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TOPIC HIGHLIGHT

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Effects of ethanol on hepatic cellular replication and cell cycle progression

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Abstract

Ethanol is a hepatotoxin. It appears that the liver is the target of ethanol induced toxicity primarily because it is the major site of ethanol metabolism. Metabolism of ethanol results in a number of biochemical changes that are thought to mediate the toxicity associated with ethanol abuse. These include the production of acetaldehyde and reactive oxygen species, as well as an accumulation of nicotinamide adenine dinucleotide (NADH). These biochemical changes are associated with the accumulation of fat and mitochondrial dysfunction in the liver. If these changes are severe enough they can themselves cause hepatotoxicity, or they can sensitize the liver to more severe damage by other hepatotoxins. Whether liver damage is the result of ethanol metabolism or some other hepatotoxin, recovery of the liver from damage requires replacement of cells that have been destroyed. It is now apparent that ethanol metabolism not only causes hepatotoxicity but also impairs the replication of normal hepatocytes. This impairment has been shown to occur at both the G1/S, and the G2/M transitions of the cell cycle. These impairments may be the result of activation of the checkpoint kinases, which can mediate cell cycle arrest at both of these transitions. Conversely, because ethanol metabolism results in a number of biochemical changes, there may be a number of mechanisms by which ethanol metabolism impairs cellular replication. It is the goal of this article to review the mechanisms by which ethanol metabolism mediates impairment of hepatic replication.

Key words: Cyclin-dependent kinases; Cell cycle; Liver regeneration; Ethanol metabolism; Acetaldehyde; Alcoholic liver disease

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INTRODUCTION

The association between alcohol consumption and liver disease has been recognized for centuries. It is now well established that ethanol is a hepatotoxin. It is thought that the liver is a target of ethanol-induced toxicity because the hepatocytes of the liver are the primary ethanol metabolizing cells in the human body. The metabolism of ethanol results in many biochemical changes that have been proposed to contribute to liver disease. Among these changes are the generation of the reactive intermediate acetaldehyde, increased generation of reactive oxygen species, anoxia, changes in mitochondrial functions and bioenergetics, as well as an increase in the redox-state of hepatocytes.

There are two major ethanol-metabolizing pathways in hepatocytes; the first is mediated by the cytosolic enzyme alcohol dehydrogenase. This enzyme is responsible for the vast majority of the ethanol metabolism by hepatocytes[1]. The second major pathway is mediated by the membrane bound enzyme cytochrome P450 2E1. The activity of this enzyme has been shown to be induced by ethanol and therefore, it has a greater contribution to the metabolism of ethanol in cases of chronic ethanol abuse[2]. Even though there is a tremendous amount of evidence indicating that ethanol metabolism is required for many ethanol-induced dysfunctions, the mechanism(s) by which ethanol metabolism causes hepatic damage are not entirely clear. Although the mechanisms of ethanol-induced liver damage are equivocal, it is obvious that recovery from ethanol-induced liver injury is dependent on the ability of cells to replicate, replacing those damaged or killed as a consequence of ethanol metabolism.

Cellular replication is a process in which a tightly orchestrated series of sequential events must occur for successful completion. This series of events is collectively referred to as the cell cycle. The ultimate goals of the cell cycle are accurate replication of DNA and cell division.

The cell cycle can be broadly divided into five stages or phases. G0 is a stage in which cells are metabolically active but are not actively involved in the replication process (hepatocytes are normally in this stage). G1 is the initial stage of the replicative cycle, this is the stage in which enzymes and substances required for DNA synthesis are produced. S is the stage where DNA synthesis occurs. G2 is the stage in which the enzymes and substances required for mitosis are synthesized or activated. M phase is the stage in which cells divide and cellular replication is completed.

Tissue repair is a balance between damage and repair. Tissue damage occurs when injury dominates the ability of the tissue to replace cells lost to injury. In the case of ethanol-induced liver damage, it appears that ethanol metabolism is not only responsible for tissue damage but also impairs the ability of hepatocytes to respond normally and replace dead or damaged cells. Whether cellular injury to the liver has been caused by the effects of ethanol metabolism or by some other means, recovery is dependent, at least in part, on the capacity of the liver to replace cells lost to toxic insult. It is the focus of this article to review the evidence and potential mechanisms by which ethanol metabolism impairs the replication of hepatic cells and initiates or enhances alcoholic liver disease.

G1 phase inhibition

The liver has a tremendous capacity to replace cells that are lost or damaged by cytotoxic injury. In fact, if part of the liver is surgically removed, the remaining portion of the liver will increase in size to replace the mass of the original organ. This surgical procedure, performed with experimental animals has been termed partial hepatectomy and has become a standard model to investigate hepatic regeneration. Following partial hepatectomy, a synchronized wave of cellular replication occurs; thus, this model of liver damage is extremely amenable to the study of individual aspects of the cell cycle.

Much of what we have learned about the effects of ethanol on the cell cycle and hepatic replication has been derived through the use of the partial hepatectomy model of liver injury. In early studies using this model of liver injury, Wands *et al*[3] found that the timing of ethanol administration after partial hepatectomy was important in determining the effects of ethanol on hepatic regeneration. [12] If the animals were administered a single dose of ethanol 4 h before, or up to 8 h after partial hepatectomy, no effect of ethanol was detected on DNA synthesis 24 h after the surgical procedure. Conversely, if ethanol was administered 12-16 h after partial hepatectomy, DNA synthesis at 24-h post surgery was significantly impaired. If the animals received multiple doses of ethanol 0-8 h afterpartial hepatectomy, the effects of ethanol were altered and more severe inhibition of DNA synthesis was observed. These results led the authors to suggest that the inhibitory effects of ethanol on liver regeneration were cell cycle dependent; hepatocytes appeared to be most sensitive to the effects of ethanol early in G1, prior to the initiation of DNA synthesis. Once DNA synthesis was initiated, hepatocytes appeared to be relatively resistant to the effects of ethanol[3]. It has also been shown that chronically feeding ethanol to experimental animals impairs the regenerative capacity of the liver[3,4]. This impairment was characterized by a delay in the peak, as well as a reduction in the magnitude of DNA synthesis, after partial hepatectomy[4].

Although these initial *in vivo* investigations established the fact that ethanol affected the regenerative capacity of the liver, they did not address the possible mechanisms involved in this impairment. Higgins and Borenfreund, using the rat liver tumor cell line 32ⅡA6/2d, showed that addition of pharmacological concentrations of ethanol (10-100 mmol/L) for up to three days resulted in a concentration-dependent reduction in the final population density of the culture[5]. Additionally, these authors demonstrated that substitution of acetaldehyde, the reactive byproduct of ethanol metabolism, for ethanol in the growth medium reproduced the ethanol-mediated impairments. This indicated that ethanol metabolism was responsible for the observed impairments and that acetaldehyde had a role in this dysfunction. To further implicate the role of ethanol metabolism, addition of the alcohol dehydrogenase inhibitor, pyrazole, ameliorated the ethanol-mediated impairments[5]. Extending these findings, Higgins and Borenfreund analyzed 32ⅡA6/2d cells exposed to ethanol by flow cytometry and found an increase in the percentage of cells in the G1 phase of the cell cycle. The authors concluded that the inhibition of cellular proliferation associated with acute ethanol exposure in these cells was a result of this accumulation of cells in the G1 phase of the cell cycle[6].

In the normal liver, the vast majority of hepatocytes are arrested in the G0 phase of the cell cycle. Hepatocytes require stimulation of hormones and growth factors to traverse G1 and begin cellular replication[7]. Using isolated rat hepatocytes, Carter *et al*[8] demonstrated that inclusion of ethanol to the growth medium dampened the proliferative response induced by hormonal and growth factor stimulation. These results indicated that the signals required to induce completion of G1 are also affected by ethanol.

Ethanol metabolism results in the production of reactive oxygen species; accumulation of reactive oxygen species results in oxidative stress[9,10]. After partial hepatectomy there is an increase in the expression of TNFα and a number of mitogens[7]. Interestingly, TNFα (which is necessary for induction of hepatocyte DNA synthesis) and mitogens both cause transient increases of H_2O_2 (a species of reactive oxygen). Transient increases in reactive oxygen species induce the expression of three important mediators of hepatocyte replication, the mitogen activated protein kinase (MAPK), extracellular regulated kinases (ERK) 1 and 2, and cyclin D1. Prolonged exposure to reactive oxygen species not only induces ERK 1 and 2, but also induces the cyclin dependent kinase inhibitor p21, and p38 MAPK, an inhibitor of cyclin D1. The combined effects of reduced cyclin D1 and expression of p21 efficiently inhibits the proliferation of cells[11].

Investigating the effect of chronic ethanol administration on liver regeneration after partial hepatectomy, Koteish *et al*[12] demonstrated that p38 MAPK and p21 were significantly increased in the livers of animals chronically fed ethanol, compared to controls after partial hepatectomy. These increases were accompanied by decreased expression

of cyclin D1, increased expression and activity of the cyclin dependent kinase inhibitor p27, and an increase in the activity of the transcriptional activator signal transducer and activator of transcription-3 (STAT-3). STAT-3 can be involved in cell cycle progression or growth arrest[13] and is capable of inducing the expression of the cyclin-dependent kinase inhibitors p21 and p27. Normally, cyclin D1 binds to and sequesters p21, effectively nullifying its inhibitory affects. When cyclin D1 is deficient, as it is after partial hepatectomy in ethanol-fed animals, the activity of p21 as a cyclin-dependent kinase inhibitor is favored and cells can be arrested at the G1/S transition. Because ethanol metabolism produces reactive oxygen species in hepatocytes, the authors suggested that the increased activity of STAT-3, induction of p21, and cell cycle arrest at the G1 stage of the cell cycle may be a form of cytoprotection, protecting the cells from the acute increases in reactive oxygen species resulting from partial hepatectomy or other oxidative injury[12].

Fat accumulation and impaired hepatic replication

The first pathologic change that occurs to the liver as a result of ethanol consumption is the appearance of fat in the liver, a condition known as steatosis. The accumulation of fat in the liver was once thought to be benign, but is now recognized as a factor that can be involved in more serious liver injury[14]. It has been proposed that ethanol sensitizes the liver to a "second hit", and that this "second hit" then induces the damage associated with alcoholic liver disease[15]. In the model proposed above, fat accumulation in the liver, as a result of ethanol consumption, sensitizes the liver to additional oxidative injury.

To determine if the accumulation of fat in the liver was sufficient to sensitize the liver to oxidative damage, Torbenson *et al*[16] investigated the effects of fatty liver on liver regeneration after partial hepatectomy. Mice with mutations in their leptin genes (leptin deficient ob/ob mice) spontaneously develop fatty livers. Using ob/ob mice, Torbenson *et al*[16] found that liver regeneration was delayed in the ob/ob mice compared with lean littermates. The authors also found that the accumulation of the activated, phosphorylated form of STAT-3 (pSTAT-3) was increased in the livers of ob/ob mice compared with lean littermates. Additionally, an inverse correlation between the nuclear location of pSTAT-3 and DNA synthesis was observed, as well as decreased percentages of nuclei labeled with the DNA analog 5-bromo deoxyuridine (BrdU), increased expression of the cyclin-dependent kinase inhibitors p21 and p27, and expression of cyclin D1[16]. These authors also suggested that this cell cycle arrest could act as a cytoprotective mechanism to protect hepatocytes from reactive oxygen species.

These results were very reminiscent of those observed in ethanol-fed animals, and leads one to speculate whether the induction of fatty liver by ethanol consumption is a causative agent of cell cycle impairment and aberrant cellular replication.

Mitochondrial dysfunction

Mitochondrial dysfunction has also been implicated as a mechanism by which ethanol metabolism mediates hepatocyte injury. The mitochondria are the sites where oxidative phosphorylation occurs and ATP is produced. The process of oxidative phosphorylation inherently generates the reactive oxygen species, superoxide anion, and therefore, is a major source for the production of reactive oxygen species. Interestingly, mitochondrial dysfunction has also been shown to have a role in impaired hepatic regeneration.

Mice deficient in the inner mitochondrial anion carrier uncoupling protein-2 (UCP2), a negative regulator of superoxide production[17], show impairment in their hepatic regenerative response after partial hepatectomy[18]. The characteristics associated with this impairment in liver regeneration are reminiscent of those observed in ethanol-fed and leptin deficient ob/ob animals, and include elevated levels of reactive oxygen species, as well as increased and sustained expression of p21 and p38 MAPK.

UCP2 is induced by fatty acids and its expression is upregulated in the livers of ethanol-fed and ob/ob animals[19,20]. Although one would expect that increased UCP2 would inhibit reactive oxygen-mediated impairment of liver regeneration, this does not appear to be the case[18]. It has been suggested that the excess fuel supply present in the fatty liver promotes increased production of superoxide to levels beyond the capacity of the increased UCP2. This results in a net increase in reactive oxygen production that leads to the reactive oxygen species-mediated activation of signals, cell cycle inhibition, and impaired hepatic regeneration[18].

G2/M transition

Using cells based on the well-differentiated hepatoblastoma cell line, Hep G2, that were genetically engineered to express alcohol dehydrogenase, our group investigated the effects of ethanol on cellular replication. Initially, it was shown that culturing these cells, termed VA-13 cells, in the presence of 25 mmol/L ethanol resulted in a decrease in the accumulation of the number of cells in the culture, and that this effect could be ameliorated by inhibiting alcohol dehydrogenase activity[21]. Additionally, it was shown that inclusion of cyanamide, an inhibitor of aldehyde dehydrogenase, accentuated this impairment and that culturing the VA-13 cells in the presence of isopropanol, had no effect on cell accumulation. Alcohol dehydrogenase-mediated metabolism of isopropanol results in the production of acetone, not acetaldehyde. These results led to the proposal that acetaldehyde was responsible for the impairment in cell accumulation[21].

Further investigation of the mechanisms of this impairment revealed an increase in the percentage of cells at the G2/M transition of the cell cycle in cultures of VA-13 cells maintained in ethanol[22]. The activity of the cyclin-dependent kinase, CDK1, is required for mitosis to occur[23]. Catalytic activity of CDK1 is negatively regulated by phosphorylation at threonine 14 (Thr 14) and tyrosine 15 (Tyr 15) and positively regulated by its interaction with cyclin B1. It was shown that the inactive phosphorylated form of CDK1 was increased by ethanol metabolism. Additionally, it was shown that there was no impairment in the ability of CDK1 to associate with cyclin B1. These findings indicated

that the G2/M cell cycle arrest was, at least in part, the result of an increase in the phosphorylated form of CDK1 and decreased activity of this cyclin-dependent kinase, the activity of which is required for mitosis[22].

Recently we have begun studies investigating the mechanisms by which ethanol metabolism results in increased phosphorylation of CDK1, and have found that ethanol metabolism activated the checkpoint kinases Chk1 and Chk2. A number of stages or transitions at which the cell cycle is delayed or arrested have been described and have been termed checkpoints[24]. These checkpoints are highly conserved evolutionarily. It is generally thought that the checkpoints have been conserved because of the importance of repairing damaged or inaccurately replicated DNA, thereby ensuring genomic integrity.

Cell cycle checkpoints result from the activation of signal transduction pathways that are mediated by ataxia telangiectasia mutated (ATM) and ATM-and Rad3-related (ATR), two closely related, highly conserved kinases with catalytic domains related to the phosphatidylinositol 3-kinase[25]. ATM and ATR are thought to share the responsibility as the apical protein kinases of all cell cycle checkpoints with the possible exception of the mitotic spindle checkpoint[25]. A variety of stimuli can initiate activation of the ATM/ATR regulated signal transduction pathways these include: DNA damage, delayed or incomplete DNA replication, viral infection, or oxidative stress[25,26]. In cells of human origin, ATM and ATR primarily mediate their actions through two downstream effector kinases Chk1 and Chk2[27]. One of the cell cycle transitions that can be regulated by this signal transduction cascade is the transition from the G2 to the M phase of the cell cycle. Although Chk1 and Chk2 are structurally unrelated serine/threonine protein kinases that are activated through phosphorylation by either ATM or ATR[28], functionally they exhibit significant overlap with respect to substrate specificity, which appears in some circumstances to function redundantly.

Chk1 and Chk2 react with a variety of substrates involved in the G2/M cell cycle arrest. Among the more important are the phosphatase Cdc25c, and the tumor suppressor p53[29-31]. Cdc25c is a phosphatase that removes the inhibitory phosphates at Tyr 15 and Thr 14 of CDK1[32]. Both Chk1 and Chk2 phosphorylate Cdc25c at Ser 216. This phosphorylation creates a 14-3-3 protein-binding site; binding of 14-3-3 to Cdc25c results in Cdc25c being sequestered in the cytoplasm, inhibiting its transport to the nucleus where it is required to dephosphorylate CDK1[32]. Using the VA-13 cells we have found that ethanol metabolism results in activation of both Chk1 and Chk2 and an increase in the phosphorylation of Cdc25c at serine 216. This may inhibit the dephosphorylation of CDK1, and contribute to the cell cycle arrest at the G2/M transition.

The tumor suppressor p53 is a transcriptional activator and therefore, binds specific DNA sites and modulates the transcriptional activity of target genes. The activity of p53 is primarily regulated by post-translational modification[33]. Chk1 and Chk2, as well as ATM and ATR, phosphorylate p53. Phosphorylation and acetylation of p53 enhances p53 activity by increasing its stability, making it a less desirable substrate for Mdm2-mediated degradation, and increasing its affinity to its target sites[34-36]. Therefore, p53 mediates its actions by modulating the expression of its downstream targets. Among the cell cycle transcriptional targets that are up-regulated by p53 are p21 and 14-3-3σ[37]. Additionally, expression of cyclin B1, the cyclin required for the G2/M activity of CDK1, is repressed[38]. We have shown that the phosphorylation of p53 is increased in VA-13 cells exposed to ethanol, and that there is an increase in the expression of the cyclin dependent-kinase inhibitor p21 indicating that p53 may also be involved in the ethanol metabolism-mediated G2/M cell cycle arrest. Thus, multiple p53-dependent and-independent pathways regulate the G2/M transition.

Cell cycle arrest and liver disease

There is little doubt that ethanol impairs the replication of hepatocytes by a number of mechanisms, and that this impairment may have detrimental consequences not only in terms of inhibiting cellular replication, but also perhaps by aberrant cellular replication. The replacement of damaged or injured hepatocytes lost from the liver parenchyma normally occurs by the replication of mature hepatocytes[39]. The normal replication of mature hepatocytes can be inhibited by cell cycle delay or arrest[11,12,40,41]. Under conditions where normal mature hepatocyte replication is inhibited, the replication of the bipotential progenitor cells known as oval cells is increased to compensate for the inhibition of normal hepatocyte replication[40,42,43]. Because oval cells are bipotential and can differentiate into either hepatocytes or bile ductular epithelium, it has been proposed that impairment of the primary pathway of hepatocyte replacement not only leads to regeneration of hepatocytes but also to increased proliferation of ductals[44]. Importantly, it has been shown that there is a very strong correlation between the increased proliferation of bile ducts and liver fibrosis in patients suffering from alcoholic liver disease[42,45]. This has led to the suggestion that impairment of mature hepatocyte replication and increased replication of oval cells contributes to portal fibrogenesis[44].

CONCLUSION

Alcoholic live disease is a complex multifactoral disease. It appears that the biochemical changes that occur in the liver as a result of ethanol are primarily responsible for the ethanol-induced toxicity to this organ. The liver has a tremendous capacity to replace cells lost to toxic insult. It is clear that the biochemical changes that occur as a result of ethanol metabolism can affect the replication of hepatic cells and potentially the recovery of the liver. The impairment in cellular replication may initiate or potentate liver damage by impairing the regeneration of cells lost to ethanol metabolism-mediated toxic injury or induction of aberrant replicative pathways. Further investigation of these dysfunctions is required to fully delineate the role of impaired cellular replication in alcoholic liver disease.

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