Research Article

JOURNAL OF HEPATOLOGY

Liver *Bid* suppression for treatment of fibrosis associated with non-alcoholic steatohepatitis

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Background & Aims: Liver fibrosis is the most worrisome feature of non-alcoholic steatohepatitis (NASH). Growing evidence supports a link between hepatocyte apoptosis and liver fibrogenesis. Our aim was to determine the therapeutic efficacy and safety of liver *Bid*, a key pro-apoptotic molecule, suppression using RNA interference (RNAi) for the treatment of fibrosis.

Methods: First, we optimized the delivery system for *Bid* siRNA in mice using ten different stealth RNAi siRNAs and two lipid formulations -Invivofectamine2.0 and a newly developed Invivofectamine3.0 – that have been designed for high efficacy accumulation in the liver, assessed via real-time PCR of *Bid* mRNA. Next, C57BL/6 mice were placed on a choline-deficient L-amino acid defined (CDAA) diet. After 19 weeks of the CDAA diet, a time point that results in severe fibrotic NASH, mice were injected with the selected *Bid* siRNA-Invivofectamine3.0 biweekly for three weeks. Additionally hepatocyte-specific *Bid* deficient ($Bid^{\Delta hep}$) mice were placed on CDAA diet for 20 weeks.

Results: A maximum *Bid* knockdown was achieved at 1.5 mg/kg siRNA with Invivofectamine3.0, whereas it was at 7 mg/kg with Invivofectamine2.0. In NASH mice, after 3 weeks of treatment, BID protein was reduced to 10% and this was associated with an improvement in liver fibrosis and inflammation associated with a marked reduction in TUNEL positive cells, caspase 3 activation, and a reduction in mitochondrial BAX and BAK. $Bid^{\Delta hep}$ mice showed similar protection from fibrotic changes.

Conclusion: Our data demonstrate that liver *Bid* suppression by RNAi technology, as well as hepatocyte-specific *Bid* deficiency, improves liver fibrosis coupled with a reduction of inflammation in experimental NASH. These findings are consistent with existing evidence that hepatocyte apoptosis triggers hepatic stellate cell activation and liver fibrosis and suggest that *Bid* inhibition may be useful as an antifibrotic NASH therapy.

Keywords: *Bid*; Liver inflammation; Liver fibrosis; Apoptosis; Mitochondrial dysfunction.

Received 13 May 2015; received in revised form 27 October 2015; accepted 2 November 2015; available online 7 November 2015

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Abbreviations: NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; CDAA, chorine-deficient L-amino acid defined; $Bid^{\Delta hep}$, hepatocyte-specific *Bid* deficient; EV, extracellular vesicle.

Introduction

Metabolic non-alcoholic fatty liver disease (NAFLD) has become one of most common forms of chronic liver disease worldwide. Growing evidence demonstrates that patients within the NAFLD spectrum who have progressed to non-alcoholic steatohepatitis (NASH), in particular NASH and fibrosis, are at a higher risk for disease-related morbidity and mortality [1–3]. The development of novel, effective therapies for patients with more advanced forms of the disease are urgently needed [4]. Hepatocellular apoptosis is emerging as an important, if not critical, mechanism contributing to the progression of fibrotic NASH [5]. In hepatocytes, certain lipids, such as free fatty acids (FFAs), can upregulate the expression of cell death receptors, as well as induce organelle stress, in particular mitochondrial dysfunction (commonly referred to as lipotoxicity), which may lead to apoptosis [5]. Fibrosis is based on the activation of hepatic stellate cells (HSCs) and experimental studies suggest that hepatocyte apoptosis and the resulting apoptotic bodies are important activators of HSCs [6]. Indeed, apoptotic bodies from hepatocytes are engulfed by HSCs, stimulating the fibrogenic activity of these cells; DNA fragments from apoptotic hepatocytes can also activate HSCs [6]. Notably, attenuation of hepatocyte apoptosis by inhibition of caspases, in particular caspase 3 and 8, reduces fibrogenesis in animal models of NASH [7,8] thus establishing the proof of concept for anti-apoptotic NASH therapy.

BID is a BH3-only BCL-2 family member that is cleaved by caspase-8 into its active form, truncated BID (tBID), which links the extrinsic and intrinsic apoptosis pathways. tBID formation is crucial for the amplification of apoptotic death signals in cells like hepatocytes (called type 2 cells), where activation of the mitochondrial pathway is essential for cell death to occur. BID, however, is dispensable for apoptosis in most other cell types (called type 1 cells). We recently demonstrated that hepatocyte-specific *Bid* deficient mice are resistant to the lethal effects of Fas activation *in vivo* [9]. Here we tested the hypothesis that selective ablation of BID in hepatocytes can effectively reduce liver injury and fibrosis associated with NASH. To test this hypothesis in this study, we used two different approaches: *Bid* knockdown in wild-type (WT) mice via RNAi technology, and hepatocyte-specific *Bid* deficient ($Bid^{\Delta hep}$) mice, both animal groups were fed a chorine-deficient L-amino acid defined (CDAA) diet.

Materials and methods

siRNA screening

Ten different stealth RNAi™ siRNAs were synthesized from Life Technologies (Life Technologies, Carlsbad, USA). *Bid* target sequences;

*Bid*1: 5′-AGCACAUCACAGACCUGCUGGUGUU-3′
*Bid*2: 5′-CCGCUCCUUCAACCAAGGAAGAAUA-3′
*Bid*3: 5′-AGGAAGAAUAGAGCCAGAUUCUGAA-3′
*Bid*4: 5′-CAGAUUCUGAAAGUCAGGAAGAAAU-3′
*Bid*5: 5′-GAAAGUCAGGAAGAAAUCAUCCACA-3′
*Bid*6: 5′-CAGCUAGCCGCACAGUUCAUGAAUG-3′
*Bid*7: 5′-GAGAACGACAAGGCCAUGCUGAUAA-3′
*Bid*8: 5′-GCCAUGCUGAUAAUGACCAUGCUGU-3′
*Bid*9: 5′-CACCAUCUUUGCUCCGUGAUGUCUU-3′
*Bid*10: 5′-CCUAUGUGAGGAACUUGGUUAGAAA-3′

To determine the best *Bid* target sequence, stealth RNAi™ siRNAs were combined with Invivofectamine2.0 (Life Technologies, Carlsbad, CA, USA) for making complexes according to the manufacturer's instruction and complexes were injected into BALB/C mice at 4 mg/kg. After 2 days of injection, liver *Bid* mRNA expression level was detected by qPCR. The three most effective stealth RNAi™ siRNA complexes (*Bid*3, *Bid*4, and *Bid*10) with Invivofectamine2.0 were further injected into BALB/C at 7 mg/kg and *Bid* mRNA expression level was checked by qPCR at 14 days post-injection. The selected *Bid* siRNA, *Bid*3, was combined with a new lipid-based delivery reagent, Invivofectamine3.0 according to the manufacturer's instruction and the complex was injected into the BALB/C mice at 1.5 mg/kg. After 2 days of injection, liver *Bid* mRNA expression level was checked by qPCR.

Animal studies

The use and care of the animals was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, San Diego (UCSD).

Male BALB/C or C57BL/6 mice, 20–25 g of body weight, were purchased from Harlan Laboratories (CA, USA) and were aged between 6 and 8 weeks at the beginning of this study. BALB/C mice were used for siRNA screening. C57BL/6 mice were fed a CDAA (Dyets, Inc., Bethlehem, PA, USA) diet for 22 weeks to induce NASH. During the last 3 weeks of the feeding course, mice fed with a CDAA diet received weekly administration of the *Bid* siRNA complex, negative siRNA complex, or control (PBS) via intravenous injection (1.5 mg/kg at 1st week, and 0.5 mg/kg at 2nd and 3rd weeks). $Bid^{\Delta hep}$ mice were fed a CDAA diet for 20 weeks to induce NASH.

Liver and blood sample preparation

All mice were sacrificed at the termination of treatment (22 weeks of CDAA diet) under anesthesia via i.p. injection using a 21G needle and a mixture of 100 mg/kg of ketamine and 10 mg/kg of xylazine dissolved in a 0.9% saline solution with euthanasia carried out by carbon dioxide exposure. Whole mouse blood was collected by cardiac puncture and disgorged into tubes with or without anticoagulant. Liver tissue was fixed in 10% formalin for 24 h and embedded in paraffin, quickly frozen in OCT (Sakura Finetek, Torrance, CA, USA), and incubated with RNAlater Solution (Life Technologies) for RNA extraction. The remaining liver tissue was quickly frozen in liquid nitrogen and stored at −80 °C. Serum was used for alanine aminotransferase (ALT) measurement via Infinity ALT (Thermo Scientific, Waltham, MA, USA) or insulin level using mouse ultrasensitive insulin ELISA (ALPCO, Salem, NH, USA).

Measurement of extracellular vesicles

Blood was centrifuged at 1200 g for 15 min and 12,000 g for 12 min at 22 °C to obtain platelet free plasma (PFP). PFP was incubated with Calcein-AM (Life Technologies) for 30 min at room temperature. EV count was performed using 2.5 μm Alignflow alignment beads (Life Technologies) as the size standards for flow cytometry, BD LSRII Flow Cytometer System, (BD Biosciences, San Jose, CA). The data were analyzed using FlowJo software (TreeStar Inc., Ashland, OR).

Liver histology and immunostaining

Tissue sections were prepared and stained for hematoxylin and eosin. Steatosis and liver fibrosis were assessed via Sirius Red staining – liver sections were incubated for 2 h at room temperature with Fast Green FCF (Fisher scientific, Pittsburgh, PA, USA) and Direct Red (Sigma–Aldrich, St. Louis, MO, USA) in saturated picric acid (Sigma–Aldrich). Immunohistochemistry staining for myeloperoxidase (Myeloperoxidase Ab-1, Thermo Scientific) or Ly6C (Abcam, Cambridge, MA, USA) was performed in paraffin embedded or frozen liver sections according to the manufacturer's instruction. All pictures were taken by NanoZoomer 2.0HT Slide Scanning System (Hamamatsu, Japan) and quantitated on ImageJ software. Frozen liver sections were stained for active BAX with anti-BAX (6A7) (Abcam) antibody followed by the Alexa 488 anti-mouse 2nd antibody (Life Technologies) according to the manufacturer's instruction, Oil Red O staining using Oil red O (Sigma–Aldrich) in 60% 2-Propanol (Sigma–Aldrich), or for terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay (Roche, Drive Pleasanton, CA, USA). Oil Red O, active BAX, or TUNEL staining was observed using immunofluorescence microscopy (Olympus, USA).

Liver cell isolation

Liver cells were collected as previously described [10]. Briefly, C57BL/6 or $Bid^{\Delta hep}$ mouse liver was digested with collagenase perfusion through portal vein and isolated parenchymal cells with centrifugation at 50 g for 1 min following centrifugation with Nycodenz gradient at 2000 *g* for 20 min for non-parenchymal cells.

In vitro cell culture studies

HepG2 cells were grown and maintained in Dulbecco's Modified Eagle Medium (Gibco, Camarillo, CA) supplemented with 10% fetal bovine serum (Cellgro, Manassas, VA), Sodium pyruvate (Gibco), penicillin and streptomycin (growth medium) at 37 °C in a 5% CO_2 incubator. HepG2 were reverse transfected with *Bid* or Negative Silencer Select siRNA (Life Technologies) with Lipofectamine RNAiMAX (Life Technologies) according to the manufacturer's instruction. At 36 h post-transfection, cells were incubated with 50 ng/ml anti-human CD95 (Jo2) (BD Biosciences) for 12 h and were collected for RNA extraction or caspase 3 activity assay (Promega, Madison, WI).

Immunoblot analysis

For immunoblot analysis 50 μg of whole liver lysate, as well as mitochondria or cytosolic fraction with mitochondria isolation kit (Thermo scientific, Rockford, IL, USA), was resolved by a 4–20% gradient gel, transferred to a nitrocellulose membrane, and blotted with the appropriate primary antibodies. Membranes were incubated with peroxidase-conjugated secondary antibody (Cell signaling, Danvers, MA, USA). Protein bands were visualized using an enhanced chemiluminescence reagent and digitized using a CCD camera (ChemiDoc®, BioRad, Hercules, CA, USA). Expression intensity was quantified by ImageLab (BioRad). A rabbit anti-BID, anti-BAX, anti-BAK, anti-cytochrome C, anti-cleaved caspase 3, anti-caspase 3, anti-cleaved caspase 8, or anti-caspase 8 antibody was purchased from Cell Signaling and anti-α-SMA and anti-GAPDH were purchased from GeneTex (Irvine, CA, USA). Protein load was verified using GAPDH (GeneTex), or PORIN (GeneTex) antibody.

Real-time PCR

Total RNA was isolated from liver tissue using Trizol (Life Technologies) followed by an RNA purification column (Life Technologies) from cultured cells using RNA purification column according to the manufacturer's instruction. The cDNA was synthesized from 1 μg of total RNA using the SuperScript VILO cDNA Synthesis kit (Life Technologies). Real-time PCR quantification for liver mRNA expression was performed using a TaqMan gene expression assay from Life Technologies, or SYBR-Green, and the CFX96 Thermal Cycler from BioRad. The sequences of the primers used for quantitative PCR are listed in Supplementary Table 1. Mean values were normalized to *β2 microglobulin* for mRNA.

Statistical analyses

All data are expressed as mean ± SEM unless otherwise noted. Data were analyzed using One-way ANOVA in siRNA screening and experimental NASH model or *t* tests in $Bid^{\Delta hep}$ mice using GraphPad (GraphPad Software Inc., CA, USA) for comparison of continuous variables. Differences were considered to be significant at $p \leqslant 0.05$.