Research Article

Arginase 2 deficiency results in spontaneous steatohepatitis: A novel link between innate immune activation and hepatic *de novo* lipogenesis

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Background & Aims: Innate immune activation has been postulated as a central mechanism for disease progression from hepatic steatosis to steatohepatitis in obesity-related fatty liver disease. Arginase 2 competes with inducible nitric oxide synthase (iNOS) for its substrate and the balance between these two enzymes plays a crucial role in regulating immune responses and macrophage activation. Our aim was to test the hypothesis that arginase 2 deficiency in mice favours progression from isolated hepatic steatosis, induced by high fat feeding, to steatohepatitis.

Methods: Arginase 2-knockout (*Arg2*$^{-/-}$) mice were studied for changes in liver histology and metabolic phenotype at baseline and after a short term course (7 week) feeding with a high fat (HFAT) diet. In additional experiments, *Arg2*$^{-/-}$ mice received tail vein injections of liposome-encapsulated clodronate (CLOD) over a three-week period to selectively deplete liver macrophages.

Results: Unexpectedly, *Arg2*$^{-/-}$ mice showed profound changes in their livers at baseline, characterized by significant steatosis as demonstrated with histological and biochemical analysis. These changes were independent of systemic metabolic parameters and associated with marked mRNA level increases of genes involved in hepatic *de novo* lipogenesis. Liver injury and inflammation were present with elevated serum ALT, marked infiltration of F4/80 positive cells, and increased mRNA levels of inflammatory genes. HFAT feeding exacerbated these changes. Macrophage depletion after CLOD injection significantly attenuated lipid deposition and normalized lipogenic mRNA profile of livers from *Arg2*$^{-/-}$ mice.

Conclusions: This study identifies arginase 2 as a novel link between innate immune responses, hepatic lipid deposition, and liver injury.

Keywords: Non-alcoholic fatty liver disease; Steatohepatitis; Arginase 2; Macrophage activation; Innate immunity; Inflammation; Liver fibrosis.

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Abbreviations: ACC, acetyl-CoA carboxylase; ACADM, acyl-coenzyme A dehydrogenase; ALT, alanine aminotransferase; Arg1, arginase 1; Arg2, arginase 2; CD11c, cluster of differentiation 11c; CD36, cluster of differentiation 36 or fatty acid translocase; CD68, cluster of differentiation 68; CLOD, clodronate; CPT1, carnitine palmitoyl-transferase 1; DIO, diet induced obesity; DAMP, damage-associated molecular pattern; DNL, *de novo* lipogenesis; F4/80, mouse macrophage marker; FABP4, fatty acid binding protein 4; FAS, fatty acid synthase; FATP2/FATP5, fatty acid transport proteins 2/5; HFAT, high fat; IL1-beta, interleukin 1 beta; IL-6, interleukin 6; iNOS, inducible nitric oxide synthase; LXR-a, liver X receptor alpha; MTTP, microsomal triglyceride transfer protein; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; ORO, Oil Red O; PAMP, pathogen-associated molecular pattern; PEMT, phosphatidylethanolamine N-methyltransferase; SCD1, stearoyl-CoA desaturase 1; SREBP-1, sterol regulatory element binding protein 1c; TNF-alpha, tumor necrosis factor alpha; WT, wild type.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is currently the most common form of chronic liver disease affecting both adults and children, and is strongly associated with obesity and insulin resistance [1,2]. One in three adults and one in ten children or adolescents in the United States have hepatic steatosis, a stage within the spectrum of NAFLD, that is characterized by triglyceride accumulation in liver cells and follows a benign non-progressive clinical course [3,4]. Non-alcoholic steatohepatitis (NASH) is defined as lipid accumulation with evidence of cellular damage, inflammation and varying degrees of scarring or fibrosis [5]. NASH is a serious condition, as approximately 25% of these patients progress to cirrhosis and its known complications that include portal hypertension, liver failure and hepatocellular carcinoma [6–8].

NAFLD pathogenesis, and specifically disease progression to NASH, has been the subject of intense investigation over the past decade, as both diagnostic and prognostic markers and treatment

options for patients with this disease remain limited [9]. A multi-hit model of NAFLD pathogenesis has emerged in recent years in which intrahepatic lipid accumulation in the setting of insulin resistance is postulated to be the first hit, which sets the stage for multiple "second hits" resulting in inflammation, hepatocellular damage and eventually fibrosis [10]. Growing evidence supports the concept that innate immune activation and increased oxidative stress in the liver are key "second hits" involved in disease progression to NASH. Activation of liver resident macrophages, or Kupffer cells, at least in part by an increase in exposure to gut-derived bacterial wall components such as endotoxin, result in an increased production of proinflammatory cytokines as well as inducible nitric oxide synthase (iNOS) and has been proposed to play a pivotal role in this process [11,12]. Expression of iNOS in cell types such as macrophages and hepatocytes is induced by proinflammatory cytokines [13], and iNOS have been implicated in the pathophysiology of NAFLD [14–16]. Mammals express two isoforms of arginase, an enzyme that competes with iNOS for its substrate, arginine, designated as types 1 and 2 [17–19]. Arginase 1 is mainly expressed in hepatocytes, and mice with a disruption of the arginase 1 gene (*Arg1*) die soon after birth. Arginase 2 is poorly expressed in hepatocytes, and most highly expressed in kidney, prostate, and immune cells, such as monocyte/macrophages [17,19]. An imbalance in iNOS/arginase ratio in favour of the former may play a crucial role in regulating immune responses and macrophage activation towards a classically "M1" activated, proinflammatory state [20–23]. The aim of our study was to test the hypothesis that arginase 2 deficiency in mice would result in progression from isolated hepatic steatosis typically seen in diet induced obesity (DIO) to steatohepatitis. Our data identify a novel link between Kupffer cell activation and hepatic steatosis and suggest that in the presence of arginase 2 deficiency, altered immune responses precede, and are responsible for lipid deposition in hepatocytes mainly by activation of *de novo* lipogenesis pathways in the liver. These findings have important implications for the pathogenesis of NAFLD and potential development of novel treatment strategies for patients with this condition.

Materials and methods

Animal studies

The experimental protocol has been approved by the Institutional Animal Care and Use Committee at Cleveland Clinic. Male C57BL/6 wild type (WT) were purchased from Jackson Laboratory. The generation of *Arg2*$^{-/-}$ mice has been described previously [24]. *Arg2*$^{-/-}$ mice are viable and indistinguishable from WT mice. Mice were fed either a diet consisting of 5% fat (TD 2918, Harlan Laboratories, Madison, WI) or a high fat (HFAT), Western-type diet (consisting of 42% of Kcal from fat, TD88137, Harlan Laboratories, Madison, WI). Total body weight was recorded on a weekly basis. At indicated time points, plasma and liver tissue were collected and weighed after an overnight fast as described previously [25].

Analyses of plasma and liver metabolic mediators

Blood was collected from *Arg2*$^{-/-}$ and WT mice after an overnight fast by cardiac puncture. Blood was spun at 2000 rpm for 15 min at 4 °C, plasma was drawn from the top layer, argon overlaid and stored at −80 °C. Plasma assays of insulin and glucose were performed using commercially available mouse insulin ELISA (ALPCO Diagnostics, Salem, NH) and glucose assay (Cayman Chemical, Ann Arbor, MI) kits. Plasma and liver triglyceride and free fatty acid levels were measured with triglyceride-GPO liquid reagent (Pointe Scientific, Inc, Canton, MI), and free fatty acid quantification (BioVision, Mountain View, CA) kits according to manufacturers' instructions. Serum alanine aminotransferase (ALT) concentrations were measured and expressed as international units per litre (Clinical Laboratory Services, Cleveland Clinic Foundation, Cleveland, OH).

Histopathology and immunostaining

Mouse tissue was diced into 5 × 5-mm sections, immersion-fixed in PBS containing 4% paraformaldehyde for 24 h at 4 °C, and embedded in paraffin. Four micrometer sections were mounted on glass slides. Haematoxylin and eosin (H&E) stained liver specimens were evaluated by light microscopy for histopathological scoring by a hepatopathologist (BGP). Steatosis, inflammation, and ballooning were scored based on NAFLD activity score [26]. Presence of macrophage infiltration was assessed by immunohistochemical staining for F4/80. Paraffin-embedded liver sections were deparaffinized and antigen retrieval, using 10 mM sodium citrate buffer, was performed. Sections were incubated with primary antibody overnight at 4 °C (1:50 dilution, Abd Serotec, Oxford). Subsequently, sections were incubated with a biotinylated anti-rat IgG secondary antibody (Vector Laboratories, Burlingame, CA) and a Vectastain ABC Elite Kit according to the manufacturer's instructions (Vector Labs). Sections were developed with ImmPACT DAB peroxidase substrate (Vector Labs) and counterstained with haematoxylin.

Oil Red O staining

Assessment of hepatic steatosis was performed by staining with Oil Red O (ORO), a fat-soluble diazo dye. Frozen liver sections (10 μm thick) were mounted on glass slides. ORO stock solution was prepared by mixing 300 mg ORO (Sigma-Aldrich, St. Louis, MO) and 100 ml 2-propanol, 99% (Fisher Scientific, Pittsburg, PA). A working solution of 1.5/1 ORO stock solution/distilled water was then prepared. Liver slides were stained with ORO working solution for 12 min, after which they were washed in distilled water twice for 20 s and rinsed in running tap water for 10 min. Finally, slides were stained with haematoxylin for 45 s and then washed in distilled water.

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from liver using RNeasy Mini kit (Qiagen, Valencia, CA). One μg RNA was reverse transcribed using random decamers and MMLV-reverse transcriptase (Applied Biosystems). Genes assayed for mRNA expression levels included inflammatory cytokines, markers of hepatic stellate cell activation, hepatic insulin signalling, and mediators of lipid trafficking and metabolism (see Supplementary Table 1 for primer sequences). The fold change over control samples was calculated using CT, ΔCT, and ΔΔCT using MaxPro software (Agilent, Santa Clara, CA). 18S ribosomal RNA was used as an endogenous control.

Immunoblot analysis

Immunoblot analysis was performed as previously described [27]. Anti-arginase 2 (ARG2, Santa Cruz, Santa Cruz, CA, USA), anti-fatty acid synthase (FAS, GeneTex, Irvine, CA, USA), and anti-stearoyl-CoA desaturase 1 (SCD-1, Cell Signaling Technology, Boston, MA, USA) antibodies were used in combination with the appropriate peroxidase-conjugated secondary antibodies. Protein load was verified with an α-tubulin antibody (dilution 1:10,000) (Hybridomabank, University of Iowa) (kindly provided by M. Kaulich). Bands were visualized with the enhanced chemiluminescence reagent and digitized using a CCD camera (ChemiDoc®, Biorad, Hercules, CA, USA). Expression intensity was quantified by ImageLab (Biorad).

Liver-specific macrophage depletion

Macrophage depletion studies were done in *Arg2*$^{-/-}$ and wild type mice using liposome-encapsulated clodronate injection as this is a validated method for the *in vivo* depletion of tissue-specific macrophages [28,29]. Mice received four tail vein injections of 100 μl/10 g body weight of 1 mg/ml PBS or clodronate (CLOD)-containing liposomes over a three-week period. Mice were sacrificed 24 h after the fourth injection, at which time plasma, and liver tissue were collected.

Statistical analysis

All data were expressed as the mean ± S.E.M. unless otherwise indicated. Differences between groups were compared by an ANOVA analysis followed by a post hoc Bonferroni test to correct for multiple comparisons. Differences were considered to be statistically significant at $p < 0.05$.