Recent Events in Alcoholic Liver Disease
V. Effects of ethanol on liver regeneration

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Diehl, Anna Mae. Recent Events in Alcoholic Liver Disease. V. Effects of ethanol on liver regeneration. Am J Physiol Gastrointest Liver Physiol 288: G1–G6, 2005; doi:10.1152/ajpgi.00376.2004.—Liver regeneration is necessary to recover from alcoholic liver injury. Herein, we review evidence that ethanol interferes with liver regeneration. Briefly, alcoholic fatty livers demonstrate increased rates of hepatocyte death. The latter provides a regenerative stimulus. However, unlike mature hepatocytes in healthy adult livers, most surviving mature hepatocytes in alcoholic fatty livers cannot replicate. Therefore, less mature cells (progenitors) must differentiate to replace dead hepatocytes. Little is known about the general mechanisms that modulate the differentiation of liver progenitors in adults. Delineation of these mechanisms and clarification of how ethanol influences them might suggest new therapies for alcoholic liver disease.

LIVER REGENERATION

As is true of other adult tissues, the mass of the liver is determined by the relative rates of liver cell death and proliferation. The mechanisms that balance mammalian hepatocyte death and proliferation have been studied extensively in rats and mice. For example, in healthy adult rats, the lifespan of mature hepatocytes is estimated to be >300 days. Because hepatocyte death occurs infrequently, hepatocyte proliferation is also unusual, as demonstrated by evidence that only about 1:10,000 hepatocytes are actively synthesizing DNA at any given point in time (17).

To study the mechanisms that regulate hepatocyte proliferation, factors that induce hepatocyte loss have been used experimentally to increase hepatocyte proliferative activity. Three general strategies have been used: 1) acute surgical resection of 70% of the liver mass [i.e., partial hepatectomy (PH)], 2) acute exposure to potent hepatotoxins, such as carbon tetrachloride (CCl4), or 3) exposure to “milder” hepatotoxins [such as 2-acetylamino-fluorene (2-AAF) or retrorsine] followed by PH. All of these liver injuries upregulate DNA synthesis in residual liver cells. The daughter cells repopulate the liver, and the mass of the organ is restored within days to weeks (5, 17). Studies of people after partial hepatic resection or patients with various types of acute or chronic liver disease confirm that similar responses occur in humans. These findings demonstrate that adult livers have enormous regenerative capacity, generally permitting rapid and complete recovery from liver injury.

MATURE AND IMMATURE LIVER CELLS CONTRIBUTE TO REGENERATION

Although all kinds of liver injury induce liver regeneration, the kinetics and certain other characteristics of the regenerative response can vary. In rats, for example, hepatocyte DNA synthesis peaks ~1 day after PH but 2 days after acute CCl4-induced liver injury. Consequently, liver mass is restored sooner after PH than after acute CCl4 exposure (17). The regenerative response is also protracted when rats are treated with a toxin (e.g., 2-AAF or retrorsine) before PH. In this situation, PH fails to induce DNA synthesis in mature hepatocytes but triggers dramatic expansion of the resident population of oval cells, progenitor cells that can differentiate into hepatocytes or cholangiocytes (5). After PH, these oval cells differentiate predominately into hepatocytes to replace the large numbers of hepatocytes that were resected.

One explanation for the model-dependent differences in liver regeneration is that the differentiation status of liver cells influences their sensitivity to injury-related factors that inhibit proliferation. In this regard, mature hepatocytes are more “fragile” than hepatic progenitor cells. Thus mature hepatocytes become growth inhibited by toxins that are unable to suppress the proliferation of the less-differentiated oval cells (5). The resultant “growth advantage” of the oval cells allows them to repopulate the liver, but this regenerative process takes longer than when more mature hepatocytes proliferate, because the hepatic progenitors must differentiate as well as proliferate (Fig. 1).

INJURY-RELATED SIGNALS MODULATE REGENERATIVE RESPONSES

The dramatic induction of hepatic DNA synthesis and liver growth that follows liver injury has somewhat obscured the fact that liver regeneration always involves some degree of hepatocyte death as well as the induction of local cytoprotective and repair responses. Thus the regenerative response to liver injury is actually an example of tissue remodeling. Hence, it is not surprising that certain kinases and transcription factors that are required during other situations when tissues are remodeled (e.g., during fetal hepatogenesis) (1, 8) are also necessary for regeneration of the adult liver (4, 10).

In general, tissue remodeling is triggered by some sort of stress and ultimately leads to the selective deletion of cells that are no longer useful. Cells that escape destruction induce protective mechanisms and/or mechanisms that permit them to repair minor damage they might have sustained. Ultimately, to restore or increase net tissue mass, surviving cells must enlarge and/or new cells must be generated (or recruited) to replace the cells that were eliminated.

PH is a convenient tool that has been used to trigger hepatic remodeling because it abruptly eliminates ~70% of the liver without overtly traumatizing the residual liver tissue that remains (17). Nevertheless, we showed that PH rapidly induces cytokines, such as TNF-α, that are potentially toxic to remaining hepatocytes (4). In previously healthy rats and mice, this TNF-α is necessary for the induction of hepatocyte DNA synthesis peaks ~1 day after PH but 2 days after acute CCl4-induced liver injury. Consequently, liver mass is restored sooner after PH than after acute CCl4 exposure (17). The regenerative response is also protracted when rats are treated with a toxin (e.g., 2-AAF or retrorsine) before PH. In this situation, PH fails to induce DNA synthesis in mature hepatocytes but triggers dramatic expansion of the resident population of oval cells, progenitor cells that can differentiate into hepatocytes or cholangiocytes (5). After PH, these oval cells differentiate predominately into hepatocytes to replace the large numbers of hepatocytes that were resected.
In healthy adult livers, mature hepatocytes replicate to replace hepatocytes that die. Mature hepatocytes become growth-inhibited by certain toxins (e.g., 2-AAF, retrotamine) that are unable to suppress the proliferation of less-differentiated progenitor cells. The resultant "growth advantage" of the progenitors allows them to repopulate the liver, but this regenerative process takes longer than when more mature hepatocytes proliferate because the hepatic progenitors must differentiate as well as proliferate.

Fig. 1. In healthy adult livers, mature hepatocytes replicate to replace hepatocytes that die. Mature hepatocytes become growth-inhibited by certain toxins (e.g., 2-AAF, retrotamine) that are unable to suppress the proliferation of less-differentiated progenitor cells. The resultant "growth advantage" of the progenitors allows them to repopulate the liver, but this regenerative process takes longer than when more mature hepatocytes proliferate because the hepatic progenitors must differentiate as well as proliferate.

Thus, even after PH, which is the "cleanest" model for inducing replication of mature hepatocytes in adults, liver regeneration is a multifaceted response to injury. Initially, defensive mechanisms are induced. Surviving hepatocytes are then surveyed for damage and repaired or deleted. Temporary hepatocyte hypertrophy helps to compensate for lost liver cells until they are replaced by the proliferation of residual liver cells and/or their progenitors. Recent studies provide additional support for this concept that the regenerative response to PH is comprehensive and involves far more than the proliferation of surviving mature hepatocytes. For example, pretreatment with pharmacological agents, such as 5-fluorouracil, which transiently inhibit hepatocyte DNA synthesis after PH, amplifies the hypertrophic component of the regenerative response in otherwise healthy rats. Other drugs, such as 2-AAF or retrotamine, which produce more profound inhibition of DNA synthesis in mature hepatocytes, accentuate the recruitment and expansion of hepatic progenitors to regenerating livers (5).

NATURE OF REGENERATIVE RESPONSE DICTED BY INTRACELLULAR RESPONSES TO STRESS

Studies of mice that are genetically deficient in type 1 TNF receptors (TNFR1) prove that interactions between this receptor and TNF-α are required for the liver to regenerate after PH (24). However, TNFR1-null mice are born viable and grow to adulthood. Therefore, this interaction is not required for liver development. Even in normal adult mice, hepatocyte proliferation can be induced without TNF-TNFR1 interactions. This is well demonstrated by evidence that certain drugs and hormones increase hepatocyte proliferation and liver growth after experimental inactivation of TNF-α (14).

These findings suggest that TNF-α and TNFR1 are merely one set of factors that regulate the true proximal (i.e., intracellular) mediators of hepatocyte proliferation. Consistent with this concept, both post-PH liver regeneration in adults and growth of the liver bud in developing embryos are abolished by genetic disruption of various TNF-regulated genes that encode transcription factors (e.g., NF-kB p65) or stress-related kinases (e.g., SEK-1) that permit adult hepatocytes to survive TNF exposure (1, 10). NF-kB and SEK-1 are also induced by various other stresses and are known to regulate cell survival after exposure to stress.

Together, these observations suggest that stress-regulated intracellular signals permit a spectrum of responses in "stressed" cells, including proliferation, survival without proliferation (i.e., senescence), and death. Why and how cells select a particular response over other options remains the subject of intensive basic research for many laboratories. However, studies in cultured cells suggest that the type and duration of reactive oxygen species (ROS) that accumulate following cellular stress play a pivotal role in this decision.

TRANSIENT INCREASES IN INTRACELLULAR ROS PROMOTE PROLIFERATION

Transfection of fibroblasts with the protooncogene v-Ha-ras rapidly increases superoxide anion (O_2^-) production. The increased ROS production is followed by increased proliferation (11). O_2^- is necessary for v-Ha-ras-mediated proliferative activity because proliferation is inhibited by treatments, such as overexpression of manganese superoxide dismutase (Mn-SOD), that detoxify O_2^-.

Interestingly, malignant transformation also requires the activation of another protooncogene, Ki-ras, that is known to activate ERKs 1–2. ERKs 1–2 are also induced by many mitogens, and they are known to activate Mn-SOD via posttranslational mechanisms. Thus acute but transient increases in O_2^- appear to be necessary for protooncogenes and growth factors to induce cellular proliferation (Fig. 2).
CHRONIC OVERPRODUCTION OF ROS RESULTS IN SENESCENCE

Fibroblasts that have been transformed by v-Ha-ras are also known to exhibit premature replicative senescence, i.e., they become refractory to the growth-stimulatory actions of mitogens. Replicative senescence (also called “mitoinhibition”) requires the accumulation of H₂O₂ because it can be prevented by agents that scavenge H₂O₂ (but not by agents that detoxify O₂⁻; Fig. 2) (15).

H₂O₂ elicits several responses that cooperate to inhibit cell cycle progression at the G-1/S checkpoint. For example, H₂O₂ activates the stress-related protein kinase p38 MAPK (13). p38 MAPK inhibits the transcription and posttranscriptional stability of cyclin D-1, a factor that is necessary for cells to move from G-1 into S phase. Cyclin D-1 protein has many functions that promote cell cycle progression. For example, it physically interacts with cyclin-dependent kinases (CDKs) to form cyclin-CDK complexes that liberate the transcription factor E2F from its inhibitor, Rb, and permit the activation of S-phase genes. Cyclin D-1 protein also interacts with the transcription factor Stat-3. This interaction sequesters Stat-3 in the cytosol and prevents activated (i.e., phosphorylated) Stat-3 from entering the nucleus, where it normally induces gene transcription (2). The target genes of Stat-3 include several cyclin-dependent kinase inhibitors, including the cyclin D-1/CDK inhibitor p21. Therefore, if Stat-3 activation occurs when cyclin D-1 protein levels are low, phosphorylated (activated) Stat-3 accumulates in nuclei and promotes p21 transcription. Increases in p21, in turn, further inhibit cyclin-CDK activity, perpetuating cell-cycle arrest (Fig. 3). This mechanism helps to explain observations that H₂O₂-mediated replicative senescence involves p21 induction as well as evidence that Stat-3 “hyperphosphorylation” is common in growth-arrested cells.

EFFECTS OF CHRONIC ALCOHOL CONSUMPTION ON LIVER REGENERATION

EtOH Increases Hepatocyte ROS and Kills Mature Hepatocytes

EtOH exposure increases cellular production of ROS, including H₂O₂. When antioxidant defenses become overwhelmed, hepatocyte viability decreases and liver damage ensues (21). In both experimental animals and humans, increased rates of hepatocyte death occur at the earliest stages of alcoholic liver disease, such as fatty livers (19). Therefore, even “mild” alcoholic liver damage presents a stimulus for liver regeneration.

EtOH Promotes Replicative Senescence in Mature Hepatocytes

Given the aforementioned evidence that excessive H₂O₂ causes replicative senescence in fibroblasts, increased H₂O₂ might help to explain observations that chronic EtOH exposure inhibits induction of DNA synthesis in mature hepatocytes. In support of this possibility, Devi et al. (3) showed that exposure of cultured rat hepatocytes to EtOH increased ROS production, decreased OSH and ATP levels, and increased lipid peroxidation, while inhibiting epidermal growth factor-induced DNA synthesis. Pretreatment of these cells with N-acetylcysteine or S-adenosylmethionine for 24 h prevented the EtOH-induced reduction of ATP and GSH levels and restored replication (3). The intracellular signals mediating these antiproliferative effects were not characterized by those investigators.

Our recent work (12) demonstrates that chronic EtOH induces many of the typical H₂O₂-regulated events that cause replicative senescence in fibroblasts (Fig. 3). Moreover, subsequent removal from EtOH and treatment with GSH precursors failed to “rescue” the mitogenic response in the senescent hepatocytes. The latter finding implies that mature hepatocytes are probably incapable of increasing their proliferative activity to compensate for EtOH-induced increases in hepatocyte death.

Liver Progenitors Accumulate in Alcohol-Damaged Livers

Evidence that EtOH provides a regenerative stimulus to the liver (by increasing the hepatocyte death rate) while inhibiting the proliferative activity of mature hepatocytes suggests that this commonly used drug may act similarly to certain hepatotoxins (e.g., 2-AAF, retrorsine) that are used experimentally to induce compensatory expansion of progenitor cell populations. Studies in rats, mice, and humans with alcoholic liver damage support this possibility by demonstrating significant hepatic accumulation of putative liver progenitor cells (i.e., “oval” cells; see Ref. 19 and references cited therein).

This finding suggests that progenitor cell differentiation becomes a critical component of the regenerative response in alcohol-damaged livers (Fig. 4). The added requirement for differentiation is predicted to lengthen the time needed to accomplish regeneration. Consistent with this concept, it is well known that the regenerative response to liver injury is significantly delayed in experimental animals and humans with alcohol-induced liver damage.

REGULATION OF LIVER PROGENITOR CELL FATE DURING LIVER REGENERATION

Identity of Liver Progenitor Populations

Although there is general consensus that various toxins that inhibit the replication of mature hepatocytes promote the expansion and differentiation of liver progenitors, very little is known about the specific mechanisms that mediate this pro-
The progenitors themselves have been characterized predominately by their morphology (i.e., small, oval cells that express biliary-type cytokeratins) and tendency to localize near the canals of Herring. These progenitor (also known as "oval") cells have been shown to differentiate into hepatocytes or cholangiocytes, depending on the microenvironment to which they are exposed. Recently, Thorgerisson and Grisham (20) has shown that the oval cell population itself is heterogeneous and suggested that this heterogeneity reflects cells that are at different stages of differentiation. However, the identity of the most primitive progenitor cells that ultimately develop(s) into hepatocytes remains obscure. Consequently, it is uncertain whether the oval cell population in adult livers derives from a resident or circulating stem cell with bonafide self-renewal capacity.

The “plasticity” of the putative liver stem cell is also unknown. For example, it is not known whether the liver stem cell in adults differentiates solely along an epithelial lineage to generate hepatocytes and cholangiocytes or is more pluripotent, giving rise to one or more of the other types of cells (e.g., endothelial cells, macrophages, lymphocytes, stellate cells) that reside in adult livers or other tissues (e.g., bone marrow or pancreas). If further study shows that various types of adult liver cells are derived from a common, pluripotent progenitor, then it will be important to learn how cell-fate decisions are regulated. On the other hand, if the progeny of liver stem cells are restricted to the epithelial lineage, then it will be important to determine whether the other cell types in adult livers have their own unique progenitors and to delineate the mechanisms that regulate the expansion and differentiation of these populations.

Control of Progenitor Cell Differentiation: Lessons from Embryonic Development

Given emerging evidence that recovery of normal liver architecture after alcohol-induced liver damage involves differentiation of liver progenitors, it will be important to determine how chronic ethanol exposure modulates the viability and differentiation of liver progenitors. At this point, however, understanding about the general mechanisms that regulate progenitor cells in adult livers is limited, and virtually nothing is known about how ethanol effects these processes. Information gathered by studying embryonic development might be useful to understand how progenitor cell biology is regulated in adult livers. Over the last decade, there have been tremendous advances in understanding the mechanisms involved in hepatic morphogenesis in developing embryos. It is now known that the liver is derived from the ventral endoderm under the guidance of signals (e.g., fibroblast growth factors and bone morphogenetic proteins that are provided by the adjacent cardiac mesenchyme and septum transversum; Fig. 5) (25). Early in this process, the condensed chromatin in primitive endodermal cells prevents their cellular DNA from interacting with transcription factors that are required to induce liver cell-specific gene expression. Specification to liver cell fate follows the binding of GATA-4 and FoxA [also called hepatocyte nuclear factor (HNF)-3] to the condensed chromatin, an event that “opens” the chromatin structure of the primitive endodermal cells sufficiently for other transactivating factors to bind to their respective DNA-binding elements. Specification to the liver cell fate is reversible initially. Hence, these cells retain their multipotency and can differentiate along various endodermally derived lineages, depending on the microenvironment. For example, endodermal cells that are bathed in Sonic hedgehog ultimately become liver cells, whereas those that are not exposed to this factor differentiate along the pancreatic lineage.

Sustained commitment to the liver lineage develops as the primitive endodermal cells migrate into the cardiac mesenchyme to form the liver bud. In their new environment, the “receptive” GATA4/FoxA-positive endodermal cells intermingle with endothelial cells and become exposed to soluble factors that induce other transcription factors (e.g., GATA 6), which, in turn, interact with the newly accessible DNA to
induce transcriptional cascades that eventually generate mature hepatocytes or cholangiocytes. Terminal differentiation into hepatocytes appears to require activation of HNF-4α. Another transcription factor, HNF-6, seems to repress hepatocyte differentiation while promoting activation of Notch/Jagged signaling and the induction of HNF-1β, a transcription factor that favors differentiation into cholangiocytes.

**Progenitors Generate Mature Liver Cells in Injured Adult Livers**

The significance of these pathways that regulate the fate of liver progenitors in embryos to adult liver regeneration is beginning to be evaluated. There have been several reports (6, 7, 22) that damaged adult mouse and rat livers can be repopulated by resident liver progenitors (i.e., oval cells) as well as by progenitors that were derived from fetal liver, adult bone marrow, or adult pancreas. In models that provoke predominately hepatocyte damage, the repopulating cells come to resemble hepatocytes, liver function is restored, and survival is improved. When transplanted into models of bile duct injury, the same cells turn into cholangiocytes, replace damaged bile ducts, and rescue the animal. Therefore, injured adult livers are clearly capable of generating signals that can transform progenitor cells into functionally mature liver cells.

Controversy rages, however, about whether these beneficial responses result from differentiation of liver stem cells, fusion of myeloid progenitors with resident liver cells, or reprogramming of “unrelated” stem cells so that they adopt a liver cell phenotype (7, 23). Which mechanism predominates may depend on the type of progenitor cell as well as factors that are related to the type/severity of the liver damage that is being studied. Reports that resident liver nonparenchymal cells, including injury-activated oval cells, express Notch and Jagged suggest that certain types of liver injury may at least partially reactivate pathways that control progenitor cell differentiation during fetal development. This area is in great need of further research.

**Effects of Ethanol on Progenitor Cell Fate**

Once the fundamental pathways that regulate progenitor cell fate in injured adult livers have been characterized, it will be important to determine how ethanol influences these mechanisms. The latter work will have broad implications. For example, it may suggest novel markers that could be used to identify individuals who are at high risk for chronic liver damage because they have insufficient reparative (regenerative) responses. In addition, a better understanding of mechanisms that regulate the generation of mature liver cells from progenitors might expand therapies to improve this response and speed recovery from alcoholic liver damage. Finally, determining how alcohol alters progenitor cell fate might provide insight into other types of alcohol-mediated pathology in adults and during fetal development.

In summary, chronic consumption of ethanol generally increases the death rate of mature hepatocytes. This stimulates a compensatory regenerative response to preserve normal liver mass and function. Most of the mature hepatocytes that survive in alcohol-damaged livers are replicatively senescent and hence incapable of proliferating to replace their dead neighbors. Hence, regeneration of alcohol-damaged livers involves the expansion and differentiation of liver progenitor cells. Research is needed to delineate the general mechanisms that modulate the fate of liver progenitor cells in adults and to characterize the effects of chronic ethanol consumption on this process. This work is likely to have broad relevance to alcohol-induced pathology in various adult and fetal tissues.

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**REFERENCES**


