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

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Abstract

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 hepatocytes-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, Jo2 injected hBid^{-/-} nearly all 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 noncanonical interleukin-1 β 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 noncanonical interleukin-1 β maturation, amplified in the absence of apoptotic Bid-mitochondrial loop, in

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hepatocytes. These findings may have important pathophysiological and therapeutic implications in a variety of liver disorders associated with Fas activation.

Keywords

hepatoprotection; inflammation; Fas-mediated hepatic apoptosis; acute liver failure; crosstalk of inflammatory and apoptotic pathways; noncanonical IL-1 β maturation

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 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 μ g 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 diet induced obesity animal model and human nonalcoholic 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 [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 of 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 versus 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 noncanonical inflammatory IL-1 β , TNF α , and NF- κ B 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 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 μ g anti-Fas antibody (clone Jo2; BD Pharmingen, San Jose, CA) or vehicle (physiological saline) control. Animals were observed over 12 h for mortality and sacrificed 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 μ g anti-Fas antibody (or vehicle controls) and sacrificed 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 μ g/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 hours in William E medium-10% FBS and further cultured for 8 hours 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 deoxynucleotidyl 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 antibody 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 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). Secondary antibody used was anti-rabbit (Cell Signaling; 1:5000). Proteins were visualized by SuperSignal West chemiluminescence substrate (Pierce Biotechnology) and quantified by ImageJ software.

Gene expression

Liver tissue (or isolated hepatocytes for the time-course study) from WT and hBid^{-/-} were homogenized using the FastPrep 24 bead homogenization system (MP Biomedicals, Santa Ana, CA). Total RNA was isolated using RNeasy kit (Qiagen, Valencia, CA) and reverse transcribed by iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions. The concentration and purity of RNA was assessed by NanoDrop (Thermo scientific). Housekeeping genes GAPDH or B2M were used as an internal control. The PCR primers used to amplify each gene are listed in Supplement Table 1. Quantitative Real-time PCR was performed on a Bio-Rad Cyclor (Bio-Rad) by using SYBRGreen real time PCR master mix (KapaBiosystems, Woburn, MA).

Flow cytometry

Livers were perfused with PBS and force filtered through a 100 µm nylon cell strainer (BD Biosciences). Whole liver cell suspensions were incubated with RBC lysis buffer (eBioscience, San Diego, CA), washed with PBS-3% FBS and a small fraction was first analyzed with CD45 (BD Biosciences) or Ly6G (Biolegend, San Diego, CA) antibodies by flow cytometry to assess the specificity of infiltrated cells in the entire liver. Next, to separate infiltrated cells, whole liver cell suspensions were centrifuged at 50 g for 1 min to remove hepatocytes. Infiltrated cells were further purified by Polymorphprep™ (CosmoBio USA, Carlsbad, CA) and layers of mononuclear and polymorphonuclear cells were recovered, following the manufacturer's protocol. Purified infiltrated cells were incubated with RBC lysis buffer (eBioscience), washed with PBS-3% FBS, incubated with antibody cocktail, FITC-CD45 (BD Biosciences), Alexa405-CD11b (eBioscience), PEcy5.5-Ly6G

(Biolegend), PE-ICAM (BD Biosciences) for 30 min on ice, and washed with PBS-3% FBS. For each time point the data obtained from Jo2-treated hBid^{-/-} mice (n = 4) were corrected to data from saline-treated hBid^{-/-} mice (n = 3). All cell suspensions were analyzed by Flow cytometer, BD LSR II (BD Biosciences), and data were analyzed by FlowJo software.

Data analysis

Two-tailed Student t-test and log-rank (Mantel-Cox) test were used for analyzing qPCR data and survival rate, respectively, when comparing two groups. For multiple-comparison tests, data were analyzed by one-way ANOVA with Bonferroni Post-hoc test among groups at each time point. Data are reported as mean ± SEM, with significance at $P < 0.05$. GraphPad software (La Jolla, CA) was used to perform analyses and to construct graphs.

RESULTS

Hepatocyte-specific Bid-deficiency mediates *in vivo* resistance to Fas-induced mortality, hepatocyte apoptosis, gross liver hemorrhage, and marked neutrophilic infiltration in the livers of hBid^{-/-} mice

To assess the role of selective hepatocyte apoptosis during Fas activation *in vivo*, we generated a hepatocyte-specific cKO model of the mBid gene using the cre/loxP system (Supplement Fig. 1A). Bid^{fl/fl} mice were born at the expected mendelian frequency, had no apparent developmental abnormalities, and had normal spleens, thymuses, brains, hearts, livers, lungs, kidneys and testes with respect to weight and histology. The selective and specific absence of Bid expression in hepatocytes in albumin-Cre Bid flox/flox (hBid^{-/-}) was demonstrated by immunoblotting. No changes in the abundance of other pro- or anti-apoptotic proteins (Bax, Bad, or Bcl-XL) in the liver, kidney, spleen, or thymus were detected (Supplement Fig. 1B). To investigate the role of Bid in hepatocyte apoptosis, hemorrhagic response, hepatic failure and lethality, mice were injected IP with 100 µg of anti-Fas antibody (clone Jo2), as it was done previously to challenge global Bid knockout [3]. All WT mice, as well as albumin-cre negative Bid^{fl/fl} controls, died within the first 3 to 6 hours (mean survival time – 5 and 4.7 hours, respectively) of acute liver failure associated with marked disruption of liver architecture, massive hepatic apoptosis and gross hemorrhage (Fig. 1A; Supplement Fig. 1C). In sharp contrast, most hBid^{-/-} mice (8 out of 9) survived the high-dose (100 µg) of anti-Fas antibody injection (Fig. 1A). Histopathological examination of hBid^{-/-} livers demonstrated nearly complete protection against hepatocellular apoptosis with intact cell nuclei, minimal focal hemorrhagic changes, similarly to the phenotype of hBid^{-/-} physiological saline (vehicle) injected controls (Fig. 1A). The low mortality in hBid^{-/-} mice and the remaining hepatocyte apoptosis is likely due to variable penetrance of cre/loxP approach and/or the activation of alternative apoptotic mechanisms independent of Bid activation, as previously described [13].

Acute liver failure in WT mice was associated with marked increase in cellular death coupled with activation of the initiator caspase 8 and the effector caspase 3, as evidenced by TUNEL assay, immunohistochemical staining, immunoblotting, and a notable redistribution of cytochrome c into the cytosol (Fig. 1B, C). In contrast, there was significantly less TUNEL-positive nuclei in hBid^{-/-} livers and cytochrome c was mostly contained in

mitochondria in hBid^{-/-} mice compared to WT controls (Fig. 1B). Despite the similar level of caspase 8 activity in WT and hBid^{-/-}, there was a significant absence of the active, cleaved fragment of caspase 3 in the livers of hBid^{-/-} mice compared to WT (Fig. 1C). Moreover, massive apoptosis of both hepatocytes and non-parenchymal cells with neutrophil infiltration was demonstrated in Bid^{fl/fl} controls with immunohistochemical serial liver sections stained against antibody markers of endothelial cells (CD31), neutrophils (MPO) and TUNEL (Supplement Fig. 2B). In contrast, serial liver sections stained with the same set of markers showed that hBid^{-/-} mice were protected from both hepatocyte and sinusoidal endothelial cell death, and that most of the TUNEL-positive nuclei co-localized with infiltrated neutrophil foci (Supplement Fig. 2B).

Despite diminished hepatocyte apoptosis, livers from anti-Fas antibody treated hBid^{-/-} mice showed a plethora of marked acute inflammatory foci composed predominantly of neutrophils and monocytes, as demonstrated by MPO immunohistochemical staining and histopathological examination (Fig. 1D and Supplement Fig. 2A, B). In addition to the presence of apoptotic neutrophils, this pattern was also associated with increased focal abundance of MIP-2 (a murine IL-8 analog; Fig. 1E) and increased expression of other chemokines often associated with hepatic inflammation, such as MIP-1 α and KC ($P < 0.05$), as well as pro-inflammatory cytokines IL-1 β ($P < 0.05$) and interferon (IFN)- γ ($P < 0.01$; Fig. 1F). There were no significant changes in expression of these genes between saline treated WT and hBid^{-/-} controls (data not shown).

Bid-deficiency facilitates hepatic protection and leads to the complete resolution of inflammation two weeks post anti-Fas antibody injection

We performed a time-course study to assess the extent of protection resulting from hepatocyte-specific Bid-deficiency. This study showed almost complete protection from lethality (90% survival rate), hepatocyte apoptosis and liver hemorrhage over the two week period post single high-dose (100 μ g) anti-Fas antibody injection in hBid^{-/-} mice (Fig. 2A, B). While neutrophils have previously been described as playing a main role in acute inflammatory liver injury and leading to hepatocellular death [17], in Jo2 injected hBid^{-/-} mice they remained confined to inflammatory foci in the acute phase (12 hours post injection) and ultimately returned to near normal levels two weeks post injection (Fig. 2B). Correspondingly, the serum levels of ALT and AST returned from supraphysiological levels at 12 hours to normal levels two weeks post Jo2 injection (Fig. 2C). Flow cytometry analysis showed the percentages of infiltrated leukocytes, especially the fold increase of neutrophils, into the liver were acutely increased at 3 and 12 hours post Jo2 injection, with almost complete resolution of inflammation two weeks post injection (Fig. 2D). In detailed flow cytometry analysis using purified infiltrated cell population, monocyte infiltration was also shown to be dramatically increased at 3 and 12 hours post injection (Fig. 2E). Notably, monocyte infiltration was completely recovered to baseline levels at 2 weeks post Jo2 injection (Fig. 2E). Similar trend was observed with ICAM⁺ cells (a marker of eosinophils; Supplement Fig. 3A). There was no apparent difference in the abundance in T-lymphocytes (CD3⁺ cells) and mature macrophages (F4/80⁺) at any of the time points, as demonstrated by immunohistochemistry (Supplement Fig. 3B).

In hBid^{-/-} mice Fas mediates inflammation via hepatocyte chemokine and IL-1 β production in RIP-independent manner

In the time-course study we sought to characterize the mechanisms of Fas-induced inflammation and its resolution. We demonstrated by fluorescent immunohistochemical staining of hBid^{-/-} liver sections that Fas stimulation does not induce activation of either RIP-1 or RIP-3, or does not lead to the *in vivo* formation RIP-1/RIP-3 complex 12 hours post Jo-2 injection (Fig. 3A), shown to be necessary for the initiation of programmed necrosis [18]. Positive control for immunofluorescent assay and the formation of RIP-1/RIP-3 complex was performed in mouse embryonic fibroblast cells, as described in the methods (Figure 3A). Furthermore, in isolated hepatocytes, we showed that there was a significant increase of MIP-1 α expression levels ($P < 0.05$) 3 hours post Jo2 as compared to saline control treated hBid^{-/-} mice, as well as MIP-2, IL-6, and KC (all $P < 0.001$) 12 hours post Jo2-treatment (Fig. 3B). It has recently been shown that these chemokines, in particular IL-8 (murine MIP-2), may serve as “find-me” signals for apoptotic cells and play essential role for migration of human neutrophils toward dying cells [19]. In addition, we showed that there is a significant increase in hepatocyte pro-IL-1 β gene expression, followed by the increase in hepatocyte pro-IL-1 β protein abundance at 12 hours post Jo2-injection ($P < 0.01$; Fig. 3C). Corresponding to the resolution of inflammation, levels of chemokines and cytokines were normalized at 2 weeks post Jo2-injection (Fig. 3B, C).

Low dose Jo2 treatment of hBid^{-/-} primary hepatocytes and mice demonstrates that Fas mediates inflammation via the activation of noncanonical IL-1 β /TNF α and NF- κ B pathways amplified in the absence of apoptotic Bid-mitochondrial loop

By introducing a low-dose Jo2 time-course treatment in WT and hBid^{-/-} primary hepatocytes and mice we aimed at determining the relationship between inflammation and apoptosis, the mechanisms involved, and the hepatocyte-specific contribution. In order to determine the lowest dose of Jo2 antibody to induce minimal apoptosis and still activate chemokine and cytokine production, we performed *in vitro* and *in vivo* Jo2 dose response experiments in primary WT hepatocytes (Supplement Fig. 4A, B) and WT mice (Supplement Fig. 4C, D). Based on these experiments, we decided to use a low-dose of 10 ng Jo2 antibody/ml cell media for the *in vitro* study and 0.25 μ g Jo2/g mouse body weight for the *in vivo* study. The levels of apoptosis and inflammation were assessed at baseline (untreated controls), 1 h, 3 h and 12 h post Jo2-treatment in primary hepatocytes extracted from WT and hBid^{-/-} mice. hBid^{-/-} primary hepatocytes showed marked protection from apoptosis as shown by reduced lactate dehydrogenase release and caspase 3 activity, and live/dead cell viability assay as compared to WT primary hepatocytes treated with 10 ng Jo2 antibody/ml media (Fig. 4A). Furthermore, MIP-2 ($P < 0.01$ at 12 h), KC ($P < 0.001$ at 12 h), and IL-6 ($P < 0.001$ at 3 h) were significantly higher in WT primary hepatocytes parallel to the amount of apoptosis (Fig. 4B). Remarkably, the expression levels of IL-1 β ($P < 0.05$ at 3 h and $P < 0.001$ at 12 h) and TNF α ($P < 0.001$ at 12 h) were significantly higher in hBid^{-/-} primary hepatocytes, suggesting that these genes were up-regulated via Fas signaling in the absence of caspase-3 activation (Fig. 4B). The upregulation of these genes was concomitant to the increased NF- κ B activation in hBid^{-/-} as compared to WT primary hepatocytes (Supplement Fig. 5). To investigate the crosstalk of apoptotic and inflammatory

pathways *in vivo* we exposed the WT and hBid^{-/-} to a low dose of Jo2 antibody and monitored apoptotic and inflammatory signals at baseline (untreated controls), 3 h and 12 h post Jo2-injection. All mice lived to 12 h without apparent physiological trauma. Histopathological examination of livers demonstrated moderate levels of liver hemorrhage in WT mice at 12 h post Jo2 (but significantly less hemorrhage that was caused by a high dose of Jo2), and the formation of focal inflammatory points in hBid^{-/-} mice at 12 h as previously shown with high Jo2 concentration (Fig. 4C). Similarly to mice treated with high dose of Jo2, there was a significant absence of the active, cleaved fragment of caspase 3 in the livers of hBid^{-/-} mice compared to WT mice at 12 h (Fig. 4D). Remarkably, there was an increase in the active IL-1 β fragment abundance especially at 3 h post Jo2-injection (7-fold over the control) and increased IL-6 levels at 12 h corresponding to the marked abundance of phosphorylated NF- κ B in both nuclear and cytosolic fractions of the livers of hBid^{-/-} mice (Fig. 4E). Both *in vitro* and *in vivo* data strongly suggest that Fas may induce differential activation of alternative inflammatory IL-1 β , TNF α , and NF- κ B pathways amplified in the absence of apoptotic Bid-mitochondrial loop (Supplement Fig. 6).

DISCUSSION

Fas (Apo-1/CD 95), a death receptors belonging to the tumor necrosis factor receptor family, was shown to function as a link between steatohepatitis and hepatocellular damage. In our present study we generated hepatocyte-specific Bid deficient mouse to investigate the role of selective hepatocyte apoptosis during Fas activation *in vivo*, a model of acute liver injury. Here, we clearly demonstrate that hepatocyte Bid is crucial for Fas-induced liver hemorrhagic changes and lethality and show dissociation between hepatocyte apoptosis and inflammation. We also reveal that inflammatory cells by themselves cannot induce liver failure, even though abundant inflammatory cells acutely and focally infiltrated into the liver via Jo2 injection. When hepatocytes were protected from apoptosis, we found a near complete recovery of liver function and the resolution of inflammation two weeks post Jo2 injection.

Fas-induced apoptosis has been postulated as a model of pro-inflammatory apoptotic cell death [20–22]. However, the role the cell-specific and molecular mechanisms linking hepatic apoptosis and inflammation during systemic administration of an anti-Fas antibody has not been characterized, partially due to the lack of adequate *in vivo* models and specific caspase inhibitors. While Faouzi et al. showed that caspase 3 inhibition reduced chemokine induction and neutrophilic infiltration in Jo2-treated livers [9], it has later been shown that the caspase 3 inhibitor used in this study (Z-DEVD-FMK) was also partially inhibiting caspase 8 [23], as well as other caspases, thus bringing the conclusion that inflammatory response is dependent on caspase 3 activation and apoptosis into question. In hBid^{-/-} mice we observed marked neutrophilic infiltration to the liver, suggesting that inflammatory pathway may be activated in parallel and differentially to apoptotic pathway.

As shown by Faouzi et al. [9] we confirmed that common neutrophilic chemokines, MIP-2 and KC, and cytokine IL-6, increased their expression level in parallel to the amount of apoptosis in primary WT hepatocytes. However, we identified that a noncanonical pathway for IL-1 β and TNF α expression was significantly increased in hBid^{-/-} compared to WT

hepatocytes. Furthermore, our *in vivo* studies with a low dose of Jo2 demonstrated that there is a significant increase in the noncanonical IL-1 β maturation and higher abundance of phosphorylated, active NF- κ B with the sustained presence in both nuclear and cytosolic fraction in the livers of Jo2-injected hBid^{-/-} compared to WT mice over a 12 h time-course. These data are supported by the findings of Bossaller et al., where it has recently been shown that Fas mediates noncanonical IL-1 β maturation via caspase 8 in an RIP-3-independent manner in macrophages [24]. In agreement with these findings, we observed the similar alternate activation and maturation of IL-1 β in the hepatocytes, in a manner dependent on caspase 8 activation, independent of RIP-1 and -3 activity and co-localization, and amplified in the absence of caspase 3 activation 12 hours post single Jo2-injection. Although RIP-1 kinase has been shown to be recruited to the Fas Death-inducing signaling complex and necessary for the optimal production of cytokines and chemokines in response to Fas receptor stimulations, its recruitment and role has been shown to be minimal in the cells with functioning caspase 8 [19], or cells stimulated with anti-Fas antibody [25]. In summary, these data suggest that Fas signaling induces differential activation of noncanonical inflammatory IL-1 β , TNF α , and NF- κ B pathways amplified in the absence of apoptotic Bid-mitochondrial loop in hepatocytes.

Most of the non-hepatocytes are the so-called type I cells, where Fas-induced death is not dependent on Bid signaling and mitochondrial amplification [13], and thus inhibiting Bid in these cells might not play a crucial protective role, but rather lead to delayed or attenuated apoptotic response. However, hepatocytes are generally considered to be type II cells (where Bid and mitochondria are necessary to amplify apoptotic signal) because global Bid knockout mice were reported to be resistant to hepatocyte apoptosis upon Fas activation [13, 26]. However, more recent studies have challenged this view. It has been shown that the high-dose of hexameric Fas ligand (a more potent activator of Fas receptor [27]) or double Bak/Bax deficiency [28] may lead to circumvention of mitochondrial apoptotic pathways and activation of type I pathway in the hepatocytes, where caspase 8 directly activates caspase 3 and induces apoptosis. However, while we used relatively high dose of Jo2 antibody in this study (~5 mg/kg), we have not seen a significant activation of apoptotic pathways in hBid^{-/-}. In addition, in contrast to double Bak/Bax knockout mice, by specifically deleting Bid from hepatocytes there was no delayed-onset hepatocyte apoptosis as evidenced by our two week time-course study. Furthermore, although Liedtke et al. have previously reported that the hepatocyte-specific deletion of caspase 8 protected from Fas-induced lethality, this model also unexpectedly triggered increased non-apoptotic liver injury and oxidative stress [16]. Another group demonstrated that caspase 8 activation is crucial to inhibit the interaction of RIP-1 and -3 which complex formation has been shown to be required for initiation of programmed necrosis [18]. In agreement, we demonstrated that hepatocyte-specific deletion of Bid, which does not interfere with Fas-induced caspase 8 activation, did not induce programmed necrosis. Taken together, these results suggest that Bid, as opposed to its upstream (caspase 8) or downstream (Bak/Bax) counterparts, is the ideal target to inhibit apoptotic pathway and reduce hepatic stress.

The engagement of Fas leads to the activation of caspase 8 by cleaving the large cytosolic Bid to its active, truncated form, tBid, which in turn translocates into the mitochondrion as

an integral membrane protein [29, 30]. tBid is a key component required for the release of cytochrome c from mitochondria [31, 32]. Cytosolic cytochrome c binds to apoptosis activating-factor 1, a scaffolding protein that facilitates activation of procaspase 9. Once activated, caspase 9 cleaves caspases 3 and 7, resulting in their activation and subsequent stereotypic apoptotic events [33, 34]. Our hepatocyte specific Bid-deficient mice demonstrate that activation of Bid in hepatocytes and selective hepatocellular apoptosis is a critical event leading to the lethality induced by Fas activation *in vivo*. Indeed, in striking contrast to WT animals or Bid^{fl/fl} controls, hBid^{-/-} mice were almost completely resistant to the mortality resulting from systemic anti-Fas antibody administration. Second, our data clearly demonstrate the importance of hepatocyte apoptosis in the hemorrhagic changes typically observed in the livers of anti-Fas antibody injected animals. In the past, these changes were proposed to be the consequence of apoptosis of sinusoidal endothelial cells of the liver which are known to express Fas and to be sensitive to Fas ligation *in vivo*. However, the fact that the lack of hepatocyte apoptosis in the hBid^{-/-} mice led to marked protection from Fas-induced liver hemorrhage, suggests that the hemorrhage and lethality are the consequences of the massive distortion of the liver architecture resulting from the hepatocyte apoptosis, rather than the apoptosis of sinusoidal endothelial cells, and that sinusoidal endothelial cells entail stronger resistance to Fas-induced apoptosis. Current studies also showed that inhibition of hepatocyte apoptosis in the hBid^{-/-} mice was in fact associated with marked increased in chemokine and pro-inflammatory cytokine production in the liver, in particular the activation of noncanonical IL-1 β maturation, resulting in acute inflammatory infiltrates composed mainly of neutrophils and monocytes. These data strongly suggest that although hepatocyte apoptosis and liver inflammation occur in parallel during Fas engagement, they are differentially regulated. In summary, our results from hepatocyte specific deletion of Bid indicate that hepatocyte apoptosis is the crucial event for the lethal effects of Fas engagement *in vivo*. These results may have important pathophysiological and therapeutic implications in a variety of liver disorders associated with Fas activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this manuscript

hBid^{-/-}	hepatocyte-specific Bid knockout
cKO	conditional knockout
Bid^{fl/fl}	albumin-cre negative Bid flox/flox control

WT	wild-type
TUNEL	terminal deoxynucleotidyl 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

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Figure 1. hBid^{-/-} mice are resistant to Fas-induced mortality, hepatocyte apoptosis and gross liver hemorrhage, but are prone to the formation of marked acute inflammatory foci and neutrophilic infiltration

(A) Survival curve of wild-type (WT; filled circles, n = 7) and hBid^{-/-} mice (circles, n = 9) in response to a single IP injection of 100 µg Jo2 over the period of 12 h post injection, $P < 0.0001$. Mice were sacrificed 12 h after Jo2-injection or shortly before death. Liver macro pictures and haematoxylin-eosin staining of liver sections of Jo2 injected WT mice and vehicle control (saline) or Jo2 injected hBid^{-/-} mice, respectively. Note the marked disruption of liver architecture, massive hepatic apoptosis and gross hemorrhage due to Fas activation in WT mice. (B) DNA fragmentation by TUNEL assay and detection of apoptosis by immunohistochemical staining specific for cleaved caspase 3 in WT and vehicle control or Jo2 injected hBid^{-/-} mice. Quantification of the representative 40x TUNEL fields of WT (n = 7) vs. hBid^{-/-} (n = 5) mice. Assessment of cytochrome c distribution in mitochondria vs. cytosol of fractionated liver lysates in WT and hBid^{-/-} mice. (C) Analysis of the activation of initiator caspase 8 and effector caspase 3 in livers by immunoblots after injection of Jo2 in WT and vehicle control or Jo2 injected hBid^{-/-} mice. Representative blots show the activated fragments: p18 of caspase-8 and p17/19 of caspase-3, in addition to their pro-caspase counterparts. (D) Haematoxylin-eosin staining of liver sections of Jo2

injected hBid^{-/-} mice, emphasizing neutrophilic infiltration; (E) immunohistochemical staining specific for MIP-2 in WT and vehicle control or Jo2 injected hBid^{-/-} mice. Note the marked neutrophilic infiltration and the concomitant focal increase in the abundance of MIP-2 in hBid^{-/-} compared to WT mice. (F) Expression profiling of chemokines and cytokines involved in Fas-mediated liver inflammation. All animals (n = 4, each group; WT, open bars; hBid^{-/-}, black bars) were injected with Jo2. All gene expression levels are shown relative to the expression levels of WT mice. Values are mean ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared to controls. Scale bar, 50 μm.

Figure 2. Time-course study: resistance of hBid^{-/-} mice to Fas-induced mortality and the complete resolution of inflammation two weeks post anti-Fas antibody injection
 (A) Survival curve of hBid^{-/-} mice (n = 10) in response to a single IP injection of 100 µg anti-Fas antibody (Jo2) over the period of 2 weeks. (B) Liver macro pictures and haematoxylin-eosin staining of liver sections of hBid^{-/-} mice post Jo2-injection. (C) Plasma levels of ALT and AST of hBid^{-/-} mice 12 h and 2 wk post Jo2-injection (hBid^{-/-} n = 4). (D) Percent of leukocytes and fold change of neutrophils (relative to saline injections at each time point) infiltrated into the liver of hBid^{-/-} post Jo2-injection by flow cytometry analysis. (E) Representative histograms of infiltrated monocytes (CD11b⁺). Red line: hBid^{-/-} Jo2 injection, Black line: hBid^{-/-} saline injection. Bar graph shows quantification of infiltrated cells as shown by fold change compared to saline injections at each time point. Values are mean ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared to baseline controls. Scale bar, 50 µm.

Figure 3. Time-course study: Fas mediates hepatocyte chemokine and IL-1β production in an RIP-independent manner

(A) Hepatic Fas signaling does not induce RIP-1/RIP-3 complex required for programmed necrosis 12 h post Jo2-injection. Immunofluorescent staining specific for RIP-1 and RIP-3 kinases and merged image in vehicle and control Jo2 injected hBid^{-/-} mice. Necrotic MEFs were used as a positive control for the formation of RIP-1/RIP-3 complex. Scale bar, 50 μm. (B) Hepatocyte (at 3 h and 12 h) and whole liver (at 2 wk) chemokine gene expression levels in Fas-mediated liver inflammation of hBid^{-/-} mice. Gene expression was measured in hBid^{-/-} mice (n = 3, each group; saline, open bars; Jo2 treated, black bars) 3 h, 12 h and 2 wk post Jo2 injection. All gene expression levels were normalized to housekeeping control and shown relative to the expression levels of saline treated mice. (C) Hepatocyte pro-IL-1β gene expression and protein levels post Jo2-injections in Fas-mediated liver inflammation of hBid^{-/-} mice (saline, open bars; Jo2 treated, black bars). Values are mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 compared to controls.

Figure 4. *In vitro* and *in vivo* low Jo2 dose time-course study dissociates apoptotic and inflammatory pathways

(A) The assessment of apoptosis and cell death in WT (open bars) and hBid^{-/-} (black bars) primary hepatocytes. From left to right, lactate dehydrogenase (LDH) cytotoxicity assay, caspase 3 activity and live/dead cell viability assay (red stains nuclei of dead cells, green stains living cells). (B) Hepatocyte inflammatory chemokine and cytokine gene expression normalized to untreated controls (n=6 each group). (C) Liver macro pictures and haematoxylin-eosin staining of liver sections of Jo2 injected WT (open bars) and hBid^{-/-} mice (black bars). Note the small levels of liver hemorrhage in WT and the formation of inflammatory foci hBid^{-/-} mice at 12 h post Jo2 injection. (D) Representative immunoblots of select apoptotic signaling pathways in Jo2 injected WT and hBid^{-/-} mice. (E) Representative immunoblots and protein levels of select inflammatory signaling pathways probed in cytosolic (cyt.) and nuclear (nuc.) fractions. Note the marked abundance of mature/active IL-1 β at 3 h and sustained activation of NF- κ B and the corresponding abundance of IL-6 through 12 h post Jo2-treatment in hBid^{-/-} mice. W, WT; hB, hBid^{-/-}. Values are mean $\pm$ SEM. * P < 0.05, ** P < 0.01, *** P < 0.001 compared to controls. Scale bar, 50 μ m.