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Sustained Phosphorylation of Bid Is a Marker for Resistance to Fas-Induced Apoptosis During Chronic Liver Diseases

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Abstract

Background & Aims—Increased rates of apoptosis have been reported to play a role in the pathophysiology of many disorders, including liver diseases. Conversely, genetic mutations that result in impairment of programmed cell death have been associated with cancer development. However, apoptosis resistance can also be the result of nongenetic stress adaptation, as seen in the cancer-prone metabolic liver disease hereditary tyrosinemia. To clarify whether stress-induced apoptosis resistance is a general feature of chronic liver diseases, an animal model of chronic cholestasis was examined.

Methods—Studies were performed with mice before and 2 weeks following bile duct ligation and with *Fah*^{−/−} and *Fah/p21*^{−/−} mice before and after NTBC withdrawal.

Results—Here we show that bile duct ligation induced profound resistance against Fas monoclonal antibody-mediated hepatocyte death. The apoptosis signaling pathway was blocked downstream of caspase-8 activation and proximal to mitochondrial cytochrome *c* release. In controls, activation of the Fas receptor resulted in rapid dephosphorylation of Bid and its subsequent cleavage, whereas Bid remained phosphorylated and uncleaved in chronic cholestasis and other models of hepatic apoptosis resistance.

Conclusions—We propose a model in which the phosphorylation status of Bid determines the apoptotic threshold of hepatocytes in vivo. Furthermore, resistance to apoptosis in chronic cholestasis may contribute to the long-term risk of cancer in this setting.

Abbreviations used in this paper

BDL, bile duct ligation; DISC, death-inducing signaling complex; EGTA, ethylene glycol-bis[β-aminoethyl ether]-N,N,N',N'-tetraacetic acid; FADD, Fas-associated death domain adaptor protein; HT-1, hereditary tyrosinemia; IAPs, inhibitors of apoptosis proteins; mAb, monoclonal antibody; NF-κB, nuclear factor κB; PP2A, protein phosphatase 2A; SDS-PAGE, sodium dodecyl sulfate/

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polyacrylamide gel electrophoresis; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling

Fas (CD95/Apo-1) belongs to the subfamily of death receptors and plays an important role in liver homeostasis. Hepatocytes are exquisitely sensitive to apoptosis mediated by the Fas receptor. Hence, cell death induced by this mechanism has been implicated in acute liver injury and liver failure.¹ However, chronic liver disease is in some cases associated with apoptosis resistance. For example, we have recently shown that the chronic injury found in the metabolic liver disease hereditary tyrosinemia (HT-1) is associated with profound cell death resistance.² This observation raised the question whether death resistance is a specific adaptation seen only in the rare genetic disease HT-1 or whether it represents a more general response in chronic liver disorders. Here, an animal model of prolonged cholestasis was used to address this question. Cholestasis is defined as an impairment of bile flow and is frequently seen in human liver disease, manifesting itself as jaundice. Many conditions associated with chronic cholestasis have not only an elevated risk of cholangiocellular carcinoma but also of hepatocellular carcinoma.^{3–6} During cholestasis, toxic bile acids accumulate within hepatocytes, leading to structural changes, inflammatory responses, and ultimately hepatotoxicity. The mechanisms leading to hepatocyte death in cholestasis are not well defined, and 2 distinct mechanisms have been reported. Several studies have proposed that Fas-receptor and Bid-dependent apoptosis is an important mechanism for cholestatic liver injury.^{7–9} In contrast, more recent studies carefully reevaluated the mode of cell death in vivo and showed that the mechanism of hepatocyte death is almost exclusively oncotic and not apoptotic following bile duct ligation (BDL).^{10,11} Despite these reports demonstrating liver cell death acutely after accumulation of bile acids, it is increasingly recognized that hepatocytes develop protective adaptations against bile acid–induced cell death during chronic cholestatic liver diseases.⁹ In HT-1, the cell death–resistant state is characterized by protection against multiple triggers, including the toxic tyrosine metabolite homogentisic acid (HGA), the drug acetaminophen, and the monoclonal anti-mouse Fas-activating antibody Jo₂ (Fas mAb).² In this study, we therefore determined whether hepatocytes in chronic cholestasis also display resistance against classic apoptosis induced by Fas mAb and analyzed the mechanism underlying this adaptive response. We found that resistance to Fas mAb–induced apoptosis is indeed a feature of chronic cholestasis. Furthermore, we show that phosphorylation/dephosphorylation of Bid correlates with this adaptive response in different apoptosis resistance models. Our data suggest a central role for Bid in the regulation of apoptosis sensitivity in the liver.

Materials and Methods

Mice

We used the *Fah*^{−/−} strain of mice previously described.¹² All *Fah*^{−/−} animals were treated with NTBC-containing drinking water at a concentration of 7.5 mg/L unless otherwise indicated. 129S1 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Common BDL was performed as previously described.¹³ In brief, mice were anesthetized and the peritoneal cavity was opened. The common bile duct was double ligated and cut between the ligatures. NTBC was withdrawn from *Fah*^{−/−} and *Fah*/p21^{−/−} mice as previously described, and the mice were kept on water for 2 weeks. At the end of each pretreatment, the animals were injected intraperitoneally with 0.4 μg/g monoclonal anti-mouse Fas-activating antibody Jo₂ (BD PharMingen, San Diego, CA) and killed at indicated time points. In the suramin experiments, suramin (5 mg/mouse; Sigma Chemical Co, St Louis, MO) was injected intraperitoneally concomitantly at a different site.¹⁴ Animal care and experiments were all in accordance with the Guidelines of the Department of Animal Care at Oregon Health Sciences University. At the time the mice were killed, a small fragment of each liver was fixed in buffered

formalin. The remaining liver tissues were flash frozen in liquid nitrogen and stored at -80°C until analysis.

Histology and Terminal Deoxynucleotidyl Transferase–Mediated Deoxyuridine Triphosphate Nick-End Labeling Assay

Liver tissues were fixed in 10% phosphate-buffered formalin (pH 7.4), dehydrated in 100% ethanol, and embedded in paraffin wax at 58°C . Five-micron sections were rehydrated and stained with H&E. The terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay (ApopTag; Serological Corp, Norcross, GA) was performed according to the manufacturer's recommendations.

DNA Fragmentation Analysis

Genomic DNA was isolated and purified from mouse livers using a genomic DNA isolation kit (Qiagen, Valencia, CA). DNA samples (1 μg each) were electrophoretically separated on 2% agarose gel containing ethidium bromide (0.5 g/L).

Aminotransferase Determinations

For aminotransferase analysis, animal blood was drawn. After spinning at 200g, plasma was recovered and stored at 80°C until used for determination of aminotransferase activities.

Mitochondria Isolation

Liver homogenates were prepared and subfractionated as described previously.¹⁵ Liver samples were homogenized in 2 mL buffer A (225 mmol/L mannitol, 75 mmol/L sucrose, 0.1 mmol/L ethylene glycol-bis[β -aminoethyl ether]- N,N,N',N' -tetraacetic acid [EGTA], 10 mmol/L HEPES, pH 7.2) with a Dounce glass homogenizer (type B pestle) and centrifuged at 4°C for 10 minutes at 600g. The supernatants were further centrifuged at 12,000g for 10 minutes. The supernatants were collected as the cytosol fraction. The pellet (heavy membranes or mitochondria) was washed once again in buffer A by centrifugation at 600g for 10 minutes and recovered by centrifugation at 12,000g for 10 minutes. The isolated mitochondria were resuspended in buffer B (395 mmol/L sucrose, 10 mmol/L HEPES-NaOH, pH 7.5).

Caspase Activity

Liver lysates were prepared by homogenization in hypotonic buffer (25 mmol/L HEPES, pH 7.5, 5 mmol/L MgCl_2 , 1 mmol/L EGTA, 1 mmol/L phenylmethylsulfonyl fluoride). Homogenates were centrifuged at 15,000 rpm for 15 minutes, and extracted proteins (50 μg) were tested in triplicate experiments by measuring the proteolytic cleavage of specific fluorogenic substrate for caspase-3 and caspase-8 (CaspACE Assay System; Promega, Madison, WI).

Antibodies

Antibodies against Fas receptor, caspase-9, Flip, and Bid were obtained from Cell Signaling Technology (Beverly, MA). Antibodies against Bak, Bax, and cytochrome *c* were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and antibodies against caspase-8, c-Iap-2, Bcl-2, Bcl-x, and phospho-Bad were from BD Biosciences (San Diego, CA). The antibody that recognizes S61 phosphorylated Bid was generously provided by J. C. Martinou (University of Geneva, Switzerland).

Western Blot Analysis

Protein extracts were resolved by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membrane (Millipore, Bedford,

MA). Coomassie staining was used to demonstrate equal protein loading. Western blotting was performed as recently described.² Detection of immunolabeled proteins was performed using the chemiluminescence kit (BioRad, Hercules, CA) and Hyperfilm enhanced chemiluminescence film (Amersham Biosciences, Piscataway, NJ).

Hepatocyte Preparation and Flow Cytometry

Livers were perfused as previously described. For cell surface Fas, cell suspensions were incubated with Fas-specific fluorescein isothiocyanate–conjugated antibody (PharMingen, San Diego, CA). Data were acquired using an Elite Epics flow cytometer (Coulter, Hialeah, FL) and analyzed with CellQuest software (Becton Dickinson, San Jose, CA). Analysis gates were set to exclude contaminating immune cells (lymphocytes and macrophages).

Nuclear Factor κ B p65 Immunofluorescence

Cryosections of livers were performed. Sections were fixed in methanol/acetone, dried, and rinsed in Tris-buffered saline/Tween 20. After incubation with a specific antibody directed against the mouse nuclear factor κ B (NF- κ B) p65 subunit (Santa Cruz Biotechnology), positive cells were detected by incubation with Alexa Fluor₅₉₄ nm donkey anti-rabbit secondary antibody (Molecular Probes, Carlsbad, CA). Staining of liver nuclei was performed by incubation with 4',6-diamidino-2-phenylindole-containing mounting medium (Vector Laboratories, Burlingame, CA).

In Vitro Cleavage of Hepatic Caspase-3 and Caspase-9 by Recombinant Caspase-8

Liver cytosols (2 μ g/ μ L) of bile duct-ligated and control mice were incubated with activated recombinant caspase-8 (1 ng/ μ L; BD PharMingen) at 30°C for 120 minutes. Extracts were then separated on a 10% SDS-PAGE followed by Western blotting with anti-caspase-3 and anti-caspase-9 antibodies.

In Vitro Induction of Mitochondrial Cytochrome c Release

Recombinant tBid at various concentrations (1, 5, 10, and 25 nmol/L) was mixed with 100 μ g of hepatocyte mitochondria (2 mg/mL) in 75 μ L buffer B. Mitochondria of 4 mice in each group were analyzed. After incubation at 30°C for 30 minutes, the supernatants were separated from the mitochondria by centrifugation at 16,000g at 4°C for 10 minutes (Biofuge Fresco; Thermo Electron Corporation, Asheville, NC.). An aliquot of 50 μ L was removed from the supernatant, transferred to a new tube, and 5 μ L of 5% Triton X-100 was added. Two control samples containing only mitochondria and no tBid were run for each assay to determine the total amount of cytochrome c that can be released from mitochondria and the amount of spontaneously released cytochrome c from untreated mitochondria. The cytosolic fractions were then subjected to a 12% SDS-PAGE followed by Western blotting with the anti-cytochrome c antibody.

In Vitro Phosphorylation of Bid

Recombinant murine Bid-His₆ (5 μ g) was in vitro phosphorylated by 3 μ g hepatocyte lysate. Control phosphorylation reactions were performed using 100 ng recombinant CK1. Reactions were incubated for 30 minutes at 30°C in a total volume of 25 μ L CK1 reaction buffer (20 mmol/L 3-[N-morpholino]propanesulfonic acid, pH 7.2, 25 mmol/L β -glycerophosphate, 5 mmol/L EGTA, 1 mmol/L sodium orthovanadate, 1 mmol/L dithiothreitol) containing 200 μ mol/L cold adenosine triphosphate and 2.5 μ Ci [γ -³²P]-adenosine triphosphate (3000 Ci/mmol; MP Biomedicals, Irvine, CA). CK1 activity was inhibited by 100 μ mol/L CKI-7 (US Biological, Swampscott, MA). Phosphorylation reactions were terminated by the addition of SDS-PAGE loading buffer. Bid protein was separated by 15% SDS-PAGE, transferred to

nitrocellulose, and analyzed by autoradiography. Additionally, membranes were probed with the phospho-specific Bid antibody.

CK1 Substrate Peptide Assay

Three micrograms of hepatocyte lysate was used to phosphorylate the CK1 substrate peptide RRKDLHDDEE-DEAMSITA (Upstate, Charlottesville, VA). A reaction containing 250 $\mu\text{mol/L}$ substrate peptide in a total volume of 25 μL CK1 assay buffer using adenosine triphosphate concentrations mentioned previously was incubated for 10 minutes at 30°C. A total of 100 $\mu\text{mol/L}$ CKI-7 was used to inhibit CK1 activity. CK1 substrate reactions were stopped by the addition of trichloroacetic acid and spotted onto P81 grade Whatman filter paper (Florham Park, NJ). Filters were washed 3 times in 0.75% phosphoric acid and once in acetone. Filters were counted with a Packard 1900 TR Liquid Scintillation Analyzer (Global Medical Instrumentation, Ramsey, MI) to measure ^{32}P incorporation into peptide bound to filter.

Microarray Analysis

Total RNA was extracted with the RNeasy kit (Qiagen, Valencia, CA). Microarray assays were performed in the Affymetrix Microarray Core of the Oregon Health & Science University Gene Microarray Shared Resource, which uses Affymetrix Expression Analysis software (<http://www.ohsu.edu/gmsr/amc/>). The screen was performed in duplicate on Gene-Chip Mouse Genome 430 2.0 arrays (Affymetrix, Inc, Santa Clara, CA).

Results

BDL Induces Resistance Against Fas-Induced Apoptosis

Cholestasis was induced in a cohort of experimental mice by surgical BDL, which was well tolerated. Two weeks after the procedure, no signs of hepatic apoptosis were present despite TUNEL-positive bile infarcts (data not shown) as reported by others.^{9–11} Apoptosis was determined by DNA laddering (Figure 1B), caspase activity assays (data not shown), and analysis of the mitochondrial apoptosis pathway (Figure 2B). At this point, bile duct-ligated mice still maintained the same weight and overall health as controls, despite clearly abnormal liver function tests including marked hyperbilirubinemia and mild elevation of transaminase levels (Figure 2A) caused by oncotic liver cell injury. Because of the absence of spontaneous apoptosis, it was next determined whether bile duct-ligated mice were protected against Fas-induced apoptosis. Remarkably, all bile duct-ligated mice survived an otherwise 100% lethal dose of the Fas mAb (Figure 1A). Histologic analysis, DNA laddering (Figure 1B), and TUNEL staining (Figure 1C) showed massive apoptosis in control mice, whereas bile duct-ligated mice were completely protected. Additionally, transaminase and bilirubin levels did not increase further (Figure 2A). Together these data show that hepatocytes in chronic cholestasis acquired resistance to Fas-induced apoptosis.

In our previous work, heat shock protein (70, 32, and 27) up-regulation and stress kinase down-regulation were implicated in the resistance to Fas mAb-induced apoptosis in the *Fah*^{-/-} model. In bile duct-ligated mice and in comparisons of *Fah*^{-/-} and *Fah/p21*^{-/-} mice, however, none of these changes were consistently seen. Heat shock proteins were also up-regulated in *Fah/p21*^{-/-} mice, which are no longer resistant against apoptosis (Willenbring et al, manuscript submitted); the stress kinase pathways, which were blocked in apoptosis-resistant *Fah*^{-/-} mice, were activated in bile duct-ligated mice (data not shown).

The transcription factor NF- κ B has been implicated in both hepatocyte proliferation and apoptosis, and recent studies have suggested that NF- κ B can be activated in some models of cholestasis.^{9,16,17} To determine whether NF- κ B signaling could account for the resistance against Fas-induced apoptosis in bile duct-ligated mice, p65 translocation into the nucleus was

used as a marker of its activation. A mild, transient activation of NF- κ B occurred only in the first 48 hours following BDL, and there was no nuclear staining of p65 at later time points. Interestingly, no consistent activation of NF- κ B was evident within the liver even at the earlier time points; only scattered hepatocytes showed nuclear accumulation of p65. In agreement with these observations, we were also unable to detect any decrease of I κ B- α and I κ B- β 2 weeks following BDL. In addition, we performed microarray analysis to ascertain the levels of known NF- κ B target genes such as *Fas receptor*,¹⁸ *A20*,¹⁹ *inos*,²⁰ *ciap1*,¹⁷ *ciap2*,²⁰ *xiap*,²¹ *bfl1*,¹⁷ *bclxb*,²² *Gadd45 β* ,¹⁷ *Afp*,²¹ *IP₃R-1*,²³ or *mSOD* (Table 1), which have been implicated in the regulation of apoptosis. Gene expression levels were compared in 2 apoptosis-resistant models: bile duct-ligated mice and *Fah*^{-/-} mice off NTBC (Willenbring et al, manuscript submitted). There was no overlap of genes differentially regulated in the 2 models, and there was no consistent up-regulation in apoptosis-resistant mice compared with apoptosis-sensitive mice (*Fah*^{-/-}/*p21*^{-/-} mice off NTBC). Finally, several publications have shown that activation or inactivation of NF- κ B does not significantly modulate Fas-induced apoptosis in the liver in contrast to the established antiapoptotic activity of NF- κ B in tumor necrosis factor- and TRAIL-induced liver injury.^{18,24–27} We concluded that NF- κ B activation could not explain the Fas resistance of bile duct-ligated mice and sought to determine at which level apoptosis was blocked and which alternate factors may be responsible.

The Apoptosis Signaling Pathway Is Blocked Above and/or at the Mitochondria

Upon ligand binding, activated death receptors engage the Fas-associated death domain adaptor protein (FADD).²⁹ FADD in turn recruits caspase-8 via a homophilic death effector domain interaction forming the death-inducing signaling complex (DISC). DISC formation activates caspase-8 through a proximity-induced dimerization mechanism requiring no autoproteolysis.^{29,30} Here, a clear increase of caspase-8 activity was detectable in both control and bile duct-ligated mice 2 hours following Fas mAb injection, indicating that the early steps of the pathway were unaltered following BDL (Figure 2A). There was no difference in baseline activation of caspase-8 and caspase-3 two weeks following BDL. All values were therefore normalized to the same wild-type controls.

In type II cells such as hepatocytes, activation of caspase-8 at the DISC alone is insufficient to efficiently trigger cell death and an amplification loop via the mitochondria is required.³¹ Previous studies have shown that this link between the Fas receptor and the mitochondria is dependent on cleavage and translocation to the mitochondria of the proapoptotic Bcl-2 family member Bid by caspase-8.^{32,33} Caspase-8-cleaved tBid was not detectable in *in vivo* experiments with various commercially available antibodies, possibly because of the small pool of Bid molecules involved or because of its rapid turnover.³⁴ Therefore, to examine this step of the apoptotic machinery, protein levels of full-length (uncleaved) Bid were compared after Fas mAb injection in bile duct-ligated mice and controls. A marked reduction of full-length Bid was visible in the Fas mAb-treated control group (Figure 2B), but full-length Bid remained unchanged in bile duct-ligated mice.

During the apoptotic process in normal type II cells, tBid activates Bax or Bak to initiate mitochondrial dysfunction and cell death; therefore, the role of these proteins was also examined.^{35,36} As expected, analysis of the intracellular distribution revealed translocation of Bax from the cytosol to the mitochondrial fraction in Fas mAb-injected control mice (Figure 2B). Surprisingly, Bax was constitutively localized to the mitochondrial fraction even before Fas mAb injection in bile duct-ligated mice, and these high levels of Bax remained unchanged after administration of Fas mAb (Figure 2B). Normally, activation and oligomerization of Bax and Bak induce structural changes at the mitochondrial membrane, resulting in release of cytochrome *c*.^{37,38} Here, cytochrome *c* was found only in the cytosol of control animals following Fas mAb injection, but not in bile duct-ligated mice despite the translocation of Bax

to the mitochondria (Figure 2B). Following release of cytochrome *c* from mitochondria, APAF-1 is known to recruit caspase-9 and the resulting assemblage of proteins activates effector caspases such as caspase-3.³⁹ As expected from the observed release of cytochrome *c*, the cleaved, activated fragment of caspase-9 and activity of caspase-3 were only detectable in controls, and no caspase activation was found in bile duct–ligated mice (Figure 2A and B).

Resistance Against Apoptosis Is Not Mediated by Direct Inhibition of Caspases

Apoptosis is a tightly regulated process that involves many proapoptotic and antiapoptotic proteins capable of altering the balance between life and death. We first wondered whether direct inhibition of caspases contributed to the observed failure of mitochondrial cytochrome *c* release in bile duct–ligated mice. Specifically, the expression of death receptor, Flice-like inhibitory proteins (c-Flip), and inhibitors of apoptosis proteins (Iap) were analyzed. In agreement with the activation of caspase-8 seen in bile duct–ligated mice (Figure 2A), messenger RNA levels for components of the DISC complex such as the Fas receptor, FADD, and caspase-8 were unaltered (Table 1). This was confirmed at the protein level for the Fas receptor and caspase-8 (Figures 2B and 3A). Furthermore, Fas receptor at the cell surface was not down-regulated as assessed by flow cytometric cell cycle analysis, indicating that the adaptive response did not result from lack of available Fas receptors (data not shown). Additionally, levels of c-Flip_{s/l}, inhibitors of caspase-8,⁴⁰ remained unchanged in bile duct–ligated mice (Table 1 and Figure 3A). Another group of proteins that function as intrinsic regulators of caspase activation are the Iaps. Iaps are known to regulate the activity of both initiator (caspase-9) and effector caspases (caspase-3 and caspase-7). However, there were no significant changes for several Iaps, including cIap-1 (Figure 3A), cIap-2, and xIap (Table 1). These findings indicated that the expressions of proteins known to have direct effects on caspase activities were not changed in BDL. This was further verified by showing that purified active recombinant caspase-8 was able to normally induce cleavage of caspase-9 and caspase-3 in cytosolic extracts of bile duct–ligated mice in vitro (Figure 3B).

Detailed Analysis of Bcl-2–like Family Members

During apoptosis, mitochondria experience specific damage that ultimately results in loss of their function. Mitochondrial changes include the release of several proapoptotic factors that trigger and amplify the cell death signal leading to activation of caspases and DNA fragmentation.⁴¹ The family of Bcl-2–like proteins plays a pivotal role in protection against such irreversible mitochondrial damage, and the interactions between the proapoptotic and antiapoptotic family members determine the overall susceptibility to a cell death signal.⁴² Antiapoptotic members include Bcl-2, Bcl-x, Bcl-w, A1, and Mcl-1. Proapoptotic members can be subdivided into more fully conserved members possessing Bcl-2 homology domains 1–3 such as Bax, Bak, and Bok and the BH3-only members such as Bid, Bad, Bim, Noxa, Puma, and Bik. Activation of Bcl-2–like family members is regulated in multiple ways, including transcriptional up-regulation, proteolytic cleavage, dimerization, phosphorylation, and translocation.⁴³ First, it was determined whether any of the Bcl-2–like genes were up-regulated in response to BDL. Interestingly, only *Bfl-1/A1* was more than 2-fold induced at the RNA level. Messenger RNA levels of all other members of the Bcl-2–like family were only slightly modulated following BDL (Table 1). In agreement with these microarray data, protein levels of Bfl-1/A1 were mildly increased in the cytosol of bile duct–ligated mice (Figure 3A), whereas Bak, Bcl-2, and Bcl-x remained unchanged.

Next, the subcellular location of Bcl-2–like proteins was studied. Bcl-x is found both in the cytosol and attached to mitochondrial membranes of most healthy cells, whereas Bcl-2 and Bak are constitutively associated with mitochondrial membranes.⁴⁴ The pro-apoptotic Bcl-2–like proteins Bax and Bid have been reported to be predominantly cytosolic in nonapoptotic cells. In the mitochondrial fraction of bile duct–ligated mice, an increase of not only Bax but

also of full-length Bid was seen (Figures 2B and 3C). On the other hand, levels of Bak, Bcl-x, and Bcl-2 in the mitochondrial fraction of bile duct–ligated mice were not significantly changed before injection of Fas mAb (Figure 3C). It has previously been shown that Bax and Bid need to be integrated into the mitochondrial membrane to be active.⁴⁵ Alkaline extraction of mitochondria was therefore used to assess membrane insertion. In contrast to Bcl-x, both Bax and Bid were only loosely attached but not integrated into the mitochondrial membrane. This suggested that these proteins were not activated in bile duct–ligated mice before Fas mAb injection (Figure 3D). Mitochondrial integrity was further tested by using different amounts (1, 5, 10, and 25 nmol/L) of caspase-8–cleaved recombinant mouse tBid to induce cytochrome *c* release from isolated liver mitochondria of apoptosis-resistant bile duct–ligated mice and controls in vitro. Importantly, both groups discharged cytochrome *c* equally well at all tested tBid concentrations (Figure 3E). This demonstrates that alterations of the mitochondria themselves did not contribute to the observed resistance against apoptosis in BDL.

Bid Is Not Dephosphorylated During Apoptosis in Bile Duct–Ligated Mice

The results described previously show that the apoptotic block in BDL primarily occurred distal to caspase-8 activation and proximal to mitochondrial damage. In addition, failure of Bid cleavage despite caspase-8 activation was observed, and we therefore sought to understand the mechanism underlying this finding. Phosphorylation of Bid has been shown to prevent its cleavage by caspase-8.^{46,47} In bile duct–ligated mice, slightly elevated levels of Bid messenger RNA and protein were detectable, indicating a transcriptional up-regulation of this BH3-only protein (Table 1 and Figure 3A). Accordingly, an increase of phosphorylated Bid (S61) was detectable in these mice 2 weeks following BDL (Figure 4A). During Fas-induced apoptosis, Bid needs to be dephosphorylated before it can be cleaved by caspase-8.^{46,47} The required net dephosphorylation of Bid could be mediated either by the inactivation of a Bid kinase or by activation of a Bid phosphatase. As expected from this model, Bid was almost completely dephosphorylated following induction of apoptosis in control mice (Figure 4A). Importantly, dephosphorylation of Bid did not occur at all in apoptosis-resistant bile duct–ligated mice (Figure 4A). To date, no phosphatase that dephosphorylates Bid during apoptosis has been identified, but protein phosphatase 2A (PP2A) is a candidate because it is cleaved and activated during apoptosis.⁴² However, here PP2A protein levels were not significantly changed before or following Fas mAb injection in control and bile duct–ligated mice (Figure 4B). In the case of murine Bid, CK1 ϵ is known to phosphorylate Bid on Ser 61 in vitro.⁴⁷ Several CK1 isoforms with different functions have been identified and are found in the cytosol, associated with membranes, and in the nucleus.^{48–50} Interestingly, CK1 ϵ was increased in the cytosol of bile duct–ligated mice (Figure 4B) and CK1 activity was slightly increased in cell extracts of bile duct–ligated mice (Figure 4C). More importantly, CK1 activity remained significantly higher in apoptosis-resistant bile duct–ligated mice compared with control mice following Jo₂ injection (Figure 4C). To further confirm this finding, Bid phosphorylation activity was measured in vitro with extracts from bile duct–ligated and control mice following Fas mAb injection. In agreement with the in vivo data, recombinant Bid was only phosphorylated in the cytosol of bile duct–ligated mice (Figure 4D). The phosphorylation on S61 was almost completely blocked by the CK1 inhibitor CKI-7.

Bid Is Not Dephosphorylated in Cell Death–Resistant *Fah*^{−/−} Mice During Apoptosis in Contrast to Apoptosis-Sensitive *Fah*^{−/−}/*p21*^{−/−} Mice

To determine the significance of the alterations of Bid phosphorylation seen here in bile duct–ligated mice, we wondered whether a similar pattern was present in other apoptosis-resistant liver models. Chronic injury found in the cancer-prone metabolic liver disease HT-1 induces profound resistance against hepatocyte death.² However, this adaptation does not occur in mice that are doubly mutant in the gene responsible for HT-1, *Fah*, and the cell cycle regulatory protein *p21/Cip/Waf*, which develop a similar liver damage as wild-type mice following Fas

mAb injection. Interestingly, *Fah*^{-/-}/*p21*^{-/-} mice were highly sensitive against Fas-induced apoptosis despite a marked increase of proliferating hepatocytes 2 weeks following NTBC withdrawal, indicating that proliferation alone does not prevent Fas-induced apoptosis in the liver (Willenbring et al, manuscript submitted).⁵¹ Our observation provides the opportunity to directly compare Bid phosphorylation in apoptosis-resistant and apoptosis-sensitive livers. First, we compared the transcriptional changes of apoptosis-related genes including proapoptotic and antiapoptotic BH3-like proteins and inhibitors of caspases between *Fah*^{-/-}/*p21*^{-/-} and *Fah*^{-/-} mice on NTBC and 2 weeks following NTBC withdrawal (Table 1). We did not observe significant changes of these genes, similar to our results in the bile duct-ligated mice. In particular, *Bfl1/A1* was less than 2-fold induced in apoptosis-resistant *Fah*^{-/-} mice and there was no significant difference in comparison to apoptosis-sensitive *Fah*^{-/-}/*p21*^{-/-} mice. Next, we wondered whether the phosphorylation pattern of Bid would correlate with the observed apoptosis sensitivity. *Fah*^{-/-}/*p21*^{-/-} and *Fah*^{-/-} mice were taken off the liver-protective drug NTBC for 14 days and then injected with Fas mAb. Phosphorylation levels of Bid were determined in mice of both groups, which at this point still had the same weight and overall fitness despite their hepatic dysfunction.⁵² Phosphorylation of Bid was not different in *Fah*^{-/-}/*p21*^{-/-} and *Fah*^{-/-} mice before Fas mAb injection. Importantly, however, Bid was not dephosphorylated in the apoptosis-resistant *Fah*^{-/-} mutants but almost completely dephosphorylated in the apoptosis-competent *Fah*^{-/-}/*p21*^{-/-} double mutant mice following Fas mAb injection (Figure 4E). Similarly, cleavage of Bid measured by decreasing levels of full-length Bid and release of cytochrome *c* occurred only in *Fah*^{-/-}/*p21*^{-/-} mice (Figure 4E). Dephosphorylation of Bid was associated with decreased cytosolic CK1ε levels in the apoptosis-sensitive mice following Fas mAb injection, and PP2A levels remained unchanged in both groups (Figure 4E).

Suramin Inhibits Fas-Induced Hepatocellular Apoptosis and Prevents Dephosphorylation of Bid

Recently, it has been shown that suramin inhibits death receptor-induced apoptosis in vitro and fulminant apoptotic liver damage in mice.¹⁴ Suramin is a polysulfonated naphthylurea compound that has been widely used for the treatment of trypanosomiasis and onchocerciasis.⁵³ Initial observation suggested that suramin acts primarily by interfering with the binding of growth factors, such as platelet-derived growth factor, transforming growth factor, and tumor necrosis factor α, to their corresponding receptors. Subsequent investigation, however, revealed a marked increase in phosphorylation of several cellular proteins in suramin-treated cancer cell lines and identified suramin as a reversible modulator of protein phosphatases and kinases.^{54,55} Previous studies have shown that suramin does have proapoptotic effects in specific tumors such as prostate carcinoma, whereas it has antiapoptotic effects in lymphoid and hepatic cell lines. Remarkably, suramin does not prevent apoptosis in type I cells such as SKW6.4 cells, whereas it inhibited apoptosis in type II cells such as hepatocytes.¹⁴ Subsequent analysis revealed that the block of apoptosis occurred upstream of the mitochondria and involves inhibition of the DISC.¹⁴ Interestingly, however, the general composition of the DISC was not changed by treatment with suramin; CD95, FADD, pro-caspase-8 (p55/53), and their initial cleavage products (p43/44) were present in almost equal amounts.¹⁴ The observations that suramin was only effective in type II cells and acts upstream of the mitochondria suggest that modulation of Bid could also contribute to the observed phenotype. We therefore wondered whether resistance against cell death induced by suramin was also associated with inhibition of Bid dephosphorylation and injected 5 mg of suramin per mouse concomitantly with Fas mAb in wild-type mice. Coadministration of suramin significantly protected mice from Fas mAb-induced death. In agreement with our hypothesis, Bid was not dephosphorylated and subsequently not cleaved in suramin-treated cells. Cytosolic CK1ε levels were again higher in the apoptosis-resistant cells, whereas PP2A levels remained unchanged (Figure 4F).

ATM Phosphorylates Bid In Vitro But Is Not Necessary for BDL-Induced Resistance Against Apoptosis

Our data indicate that phosphorylation/dephosphorylation of Bid strictly correlates with the apoptosis sensitivity in different apoptosis resistance models. Furthermore, apoptosis-resistant mice revealed higher cytosolic CK1 ϵ levels in contrast to apoptosis-sensitive mice either before or following Jo₂ injection, implying CK1 ϵ in this adaptive response. To test whether other kinases also phosphorylate Bid, the Scansite of the Massachusetts Institute of Technology was used.⁵⁶ The current release of Scansite (version 2.0) includes 63 motifs characterizing the binding and/or substrate specificities of many families of Ser/Thr or Tyr kinases, SH2, SH3, PDZ, 14-3-3, and PTB domains, together with signature motifs for PtdIns(3,4,5)P(3)-specific PH domains. Out of these 63 kinases, only Atm (ataxia telangiectasia-mutated) was identified to phosphorylate Bid in silico. Loss of Atm function causes ataxia telangiectasia, a rare autosomal recessive disorder characterized by clinical manifestations that include progressive cerebellar ataxia, neuronal degeneration, hypersensitivity to ionizing radiation, and an increased risk for cancer.⁵⁷ Atm encodes a 370-kilodalton protein that is a member of a family of proteins related to phosphatidylinositol 3-kinase, which transduce signals through the phosphorylation of proteins on serine or threonine residues.⁵⁸ Interestingly, the ATM-p53-p21 DNA damage pathway is activated in both *Fah*^{-/-} mice off NTBC (Willenbring et al, manuscript submitted) and bile duct-ligated mice (Figure 5A). Furthermore, recombinant Atm phosphorylates Bid in vitro (Figure 5B). To test whether Atm significantly contributes to the observed resistance against apoptosis, *Atm*^{-/-} mice underwent BDL. Two weeks following this procedure, *Atm*^{-/-} and control mice were injected with a lethal dose of Fas mAb. However, bile duct-ligated *Atm*^{-/-} mice showed the same resistance against apoptosis as the control bile duct-ligated mice and Bid was not dephosphorylated following Fas mAb injection (Figure 5C–E). Subsequently, cleavage of Bid and release of cytochrome *c* occurred only in the non—bile duct-ligated controls (Figure 5C). Cytosolic CK1 ϵ levels were slightly higher in the bile duct-ligated *Atm*^{-/-} mice than in the bile duct-ligated control mice (Figure 5F). PP2A levels were, similar to the previous experiments, unchanged (Figure 5F).

Discussion

We have recently shown that mice modeling the genetic disease HT-1 develop resistance against cell death during chronic liver injury,² an adaptation that does not occur in mice that are doubly mutant in the gene responsible for HT-1, *Fah*, and the cell cycle regulatory protein *p21/Cip/Waf* (Willenbring et al, manuscript submitted). In the current study, we therefore asked whether apoptosis resistance could also be a feature of other, more common liver disorders. Jaundice and obstruction of bile flow are found in many hepatic diseases; therefore, an animal model of chronic cholestasis was used to address this question. Here, we show that bile duct-ligated mice exhibit profound resistance against apoptosis. Our observations are not contradictory to previous reports showing that bile acids acutely induce hepatocyte cell death. In HT-1, several studies have similarly shown that homogentisic acid (HGA) acutely induces apoptosis in hepatocytes. However, in both *Fah* deficiency and chronic cholestasis, already injured hepatocytes develop resistance to additional cell death triggers over time. We found in both models that the apoptotic block was primarily related to an inhibition of the mitochondrial amplification loop of Fas-induced apoptosis. We therefore wondered which specific factors contribute to the observed resistance against Fas-induced apoptosis.

Analysis of proapoptotic and antiapoptotic proteins identified several changes to Bcl-2-like proteins. These alterations included not only quantitative changes but also posttranslational modifications such as phosphorylation and subcellular localization. First, the antiapoptotic protein Bfl-1/A1 was transcriptionally unregulated in bile duct-ligated mice compared with controls. However, we did not observe a significant difference between apoptosis-sensitive

Fah^{-/-}/*p21*^{-/-} and apoptosis-resistant *Fah*^{-/-} mice. Additionally, previous studies have shown that Bfl-1/A1 did not prevent processing of Bid or tBid translocation to the mitochondria but prevented subsequent collaboration between tBid and Bax/Bak in the mitochondrial membrane.³⁴ It is therefore unlikely that the mild elevation of Bfl-1/A1 can completely explain the lack of Bid cleavage seen in all 3 apoptosis-resistant models examined here. Furthermore, we did not observe any additional consistent changes of proapoptotic and antiapoptotic Bcl-2-like proteins in bile duct-ligated, *Fah*^{-/-}, and *Fah*^{-/-}/*p21*^{-/-} mice, indicating that the resistance against Fas-induced apoptosis is not mediated by transcriptional regulation of these proteins. Another change was the increase of Bax and Bid in the mitochondrial fraction of bile duct-ligated mice even before injection of Fas. Translocation of Bax to the mitochondria is generally associated with cytochrome *c* release and/or mitochondrial dysfunction and apoptosis. For its proapoptotic functions, Bax needs to be activated and inserted into the mitochondrial membrane similar to Bid to induce release of cytochrome *c*. In bile duct-ligated mice, however, both proteins were only loosely attached to the membrane. To clarify the potential role of the high levels of mitochondrially located Bax, in vitro cytochrome *c* release assays were performed. Mitochondrial cytochrome *c* release was completely normal in bile duct-ligated mice, and therefore these changes are also unlikely to be the main cause of enhanced hepatocyte survival.

Instead, failure of Bid cleavage was observed consistently in all 3 models of hepatic cell death resistance: BDL, *Fah* deficiency, and suramin. Previously, it has been shown that phosphorylation of Bid regulates its cleavage by caspase-8.^{46,47} In type II cells, the mitochondrial amplification loop necessary for Fas-induced apoptosis is triggered through cleavage of Bid at Asp 59 by caspase-8. In the case of murine Bid, CK1ε phosphorylates Bid in vitro on Ser 61 located exactly next to the cleavage site of caspase-8. Phosphorylation of Bid by CK1 renders Bid resistant to cleavage by caspase-8. Moreover, inhibition of CK1 accelerated Fas-induced apoptosis, whereas hyperactivity of the kinase inhibited apoptosis.⁴⁷ More recent data additionally indicate that phosphorylation of Bid does not only prevent cleavage by caspase-8 but also the translocation of Bid to the mitochondria.⁶¹ The mechanisms underlying the trafficking of Bid to the mitochondria are poorly defined at this time; the current model suggests that cleavage and myristoylation of Bid induces a molecular switch targeting tBid to the mitochondria.^{62,63} However, a recent study suggests that the sorting protein PACS-2 is required for the apoptosis-induced targeting of Bid. The PACS-2-dependent translocation of Bid to the mitochondria appears to occur before caspase cleavage and requires the dephosphorylation of Bid.⁶¹ Phosphorylated Bid (S61) was readily detectable in wild-type, bile duct-ligated, *Fah*^{-/-} and *Fah*^{-/-}/*p21*^{-/-} mice. The constitutive phosphorylation of Bid in hepatocytes implies that it must be dephosphorylated during Fas-induced apoptosis to transduce and amplify the death signal. Here, however, Bid was not dephosphorylated following Fas mAb injection and CK1 activity remained higher in apoptosis-resistant bile duct-ligated mice. This phosphorylation/dephosphorylation pattern of Bid was not only specific for BDL but was also seen in the adaptive response in the cancer-prone metabolic liver disease HT-1 and after suramin injection. Furthermore, Bid was only dephosphorylated in the apoptosis-sensitive *Fah*^{-/-}/*p21*^{-/-} double knockout mice following induction of apoptosis but not in the resistant *Fah*^{-/-} mice. Finally, the effect of suramin was analyzed, which has been shown to inhibit Fas-induced hepatocellular apoptosis.¹⁴ Interestingly, suramin does not alter the composition of the DISC in the liver following challenge with APO-1 antibody but prevents release of cytochrome *c*.¹⁴ Additionally, the antiapoptotic effect of suramin is specific for type II cells, suggesting a role for the mitochondrial pathway.¹⁴ Here, we show that Bid is not dephosphorylated and subsequently not cleaved in suramin-treated mice following Fas mAb injection.

Interestingly, lack of dephosphorylation of Bid following Fas mAb injection correlates with increased cytosolic levels of CK1ε in all models analyzed in this study. However, at this point,

we cannot exclude that additional kinases and/or phosphatases also contribute to the increased Bid phosphorylation level in vivo. We found that Atm phosphorylates Bid in vitro but is not necessary to sustain phosphorylation levels of Bid in bile duct–ligated mice following induction of apoptosis with Fas mAb. This finding implicates CK1 ϵ as the candidate kinase. Accumulating data suggest that CK1 is involved in negative regulation of apoptosis. Phosphorylation of different proteins by CK1 prevents their cleavage by caspases.^{64,65} Additionally, CK1 acts as a molecular switch for certain proteins such as galectin-3.⁶² Phosphorylation of galectin-3 regulates its cellular translocation from the nucleus to the cytoplasm and, as a result, its antiapoptotic function. Finally, CK1 phosphorylates several membrane receptors and it has been shown that the p75 tumor necrosis factor receptor is phosphorylated and associated with CK1, which negatively regulates p75-mediated tumor necrosis factor signaling to apoptosis.⁶⁶ The data shown here establish a strong correlation between the status of Bid phosphorylation and hepatic apoptosis resistance and suggest a coherent model explaining this phenomenon in distinct models. However, future experiments using phosphorylation-resistant Bid mutants and mutant mimicking constitutive phosphorylation will be needed to determine conclusively whether this is the central mechanism.

In summary, our data show that resistance against apoptosis is a common feature of chronic liver diseases such as HT-1 and chronic cholestasis. In terms of evolution, this can be viewed as an adaptive response, allowing survival during chronic liver injury. However, survival might come at a price, because hepatocytes can retain DNA damage and become malignantly transformed. Our data show that the Fas apoptosis signaling pathway is blocked at the level of Bid cleavage. We did not observe consistent transcriptional changes to pro-apoptotic and antiapoptotic genes, including BH3-like genes and inhibitors of apoptosis, and therefore propose a model in which phosphorylation/dephosphorylation of Bid determines the apoptotic threshold of hepatocytes during chronic liver diseases. Importantly, *Bid*^{-/-} mice are not only resistant against Fas mAb–induced hepatocellular apoptosis but also spontaneously develop a myeloproliferative disorder, proving that loss of function of a single BH3-only molecule can predispose to cancer.⁶⁷ Therefore, understanding the changes in apoptosis sensitivity during chronic diseases might help to reduce the long-term risk of cancer development.

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Acknowledgements

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Figure 1.

Bile duct–ligated mice develop a resistance against Fas-induced apoptosis. (A) Wild-type mice underwent BDL and were challenged with the Fas mAb Jo₂ (0.35 µg/g) 14 days later. Survival of control (*squares*) and bile duct–ligated (*triangles*) mice are shown. All control mice (n = 10) died from acute liver failure within 12 hours following injection of Jo₂, whereas all bile duct–ligated mice survived (n = 10). (B) DNA laddering following challenge with Jo₂ was only seen in control mice. Results are shown for duplicate animals under each condition. A standard DNA ladder marker (M) is shown. (C) TUNEL staining (*brown color*) of liver sections is shown (original magnification 200×) in control and bile duct–ligated mice before and after Jo₂ injection. TUNEL-positive cells were only visible in control mice following Jo₂ injection.

Figure 2.

The mitochondrial pathway is blocked in bile duct–ligated mice. (A) Liver injury after Jo₂ challenge in control (WT; *open columns*) (n = 4) and bile duct–ligated mice (*filled columns*) (n = 4). Alanine aminotransferase and bilirubin levels were measured in plasma before (F.0) and after (F.4) challenge with Jo₂. Alanine aminotransferase levels increased only in control mice following Jo₂ injection. Bilirubin levels were significantly elevated in bile duct–ligated mice. Caspase-8 and caspase-3 activities were determined in liver homogenates 2 hours (F.2; caspase 8) or 4 hours (F.4; caspase 3) following Jo₂ injection (n = 4 each). In both groups, activation of caspase-8 was observed, whereas caspase-3 activity was only detectable in control mice. (B) Western blots of Bid, Bax, cytochrome c, and caspase-9 before (basal) and 4 hours after (Fas) induction of apoptosis with Jo₂ in control and bile duct–ligated mice (n = 4 each; 2 of 4 samples shown here) to determine activation of the mitochondrial pathway of apoptosis. Cleavage of Bid (decrease of full-length Bid), translocation of Bax, release of cytochrome c, and cleavage of caspase-9 following Jo₂ occurred only in control mice. F.0, before Fas mAb injection, F.2/F.4, 2/4 hours following Fas mAb injection.

Figure 3.

Analysis of the mitochondrial pathway in wild-type and bile duct-ligated mice. (A) Western blot analysis of liver tissues in control and bile duct-ligated mice in the basal state without Fas mAb injection. Shown are data from 2 of 4 mice in each group. Liver tissues were analyzed for total cellular levels of Fas-R, Flip_s/I, clap-2, Bax, Bid, Bak, Bcl-2, Bcl-x, and A1/Bfl-1. Of all the proteins analyzed, only A1/Bfl-1 was significantly induced 2 weeks following BDL. (B) Liver extracts of control and bile duct-ligated mice were incubated with in vitro activated recombinant caspase-8. Western blots for in vitro cleavage of caspase-3 and caspase-9 revealed no difference in both groups. (C) The mitochondrial fraction of control and bile duct-ligated mice was analyzed for BH-3-like proteins. Levels of Bak, Bcl-2, and Bcl-x were not significantly changed before and after challenge with Jo₂, whereas Bid was significantly increased in this fraction 2 weeks following BDL even before Jo₂ injection. (D) Alkaline extraction of mitochondria from bile duct-ligated mice and controls. Bid and Bax were only loosely attached and not inserted into the mitochondrial membrane of bile duct-ligated mice in contrast to Bcl-x. (E) Isolated liver mitochondria (n = 4 each; 2 of 4 samples shown here) were incubated with different concentrations of in vitro cleaved tBid, and translocation of cytochrome c from the mitochondrial (mito) fraction to the cytosol (released) was monitored by immunoblotting with a cytochrome c antibody. Cytochrome c release following incubation with 5 nmol/L tBid is shown. First row (–), spontaneous cytochrome c release without tBid incubation; second to ninth row, the overall mitochondrial cytochrome c content of the respective sample (n = 2 per group) is shown followed by the released cytosolic cytochrome c fraction following incubation with tBid. There was no difference in cytochrome c release between bile duct-ligated mice and controls.

Figure 4.

Bid is not dephosphorylated following Fas injection in bile duct-ligated and in *Fah*^{-/-}/*p21*^{-/-} mice. (A) Phosphorylation of Bid at S61 was measured in cytosolic liver extracts from control and bile duct-ligated mice by Western blot. Increased phosphorylation of Bid in bile duct-ligated mice (*n* = 4 each; 2 of 4 samples shown here) before challenge with Jo₂ and, more importantly, no dephosphorylation following Jo₂ injection were seen. In contrast, Bid was almost completely dephosphorylated following induction of apoptosis in control mice. (B) Western blot analysis of CK1ε and PP2A levels in cell extracts from control and bile duct-ligated mice. The cytosolic CK1ε levels were higher in bile duct-ligated mice. Levels of PP2A were not changed in cytosolic extracts (data not shown). (C) CK1 activity was measured in

cell extracts of control and bile duct–ligated mice with a CK1-specific peptide ($n = 4$). Note that activity assay is not specific for CK1 ϵ but measures overall CK1 activity. CK1 activity was higher in bile duct–ligated mice at all time points. Interestingly, CK1 activity was reduced in control mice following challenge with Jo₂, but this reduction was not seen in bile duct–ligated mice. (D) Cellular extracts of control and bile duct–ligated mice following Jo₂ injection were analyzed for in vitro phosphorylation activity of His6-Bid (representative blot, $n = 4$ each); negative control (–) contains no cell extract, and positive control (CK1) contains recombinant CK1. Shown is a Western blot of the membranes probed with the phosphor-specific Bid (S61) antibody. Phosphorylation of Bid was only detectable in bile duct–ligated mice and was almost absent in controls. CK1 inhibitor (CKI-7) significantly inhibited phosphorylation of His6-Bid in bile duct–ligated mice. Total His6-Bid levels of non–CKI-7–treated reactions are shown. (E) *Fah*^{–/–} and *Fah*^{–/–}/*p21*^{–/–} mice were challenged with Jo₂ 2 weeks following NTBC withdrawal. Dephosphorylation and subsequent cleavage of Bid and cytochrome *c* release occurred only in the double-knockout mice. Total CK1 ϵ levels were unchanged, whereas cytosolic levels were lower in *Fah*^{–/–}/*p21*^{–/–} mice following Jo₂ injection. (F) Wild-type mice were injected with Jo₂ alone or concomitantly with suramin. Suramin prevents dephosphorylation of Bid and subsequent Bid cleavage and cytochrome *c* release. Cytosolic CK1 ϵ levels are higher in suramin-treated mice. (G) In suramin-treated mice, only a few scattered TUNEL-positive hepatocytes were visible following Jo₂ injection.

Figure 5.

Atm phosphorylates Bid in vitro but is not necessary to prevent Fas-induced apoptosis during chronic liver diseases. (A) The ATM-p53 DNA damage pathway is activated in bile duct-ligated mice, measured by increased phosphorylation of p53 and increased overall levels of p53 and effector proteins p21 and Bax. (B) Recombinant Atm phosphorylated Bid-GST in vitro. (C) Bile duct-ligated Atm^{-/-} mice are protected against Fas mAb-induced apoptosis similar to wild-type mice. Dephosphorylation of Bid and subsequent cleavage of Bid, release of cytochrome c, and cleavage of caspase-9 and caspase-3 occurs only in wild-type mice. (D and E) TUNEL staining (brown color) of liver sections is shown (original magnification 200×). TUNEL-positive cells are only visible (D) in control mice 6 hours after Jo₂ injection but not

(*E*) in $Atm^{-/-}$ mice. (*F*) Cytosolic CK1 ϵ levels are again higher in bile duct–ligated mice, whereas PP2A levels remain unchanged.

Table 1
Comparison of Gene Expression in Apoptosis Resistant Versus Sensitive Livers

	BDL/wild-type	<i>Fah</i> ^{-/-} off/ <i>Fah</i> ^{-/-} on	<i>Fah/p21</i> ^{-/-} off/ <i>Fah/p21</i> ^{-/-} on	<i>Fah</i> ^{-/-} off/ <i>Fah/p21</i> ^{-/-} off
<i>Fas receptor</i> ^a	1.09	0.89	1.46	0.61
<i>Fadd</i>	1.27	0.69	0.87	0.79
<i>Trif-R</i>	1.30	1.13	0.88	1.28
<i>Caspase-9</i>	1.05	0.77	0.79	0.97
<i>Caspase-8</i>	1.10	1.68	1.60	1.05
<i>Caspase-7</i>	0.90	0.69	0.64	1.08
<i>Caspase-6</i>	0.87	0.64	0.69	0.93
<i>Caspase-3</i>	1.22	1.14	0.75	1.52
<i>Smac/Diablo</i>	1.02	1.04	0.89	1.17
<i>Cytochrome c</i>	0.66	1.53	1.90	0.81
<i>Apaf-1</i>	1.67	1.45	1.74	0.83
<i>Bak</i>	1.59	0.85	0.70	1.21
<i>Bax</i>	1.37	2.53	2.35	1.08
<i>Bcl</i>	1.13	0.90	0.56	1.61
<i>Bad</i>	1.01	0.57	0.74	0.77
<i>Bid</i>	1.77	3.06	1.82	1.68
<i>Bim</i>	1.16	2.12	2.56	0.83
<i>Bag-1</i>	1.16	1.39	1.02	1.36
<i>Bcl-2</i>	1.49	0.72	2.39	0.0
<i>Bcl-xl</i> ^a	1.01	1.67	1.38	1.21
<i>Bcl-w</i>	2.04	0.90	1.14	0.79
<i>Bfl1/A1</i> ^a	3.16	1.91	1.77	1.08
<i>Mcl-1</i>	1.43	0.82	0.75	1.09
<i>Bis</i>	1.03	1.79	1.26	1.42
<i>clap-1</i> ^a	1.64	0.99	1.22	0.81
<i>clap-2</i> ^a	1.31	0.57	0.82	0.70
<i>clap</i> ^a	1.03	1.00	1.30	0.72
<i>Flip</i>	0.68	0.69	0.96	0.72
<i>Survivin</i>	ND	ND	37.41	—
<i>Nox-2</i> ^a	ND	ND	ND	—
<i>Afp</i> ^a	1.66	50.09	52.48	0.95
<i>A20</i> ^a	ND	ND	ND	—
<i>Cox-2</i> ^a	1.16	0.85	1.37	0.62
<i>Sod-2</i> ^a	0.93	0.75	0.71	1.05
<i>IP₃R-1</i> ^a	1.13	0.79	0.65	1.21

NOTE. Values are expressed as fold change. Transcriptional regulation of several pro- and anti-apoptotic genes were analyzed for (column 1) apoptosis-resistant BDL mice in comparison to apoptosis-sensitive WT mice, (column 2) apoptosis-sensitive *Fah*^{-/-} on NTBC in comparison to apoptosis-resistant *Fah*^{-/-} off NTBC, (column 3) apoptosis-sensitive *Fah*^{-/-}/*p21*^{-/-} on NTBC in comparison to apoptosis-sensitive *Fah*^{-/-}/*p21*^{-/-} off NTBC withdrawal and (column 4) apoptosis-resistant *Fah*^{-/-} off NTBC in comparison to apoptosis-sensitive *Fah*^{-/-}/*p21*^{-/-} off NTBC.

ND, not detectable.

^a NFκB target genes.