Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

Differential regulation of inflammation and apoptosis in Fas-resistant hepatocyte-specific *Bid*-deficient mice

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Background & Aims: Activation of Fas death receptor results in apoptosis in multiple organs, particularly liver, in a process dependent on Bid cleavage. Mice injected with an anti-Fas antibody die within hours of acute liver failure associated with massive apoptosis and hemorrhage. Our aim was to investigate the crosstalk of apoptotic and inflammatory pathways and the contribution of selective hepatocellular apoptosis during *in vivo* Fas activation.

Methods: We generated hepatocyte-specific *Bid* deficient mice ($hBid^{-/-}$). Acute liver injury was induced by Fas-activating antibody (Jo2) in a time-course study.

Results: In contrast to controls, nearly all Jo2 injected $hBid^{-/-}$ survived. Their livers showed complete protection against hepatocellular apoptosis with minimal focal hemorrhagic changes and mainly non-parenchymal cell apoptosis. In agreement, the hepatocytes had no mitochondrial cytochrome c release in cytosol, or caspase 3 activation. $hBid^{-/-}$ livers showed marked increase in acute inflammatory foci composed of neutrophils and monocytes associated with the increased expression of proinflammatory chemokines and cytokines, in the manner dependent on non-canonical interleukin-1β activation and amplified in the absence of caspase-3 activation. In addition, $hBid^{-/-}$ mice were completely protected from hepatotoxicity and the infiltrated cells were cleared 2 weeks post single Jo2 injection.

Conclusions: Hepatocyte Bid suppression is critical for the resistance to the lethal effects of Fas activation *in vivo*. Fas signaling induces differential activation of non-canonical interleukin-1β maturation, amplified in the absence of apoptotic Bid-mitochondrial loop, in hepatocytes. These findings may have important pathophysiological and therapeutic implications in a variety of liver disorders associated with Fas activation.

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Introduction

Fas (Apo-1/CD 95) is one of the death receptors belonging to the tumor necrosis factor receptor family that are potent inducers of apoptosis and constitutively expressed in multiple tissues including the liver, spleen, thymus, heart, and lungs [1,2]. Injection of Jo2 agonistic anti-Fas antibody to mice results in death within a few hours of acute liver failure associated with marked disruption of liver architecture, massive hepatic apoptosis and gross hemorrhage [3]. Although Fas activation by the anti-Fas antibody may result in apoptotic cell death in various tissues, the lethality of this treatment has been mainly thought to be the result of acute hepatic failure secondary to massive hepatocyte apoptosis in a process dependent on Bid cleavage, a BH3-only Bcl-2 family member [4–6]. Acute inflammation and apoptosis of non-parenchymal cells of the liver have also been described [7–9]. In particular, anti-Fas antibody induced injury and apoptosis of sinusoidal endothelial cells was observed leading to the peliosis and hemorrhage in the entire liver lobule [7] and also caused rapid, extensive and disseminated endothelial cell apoptosis throughout the body [8], when the 10 and 20 μg of Jo2 anti-Fas antibody, respectively, were delivered intravenously into mice. Faouzi *et al.* also showed a Jo2 dose-dependent induction in hepatic chemokine production, dependent on caspase 3 activation [9].

Fas was shown to function as a link between obesity associated fatty liver and increased susceptibility to liver damage. We have previously reported that in both a diet induced obesity animal model and human non-alcoholic steatohepatitis there is a significantly increased abundance of hepatic Fas, leading to increased liver sensitivity to endogenous Fas ligand [10,11]. Pharmacologically blocking Fas signaling with an inhibitory peptide (YLGA 12-mer) reverses liver damage in two established models of fatty liver [12]. Global Bid knockout is resistant to Fas-induced hepatocellular apoptosis, gross liver hemorrhage and lethality

Keywords: Hepatoprotection; Inflammation; Fas-mediated hepatic apoptosis; Acute liver failure; Crosstalk of inflammatory and apoptotic pathways; Non-canonical IL-1β maturation.

Received 4 July 2013; received in revised form 3 March 2014; accepted 16 March 2014; available online 27 March 2014

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Abbreviations: $hBid^{-/-}$, hepatocyte-specific Bid knockout; cKO, conditional knockout; $Bid^{fl/fl}$, albumin-cre negative Bid flox/flox control; WT, wild-type; TUNEL, terminal desoxynucleotidyl transferase-mediated dUTP-biotin nick and labeling; MIP, macrophage inflammatory protein; MPO, myeloperoxidase; RIP, receptor-interacting protein; IL, interleukin; IFN, interferon; IP, intraperitoneal; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells.

Molecular and Cell Biology

[13]. Studies to further characterize the histopathological changes in the liver of Jo2-treated mice have shown that in addition to the well-defined massive hepatocellular apoptosis and hemorrhage, there are also significant levels of apoptosis in non-parenchymal cells of the liver, mainly sinusoidal endothelial cells and inflammatory cells [8,14]. Furthermore, these mice show the features of acute inflammation associated with neutrophilic infiltration and increased production of certain chemokines [9]. The relative contribution of hepatocyte apoptosis *vs.* the other lesions, and the crosstalk of apoptotic and inflammatory pathways, observed in the liver during Fas-induced liver hemorrhage *in vivo* remain poorly understood.

In the present study, we aimed at addressing these issues by generating hepatocyte-specific *Bid* knockout mice – $hBid^{-/-}$. Here, we demonstrate that hepatocyte Bid is crucial for Fas-induced liver hemorrhagic changes and lethality. Furthermore, our data suggest that Fas signaling induces differential activation of non-canonical inflammatory IL-1β, TNFα, and NF-κB pathways amplified in the absence of apoptotic Bid-mitochondrial loop in hepatocytes. This study has confirmed a crucial role of hepatocyte Bid activation in Fas induced liver failure and lethality and that hepatocyte Bid-inhibition *in vivo* facilitates nearly full hepatic protection and leads to the complete resolution of Fas-induced inflammation.

Materials and methods

Generation of $hBid^{-/-}$ mice

Six exons were identified with the ATG start codon in exon 2 and the TGA stop codon in exon 6. We selected exons 2–4 (encoding the N-terminal 121 amino acids) as our conditional knockout (cKO) region. In the targeting vector, the Neo cassette is flanked by Frt sites and the cKO region is flanked by LoxP sites. The final vector was confirmed by both restriction digestion and end sequencing analysis. NotI was used to linearize the vector prior to electroporation into mouse C57BL/6 embryonic stem cells. Cells that have undergone homologous recombination were identified and correctly targeted clones have been confirmed. Targeted deletion of Bid in hepatocytes was created by crossing the Bid cKO with Cre recombinase expression driven by the Albumin promoter. Mouse genotypes were determined by PCR approach and confirmed by Southern blotting of genomic DNA isolated from tail biopsies. The use of the Alb-Cre promoter approach also partially deletes Bid from cholangiocytes and progenitor oval cells, thus their role, although unlikely, cannot be completely excluded in this model.

Animal studies

These experimental protocols were approved by the Institutional Animal Care and Use Committee at the Cleveland Clinic and University of California, San Diego. All efforts were made to minimize pain and distress during animal husbandry and experimental assessments. Six to 8 weeks old $hBid^{-/-}$ (n = 9), *Bid* flox/flox albumin-cre negative ($Bid^{fl/fl}$; n = 12), and wild-type C57BL/6 (WT; n = 7) mice (Jackson Laboratory, Bar Harbor, ME) were injected once intraperitoneally (IP) with a single dose of 100 μg anti-Fas antibody (clone Jo2; BD Pharmingen, San Jose, CA) or vehicle (physiological saline) control. Animals were observed over 12 h for mortality and euthanized at a point near death or at 12 h for liver histology or biochemical studies. For the time-course study $hBid^{-/-}$ (n = 27) mice were injected IP with 100 μg anti-Fas antibody (or vehicle controls) and euthanized at time 0 h (baseline), 3 h, 12 h, and 2 wk. Serum samples were collected to measure ALT and AST by using a linearity test kit Infinity ALT/AST kits (Thermo Scientific, Waltham, MA) according to the manufacturer's specifications. For the low-dose Jo2 time-course study, WT (n = 12) and $hBid^{-/-}$ mice (n = 12) were IP injected with 0.25 μg/g body weight of Jo2 antibody and the assessments of pathology, apoptosis, and inflammation were made at 3 h and 12 h post Jo2-injection (n = 6 at each time point). Untreated mice (n = 6, each group) were used as baseline controls.

Cell culture and treatment

For primary hepatocytes, WT and $hBid^{-/-}$ mice were perfused with collagenase D (Roche, Indianapolis, IN), described in detail in the reference [15] and digested liver was filtered on 100 μm nylon cell strainer (BD Biosciences, San Jose, CA). Primary hepatocytes were isolated at 500*g* for 1 min with two washes, cultured in Collagenase I coated dish for 3 h in William E medium-10% FBS and further cultured for 8 h in serum free William E medium. Primary hepatocytes were treated with 10 ng Jo2 antibody/ml media and the assessments of cell death/apoptosis and inflammation were made at 1 h, 3 h, and 12 h post jo2-treatment (n = 6 at each time point). Untreated primary hepatocytes were used as baseline controls. Mouse embryonic fibroblasts (MEFs) were cultured in DMEM-10% FBS. To initiate necrosis, MEFs were pre-incubated for 1 h in 20 μM Z-VAD-FMK (global caspase inhibitor) and 1 μg/ml cycloheximide, and treated with 100 ng/ml of TNFα for additional 5 h.

Histology, apoptosis assessment, immunohistochemistry, and immunofluorescence

Livers were weighted, cut into small pieces and fixed in 10% formalin up to 24 h. After wash and dehydration, livers were paraffin embedded and cut (4 μm) by the UCSD Histology core. Haematoxylin and eosin stained sections were used for histopathological evaluation of hepatic injury. Assessment of apoptosis were performed by terminal desoxynucleotidyl transferase-mediated dUTP-biotin nick and labeling (TUNEL) staining with ApopTag peroxidase *in situ* apoptosis detection kit (Millipore, Billerica, MA) on paraffin-embedded liver specimens and *DeadEnd* Fluorometric System (Promega, Madison, WI) on frozen sections according to the manufacturer instructions. TUNEL positive nuclei were quantified by ImageJ software (National Institutes of Health, Bethesda, MD). In primary hepatocyte, cell death/apoptosis was assessed by (1) measuring the release of lactate dehydrogenase (LDH) in cell culture media via CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega), (2) assessing caspase 3 activity via Apo-ONE® Homogenous Caspase 3/7 assay, and (3) LIVE/DEAD® Cell Viability Assay (Invitrogen/Life technologies, Carlsbad, CA), according to the manufacturers' protocols. Immunostaining for cleaved caspase 3 (apoptosis; Cell Signaling; 1:250), CD31 (marker of endothelial cells; Abcam, Cambridge, MA; 1:50), macrophage inflammatory protein (MIP)-2 (AbD Serotec, Raleigh, NC; 1:50), myeloperoxidase (MPO; marker of neutrophils; Abcam; 1:50), CD3 (marker of T-cells; Abcam; 1:50), and F4/80 (marker of mouse macrophages; AbD Serotec; 1:50) were performed according to the manufacturers' protocols. Necrosis was assessed by co-localization of kinase receptor-interacting protein (RIP)-1 (BD Transduction Laboratories; 1:50) and RIP-3 (Santa Cruz, Santa Cruz, CA; 1:50) with Alexa Flour 488 and 594 (Life Technologies), as secondary antibodies, by immunofluorescence on frozen sections as previously described [16]. Treated MEFs were incubated with RIP-1 and RIP-3 antibodies as described above. All pictures were taken by NanoZoomer 2.0HT Slide Scanning System (Hamamatsu, Japan).

Western blots

Whole cell liver lysates were digested in RIPA buffer (Cell Signaling, Danvers, MA) containing Protease Inhibitor Cocktail Tables (Roche). Liver lysates were continuously rotated for 30 min at 4 °C, followed by centrifugation at 14,000*g* for 10 min at 4 °C. Liver lysates (80 μg) were resolved by Criterion™ TGX™ Any kD™ Precast Gel (Bio-Rad, Hercules, CA). Proteins were transferred to 0.2 μm nitrocellulose membrane (Bio-Rad) and blocked for 1 h with 5% non-fat milk or bovine serum albumin in TBS, 0.05% Tween 20. Blots were then hybridized overnight using antibodies specific for total caspase 8, cleaved caspase 8 (D387), total caspase 3, cleaved caspase 3 (D175), phospho-NF-κB, interleukin (IL)-6 (all: Cell Signaling; 1:1000), and Fas receptor/CD95 (GeneTex, Irvine, CA; 1:1000). Mitochondrial and cytosolic fractionation was performed by Mitochondrial Isolation kit for tissues (Thermo Scientific), proteins were transferred to membranes as above and hybridized overnight using anti-cytochrome c antibody (136F3; Cell Signaling; 1:1000). Nuclear and cytosolic fractionation was performed by NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific). Actin (Santa Cruz; 1:1000), porin (VDAC1; Abcam; 1:1000), and p84 (GeneTex; 1:1000) were used as loading controls for the whole liver lysates/cytoplasmic fraction, mitochondrial fraction and nuclear fraction, respectively. For the time-course study (with the high Jo2 dose), cell lysates were made from isolated hepatocytes (or whole liver lysates at 2 wk) following the same protocol as above and proteins were hybridized using an antibody specific for IL-1β (Abcam; 1:1000). Antibodies used to test selectivity and specificity of Bid absence were specific for mouse Bid, Bax and BAD (Cell Signaling), and Bcl-Xl (BD Biosciences). The secondary antibody used was

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