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Drinks like a fish: Using zebrafish to understand alcoholic liver disease

Deanna L. Howarth, Mike Passeri, and Kirsten C. Sadler¹

Division of Liver Diseases/Department of Medicine, Department of Developmental and Regenerative Biology, Mount Sinai School of Medicine, Box 1020, 1 Gustave L. Levy Place, New York, NY 10029

Abstract

Steatosis is the most common consequence of acute alcohol abuse, such as occurs during a drinking binge. Acute alcohol induced steatosis may predispose to more severe hepatic disease. We have developed a model of alcoholic liver disease (ALD) in zebrafish larvae to provide a system in which the genes and pathways that contribute to steatosis can be rapidly identified. Zebrafish larvae represent an attractive vertebrate model for studying acute ALD because they possess the pathways to metabolize alcohol, the liver is mature by 4 days post-fertilization (dpf), and alcohol can be simply added to their water. Exposing 4 dpf zebrafish larvae to 2% ethanol (EtOH) for 32 hours achieves ~80 mM intracellular EtOH and upregulation of hepatic *cyp2e1*, *sod* and *bip*, indicating that EtOH is metabolized and provokes oxidative stress. EtOH-treated larvae develop ALD as demonstrated by hepatomegaly and steatosis. Increased lipogenesis driven by the sterol response element binding protein (SREBP) transcription factors is essential for steatosis associated with chronic alcohol ingestion but it has not been determined if the same pathway is essential for steatosis following a drinking binge. We report that several Srebp target genes are induced in the liver of zebrafish exposed to EtOH. We used fish which harbor a mutation in the gene encoding the membrane bound transcription factor protease 1 (*mbtps1*; also called site-1 protease) and embryos in which the Srebp cleavage activating protein (*scap*) is knocked down to determine the requirement of this pathway in acute ALD. We find that both means of blocking Srebp activation prevents steatosis in response to 2% EtOH. Moreover, this is accompanied by the failure to activate several Srebp target genes in response to alcohol. We conclude that Srebps are required for steatosis in response to acute alcohol exposure. Moreover, these data highlight the utility of zebrafish as a useful new vertebrate model to study ALD.

Introduction

Lipid accumulation (steatosis) in hepatocytes is the most common and immediate hepatic manifestation of excessive alcohol ingestion. While sustained steatosis due to chronic alcohol abuse is frequently coupled with inflammation and liver damage (i.e. hepatitis), binge drinking typically results in transient steatosis that resolves if drinking ceases. While steatosis from binge drinking is reversible and likely benign, when coupled with other agents that damage the liver such as diabetes or viral infection, it can precipitate more serious liver disease.

Studies in mammalian models provide a basis for understanding the pathways leading to steatosis and steatohepatitis resulting from chronic alcohol exposure, but it is not known whether these same pathways cause lipid accumulation due to binge drinking. Steatosis due

¹Corresponding author: Kirsten.edepli@mssm.edu, Phone: 212-241-7152, Fax: 212-860-9279.

to chronic alcohol abuse is largely attributed to two main causes. First, alcohol metabolism leads to oxidative stress, which results in mitochondrial damage and reduced lipid oxidation (Cederbaum et al., 1975; Hoek et al., 2002). Second, lipid synthesis is induced by activation of the sterol response element binding proteins (SREBP) transcription factors (Ji et al., 2006; You and Crabb, 2004; You et al., 2002) (Fig. 1). It is likely that these and other mechanisms collaborate to generate fatty liver in chronic alcoholics. Reports suggest that mitochondrial damage following an alcohol binge in rodents (Demeilliers et al., 2002; Mansouri et al., 1999) may contribute to acute alcoholic liver disease (ALD), but the role of SREBPs in an acute ALD context has not been investigated.

Zebrafish are a powerful vertebrate system for modeling human disease (Lieschke and Currie, 2007). Zebrafish larvae are fully mature by 5 days post-fertilization (dpf), develop outside of the mother and each clutch is comprised of hundreds of transparent embryos that can be maintained in a simple salt solution in a Petri dish (Chu and Sadler, 2009; Lieschke and Currie, 2007). By 5 dpf, the liver is functional and capable of developing signs of hepatic disease, including fatty liver (Chu and Sadler, 2009; Matthews et al., 2009; Sadler et al., 2005). Importantly, several genetic tools, including mutants, transgenics and gene knock-down via morpholino injection provide a rapid and cost effective means to investigate the genetic basis of ALD. We have established zebrafish larvae as a new vertebrate model for studying acute ALD (Passeri et al., 2009).

Results

Zebrafish respond to acute exposure to alcohol

Mammalian models used to study the effects of alcohol allow animals to drink *ad libitum* or require intragastric infusion. In fish, however, simply adding ethanol (EtOH) to the water provides a means for continual exposure, as the fish swallow water while breathing. The advantages of working with zebrafish, namely their optical transparency and the ease of genetic manipulation, are primarily available when working embryos and young larvae. Liver development in zebrafish begins on 1 dpf. Hepatocytes are histologically identifiable by 3 dpf and by 5 dpf the liver has undergone morphogenesis, reaches the appropriate proportional size relative to the rest of the organism, and the liver at this stage carries out virtually all of the functions found in a mature liver (Chu and Sadler, 2009). Moreover, since zebrafish development is supported by nutrients provided by the yolk, larvae do not require any external nutrients until after 5 dpf. Therefore, young zebrafish larvae (4–5 dpf) have a functional liver, can ingest externally provided substances and represent an ideal system to study the effects of EtOH on the liver.

We developed an alcohol exposure protocol using 4–5 dpf larvae by simply adding alcohol to their water (Passeri et al., 2009). We have further refined this protocol by exposing larvae to EtOH concentrations ranging from 0.5%–4% and assessing the effects on behavior, morphology and viability. Most larvae do not survive more than 24 hours of exposure to alcohol concentrations greater than 3%, while almost all larvae survive for 32 hours in 2% EtOH. Nearly all larvae exposed to greater than 2% EtOH develop severe morphological abnormalities after 24 hours of exposure. The morphological abnormalities induced by a EtOH include edema, lordosis and hepatomegaly (see Fig. 2A), and the percentage of larvae displaying these changes increase according to the length of the exposure, with over 80% of affected fish observed in those exposed to 2% EtOH for 32 hours. Based on these data, we established that the maximal tolerable dose is 2% EtOH and the optimal exposure duration is for 32 hours. This protocol results in a 60–80 mM intracellular concentration over the duration of the exposure (Passeri et al., 2009). Interestingly, we found that behavioral changes induced by alcohol, including lethargy, hyperactivity and non-directional swimming were induced by much lower concentrations (Passeri et al., 2009), suggesting that this

system can be used to study the effects of alcohol on other systems, including the brain. This is supported by our preliminary results indicate that while alcohol exposed fish do not have significant hepatocyte apoptosis, but there is a marked increase in cell death in the brain. Similar studies have been initiated by other groups (Gerlai et al., 2009; Peng et al., 2009), and promise to be of great interest.

Zebrafish develop fatty liver after acute ethanol exposure

To investigate whether zebrafish larval livers are affected by alcohol in a manner similar to mammals, we assessed changes in liver size, cellular architecture, gene expression patterns and lipid accumulation. Transgenic fish expressing red fluorescent protein in hepatocytes (*Tg(fabp10:RFP)*) were treated with alcohol concentrations ranging from 0.5–3% on 4 dpf. The larvae were imaged after 32 hours of continuous exposure (i.e. on 5.5 dpf). The liver in untreated larvae is composed of two lobes, and the left lobe typically has a crescent shape that can be observed in live fish (Fig. 2A). Treatment with 2% alcohol causes the liver to become circular (Fig. 2A-B), indicative of hepatomegaly. Histological analysis reveals that hepatocytes are enlarged with clear cytoplasmic vesicles (Passeri et al., 2009), suggestive of lipid. Quantitative PCR analysis of dissected livers reveals that genes involved in lipid and cholesterol biogenesis are upregulated following exposure to 2% EtOH, as are genes indicative of alcohol metabolism and oxidative stress, such as *cyp2e1* and *sod1* (Passeri et al., 2009). These data suggest that alcohol is metabolized in zebrafish larval livers, resulting in oxidative stress and steatosis.

To directly assess whether EtOH causes steatosis, we stained whole larvae with oil red O. Larvae exposed to EtOH for 24–32 hours had large livers that were full of fat (Fig. 2C). The percent of larvae which develop steatosis increases directly with EtOH concentration (Fig. 2D). Therefore, as in mammals, alcohol zebrafish larvae exposed to alcohol develop steatosis, suggesting they have acute alcoholic liver disease.

EtOH-induced steatosis depends on SREBP activation

Studies in mammals have demonstrated that lipogenesis via activation of the SREBP transcription factors is a key mechanism by which chronic alcohol exposure leads to steatosis (Ji et al., 2006; You and Crabb, 2004; You et al., 2002). We found several Srebp target genes upregulated in the livers of EtOH treated larvae and hypothesized that this pathway may contribute to steatosis in this acute EtOH exposure model. To test this, we utilized two genetic tools to block Srebp activation and assessed their effects on the ability of 2% EtOH to generate steatosis. SREBP activation depends on the escort protein, Scap, to transport the inactive SREBP from the endoplasmic reticulum to the Golgi complex, where it is sequentially cleaved by the site 1 and site 2 proteases, which are encoded by the *mbtps1* and *mbtps2* genes (Fig. 1). EtOH-induced steatosis was blocked in zebrafish bearing a loss of function mutation in the *mbtps1* gene (Passeri et al., 2009). Similarly, larvae injected with a morpholino to knock-down Scap were protected from steatosis in response to 2% EtOH. Moreover, the *mbtps1* mutants exposed to EtOH failed to increase the expression of Srebp target genes (Passeri et al., 2009). Taken together, we conclude that acute exposure to EtOH causes steatosis in an Srebp-dependant fashion.

Several testable questions arise from this work. For instance, does the lack of lipid in the *mbtps1* mutant livers protect them from other hepatic abnormalities? Since the *mbtps1* mutants still develop hepatomegaly in response to EtOH as well as the other EtOH-induced phenotypes and behavioral changes, we predict that steatosis does not cause all the negative effects of alcohol ingestion. Finally, identifying the mechanism by which SREBP activation occurs in response to acute alcohol is an important goal for future studies.

Conclusions

Our data demonstrate the power of zebrafish to study alcoholic liver disease. We have established a simple and cost-effective protocol for exposing large numbers of zebrafish larvae to alcohol for up to 32 hours. EtOH causes hepatic changes including the changes in gene expression, hepatocyte morphology and, most importantly, steatosis. Two genetic tools, *mbtps1* mutants and *scap* morphants, demonstrate the requirement for Srebp activation in the development of EtOH-induced steatosis in this model. The mechanism by which Srebps are activated in response to EtOH and the contribution of other pathways to steatosis in acute ALD is currently under investigation. While we do not find any hepatocyte death in this model, nor do we have evidence of inflammation or fibrosis, more detailed analysis is required to establish whether acute EtOH exposure is sufficient to induce such hepatocyte damage or dysfunction. Finally, the effects of alcohol are not limited to the liver. The behavioral and morphological abnormalities exhibited by “drunk” zebrafish larvae may be exploited in future studies that examine other tissues, such as the brain and pancreas, that are affected by binge drinking.

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Figure 1.

Schematic of SREBP activation and signaling. SREBPs are retained in the endoplasmic reticulum by SCAP and are activated when cellular sterol concentrations are low. This results in SCAP mediated transport to the Golgi complex, where SREBPs are cleaved by the proteases MBTPS1 and MBTPS2 into its active form, which translocates to the nucleus to induce transcription of target genes.

Figure 2.

A: Acute EtOH administration (2% for 32 hours) causes hepatomegaly in 5.5 dpf zebrafish larvae visible in transgenic fish that express RFP in hepatocytes (Tg(fabp10:RFP). B: Quantitative analysis of liver circularity following EtOH exposure. Average liver circularity was calculated using ImageJ software using RFP to distinguish the liver, as shown in A. C: Representative oil red O whole mount staining of control and 2% EtOH exposed 5.5 dpf larvae, showing numerous lipid droplets in the livers of treated animals. D: The percent of individuals with steatosis increases in a dose-responsive fashion. Statistics: ANOVA with Tukey's post-hoc test, $p < 0.05$. Data points that have the same letter are not significantly different from each other.