Acute Injury in Natural Diet-Induced Fatty Livers - A Model for Therapy Development

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Abstract: Given the diabesity and Metabolic Syndrome epidemics, fatty liver disease is reaching epidemic proportions. Relatively indolent, this disease is often asymptomatic and the patient is often made aware of its presence only during a routine physical exam. Nevertheless, fatty livers are more susceptible to insult compared to their non-fatty counterparts and persons with fatty livers are at increased risk for morbidity and mortality following consumption of commonly used substances such as alcohol (EtOH) and acetaminophen (APAP). We have developed a rat model of natural diet-induced fatty liver disease and evaluated the effects of two commonly used substances viz. EtOH and APAP in this phenotype. High fat diet (HFD) fed animals exhibited steatosis, liver inflammation and liver fibrosis with an increase in serum aspartate aminotransferase. Bolus administration of EtOH, which was without effect on the livers from standard diet fed animals, had a profound and adverse impact on the HFD fatty liver. Similarly, APAP administration which was without effect on liver function tests in control animals, also provoked an increase in liver enzymes in HFD animals. Treatment with the poly(ADP-ribose) polymerase-1 inhibitor (PARP-1), veliparib, reduced the increase in liver function tests secondary to EtOH and APAP. This model forms the framework for identification of fatty liver disease biomarkers given that this disease is relatively asymptomatic but fraught with risk for acute injury. This model also forms the framework for evaluation of novel drugs for acute injury in fatty livers especially given that current strategies for management of acute liver failure in non-fatty livers are inadequate. Also, relevant patents related to the use of liver biomarkers as diagnostic are discussed.

Keywords: Acetaminophen, acute, alcohol, fibrosis, injury, liver, steatosis.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is accumulation of excess fat in hepatocytes or liver cells that is not caused by alcohol (EtOH) [1]. If more than 5-10% of the liver’s mass is fat, then it is termed fatty liver or steatosis. Obesity, diabetes and Metabolic Syndrome are key contributors to the NAFLD epidemic that afflicts up to 60 million Americans [2-4]. Left uncorrected, NAFLD can progress to non-alcoholic steatohepatitis (NASH) [5, 6]. In addition to fat within the liver, NASH is characterized by inflammation and damage and affects 5-10 million persons in the US alone [1]. Left untreated, NASH can progress to liver fibrosis or NASH-fibrosis [5, 6].

Consumption of EtOH is prevalent in most cultures including Western cultures. While binge drinking can lead to acute hepatic insufficiency [7, 8], lower amounts of EtOH are largely without hepatic effects in healthy individuals. Acetaminophen (APAP), a commonly used analgesic, is another agent that is without hepatotoxic effects unless consumed at very large doses [8, 9]. By contrast to their non-fatty counterparts, fatty livers have poor tolerance for insult [10]. The increased sensitivity and susceptibility of fatty livers to insult is especially of concern given that NAFLD and more often than not, NASH and NASH-fibrosis, are “silent” liver diseases [11-14]. Indolent, fatty liver disease takes years to progress and is often asymptomatic. In fact, up until the later stages of NASH the patient is often unaware of the presence of the diseased liver with diagnosis only made at the time of a routine physical exam [14]. Second, let alone the fatty liver, interventional strategies for acute injury in non-fatty livers are inadequate and suffer from a short opportunistic window [15].

Development of biomarkers for early detection of fatty livers and translational research strategies for development and screening of drugs for acute fatty liver failure could benefit from development of a model that recapitulates the clinical landscape. In the present study, we report on a clinically relevant model of a natural diet-induced NAFLD-NASH-fibrosis and the effects of EtOH and APAP on this population. We also show that this model is amenable to pharmacological intervention with a PARP-1 inhibitor. This clinically relevant model forms the framework for identification of fatty liver disease biomarkers given that this disease is relatively asymptomatic but fraught with risk for acute injury, and for screening of novel drugs for acute injury in fatty livers especially given that current strategies for management of acute liver failure in non-fatty livers are inadequate.

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MATERIALS AND METHODS

Animals: All studies relating to animals were approved by our institutional animal use and care committee. Adult male Sprague-Dawley rats (Charles River, NY) were randomized to a standard laboratory diet (control group, n = 8) or a high fat diet (HFD, n = 8) containing 10% lard, 2% cholesterol and 5% corn oil (Research Diets, NJ). Animals had access to water ad libitum. Six weeks later animals were sacrificed and their livers removed for analysis of disease. In a subsequent study, 6 weeks after randomization to standard diet or HFD, animals were administered EtOH (5g/kg, intragastric, n = 5/diet group) or APAP (1g/kg, PO, n = 5/diet group) and sacrificed 24hr later.

For the PARP-1 inhibitor study, 6 weeks after randomization to HFD, animals were administered EtOH (5g/kg, intragastric, n = 10) or APAP (1g/kg, PO, n = 10), 6hr after which animals (n = 5/group) were randomized to the PARP-1 inhibitor veliparib (15mg/kg, IP) or saline. Animals were sacrificed 18hr after veliparib administration.

Liver Function Tests: Serum samples were sent to North Shore-LIJ Core Services (NY) for analysis of liver transaminases (AST/ALT).

Liver Lipid Content: Pre-weighed samples of liver were homogenized and probed with Oil Red O. The stained Oil Red O was extracted with isopropanol and absorbance of the extracted Oil Red O was spectrophotometrically determined at 570nm to measure hepatic lipid content.

Histopathology: Liver sections were stained with hematoxylin and eosin (H&E) and examined under light microscopy (40 X, Olympus IX 100, Melville, NY) for evaluation of NAFLD Activity Scores (NAS; 0-8 scale) [5].

Fibrosis: After staining liver sections with 0.1% Picosirisir red in picric acid, the amount of collagenous protein was assessed using the Bioquant image analysis system (Bioquant, Nashville TN). The stained area was expressed as a percentage of the total field area.

Data Analysis

Between groups differences were analyzed by one-tailed Student’s T-test. A p < 0.05 was considered significant.

RESULTS

Compared to rats on a standard/control diet, rats fed a HFD over 6 weeks exhibited a fatty liver phenotype as seen in Fig. (1A). Fatty streaks were evident across the hepatic parenchyma and animals presented with pronounced hepatomegaly. The HFD cohort exhibited a slight but highly significant increase in body mass (Fig. 1B). However the in-
crease in hepatic mass was more exaggerated in the HFD cohort with average liver mass ~ twice that of the standard diet cohort (Fig. 1B). The liver to body mass ratio was also highly exaggerated in the HFD cohort compared to the standard diet cohort (Fig. 1B).

To further characterize this model of fatty liver disease, we examined several cellular and biochemical disease markers in these animals. As seen in Fig. (2A), compared to livers from the control cohort, HFD livers exhibited macrovesicular steatosis, inflammation and infiltration. Filigree Picosirius Red staining was also evident within the parenchyma of HFD livers (Fig. 2B). As seen in the panel shown in Fig. (2C), HFD livers also exhibited a > 10-fold increase in lipid content evidenced by the Oil Red O readout. Analysis of liver damage using the NAS system [5] indicated that rats fed a HFD for 6 weeks had hallmark symptoms of NASH. Consistent with a NASH phenotype, serum aspartate aminotransferase was elevated in the HFD cohort. Left untreated NASH progresses to fibrosis. Quantification (Fig. 2C) of the Picosirius Red staining in these livers confirmed increased collagen content in within the HFD livers, a hallmark indicator of fibrosis.

Having established a clinically relevant model of diet-induced NAFLD-NASH-fibrosis we next sought to study in these fatty livers the impact of agents that can cause drug-induced liver injury. In animals randomized to standard diet, fed EtOH (5g/kg) and sacrificed 24hr later, liver microarchitecture appeared relatively normal (Fig. 3A). Consistent with this observation, and as shown in Fig. (3B), there was no biochemical (liver enzymes) or histopathological (NAS) evidence of hepatic injury in these animals vs the non-EtOH controls. By contrast, the same quantity of EtOH had a major and adverse impact in animals fed a HFD. As seen in Fig. (3A), the H&E stained liver section from the HFD + EtOH cohort shows significant parenchymal injury comprising hepatocyte loss, lipid droplets, infiltration and inflammation. By contrast to its lack of effects in the non-fatty liver cohort, EtOH provoked a towering increase in liver enzymes in rats fed HFD and significantly increased NAS (Fig. 3C). Next we determined the effect of APAP on the NAFLD-NASH-fibrosis phenotype. As seen in Fig. (4), APAP (1mg/kg PO) had no effect on serum liver enzymes in rats fed a standard diet. By contrast, APAP provoked an increase in liver enzymes in rats on HFD.

To determine whether this model is amenable to pharmacotherapy, we evaluated the effects of anti-inflammatory and cyroprotective drug veliparib, a PARP-1 inhibitor, on liver

Fig. (2). Diet-induced NAFLD-NASH-fibrosis: Six weeks of HFD produced all the hallmark characteristics of NAFLD-NASH-fibrosis. 2A. Representative sections (H&E, 40X) from control and HFD livers. The HFD livers show steatosis, inflammation, infiltration and hepatocyte loss. 2B. Picosirius Red staining demonstrated mild fibrosis within the hepatic parenchyma in HFD but not control livers. 2C. Liver lipid content (Oil Red O) was increased in the HFD livers as was serum AST, NAS and collagen content. *, p < 0.05 vs control liver; **, p < 0.01 vs control liver.
function tests. As seen in Fig. (5), veliparib reduced the increase in liver enzymes secondary to EtOH and APAP.

**DISCUSSION**

In the present study we have characterized a potentially clinically relevant model of natural diet-induced fatty liver disease. Consistent with the clinical landscape, these fatty livers exhibit increased sensitivity to the toxic effects of EtOH and APAP. Acute injury in this model is amenable to pharmacologic intervention with an agent such as a PARP-1 inhibitor.

To the best of our knowledge this is the first report of a potentially clinically relevant model of a natural diet-induced liver disease and fibrosis. Previous reports of diet-induced fatty liver disease have typically included an EtOH component within the diet, used a modified unnatural diet such as methionine-choline deficient diet, used genetically susceptible animals or have not observed the fibrotic stage of the disease continuum [16-20]. In the present study, rats were fed a diet comprising 10% lard, 2% cholesterol and 5% corn oil over 6 weeks. The diet was selected to mimic the fast food Western diet culture currently prevalent in most countries. Animals on this diet developed hallmark characteristics of NAFLD-NASH-fibrosis. In addition to frank hepatomegaly, livers exhibited significant lipid accumulation, inflammation and infiltration. Serum AST was elevated and livers exhibited a filigree fibrotic phenotype.

A second and important feature of this model is that this natural diet-induced NAFLD-NASH-fibrosis liver is extremely susceptible to injury. The relatively increased sensitivity of these livers to insult was confirmed by challenging animals with commonly used substances that have hepatotoxic potential at higher doses. When administered EtOH at a quantity without effect on healthy livers, rats exhibiting a NAFLD-NASH-fibrosis phenotype presented with a towering increase in liver enzymes. Consistent with clinical reports [5, 21], microscopic examination of these livers revealed extensive macrovesicular steatosis, inflammation, infiltration and hepatocyte dropout. To determine whether the fatty liver responds similarly to another insult or whether

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**Fig. (3). Binge EtOH and Fatty Liver Injury: 3A.** In rats on standard diet, a bolus of EtOH (5g/kg) had no effect on liver microarchitecture (40 X H&E) 24 hr later. By contrast, in the HFD cohort, this amount of EtOH was associated with massive hepatic parenchymal injury. 3B. In control animals, EtOH was without effect on serum liver function tests or NAS but provoked significant increases in the HFD cohort, !, p < 0.01 vs control liver, !, **, p < 0.01 vs HFD liver.
this finding is unique to EtOH, rats randomized to standard or HFD were administered APAP. The dose of APAP selected had no effect on liver enzymes in healthy animals. By contrast, HFD animals with the NAFLD-NASH-fibrosis phenotype responded to APAP with an increase in serum liver enzymes. In fact, the results presented herein are novel.

In previous studies [10], the quantities of APAP or EtOH used did have some deleterious effects in control animals that were exacerbated in animals with fatty livers. Some of these earlier studies also evaluated the effects of chronic alcohol use in fatty livers. In our report, the quantities of APAP and EtOH used were without any effect on animals fed a standard diet, and just a single dose of APAP or EtOH was used.

Hepatocyte injury depends on the nature, duration, and severity of action of the noxious agent and can culminate in death. In addition to the primary damage caused by the toxin, secondary damage results from the release of cytokines and other cytotoxic mediators from activated cells belonging to the reticuloendothelial system [22-26]. Pro-inflammatory cytokines, TNFα, IL-1, and IL-6, released from Kupffer cells lead to a potentiated interaction between neutrophil granulocytes and the endothelium of the sinusoids, resulting in migration of neutrophils into the liver parenchyma. These activated neutrophil granulocytes release large amounts of free radicals and proteases, which cause damage to the liver parenchyma via lipid peroxidation of the cytoplasmic cell membrane and subcellular organelles. Activation and damage of sinusoidal endothelial cells also leads to abnormalities of the hepatic microcirculation with sinusoidal vasoconstriction and perfusion failure, tissue hypoxemia, and ultimately cell death. Quite often, cytochrome P450 2E1 (CYP2E1) is

**Fig (5). A PARP-1 Inhibitor Attenuates Acute Injury in Fatty Livers** - In HFD animals, the PARP-1 inhibitor veliparib, administered 6 hr after EtOH or APAP dosing, mitigated the increase in serum liver enzymes secondary to EtOH or APAP. *, p<0.05 vs HFD liver +EtOH or APAP.
induced and glutathione is depleted causing the formation of a relatively large amount of the radical N-acetyl-p-benzoquinoneimine and a reduced capacity to detoxify metabolites [27]. While the specific mechanism(s) underlying the enhanced susceptibility of the NAFLD-NASH-fibrosis liver to hepatotoxins was not investigated in the present study, it is possible that these livers contain reduced reserve to clear toxic substances. Regardless of the mode of action, these data suggest that diet-induced fatty livers have a reduced tolerance threshold for potential hepatic insult.

Consistent with the necro-inflammatory sequelae of injury, use of the anti-inflammatory and cytoprotective PARP-1 inhibitor [28, 30], veliparib, attenuated the increase in liver injury, use of the anti-inflammatory and cytoprotective PARP-1 inhibitor [28, 30], veliparib, attenuated the increase in liver enzymes secondary to EtOH or APAP. In addition to potentially confirming the mechanism of action underlying acute injury in these livers, these data suggest that this model is indeed amenable to pharmacotherapy.

CURRENT AND FUTURE DEVELOPMENTS

Findings reported in this study are both novel and of tremendous translational significance. The model reported herein forms the framework for urgent identification of fatty liver disease biomarkers given that this disease is relatively asymptomatic but fraught with risk for acute injury. Findings from this study have significant clinical implications. As described earlier, fatty liver disease is often “silent” and remains undiagnosed until the later stages. Patients with undiagnosed fatty livers are therefore at heightened susceptibility to acute liver injury following consumption of otherwise “normal” amounts of EtOH or APAP or perhaps even other potentially hepatotoxic substances such as certain mushrooms [8]. Known as the EtOH-APAP syndrome, combining EtOH and APAP might prove especially lethal in the NAFLD-NASH-fibrosis population [8]. Another important ramification of these findings relates to the transplantability of these livers. In youth with fatty livers, EtOH-related accidents leading to brain death might render their livers non-transplantable due to their “marginal” nature. In fact, given the ongoing obesity, diabetes and Metabolic Syndrome epidemics it is imperative that patients, both pediatric and adult, at enhanced risk for fatty liver disease be screened for the same and use extreme caution in consuming alcohol or analgesics such as APAP. Our model also forms the framework for evaluation of novel drugs for acute injury in fatty livers especially given that current strategies for management of acute liver failure in non-fatty livers are inadequate and suffer from a short opportunist window. Such novel cytoprotective agents can promote hepatic restitution or at the very least, serve as a bridge toward transplantation.

RECENT PATENTS ON FATTY LIVER DISEASE BIOMARKERS

From the perspective of diagnostics providers and given the Food and Drug Administration’s (FDA) increasing proclivity towards use of biomarkers as a surrogate for clinically meaningful endpoints, there is growing interest in patenting inventions that use a biomarker or combination of biomarkers as a readout of NAFLD. Several examples listed below provide insight into the growth potential of this area.

EP2333552 (also published as WO2010032458) [31] presents methods to detect nonalcoholic fatty liver disease including nonalcoholic steatohepatitis by using the protein inter-alpha-trypsin inhibitor heavy chain H4 precursor, a 35kDa protein fragment, or other fragments thereof, whose levels differ in presence or absence, or in quantity between healthy human subjects and patients with nonalcoholic fatty liver disease or nonalcoholic steatohepatitis.

EP2546649 (also published as WO2009059150) [32] describes a very large number of biomarkers of fatty liver disease, including steatosis and steatohepatitis. Methods include diagnosis of fatty liver disease, methods of determining predisposition to fatty liver disease, methods of monitoring progression/regression of fatty liver disease, methods of assessing efficacy of compositions for treating fatty liver disease, methods of screening compositions for activity in modulating biomarkers of fatty liver disease, methods of treating fatty liver disease, as well as other methods based on biomarkers of fatty liver disease, by comparing levels of one or more of these markers between levels from known healthy and known diseased individuals.

US2014303018 [33] discloses methods, compositions, and kits for determining whether a subject has non-alcoholic fatty liver disease (NAFLD) by creating a biomarker panel having one or more biomarker proteins from a list provided of biomarker proteins and detecting the level of each of the NAFLD biomarker proteins of the panel in a sample from the subject. Preferred biomarker proteins for diagnosing NAFLD include an elevation in one or more of ACY, CTSZ, LGALS3BP, SIGLEC7, SIGLEC14, and a decrease in at least one biomarker selected from SHBG, MET, GSN, CHL1, and SERPINC1.

CONFLICT OF INTEREST

Bin Duan, Jingsong Li, Ping Zhou, Latha Paka, Michael Yamin, Itzhak D. Goldberg and Prakash Narayan, are stock or stock option holders in Angion Biomedica Corp.

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Fatty Liver Injury

Recent Patents on Biomarkers


