-induced ALF. The findings from several recent studies suggest that liver regeneration after APAP overdose involves a complex, time- and dose-dependent interplay of several mediators, including growth factors, cytokines, angiogenic factors, and other mediators such as β-catenin signaling (Figure 2 and Table 2). Liver regeneration after APAP overdose is a dose-dependent phenomenon, and liver regeneration is inhibited after severe APAP overdose, which may contribute to the progression of ALI to ALF. A lack of activation of crucial proliferative signaling and/or activation of antiproliferative signaling might be involved in this inhibition of liver regeneration. Most studies on liver regeneration after APAP overdose have used moderate APAP overdose in animal models in which tissue eventually regenerates spontaneously. It is important to study the mechanisms of impaired liver regeneration after severe APAP overdose, which simulates the pathophysiology of clinical APAP-induced ALF, in which patients cannot recover spontaneously. Thus, considering dose response of APAP in future study designs will be crucial in improving the mechanistic understanding of the differences between spontaneous transplant-free survival versus transplant-assisted survival and ALF-related deaths observed in APAP overdose patients. Apart from dose, several other factors such as nutritional status, age, and disease conditions can affect liver regeneration after APAP overdose, which might be relevant in clinical practice, need to be characterized in animal models. Furthermore, in any study designed to address the role of any mediator/intervention in liver regeneration after APAP overdose, it is important to consider the effect on initial liver injury because the level of liver regeneration can be indirectly affected by the extent of initial liver injury. Thus, studies should be carefully designed to investigate a direct role of any mediator on liver regeneration. Overall, stimulating liver regeneration in patients with APAP-induced ALF holds great therapeutic potential. More mechanistic studies are required for the identification of potential targets to stimulate liver regeneration after APAP overdose. Regenerative therapies at least could be helpful in bridging the time gap before liver is available for transplantation to ALF patients.

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