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Research Paper

Transcriptome Analysis Uncovers a Growth-Promoting Activity of Orosomucoid-1 on Hepatocytes

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ABSTRACT

The acute phase protein orosomucoid-1 (Orm1) is mainly expressed by hepatocytes (HPCs) under stress conditions. However, its specific function is not fully understood. Here, we report a role of Orm1 as an executer of HPC proliferation. Increases in serum levels of Orm1 were observed in patients after surgical resection for liver cancer and in mice undergone partial hepatectomy (PH). Transcriptome study showed that Orm1 became the most abundant in HPCs isolated from regenerating mouse liver tissues after PH. Both *in vitro* and *in vivo* siRNA-induced knockdown of Orm1 suppressed proliferation of mouse regenerating HPCs and human hepatic cells. Microarray analysis in regenerating mouse livers revealed that the signaling pathways controlling chromatin replication, especially the minichromosome maintenance protein complex genes were uniformly down-regulated following Orm1 knockdown. These data suggest that Orm1 is induced in response to hepatic injury and executes liver regeneration by activating cell cycle progression in HPCs.

1. Introduction

Orosomucoid-1 (Orm1), also known as alpha-1-acid glycoprotein 1 (AGP1), is a member of lipocalin protein family, which acts as the carrier of basic and neutrally charged lipophilic compounds under normal physiological conditions. In addition, it is known as an acute phase protein, which is expressed in response to stressful conditions such as tissue injury, inflammation, or infection (Lee et al., 2010). There are emerging evidences from proteomic studies that both the urinary and serum Orm1 may serve as predictors of therapeutic response and diagnostic and prognostic biomarkers for inflammatory diseases such as chronic heart failure (Agra et al., 2017) and cancers such as bladder cancer (Li et al., 2016), lung squamous cell carcinoma (Ayyub et al., 2016), breast cancer (Alexander et al., 2004) as well as hepatocellular carcinoma (HCC) (Falleti et al., 1993). However, the specific function of Orm1 under stresses has not yet been fully elucidated.

Liver regeneration is a complicated but coordinated multistep process mediated by integration of multiple signals involved in cytokines, growth factors and metabolic networks (Michalopoulos, 1990). Normal liver regeneration requires spatially and temporally precise interactions between different populations of liver-composing cells, including liver sinusoidal endothelial cells (LSECs) and hepatocytes (HPCs), to

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reconstitute liver structure and function (Ding et al., 2014; Hu et al., 2014). However, its executers have not been fully identified. Loss of liver function caused by viral hepatitis, cirrhosis, and liver damage from alcohol or drugs is a life-threatening condition. Increased understanding of the regenerative process should also shed light on clinical applications such as the treatment of acute liver failure (ALF), which is a highly lethal disorder with abrupt loss of hepatic metabolic and immunological function (Bernal et al., 2010; Mao et al., 2014). It would also have clinical implication for the development of predictive biomarkers for the prognosis of portal vein embolization (PVE) before surgical resection for liver cancer. PVE is a technique used to increase future remnant liver volume. However, a few patients fail to achieve sufficient growth of the liver tissue or suffer from tumor progression following PVE (de Graaf et al., 2009; Treska et al., 2011).

Here, we addressed a role of Orm1 as an executer promoting cell cycle of HPCs during liver regeneration. We observed increases in serum levels of Orm1 in patients after surgical resection for HCC and in mice undergone partial hepatectomy (PH), implying that Orm1 might be induced in order to promote liver regeneration. Using Cap analysis of gene expression (CAGE)-based transcriptome analysis (Carninci et al., 2005), we showed that lipocalin family genes were mostly enriched in mouse primary HPCs during liver regeneration and among these genes Orm1 was characterized as the most major biomarker of liver regeneration by Bayesian network analysis. Consistently, knockdown of Orm1 in mice resulted in decreases in HPC growth accompanying suppressed signaling in controlling chromatin replication. These results highlight that the HPC-derived lipocalin protein, Orm1 has potential to be a prognostic biomarker and potential therapeutic target for impaired regeneration in liver.

2. Materials and Methods

2.1. Clinical Samples

The study was performed in accordance with the Declaration of Helsinki and was approved by the Ethics Committee for Biomedical Research of the Jikei University School of Medicine, the Hospital Ethics Committee of Tokyo University, and the RIKEN Institute Research Ethics Committee. The patients had signed a written informed consent prior to study. Serum samples were collected from 10 patients who had undergone liver resection for HCC. Formalin-fixed paraffin-embedded liver tumor tissues and normal adjacent tissues obtained from HCC patients were purchased from Proteogenex (Culver City, CA, USA).

2.2. Experimental Animals

All experiments were performed in accordance with protocols approved by the RIKEN Institutional Animal Use and the Care Administrative Advisory Committees and adhered to the guidelines in the Institutional Regulation for Animal Experiments and Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology, Japan. Male C57BL6/J mice (age, 6–14 weeks) were housed under constant temperature (22 °C ± 1 °C) with free access to food and water.

2.3. PH Experiment

The mice were randomly divided into PH or control sham groups with no blinding. For transcriptome analysis, three independent biological replicates at each time point were analyzed. For *in vivo* RNA interference experiment, four independent biological replicates at each time point were analyzed. After anesthetized with isoflurane gas and disinfection of the skin with 70% ethanol, the abdomen is incised in the median line (15–20 mm) and opened with clip forceps. The beginning of the median and left lateral lobes is ligated with 5-0 silk thread, and the lobes are hepatectomized. After spraying antibiotics in the cavity, the abdominal wall is sutured with 6-0 nylon thread. The animals are kept warm while recovering from anesthesia (Akita et al., 2002).

2.4. Primary Cell Isolation and RNA Extraction

Primary mouse LSECs and HPCs were isolated from mouse livers at 2 h, 30 h, 48 h and 1 week after PH and at 2 h after the sham operation. The liver was perfused with collagenase solution and HPCs were collected by centrifugation at 50 ×*g* for 2 min at 4 °C for 3 times. The pelleted HPCs were then cultured in William's Medium E (Sigma Chemical Company, St. Louis, MO, USA). LSECs were isolated using purified anti-mouse CD146 (RRID: AB_1731991, ME-9F1, BioLegend, San Diego, CA, USA) and dynabeads labelled with M-450 sheep anti-rat IgG (Life Technologies, Gaithersburg, MD, USA) (Akita et al., 2002). The isolated LSECs were cultured in DMEM/F-12 medium (Gibco, Invitrogen, Grand Island, NY, USA) supplemented with 10% FBS, 1% penicillin/streptomycin and 50 mg/mL endothelial mitogen. Total RNA samples were then extracted from the cells using an RNeasy Kit (Qiagen, Valencia, CA, USA), and the amount and purity of the isolated RNA was evaluated using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

2.5. In Vivo RNA Interference

A Stealth RNAi™ Pre-Designed siRNA targeting mouse Orm1 (siOrm1), UUGAGACUCCCGAAGCUCUAUUGUG, and a paired control siRNA (siCtl), CACAAUAGAGCUUCGGGAGUCUCAA, were purchased from Life Technologies (Gaithersburg, MD, USA). Transfection was performed *via* a single tail vein injection of siRNA (1 mg/kg) using a next generation lipid based carrier Invivofectamine 3.0 reagent (Life Technologies) (Eguchi et al., 2016). Knockdown efficiency was verified 3 days post-siRNA injection in serum protein levels and in transcript levels in whole liver tissues.

2.6. CAGE Transcriptome Analysis

A simplified version of the CAGE protocol, deepCAGE, using a single-molecule sequencer HeliScope, which can avoid linker ligation, PCR, and enzymatic cleavage, was applied to generate transcriptional profiling of LSECs and HPCs during liver regeneration (Kanamori-Katayama et al., 2011). Detailed methods for the CAGE data generation and normalization are provided in the FANTOM5 main paper (Forrest et al., 2014). Data downloads and freely available genomic tools are summarized in the FANTOM database (RRID: SCR_000788, http://fantom.gsc.riken.jp/5/) (Severin et al., 2014) and are available in Table S1.

2.7. IHC Staining and Immunofluorescence Staining of Orm1 in Liver Sections

Tissue staining was performed as previously described (Hara et al., 2014). Liver sections were deparaffinized and heated to 98 °C in Target Retrieval Solution (DAKO Corporation, Carpinteria, CA, USA) in a microwave for 10 min for antigen retrieval. For histology, the sections were stained with Myer's hematoxylin solution and 1% Eosin Y solution (H&E) (Muto Pure Chemicals, Tokyo, Japan). For mouse liver sections, after blocking with 5% normal goat serum in PBS containing 0.1% Tween-20 (PBST) for 30 min at room temperature, the sections were incubated with rabbit anti-mouse Orm1 (2 μg/mL; PAA816Mu01; Cloud-Clone Corp) or control rabbit IgG overnight at 4 °C. For human liver sections, the sections were blocked in PBS containing 10% FBS for 1 h at room temperature and incubated with goat anti-human Orm1 (RRID: AB_2158195, 0.5 μg/mL; sc-51018; Santa Cruz Biotechnology; Santa Cruz, CA, USA) and goat IgG overnight at 4 °C. For blocking/competition, Orm1 antibody was treated with a five-fold (by weight) excess of blocking peptide (sc-51018 P; Santa Cruz Biotechnology) in 500 μL PBS overnight at 4 °C. EnVision + System-HRP (DAKO Corporation)

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