Original Article

MOLECULAR METABOLISM

Deficiency of fibroblast growth factor 21 (FGF21) promotes hepatocellular carcinoma (HCC) in mice on a long term obesogenic diet

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ABSTRACT

Objective: Non-alcoholic fatty liver (NAFL) associated with obesity is a major cause of liver diseases which can progress to non-alcoholic steatohepatitis, cirrhosis, and hepatocellular carcinoma (HCC). Fibroblast growth factor 21 (FGF21) plays an important role in liver metabolism and is also a potential marker for NAFL. Here we aimed to test the effect of FGF21 deficiency on liver pathology in mice consuming a conventional high fat, high sucrose (HFHS) obesogenic diet for up to 52 weeks.

Methods: C57BL6 WT and FGF21 KO mice were fed a conventional obesogenic diet and were evaluated at 16 and 52 weeks. Evaluation included metabolic assessment, liver pathology, and transcriptomic analysis.

Results: With consumption of HFHS diet, FGF21 deficient mice (FGF21 KO) develop excess fatty liver within 16 weeks. Hepatic pathology progresses and at 52 weeks FGF21 KO mice show significantly worse fibrosis and 78% of mice develop HCC; in contrast only 6% of WT mice develop HCC. Well differentiated hepatocellular carcinomas in FGF21 KO mice were characterized by expanded hepatic plates, loss of reticulin network, cytologic atypia, and positive immunostaining for glutamine synthetase. Microarray analysis reveals enrichment of several fibroblast growth factor signaling pathways in the tumors.

Conclusions: In addition to attenuating inflammation and fibrosis in mice under a number of dietary challenges, we show here that FGF21 is required to limit the progression from NAFL to HCC in response to prolonged exposure to an obesogenic diet. The induction of hepatic FGF21 in response to the high fat, high sucrose obesogenic diet may play an important role in limiting progression of liver pathology from NAFL to HCC.

Keywords Fibroblast growth factor 21; Hepatocellular carcinoma; Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; High fat high sucrose diet; Liver fibrosis

1. INTRODUCTION

Multiple lines of evidence indicate that Fibroblast growth factor 21 (FGF21) plays an important role in liver metabolism. FGF21 was originally identified as an endocrine member of the fibroblast growth factor family as it can be released into the circulation [1]. FGF21 was initially assigned a purely metabolic role as infusions led to weight loss and increased glucose clearance through induced expression of the GLUT1 transporter. However, FGF21 biology is now understood to be extremely complex, as FGF21 is expressed in many metabolically active tissues including, liver, white (WAT) and brown adipose tissue (BAT), muscle, and pancreas. Functions of FGF21 are distinct in all these tissues [2].

In mice, multiple manipulations lead to significant increase in FGF21 levels, including fasting and consumption of a ketogenic diet [3,4], protein insufficiency, and amino acid deprivation [5]. When mice are fed a high fat diet or a lipotoxic methionine-choline deficient diet, hepatic FGF21 expression rises dramatically and mice lacking FGF21 develop significantly worse hepatotoxicity as assessed by inflammation and fibrosis [6–8]. Similar liver pathologies are seen with high fructose diets [9], dietary ethanol [10], acetaminophen overdose [11], and after partial hepatectomy and CCl4 administration [12]; hepatic expression of FGF21 rises in wild type mice with these stimuli, and absence of FGF21 leads to significantly worsened pathology while overexpression is protective. Taken together these data suggest that

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FGF21 has important effects to limit hepatic toxicity in response to a variety of stimuli.

Understanding of integrated FGF21 physiology is complex and challenging. Circulating FGF21 levels rise with obesity and likely reflect increased hepatic lipid accumulation in humans [13–16]. Regulation of FGF21 expression and levels may differ between rodents and humans. Unlike rodents, in humans, circulating FGF21 is not induced by either short term fasting or consumption of ketogenic diets [17], although two studies of extreme fasting (7–10 days) showed increase in circulating FGF21 in humans [18,19]. However, acute consumption of both fructose and alcohol markedly increase serum FGF21 levels, with a time course similar in rodents and humans [9,10,20]. These data suggest that FGF21 may play a regulatory role in human liver pathology.

The accumulation of excess triglycerides, which characterizes NAFLD, is a common complication of obesity with a prevalence of up to 53% in morbidly obese populations [21,22]. While in itself benign, fatty liver can progress to non-alcoholic steatohepatitis (NASH), which is characterized by apoptosis, inflammation, and fibrosis, in 10–20% of individuals. Progression to NASH increases the risk of further deterioration to cirrhosis and hepatocellular carcinoma (HCC). However progression is unpredictable in any given individual and no risk factors predisposing to progression have been identified.

Studies to date suggest that FGF21 plays a protective role in mice challenged with a variety of diets. However, diets studied thus far are not physiologically representative of human diets that lead to NAFL. To evaluate the effects of a diet that mimics the composition of Western diets that contribute to human obesity, and thus to fatty liver, we examined the long-term consequences of consumption of an obesogenic, high fat, high sugar diet (HFHS). We speculated that such a diet would lead to significantly more pathology in mice lacking FGF21 compared to wild type animals. Indeed, mice lacking FGF21 and consuming the HFHS diet developed more severe steatosis at 16 weeks. With ongoing consumption of this diet, NAFL ultimately progressed to HCC in a high percentage of FGF21 deficient, but not wild-type mice. Microarray analysis revealed involvement of aberrant fibroblast growth factor signaling in tumors.

These data reveal for the first time that FGF21 plays a critical role in the progression of liver disease from the early and relatively benign state of lipid accumulation to the development of neoplastic hepatocellular lesions. FGF21 is known to be hepatoprotective in the context of several short term hepatic stresses. Here we show that FGF21 is required to protect the liver long term. FGF21 has potential as a therapeutic agent against obesity-related progression of NAFLD, and these results suggest it may also provide benefits against more severe complications such as HCC. These potential benefits of FGF21 in liver disease are currently being evaluated in humans [23,24].

2. MATERIALS AND METHODS

2.1. Animals

All experiments were carried out with male WT and FGF21 deficient (FGF21 KO) mice, originally generated at Lilly Research Laboratories, Indianapolis, Indiana, by a targeted disruption of the FGF21 locus, as previously described [4]. These mice have had more than 10 backcrosses to C57BL/6 mice from Jackson labs. Mice were maintained on a 12:12-hr light–dark cycle and an ambient temperature of 22 °C ± 2 °C. Colonies are maintained as het X het breedings. For chronic high-fat feeding studies, WT and FGF21 KO male mice (age 8–10 weeks) were fed either standard laboratory chow (5008; Lab Diet, St Louis, MO) or a commonly used obesogenic diet (D12451; fat calories 45%, sucrose calories 17%; Research Diets, New Brunswick, NJ). Mice were allowed *ad libitum* access to food unless otherwise stated. Body weights were recorded weekly. Blood was collected for measurement of glucose and serum analysis at the time of sacrifice at either 16 or 52 weeks. Livers were harvested, weighed, snap-frozen in liquid nitrogen, and stored at −80 °C until further molecular/biochemical analysis and a segment of liver was saved for histology. All procedures were performed in accordance with National Institute of Health Guidelines for the Care and Use of Animals and were approved by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee.

2.2. Histological analysis

Livers were fixed in 10% formalin at 4 °C overnight. Paraffin embedding and sectioning was performed by the Histology Core at Beth Israel Deaconess Medical Center. 5 μM sections were stained with hematoxylin and eosin to assess the liver architecture and histology or Sirius Red to assess fibrosis. Reticulin/Nuclear Fast Red Stain Kit (AR17992-2, Dako, Santa Clara, CA) was used to assess the tumor fiber network. Slides were analyzed by two experienced liver pathologists in a blinded fashion (HGV and IAN). Slides were graded based on the NAFLD Activity Score (NAS) [25], which looks at degree of steatosis, inflammation, and ballooning, as well as the METAVIR score [26], which assesses the degree of fibrosis.

2.3. Hepatic tissue analysis

Liver triglycerides were extracted using a modified Folch method [27]. Briefly, liver was homogenized in chloroform:methanol (2:1) and incubated overnight at room temperature. 0.9% saline was added and each sample was centrifuged for 10 min. The organic phase was removed, vacuum dried, and resuspended in butanol:Triton X:methanol solution. Triglycerides (StanBio Laboratory, Boerne, TX), free cholesterol (StanBio Laboratory, Boerne, TX), and nonesterified fatty acid content (Wako Diagnostics, Richmond, VA) were determined using colorimetric assays and normalized to the weight of the liver.

2.4. Measurement of serum markers

Blood was allowed to clot, and supernatant was collected after centrifugation at 4 °C. Serum FGF21 levels were determined using a Rat/Mouse FGF-21 ELISA kit (R&D Systems, Minneapolis MN), according to the manufacturer's instructions. α-Feto protein/AFP was measured using a mouse Quantikine ELISA Kit (R&D Systems, Minneapolis MN). Alanine aminotransferase (ALT) (Pointe Scientific, Canton MI), aspartate aminotransferase (AST) (Pointe Scientific, Canton MI), triglycerides, total cholesterol (StanBio Laboratory, Boerne, TX) and nonesterified fatty acids (Wako Diagnostics, Richmond, VA) were measured in duplicate using enzyme colorimetric assays as per the directions provided by each manufacturer.

2.5. Immunohistochemical staining

Slides were deparaffinized with xylene and hydrated through an ethanol gradient and rinsed with phosphate-buffered saline (PBS). Antigen was retrieved by citric acid buffer (pH 6.0). Sections were subsequently treated with 0.3% H_2O_2 and washed by tris-buffered saline containing 1% Tween 20 (TBST) for 15 min followed by a 60 min blocking with normal goat serum (Vector Laboratories, Burlingame, CA). Slides were incubated with glutamine synthetase, primary antibody (1:100) (ab73593, Abcam, Cambridge, MA) overnight at 4 °C. Slides were washed and incubated with a secondary antibody

