Investigating the Role of Tenascin C in Nonalcoholic Fatty Liver Disease

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Introduction

Role of Obesity in HCC

The prevalence of obesity in western countries has dramatically increased, it was estimated that over 30% of Americans were classified as obese, or having a BMI \( \geq 30 \) in 2012 [1]. The link between many obesity-related health conditions, such as diabetes, are well understood. However, a strong link between obesity and cancer, most specifically hepatocellular carcinoma (HCC), has emerged in the literature [2]. Increasing evidence demonstrates overweight and obese individuals have a higher risk of developing HCC [3, 4]. Because HCC is the fifth most common cancer and the third leading cause of cancer-related deaths, it is critical to better understand the mechanisms underlying the manifestation of obesity-induced HCC.

Obesity itself is not a direct cause of HCC, but rather initiates Nonalcoholic Fatty Liver Disease (NAFLD), the hepatic manifestation of metabolic syndrome [5]. NAFLD is a condition that affects up to 30% of the US population, mirroring the prevalence of obesity in the US [6]. This liver disease is generally asymptomatic, characterized by the accumulation of triglycerides in the hepatocytes, which is clinically referred to as hepatic steatosis [7]. The continued deposition of fat can cause inflammation, transitioning a patient’s diagnosis from NAFLD to Nonalcoholic Steatohepatitis (NASH). 25% of individuals with NASH will further progress to liver cirrhosis, an irreversible major clinical concern for the development of HCC [7]. While it is most common for obese patients to progress from NAFLD to HCC in this fashion, the ability for a patient to progress to HCC directly from NAFLD, although rare, is possible [8].

Therefore, it is important to identify biomarkers that will allow clinicians to determine which patients who have NAFLD will not only be more likely to progress to HCC through hepatic inflammation and cirrhosis, but also from NAFLD directly to cirrhosis. Thus, the discovery of a biomarker as a clinical determinant is crucial to the prevention of HCC.

Potential Biomarker in NAFLD Patients

One potential biomarker for NAFLD patients is Tenascin-C (TnC). TnC is an extracellular matrix (ECM) glycoprotein that interacts with other ECM molecules and cell receptors to modulate cell migration, proliferation, and signaling. Its use as a biomarker has been seen in literature for brain and lung cancers, as well as rheumatoid arthritis because it is an injury marker that promotes tissue repair during injury and is minimally detected in healthy tissues [9, 10].

Due to its nature as an injury marker, its possible use as a biomarker for obesity-induced liver injuries, such as NAFLD, is highly appropriate. The pathophysiology of NAFLD strongly supports the research of TnC in the field of hepatology. The liver is composed of several different cell types: hepatocytes, hepatic stellate cells (HSCs) (sinusoidal epithelial cells), and resident macrophages, Kupffer cells. HSCs are located in the Space of Disse in the liver, playing a key role in the regulation of flow through the sinusoids [11]. In a healthy liver environment, HSCs will reside in the quiescent state; however, upon hepatic injury, HSCs can become activated and transform into myofibroblast-like cells. These activated HSCs secrete ECM molecules such as TnC, which can
affect surrounding hepatocytes, potentially contributing to HCC progression, as shown in the figure below.

**Hypothesis and Aims**

To study validity of TnC as a possible biomarker, it was hypothesized that the activation of HSCs by obesity-induced liver injury increases secretion of TnC, leading to hepatocyte transformation. We examined this hypothesis through two specifically aims, first by analyzing circulating TnC in plasma from obese patients at Carolinas Medical Center and second, mechanistically by analyzing TnC secretion and hepatocyte transformation using an *in vitro* model of obesity-induced hepatic injury.

**Experimental Approach**

**Methods**

*Aim 1: Analyzing Tenascin C in the Serum of Morbidly Obese Patients*

Whole blood was collected and submitted to the Liver, Biliary and Pancreatic (LBP) Repository according to an IRB approved protocol for Carolinas HealthCare System. Patients undergoing bariatric surgery, LBP surgery or with chronic liver disease were eligible for enrollment. Patients were HBV and HCV negative. Plasma samples were obtained from healthy volunteers (BMI<30) as a control group. Levels of TnC in plasma were determined using the human TnC (FN III-C) Assay ELISA kit (IBL, Minneapolis, MN). For statistical analysis, TnC results were stratified by fibrosis stage and NAFLD activity score determined by liver biopsy at the time of bariatric surgery. Patients with NASH cirrhosis with HCC met radiographic criteria for HCC diagnosis by cross-sectional imaging.
Aim 2: Measuring TnC Secretion and Hepatocyte Transformation in an in-vitro Model of Obesity-Induced Hepatic Injury

Oil-Red-O

Huh 7.0 and HepG2 cells (hepatoma cell lines) were treated with 0.2mM of 10% BSA control, palmitic acid (saturated free fatty acid), and oleic acid (unsaturated free fatty acid) for 18 and 24 hours to induce lipid deposition in the hepatocytes. Hepatocytes were then formalin fixed and treated with Oil-Red-O to stain lipid deposition in the cytoplasm and then counterstained with hematoxylin. Images were then taken with an Olympus IX51 microscope at 20x and 40x.

Co-Culture Approach

An in-vitro model to mimic hepatic injury was accomplished using Transwell 4 millipore insert filters in a 6-well cell plate (Corning, Tewksbury MA). Transwells were incubated in 10% FBS media for a minimum of two hours prior to seeding. Huh 7.0 cells were seeded at 2.5E5 cells/insert and LX2 (activated HSC cell line) at 0.9E6 cells/well in 10% FBS media. LX2 cells were also plated in the absence of Huh 7.0 alone and treatment mirrored the co-culture system.

Cells were then switched to 1% FBS media in order to serum-starve before treatment with 0.2mM palmitic acid, oleic acid, or 10% BSA control for 18 and 24 hours. Cells were harvested using Proprep solution and spent media was used for analysis with western blot and ELISA.

Protein Analysis

Western Blot

Cell lysate and media samples were probed for TnC and were normalized to GAPDH. Relative TnC secretion levels were analyzed via densitometry through ImageJ.

ELISA

Levels of TnC in spent media were determined using the human TnC (FN III-C) Assay ELISA kit (IBL, Minneapolis, MN).

Real time PCR (qRT PCR)

Huh 7.0 cells were treated for 15 and 24 hours with spent media from FFA and 10% BSA control treated LX2 cells. qRT-PCR was preformed to analyze hepatocyte transformation by examining mRNA levels of proteins involved in epithelial mesenchyme transition (EMT) Cdh1, COL4A1, and Vimentin, and were normalized to GAPDH.
**Expected Results**

**Aim 1: Analyzing Tenasin C in the Serum of Morbidly Obese Patients**

Because TnC is known to be an injury marker, it is expected that there will be increased TnC measured in plasma from obese patients with NAFLD.

**Aim 2: Measuring TnC Secretion and Hepatocyte Transformation in an in-vitro Model of Obesity-Induced Hepatic Injury**

We expect there will be increased lipid deposition in the cytoplasm in Huh 7.0 after treatment with both palmitic and oleic acids. Because palmitic acid is a saturated free fatty acid, lipid deposition may be more severe in palmitic acid versus oleic acid treated Huh 7.0 cells.

In the co-culture experiments, we expect an increase in TnC protein secretion in the media and increased TnC production in cell lysate after treatment, due to cell injury caused by the FFAs. Previous experiments have shown that TnC secretion is primarily derived from activated HSCs. Therefore, it is expected that increased TnC secretion from activated HSCs due to liver injury by FFAs will contribute to hepatocyte transformation or progression of HCC. Epithelial to mesenchymal (EMT) transition, often seen in cancer progression, will be identified in the hepatocytes exposed to HSC derived TnC as measured by downregulation of Cdh1 and COL4A1 and concomitant upregulation of vimentin.
Work Cited