Research Article

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Hepatic B cell leukemia-3 promotes hepatic steatosis and inflammation through insulin-sensitive metabolic transcription factors

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Background & Aims: The pathomechanisms underlying non-alcoholic fatty liver disease (NAFLD) and the involved molecular regulators are incompletely explored. The nuclear factor-kappa B (NF-κB)-cofactor gene B cell leukemia-3 (*Bcl-3*) plays a critical role in altering the transcriptional capacity of NF-κB – a key inducer of inflammation – but also of genes involved in cellular energy metabolism.

Methods: To define the role of Bcl-3 in non-alcoholic steatohepatitis (NASH), we developed a novel transgenic mouse model with hepatocyte-specific overexpression of Bcl-3 (*Bcl-3*^{Hep}) and employed a high-fat, high-carbohydrate dietary feeding model. To characterize the transgenic model, deep RNA sequencing was performed. The relevance of the findings was confirmed in human liver samples.

Results: Hepatocyte-specific overexpression of Bcl-3 led to pronounced metabolic derangement, characterized by enhanced hepatic steatosis from increased *de novo* lipogenesis and uptake, as well as decreased hydrolysis and export of fatty acids. Steatosis in *Bcl-3*^{Hep} mice was accompanied by an augmented inflammatory milieu and liver cell injury. Moreover, Bcl-3 expression decreased insulin sensitivity and resulted in compensatory regulation of insulin-signaling pathways. Based on *in vivo* and *in vitro* studies we identified the transcription factors PPARα, PPARγ and PGC-1α as critical regulators of hepatic metabolism and inflammation downstream of Bcl-3. Metformin treatment improved the metabolic and inflammatory phenotype in *Bcl-3*^{Hep} mice through modulation of PPARα and PGC-1α. Remarkably, these findings were recapitulated in human NASH, which exhibited increased expression and nuclear localization of Bcl-3.

Conclusions: In summary, Bcl-3 emerges as a novel regulator of hepatic steatosis, insulin sensitivity and inflammation in NASH.

Lay summary: Non-alcoholic fatty liver disease (NAFLD) is considered the most prevalent liver disease worldwide. Patients can develop end-stage liver disease resulting in liver cirrhosis or hepatocellular carcinoma, but also develop complications unrelated to liver disease, e.g., cardiovascular disease. Still there is no full understanding of the mechanisms that cause NAFLD. In this study, genetically engineered mice were employed to examine the role of a specific protein in the liver that is involved in inflammation and the metabolism, namely Bcl-3. By this approach, a better understanding of the mechanisms contributing to disease progression was established. This can help to develop novel therapeutic and diagnostic options for patients with NAFLD.

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Introduction

The prevalence of non-alcoholic fatty liver disease (NAFLD) has steadily increased over the last years. Its incidence is strongly associated with metabolic risk factors including insulin resistance, visceral obesity and dyslipidemia. NAFLD encompasses a disease spectrum that is histologically defined by accumulation of lipids in hepatocytes exceeding 5% and ranges from simple steatosis to non-alcoholic steatohepatitis (NASH) with liver cell injury histologically characterized by ballooned hepatocytes and inflammation. NASH can progress to end-stage liver disease and hepatocellular carcinoma (HCC). Considerable gaps in the knowledge of the molecular regulators that trigger the development and progression of the disease exist and the therapeutic options are limited [1]. Overall, the subgroup of patients that

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Abbreviations: NAFLD, non-alcoholic fatty liver disease; NF-κB, nuclear factor-kappa B; Bcl-3, B cell leukemia-3; NASH, non-alcoholic steatohepatitis; HCC, hepatocellular carcinoma; TNF, tumor necrosis factor-alpha; JNK, c-jun N-terminal protein kinase; PPARα, peroxisome proliferator-activated receptor gamma; PGC-1α, coactivator 1α; HFD, high-fat, high-carbohydrate diet; ROS, reactive oxygen species; KEGG, Kyoto Encyclopedia of Genes and Genomes.

advance from hepatic steatosis and develop inflammation and fibrosis defining NASH and exhibit an increased overall mortality is poorly defined. Current models suggest that insulin resistance promotes lipotoxicity which is among the main causes of liver injury, inflammation and disease progression [2]. Key players that trigger insulin resistance and regulate cellular survival include tumor necrosis factor-alpha (TNF), nuclear factor-kappa B (NF-κB), c-jun N-terminal protein kinase (JNK) and adipocytokines [3].

NF-κB is a key regulator of inflammation, proliferation and cell death in liver tissue and comprises of five cellular DNA-binding subunit-proteins: p50/p105 (NF-κB1), p52/p100 (NF-κB2), c-Rel (Rel), p65 (RelA) and RelB [4]. Their activity is regulated through IκB proteins including among others B cell leukemia-3 (Bcl-3) [5]. *Bcl-3* was originally identified as a proto-oncogene in B cell leukemia and was later discovered to act both as an inhibitor as well as a coactivator of the NF-κB subunits p50 and p52 [6]. Functionally it was demonstrated *in vitro* that Bcl-3 enhances cellular proliferation through activation of the cyclin D1 promoter [7]. Just recently, a novel transgenic mouse was developed that allows to study tissue-specific functions of Bcl-3 and which confirmed its role in lymphoid tissue development [8].

Interestingly, in a yeast two-hybrid screen Bcl-3 was identified to synergize with the peroxisome proliferator-activated receptor gamma (PPARγ) coactivator 1α (PGC-1α) to activate the estrogen-related receptor (ERR) α and PPARα thus exerting metabolic functions through these nuclear receptors [9]. A clinical relevance in this metabolic context was recently discovered in a genome-wide association study, which identified a single nucleotide polymorphism (SNP) in the *Bcl-3* gene (rs8100239 A) and linked it to metabolic abnormalities, while the rs2965169 G allele was independently associated with coronary artery disease [10].

Based on these findings, we investigated the role of Bcl-3 in NASH in a novel transgenic mouse model with hepatocyte-specific overexpression of Bcl-3 ($Bcl\text{-}3^{Hep}$) using a high-fat, high-carbohydrate diet (HFD). Additionally, next-generation sequencing, *in vitro* experiments using $Bcl\text{-}3^{Hep}$ and Bcl-3 knockout mice and human liver tissue were studied to validate the role of Bcl-3 in NASH. Hepatocyte-specific overexpression of Bcl-3 exacerbated the dysmetabolic and inflammatory phenotype in mice on a high-fat diet (HFD), laying the grounds for the progression of hepatic steatosis to NASH through impaired insulin-signaling as well as PPARα, PPARγ and the transcriptional coactivator PGC-1α.

Materials and methods

Animal model

All animals were bred at the animal facility of the University Medical Center Mainz, according to the criteria outlined by the "Guide for the Care and Use of Laboratory Animals". Studies were approved by the Landesuntersuchungsamt Rheinland-Pfalz. Transgenic $Bcl\text{-}3^{Hep}$ mice were generated as described in Supplementary methods (Supplementary Fig. 1). At 8–12 weeks of age, male $Bcl\text{-}3^{Hep}$ mice and wild-type littermates were fed a HFD; 35.5% w/w crude fat (58 kJ%) and drinking water enriched with fructose (55% w/v) and glucose (45% w/v). When indicated, metformin (0.5 mg/ml, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added. Age-matched controls received a corresponding control diet (CD; 5.4% w/w crude fat (13 kJ%)) and plain water. The dietary composition (ssniff Spezialdiäten GmbH, Soest, Germany) is listed in Supplementary Table 1. Mice were fasted over night before sacrifice. *Bcl-3* knockout mice (*Bcl-3* KO) were kindly provided by Dr. Hana Algül (Technical University Munich, Germany).

For glucose tolerance testing animals were fasted for 6 h, then a glucose solution (2 mg/kg) was administered by intraperitoneal (i.p.) injection and blood glucose level was measured.

Total RNA sequencing and analysis

Total RNA was extracted from liver tissue of $Bcl\text{-}3^{Hep}$ and wild-type mice at 8 weeks of age using the RNeasy Plus Universal Mini Kit (Qiagen, Hilden, Germany). RNA quality was confirmed using Agilent Bioanalyzer with an RNA integrity number (RIN) of 8-10. Illumina TruSeq Stranded mRNA Library Preparation with poly A selection and sequencing (seq) to a coverage of approximately 40 million single-end reads of 50 bp were performed using Illumina HiSeq. Analysis and statistics are described in Supplementary methods.

Serological analysis

Serum was measured using standard analyzer (Hitachi 917, Roche, Mannheim, Germany). Insulin was measured using the Rat/Mouse Insulin ELISA Kit (EMD Millipore, St. Charles, Missouri, USA).

Patient samples, histological analyses and immunohistochemistry (IHC)

Formalin-fixed and paraffin-embedded human liver samples of patients were stained for Bcl-3 (Abgent, San Diego, CA, USA) followed by Biotin-SP (long spacer)-conjugated AffiniPure goat anti-rabbit IgG (H + L, Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) combined with TSA™ Cyanine 3 System (PerkinElmer Inc., Boston, MA, USA). Controls were stained in the absence of the primary Bcl-3 antibody. Tissue was derived from therapeutically indicated liver biopsies following informed consent. The study was approved by the Ethical Committee of the Landesärztekammer Rheinland-Pfalz.

In murine tissue immunohistochemistry for activated/cleaved caspase 3 (Cell Signaling Technology Inc., Danvers, MA, USA) and liver Adipophilin/Perilipin 2 (PLN2) staining were performed as previously described [11,12].

Semiquantitative evaluation of steatosis, inflammation and ballooning in human and murine liver tissue was performed blinded by BKS using the NAFLD activity score (NAS) score [13].

Quantitative real-time PCR

Isolation of total RNA, cDNA synthesis and qRT-PCR were performed as previously described [11]. Roche LightCycler software (LightCycler 480 Software Release 1.5.0) was used to perform advanced analysis relative quantification using the $2^{(-\Delta\Delta C(T))}$ method. Expression data were normalized to the housekeeping gene *Gapdh* and the mean of wild-type mice on CD, resp. untreated hepatocytes was considered 1.

Immunoblotting

Primary antibodies included: insulin receptor β, insulin receptor substrate (IRS)-1, phosphorylated (p)-IRS-1 (all Cell Signaling Technology Inc.), Bcl-3 (Abgent, San Diego, CA, USA), actin, p-mixed lineage kinase domain-like (MLKL) (Abcam, Cambridge, UK), PGC-1α, PPARγ, RIP3 (Santa Cruz Biotechnology,) and alpha-tubulin (Sigma-Aldrich, Steinheim, Germany). Membranes were exposed to anti-mouse, anti-goat (both DAKO Denmark A/S, Glostrup, Denmark) or anti-rabbit (Santa Cruz Biotechnology) secondary antibodies conjugated with horseradish peroxidase.

Determination of the hepatic triglyceride content

Hepatic triglycerides were measured using the Triglyceride Quantification Kit (BioVision, Milpitas, CA, USA).

Determination of the NF-κB activity

NF-κB activity was determined using the TransAM NF-κB Family Kit (Active Motif, Carlsbad, CA, USA).

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