The Bcl-2 Homology Domain 3 (BH3)-only Proteins Bim and Bid Are Functionally Active and Restrained by Anti-apoptotic Bcl-2 Family Proteins in Healthy Liver*

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Background: A fine balance between the anti- and pro-apoptotic multidomain Bcl-2 family proteins controls hepatocyte apoptosis in the healthy liver. Results: Disruption of the BH3-only proteins Bim and Bid prevents spontaneous hepatocyte apoptosis in the absence of anti-apoptotic Bcl-2 family proteins. Conclusion: Hepatocyte integrity is maintained by the well orchestrated Bcl-2 network. Significance: We demonstrated the novel involvement of BH3-only proteins in the healthy Bcl-2 network of the liver.

An intrinsic pathway of apoptosis is regulated by the B-cell lymphoma-2 (Bcl-2) family proteins. We previously reported that a fine rheostatic balance between the anti- and pro-apoptotic multidomain Bcl-2 family proteins controls hepatocyte apoptosis in the healthy liver. The Bcl-2 homology domain 3 (BH3)-only proteins set this rheostatic balance toward apoptosis upon activation in the diseased liver. However, their involvement in healthy Bcl-2 rheostasis remains unknown. In the present study, we focused on two BH3-only proteins, Bim and Bid, and we clarified the Bcl-2 network that governs hepatocyte life and death in the healthy liver. We generated hepatocyte-specific Bcl-xL- or Mcl-1-knock-out mice, with or without disrupting Bim and/or Bid, and we examined hepatocyte apoptosis under physiological conditions. We also examined the effect of both Bid and Bim disruption on the hepatocyte apoptosis caused by the inhibition of Bcl-xL and Mcl-1. Spontaneous hepatocyte apoptosis in Bcl-xL- or Mcl-1-knock-out mice was significantly ameliorated by Bim deletion. The disruption of both Bim and Bid completely prevented hepatocyte apoptosis in Bcl-xL-knock-out mice and weakened massive hepatocyte apoptosis via the additional in vivo knockdown of mcl-1 in these mice. Finally, the hepatocyte apoptosis caused by ABT-737, which is a Bcl-xL/Bcl-2/Bcl-w inhibitor, was completely prevented in Bim/Bid double knock-out mice. The BH3-only proteins Bim and Bid are functionally active but are restrained by the anti-apoptotic Bcl-2 family proteins under physiological conditions. Hepatocyte integrity is maintained by the dynamic and well orchestrated Bcl-2 network in the healthy liver.

These members are divided into two groups as follows: core Bcl-2 family proteins, which possess three or four Bcl-2 homology domains (BH1–BH4) and the Bcl-2 homology domain 3 (BH3)-only proteins (1). The former, which are multidomain proteins, are subdivided into pro- and anti-apoptotic proteins. Pro-apoptotic core Bcl-2 family members, such as Bax and Bak, serve as effector molecules of this apoptotic machinery. Upon activation, these members can form pores to permeabilize the mitochondrial outer membrane. Apoptogenetic factors, such as cytochrome c, can then be released through this membrane into the cytosol, leading to the activation of the caspase cascade and to cellular demise (2). Anti-apoptotic core Bcl-2 family members, including Bcl-2, Bcl-xL, Mcl-1, Bcl-w, and Bfl-1/A1, inhibit the intrinsic pathway of apoptosis by either directly or indirectly antagonizing Bak/Bax activity (3–5). In the original rheostasis model, cellular life and death are regulated by a balance between these anti- and pro-apoptotic core Bcl-2 family proteins (6). We previously reported that the hepatocyte-specific deletion of the bcl-x gene resulted in spontaneous hepatocyte apoptosis, and this effect could be completely prevented by the additional deletion of the bak and bax genes (7). These findings elucidated the importance of the rheostatic balance of the core Bcl-2 family proteins in controlling hepatocyte apoptosis in the healthy liver.

The BH3-only proteins, which include at least eight members, are considered to function as pro-apoptotic sensors, and these proteins set this rheostatic balance toward apoptosis upon activation by a variety of apoptotic stimuli (8, 9). It has been reported that hepatocyte apoptosis through the activation of these BH3-only proteins is involved in the pathophysiology of various liver diseases (10–12). Alternatively, we previously reported that the slight activation of Bid, which can trigger hepatocyte apoptosis, occurs even in the healthy liver and that the inactivation of Bid partially ameliorated spontaneous hepatocyte...
cyte apoptosis in Bcl-xL- or Mcl-1-knock-out mice (7, 13). In the present study, we focused on another BH3-only protein, Bim, which promotes hepatocyte apoptosis upon activation by free fatty acids or by reactive oxygen species in pathological settings, and we further clarified the orchestration of the Bcl-2 network, which governs hepatocyte life and death in the physiological state (10, 11, 14, 15). We found that the disruption of Bim ameliorated hepatocyte apoptosis in Bcl-xL- or Mcl-1-knock-out mice, indicating the involvement of Bim in this hepatocyte apoptosis machinery in the healthy liver as well as that of Bid. Additionally, the deletion of both Bim and Bid prevented the massive hepatocyte apoptosis caused by the inhibition of both Bcl-xL and Mcl-1, suggesting that Bim and Bid are functionally active in the healthy liver and are essential regulators for promoting the intrinsic pathway of apoptosis in hepatocytes in the absence of anti-apoptotic Bcl-2 family proteins. Our present study unveiled the fine and dynamic Bcl-2 networks, the orchestration of which determines hepatocyte life and death in the healthy liver.

**Experimental Procedures**

**Mice**—Mice carrying a bcl-x gene with two loxP sequences at the promoter region and a second intron (bcl-x<sup>fl/fl/loc</sup>), mice carrying an mcl-1 gene encoding amino acids 1–179 flanked by two loxP sequences, and heterozygous alb-cre transgenic mice expressing the Cre recombinase gene under regulation of the albumin gene promoter have been described previously (16–18). Hepatocyte-specific Bcl-xL-knock-out mice (bcl<-<sup>x<sup>fl/fl/loc</sup>alb-cre<sup></sup>) (17), hepatocyte-specific Mcl-1-knock-out mice (bcl<-<sup>x<sup>fl/fl/loc</sup>alb-cre<sup></sup>) (13), systemic Bid-knock-out mice (bid<sup><sup>−</sup><sup>−</sup></sup>) (12), and Bcl-xL/Bid double knock-out mice (bid<sup><sup>−</sup><sup>−</sup>+<sup>−</sup></sup>bcl<-<sup>x<sup>fl/fl/loc</sup>alb-cre<sup></sup>) (7) have also been described previously. We purchased C57BL/6J mice from Charles River (Osaka, Japan), systemic Bim-knock-out mice (bim<sup><sup>−</sup><sup>−</sup></sup>) from the Jackson Laboratory (Bar Harbor, ME), and NOD/ShiJic-prkdc<sup>−</sup><sup>−</sup>/H11002 Jcl mice from Clea Japan Inc. (Osaka, Japan). We generated Bcl-xL/Bim double knock-out mice (bim<sup><sup>−</sup><sup>−</sup>+<sup>−</sup>bcl<-<sup>x<sup>fl/fl/loc</sup>alb-cre<sup></sup>) Mcl-1/Bim double knock-out mice (bim<sup><sup>−</sup><sup>−</sup>+<sup>−</sup>mcl<-<sup>-<sup>-<sup>-/loc</sup>alb-cre<sup></sup>) Bcl-xL/Bim/Bid triple knock-out mice (bim<sup><sup>−</sup><sup>−</sup>+<sup>−</sup>bcl<-<sup>x<sup>fl/fl/loc</sup>alb-cre<sup></sup>) and Bim/Bid double knock-out mice (bim<sup><sup>−</sup><sup>−</sup>+<sup>−</sup>bcl<-<sup>x<sup>fl/fl/loc</sup>alb-cre<sup></sup>) by mating the strains. We generated mice with a hepatocyte-specific deletion of Mcl-1 and homozygote severe combined immune deficiency (SCID) mutations (mcl<-<sup>-<sup>-/loc</sup>prkdc<sup>−<sup>−</sup>/loc</sup>alb-cre<sup></sup>) by mating hepatocyte-specific Mcl-1-knock-out mice (bcl<-<sup>x<sup>fl/fl/loc</sup>alb-cre<sup></sup>) and NOD/ShiJic-scid Jcl mice. Genotyping of prkdc<sup>−<sup>−</sup>/loc</sup> gene mutation was performed by the PCR-confronting two-primer primer (PCR-CTPP) method reported previously (19). The mice were maintained in a specific pathogen-free facility and were afforded humane care under approval from the Animal Care and Use Committee of Osaka University Medical School.

**Histological Analyses**—Liver sections were stained with hematoxylin and eosin (H&E). To detect apoptotic cells, the liver sections were subjected to a procedure reported previously (20). For immunohistochemical detection of cleaved caspase-3, the liver sections were incubated with the polyclonal rabbit anti-cleaved caspase-3 antibody (Cell Signaling Technology, Beverly, MA) according to a procedure reported previously (20).

**Caspase-3/7 Activity**—Serum caspase-3/7 activity was measured by a luminescent substrate assay for caspase-3 and caspase-7 (Caspase-Glo assay, Promega) according to the manufacturer’s protocol.

**Western Blot Analysis**—Liver tissue was lysed in lysis buffer (1% Nonident P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1× protein inhibitor mixture (Nacalai tesque, Kyoto, Japan), 1× phosphatase inhibitor mixture (Nacalai tesque), and phosphate-buffered saline, pH 7.4). The liver lysates were cleared by centrifugation at 10,000 × g for 15 min at 4 °C. The protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce). The protein lysates were electrothermally separated with SDS-polyacrylamide gels and were transferred onto a polyvinylidene fluoride membrane. For immunodetection, the following antibodies were used: a rabbit polyclonal antibody to Bcl-xL (Santa Cruz Biotechnology, Inc.), a rabbit polyclonal antibody to Bid, a rabbit polyclonal antibody to Bax, a rabbit polyclonal antibody to cleaved caspase-3, a rabbit polyclonal antibody to cleaved caspase-7, a rabbit polyclonal antibody to Puma (Cell Signaling Technology, Beverly, MA), a rabbit monoclonal antibody to Bad, a rabbit polyclonal antibody to Noxa (Abcam, Cambridge, MA), a rabbit polyclonal antibody to Bak (Millipore, Billerica, MA), a rabbit polyclonal antibody to Bim (Enzo Life Sciences Inc., Farmingdale, NY), a rabbit polyclonal antibody to Mcl-1 (Rockland, Gilbertsville, PA), and a mouse monoclonal antibody to β-actin (Sigma-Aldrich).

**Real-time Reverse Transcription Polymerase Chain Reaction (Real-time RT-PCR) for mRNA**—Total RNA was extracted from liver tissues using an RNasey minikit (Qiagen, Valencia, CA), was reverse-transcribed, and was subjected to real-time RT-PCR as described previously (21). The mRNA expression of specific genes was quantified using TaqMan gene expression assays (Applied Biosystems, Foster City, CA) as follows: murine bcl2 (assay ID: Mm012043796_m1), murine fas (assay ID: Mm01204974_m1), murine bik (assay ID: Mm01267123_m1), murine hrk (assay ID: Mm01208086_m1), murine bmf (assay ID: Mm00567773_m1), and murine actb (assay ID: Mm02619580_g1 or Mm00607939_s). The transcript levels are presented as -fold inductions.

**siRNA-mediated in Vivo Knockdown**—The hepatocyte-specific Bcl-xL-knock-out mice (bcl<-<sup>x<sup>fl/fl/loc</sup>alb-cre<sup></sup>) and the Bcl-xL/Bim/Bid triple knock-out mice (bim<sup><sup>−</sup><sup>−</sup>+<sup>−</sup>bcl<-<sup>x<sup>fl/fl/loc</sup>alb-cre<sup></sup>) were injected with 5 mg/kg in vivo grade siRNA against mcl-1 (MSS257671_e0N), which was mixed with Invivofectamine (Invitrogen), via the tail vein according to the manufacturer’s protocol. The mice were sacrificed and examined as indicated by the time courses. The Stealth RNAi negative control with low GC content (Invitrogen) was used as the control.

**In Vivo ABT-737 Experiment**—ABT-737 was dissolved in a mixture of 30% propylene glycol, 5% Tween 80, and 65% D5W (5% dextrose in water) with pH 4–5. ABT-737 (100 mg/kg) was intraperitoneally administered to the Bim/Bid double knock-
out mice (bim<sup>-/-</sup>bid<sup>+/+</sup>) or to the Bid-knock-out mice (bid<sup>++</sup>). The mice were sacrificed and examined 6 h later.

Statistical Analysis—All of the data are expressed as means ± S.D. unless otherwise indicated. Statistical analyses were performed using an unpaired Student’s t test or a one-way analysis of variance unless otherwise indicated. When the analyses of variance were applied, the differences in the mean values among the groups were examined by Scheffe’s post hoc correction unless otherwise indicated. p < 0.05 was considered statistically significant.

RESULTS

The Disruption of Bim Alleviated Spontaneous Hepatocyte Apoptosis in Hepatocyte-specific Bcl-xL-knock-out Mice—To investigate the involvement of the BH3-only protein Bim in the hepatocyte apoptosis caused by Bcl-xL deficiency, hepatocyte-specific Bcl-xL-knock-out mice (bcl-x<sup>fl/fl</sup>alb-cre) were mated with systemic Bim-knock-out mice (bim<sup>-/-</sup>). Offspring from the mating of bim<sup>++/+</sup>bcl-x<sup>fl/fl</sup>alb-cre mice and bim<sup>++/-</sup>bcl-x<sup>fl/fl</sup> mice were examined at 6 weeks of age. A Western blot study confirmed the disappearance of both Bcl-xL and Bim protein expression in the liver tissue of the double knock-out mice (bim<sup>++/-</sup>bcl-x<sup>fl/fl</sup>alb-cre) (Fig. 1A). In agreement with our previous report (7, 17), H&E staining of the liver sections showed an increase in the number of hepatocytes, with chromatin condensation and cytosolic shrinkage in the liver lobules of the Bcl-xL-knock-out mice (Fig. 1B). The staining also showed a significant increase in TUNEL-positive cells and cleaved caspase-3-positive cells in the liver (Fig. 1, B–D). Consistent with these histological observations, the levels of serum caspase-3/7 activity and serum alanine aminotransferase (ALT), which can be used as indicators of hepatocyte apoptosis (22, 23), were significantly higher in the Bcl-xL-knock-out mice than in their wild-type littermates (Fig. 1, E and F). Additionally, cleaved caspase-3 and -7 were detected in the livers of the Bcl-xL-knock-out mice by Western blotting (Fig. 1A). All of these findings indicated spontaneous hepatocyte apoptosis in these mice. Bim-knock-out mice did not show any phenotypes in the liver under physiological conditions (Fig. 1, B–F). Alternatively, the disruption of Bim significantly improved all of the parameters that are indicative of hepatocyte apoptosis in Bcl-xL-knock-out mice, including the TUNEL-positive cell counts, cleaved caspase-3-positive cell counts, ALT levels, and serum caspase-3/7 activity (Fig. 1, B–F). These findings clearly demonstrated that Bim was involved in the hepatocyte apoptosis caused by Bcl-xL disruption. It should be noted that the gene and protein expression levels of Bim were not different between the Bcl-xL-knock-out mice and their wild-type littermates (Fig. 1, A and G), indicating that the Bim expression levels observed in the healthy liver could induce hepatocyte apoptosis in the absence of the Bcl-2 family proteins.

The Disruption of Bim Alleviated Spontaneous Hepatocyte Apoptosis in Hepatocyte-specific Mcl-1-knock-out Mice—Of the five members of the anti-apoptotic Bcl-2 family proteins, we previously reported that Mcl-1 and Bcl-xL played a pivotal anti-apoptotic role in maintaining hepatocyte integrity in the healthy liver (13). We thus examined the role of Bim in the hepatocyte apoptosis caused by Mcl-1 deficiency. We generated Mcl-1/Bim double knock-out mice (bim<sup>++/-</sup>mcl-1<sup>fl/fl</sup>alb-cre) by mating the hepatocyte-specific Mcl-1-knock-out mice (mcl-1<sup>fl/fl</sup>alb-cre) with the systemic Bim-knock-out mice (bim<sup>++/-</sup>). A Western blot study confirmed the disappearance of both Mcl-1 and Bim protein expression in the liver tissue of the double knock-out mice (bim<sup>++/-</sup>mcl-1<sup>fl/fl</sup>alb-cre) (Fig. 2A). Consistent with our previous report (13), hepatocyte-specific Mcl-1-knock-out mice showed apoptosis phenotypes very similar to those of the Bcl-xL-knock-out mice, as assessed by TUNEL staining (Fig. 2, B and C), cleaved caspase-3 staining (Fig. 2, B and D), serum caspase-3/7 activity (Fig. 2E), and serum ALT levels (Fig. 2F). In contrast, Mcl-1/Bim double knock-out mice showed significant improvement in these parameters (Fig. 2, B–F), indicating that Bim is also involved in the hepatocyte apoptosis induced by the disruption of Mcl-1.

The Disruption of Bim and Bid Prevented Spontaneous Hepatocyte Apoptosis in Hepatocyte-specific Bcl-xL-knock-out Mice—We previously reported that a small amount of Bid, which is another BH3-only protein, was constitutively active and was involved in the spontaneous hepatocyte apoptosis in Bcl-xL- or Mcl-1-knock-out mice (7, 13). We thus examined whether these BH3-only proteins redundantly or cooperatively promoted hepatocyte apoptosis in the absence of Bcl-xL. To this end, Bim/Bid/Bcl-xL triple knock-out mice (bim<sup>++/-</sup>bcl-x<sup>fl/fl</sup>alb-cre) were generated by mating the Bim/Bcl-xL double knock-out mice (bim<sup>++/-</sup>bcl-x<sup>fl/fl</sup>alb-cre) with the Bid/Bcl-xL double knock-out mice (bid<sup>++/-</sup>bcl-x<sup>fl/fl</sup>alb-cre). The offspring from the mating of bim<sup>++/-</sup>bcl-x<sup>fl/fl</sup>alb-cre mice with bid<sup>++/-</sup>bcl-x<sup>fl/fl</sup>alb-cre mice were examined at 6 weeks of age. A Western blot study confirmed that Bcl-xL, Bid, and Bim protein expression disappeared from the liver tissue of the triple knock-out mice (bim<sup>++/-</sup>bcl-x<sup>fl/fl</sup>alb-cre) (Fig. 3A). Liver sections of the Bim/Bid/Bcl-xL triple knock-out mice were histologically normal compared with those of the Bid/Bcl-xL double knock-out mice (bim<sup>++/-</sup>bcl-x<sup>fl/fl</sup>alb-cre), which still contained some hepatocytes with apoptotic morphologies (Fig. 3B). Both the number of TUNEL-positive cells and the serum caspase-3/7 activity in the triple knock-out mice were significantly lower than those in the Bid/Bcl-xL double knock-out mice and did not differ from their control Bid-knock-out or Bid double knock-out littermates (Fig. 3, B–D). Moreover, in contrast to the mild elevation of serum ALT levels in the Bid/Bcl-xL double knock-out mice, the levels in the triple knock-out mice were completely normal (Fig. 3E). These findings demonstrated that hepatocyte apoptosis in the absence of Bcl-xL was completely dependent on these two BH3-only proteins.

Bim and Bid Are Essential Regulators for the Promotion of the Intrinsic Pathway of Apoptosis in Hepatocytes in the Absence of Anti-apoptotic Bcl-2 Family Proteins—We then attempted to further examine the involvement of Bim and Bid in hepatocyte apoptosis in the absence of both Bcl-xL and Mcl-1, which are two major anti-apoptotic proteins in the liver. Because, as we reported (13), the hepatocyte-specific Bcl-xL and Mcl-1 double knock-out mice died within 1 day after birth due to impaired liver development, we performed an siRNA-mediated in vivo knockdown of mcl-1 in the Bcl-xL-knock-out mice and in the Bim/Bid/Bcl-xL triple knock-out mice. mcl-1 siRNA administration efficiently reduced Mcl-1 protein expression in the liver.
The disruption of Bim alleviated spontaneous hepatocyte apoptosis in the absence of Bcl-xL. A–F, the offspring from the mating of bim<sup>±</sup>bcl-x<sup>L</sup><sub>-Tie2cre</sub> alb-cre mice with bim<sup>±</sup>bcl-x<sup>L</sup><sub>-Tie2cre</sub> mice were examined at 6 weeks of age. Bcl-xL<sup>+/+</sup> and Bcl-xL<sup>-/-</sup>, bcl-x<sup>L</sup><sub>-Tie2cre</sub> and bcl-x<sup>L</sup><sub>-Tie2cre</sub>alb-cre, respectively. A, Western blot analysis of whole liver lysates for the expression of Bim, Bad, Bcl-xL, Mcl-1, Bak, Bax, Noxa, Puma, cleaved caspase-3, cleaved caspase-7, and β-actin. B, representative images for liver histology stained with hematoxylin-eosin (HE), TUNEL, and cleaved caspase-3 (original magnifications, ×100 (large panels) and ×400 (insets)); black arrows indicate apoptotic bodies. C, TUNEL-positive cell ratio; n = 8 mice/group; *, p < 0.05 versus all. D, cleaved caspase-3-positive cell ratio; n = 3 mice/group; *, p < 0.05 versus all. E, serum caspase-3/7 activity; n = 11 mice/group; *, p < 0.05 versus all. F, serum ALT levels; n = 13 mice/group; *, p < 0.05 versus all. G, offspring from the mating of bcl-x<sup>L</sup><sub>-Tie2cre</sub> alb-cre mice with bcl-x<sup>L</sup><sub>-Tie2cre</sub> mice were examined at 6 weeks of age. Bcl-xL<sup>+/+</sup> and Bcl-xL<sup>-/-</sup>, bcl-x<sup>L</sup><sub>-Tie2cre</sub> and bcl-x<sup>L</sup><sub>-Tie2cre</sub>alb-cre, respectively. bim mRNA levels in the whole liver tissue were determined by real-time RT-PCR; n = 6 mice/group. Error bars, S.D. RLU, relative light units; IU, international units.

The presence of Bim- and Bid-induced constant BH3 stress in the healthy liver causes hepatotoxicity with the use of anticancer agents that target the anti-apoptotic Bcl-2 family proteins—Recent advances in cancer therapy have enabled the selective targeting of some anti-apoptotic Bcl-2 family proteins,
which are often dysregulated in malignant cells. ABT-737, which is a BH3 mimetic, could inhibit Bcl-xL, Bcl-2, and Bcl-w, and it has induced the regression of solid tumors (23). We previously reported that high dose ABT-737 administration caused hepatocyte apoptosis even in a normal liver, which was partly due to constitutive Bid-mediated BH3 stress (7). This finding led us to investigate the involvement of Bim and Bid in this ABT-737-mediated hepatotoxicity. Bim/Bid double knock-out mice (\(\text{bim}^{-/-}\text{bid}^{-/-}\)) were generated by mating Bim knock-out mice (\(\text{bim}^{-/-}\)) with Bid knock-out mice (\(\text{bid}^{-/-}\)), and the offspring were then treated with this drug. Western blot analysis confirmed the efficient deletion of Bim and Bid from the liver tissue of the double knock-out mice (Fig. 5A). Upon ABT-737 treatment, the Bim/Bid double knock-out mice showed complete prevention of ABT-737-induced hepatocyte apoptosis and hepatotoxicity (Fig. 5, B–F), in sharp con-
contrast to their Bid-knock-out littermates, which still showed moderate hepatocyte apoptosis (Fig. 5, C–E) and increased serum ALT levels (Fig. 5F). These findings suggested that Bim- and Bid-mediated constant BH3 stress evoked hepatotoxicity by promoting the intrinsic pathway of apoptosis with the use of the inhibitors of the Bcl-2 family.

**DISCUSSION**

At least eight BH3-only proteins are known, and five have been reported to exist in hepatocytes: Bid, Bim, Noxa, Puma, and Bad (22). We also confirmed these five proteins in the liver tissue of our mice (Fig. 1A), and we detected at least the mRNA expression of three other genes (supplemental Fig. 1). These proteins are considered to function as pro-apoptotic sensors upon activation by a variety of apoptotic stimuli, thereby promoting an intrinsic pathway of apoptosis in a manner that is dependent on the presence of Bak and Bax. In previous studies, bile acids or death receptor stimuli activated Bid and induced liver injury, which was alleviated by Bid disruption (12, 22). Bim activation was involved in hepatocyte lipoapoptosis, which is a critical feature of non-alcoholic steatohepatitis, and in reactive oxygen species-induced hepatocyte apoptosis (10, 11, 14). Additionally, a recent in vivo study revealed that the activation of Bid and Bim played a central pro-apoptotic role in fatal TNF-α-induced hepatitis (24). Taken together, these findings indicated the importance of these two BH3-only proteins in the pathogenesis of various liver diseases (12, 24, 25). Conversely, the systemic knock-out of Bim or Bid in mice did not result in any liver abnormalities under normal conditions; therefore, there has not been much interest in studying their physiological involvement in the healthy liver (12, 26). However, our present study showed that spontaneous hepatocyte apoptosis in the absence of Bcl-xL was alleviated by the deletion of either Bim or Bid, and it was diminished by the deletion of both. These results indicated that these BH3-only proteins are functionally active even in the healthy liver, but they are fully restrained by the anti-apoptotic Bcl-2 family proteins in the physiological state.

What type of stimuli constitutively activate these BH3-only proteins remains unknown. The liver is a specific organ that can be continuously exposed to a variety of stimuli, such as bile acids and enteric endotoxin, as well as interactions with immune cells. These stimuli might cause constitutive BH3-only stress through the activation of death receptors, such as Fas, tumor necrosis factor (TNF), and TNF-related apoptosis-inducing ligand (TRAIL) receptors. To explore the involvement of Fas signaling in generating this BH3-only stress, we studied the effect of fas inhibition in the hepatocyte apoptosis induced by the genetic disruption of Bcl-xL or ABT-737 administration. siRNA-mediated in vivo knockdown of fas did not alleviate their hepatocyte apoptosis (supplemental Fig. 2, B and D), suggesting that Fas signaling may not be the origin of this BH3-only

![Figure 3. The disruption of Bim and Bid prevented spontaneous hepatocyte apoptosis in the absence of Bcl-xL. The offspring from the mating of bim<sup>−/−</sup> bcl-xL<sup>−/−</sup> alb-cre mice with bim<sup>−/−</sup> bcl-xL<sup>−/−</sup> alb-cre mice were examined at 6 weeks of age. Bcl-xL<sup>−/−</sup> and Bcl-xL<sup>−/−</sup> bcl-xL<sup>−/−</sup> alb-cre, respectively. A, Western blot analysis of whole liver lysates for the expression of BimEL, Bid, Bcl-xL, Mcl-1, Bak, Bax, and β-actin. B, representative images of liver histology stained with hematoxylin-eosin (HE) and TUNEL (original magnifications, ×100 (large panels) and ×400 (insets)). Black arrows indicate apoptotic bodies. C, TUNEL-positive cell ratio; more than 5 mice/group; *, p < 0.05 versus all. D, serum caspase-3/7 activity; more than 6 mice/group; *, p < 0.05 versus all. E, serum ALT levels; more than 6 mice/group; *, p < 0.05 versus all. Error bars, S.D. RLU, relative light units; I/U, international units.](https://www.jbc.org/content/288/42/30014/F3)
stress. However, it should be noted here that siRNA administration only decreased \textit{fas} mRNA levels to around half (supplemental Fig. 2, \textit{A} and \textit{C}). Therefore, genetic study is still necessary to clarify its involvement. In order to examine the involvement of T and B cells, which comprise about 50% of intrahepatic resident immune cells (27), in producing the BH3-only stress in the healthy liver, we crossed hepatocyte-specific Mcl-1 knock-out mice with homozygous SCID mutant mice, which are characterized by an absence of functional T cells and B cells (28). The spontaneous hepatocyte apoptosis of the Mcl-1 knock-out mice was unchanged even in the homozygous SCID mutant background, monitored by serum ALT levels and serum caspase-3/7 activity (supplemental Fig. 3, \textit{A}–\textit{D}). These data indicate that these immune cells are not the major source of the BH3-only stress in the liver under physiological conditions. Therefore, further study is required to identify the main source of constitutive BH3-only stress in the healthy liver. We previously reported that Mcl-1 and Bcl-xL individually worked as apoptotic antagonists in differentiated hepatocytes (13). However, the hepatocyte-specific deletion of both led to early postnatal death due to the failure of hepatocyte development in the fetal liver (13), thus hampering the clarification of their

\textbf{FIGURE 4. Bim and Bid are essential regulators involved in the intrinsic pathway of apoptosis in hepatocytes in the absence of anti-apoptotic Bcl-2 family proteins.} \textit{bcl-x}\textsuperscript{flox/flox}\textit{alb-cre} mice and \textit{bim}\textsuperscript{−/−} \textit{bcl-x}\textsuperscript{flox/flox}\textit{alb-cre} mice were injected with \textit{mcl-1} or with negative control siRNA via the tail vein and were sacrificed 24 h (\textit{A} and \textit{C}–\textit{F}) or 48 h (\textit{B}) later. \textit{Bcl-xL}\textsuperscript{−/−} and \textit{Bcl-xL}\textsuperscript{−/−} \textit{mcl-1}\textsuperscript{−/−} \textit{bcl-x}\textsuperscript{flox/flox} and \textit{bcl-x}\textsuperscript{flox/flox}\textit{alb-cre}, respectively. \textit{NC}, negative control. \textit{A}, Western blot analysis of whole liver lysates for the expression of Bim\textsubscript{EL}, Bid, Bcl-xL, Mcl-1, Bak, Bax, and \beta-actin. \textit{B}, representative images of liver histology stained with hematoxylin-eosin (original magnifications, \times 100 (large panels) and \times 400 (insets)). \textit{C}, representative images of liver histology stained with TUNEL (original magnification, \times 100). \textit{D}, serum caspase-3/7 activity; \textit{n} = 3–4 mice/group. \textit{E}, serum ALT levels; \textit{n} = 4 mice/group; data are presented as means ± S.E. (error bars). \textit{F}, serum T-bilirubin levels; \textit{n} = 4 mice/group. \textit{RLU}, relative light units; \textit{I/U}, international units.
cooperative involvement in the adult liver. In the present study, the combination of genetically engineered mice and in vivo siRNA technology enabled the investigation of their cooperative roles for the first time, and we found that the inhibition of Mcl-1 caused sublethal liver injury with massive hepatocyte apoptosis in Bcl-xL-knock-out mice. Meanwhile, we also found that sublethal apoptosis was prevented in a Bim/Bid double knock-out background, suggesting that, of the BH3-only proteins, Bim and Bid are important for activating the intrinsic pathway of hepatocyte apoptosis in the absence of anti-apoptotic Bcl-2 family proteins. It would also be interesting to determine whether other anti-apoptotic Bcl-2 family proteins or BH3-only proteins are involved in this healthy Bcl-2 rheostasis.

The anti-apoptotic Bcl-2 family proteins are often dysregulated in a variety of malignancies, and they have been recog-

FIGURE 5. The presence of Bim- and Bid-induced constant BH3 stress in the healthy liver causes hepatotoxicity with the use of anti-cancer agents that target anti-apoptotic Bcl-2 family proteins. The offspring from bim−/− x bid−/− mating pairs were given an intraperitoneal injection of ABT-737 (100 mg/kg) or vehicle and were examined after 6 h. A, Western blot analysis of whole liver lysates for the expression of BimEL, Bid, Bcl-xL, Mcl-1, Bak, Bax, and β-actin. B and C, representative images of liver histology stained with hematoxylin-eosin and TUNEL (original magnifications, ×100 (large panels) and ×400 (insets)). D, TUNEL-positive cell ratio; n = 5–6 mice/group; *, p < 0.05 versus all. E, serum caspase-3/7 activity; more than 5 mice/group; *, p < 0.05 versus all. F, serum ALT levels; more than 5 mice/group; *, p < 0.05 versus all. Error bars, S.D. RLU, relative light units; I/U, international units.
nized as important oncogenes (29). ABT-737, which was recently developed to inhibit the Bcl-xL, Bcl-w, and Bcl-2 proteins, displays anti-tumor activity against lymphoid malignancies and small-cell lung carcinoma (23). These drugs were considered to selectively target tumor cells because malignant cells receive many genotoxic and environmental stress-induced BH3-only signals, so these cells are thus dependent on the anti-apoptotic Bcl-2 family members for their survival. However, we previously reported that the high-dose administration of ABT-737 (100 mg/kg) elicited hepatotoxicity via Bak/Bax-dependent apoptosis in normal hepatocytes (7), suggesting that dependence on the anti-apoptotic Bcl-2 family proteins is not a specific feature of tumor cells but is the case in healthy liver cells. In the present study, we demonstrated that the disruption of Bim and Bid completely prevented hepatocyte apoptosis and hepatotoxicity induced by high dose ABT-737 (100 mg/kg), suggesting that these proteins are responsible for this hepatotoxicity. Meanwhile, although 25 mg/kg ABT-737, which is relatively close to the clinical dose, caused moderate hepatocyte apoptosis, this apoptosis was completely blocked by Bid inhibition (supplemental Fig. 4). Therefore, it is unclear whether both Bid and Bim are truly involved in hepatotoxicity when using ABT-737 at clinically relevant doses.

This study demonstrated that Bim was also involved in the hepatocyte apoptosis caused by Mcl-1 deficiency in addition to Bid, which was noted in our previous report (13). Several previous human studies have reported that Mcl-1 proteins were down-regulated in the liver tissues of non-alcoholic steatohepatitis and primary biliary cirrhosis patients (30, 31), and experimental studies have demonstrated that Mcl-1 down-regulation by saturated fatty acids caused hepatocyte lipoapoptosis, which plays an important role in the development of fatty liver disease (32, 33). Taken together with our findings, these reports suggest the possibility that Bim- and Bid-mediated constant BH3 stresses might constitute therapeutic targets of the hepatotoxicity observed in these human liver diseases.

In conclusion, we have demonstrated that the novel rheostatic balance between the pro-apoptotic BH3-only proteins Bim and Bid and the anti-apoptotic Bcl-2 family proteins Bcl-xL and Mcl-1 regulates hepatocyte life and death in the physiological state. Our present study sheds new light on the dynamic and well orchestrated Bcl-2 networks in the healthy liver.

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REFERENCES

1. Youle, R. J., and Strasser, A. (2008) The BCL-2 protein family. Opposing activities that mediate cell death. Nat. Rev. Mol. Cell Biol. 9, 47–59

2. Chipuk, J. E., and Green, D. R. (2008) How do BCL-2 proteins induce mitochondrial outer membrane permeabilization? Trends Cell Biol. 18, 157–164

3. Adams, J. M., and Cory, S. (2007) Bcl-2-regulated apoptosis. Mechanism and therapeutic potential. Curr. Opin. Immunol. 19, 488–496

4. Kim, H., Rafuiddin-Shah, M., Tu, H. C., Jeffers, J. R., Zambetti, G. P., Hsieh, J. J., and Cheng, E. H. (2006) Hierarchical regulation of mitochondrion-dependent apoptosis by BCL-2 subfamilies. Nat. Cell Biol. 8, 1348–1358

5. Willis, S. N., Fletcher, J. I., Kaufmann, T., van Delft, M. F., Chen, L., Czabotar, P. E., Ierino, H., Lee, E. F., Fairlie, W. D., Bouillet, P., Strasser, A., Kluck, R. M., Adams, J. M., and Huang, D. C. (2007) Apoptosis initiated when BH3 ligands engage multiple Bcl-2 homologs, not Bax or Bak. Science 315, 856–859

6. Korsmeyer, S. J., Shutter, J. R., Veis, D. J., Merry, D. E., and Oltvai, Z. N. (1993) Bcl-2/Bax. A rheostat that regulates an anti-oxidant pathway and cell death. Semin. Cancer Biol. 4, 327–332

7. Hikita, H., Takehara, T., Kodama, T., Shimizu, S., Hosui, A., Miyagi, T., Tatsumi, T., Ishida, H., Okawa, K., Li, W., Kanto, T., Hiramatsu, N., Hennighausen, L., Yin, X. M., and Hayashi, N. (2009) BH3-only protein bid participates in the Bcl-2 network in healthy liver cells. Hepatology 50, 1972–1980

8. Giam, M., Huang, D. C., and Bouillet, P. (2008) BH3-only proteins and their roles in programmed cell death. Oncogene 27, Suppl. 1, S128–S136

9. Lomonosova, E., and Chinnadurai, G. (2008) Oncogene 27, Suppl. 1, S2–S19

10. Baryo, F. J., Kobayashi, S., Brong, S. F., Werneburg, N. W., Malhi, H., and Gores, G. J. (2007) Transcriptional regulation of Bid by FoxO3A mediates hepatocyte lipoapoptosis. J. Biol. Chem. 282, 27141–27154

11. Ishihara, Y., Takeuchi, K., Ito, F., and Shimamoto, N. (2011) Dual regulation of hepatocyte apoptosis by reactive oxygen species. Increases in transcriptional expression and decreases in proteasomal degradation of Bid. J. Cell Physiol. 226, 1007–1016

12. Yin, X. M., Wang, K., Gross, A., Zhao, Y., Zinkel, S., Klocke, B., Roth, K. A., and Korsmeyer, S. J. (1999) Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis. Nature 400, 886–891

13. Hikita, H., Takehara, T., Shimizu, S., Kodama, T., Li, W., Miyagi, T., Hosui, A., Ishida, H., Okawa, K., Kanto, T., Hiramatsu, N., Yin, X. M., Hennighausen, L., Tatsumi, T., and Hayashi, N. (2009) Mcl-1 and Bcl-xL cooperatively maintain integrity of hepatocytes in developing and adult murine liver. Hepatology 50, 1217–1226

14. Ishihara, Y., Ito, F., and Shimamoto, N. (2011) Increased expression of c-Fos by extracellular signal-regulated kinase activation under sustained oxidative stress elicits BimEl upregulation and hepatocyte apoptosis. FEBS J. 278, 1873–1881

15. Malhi, H., Brong, S. F., Werneburg, N. W., and Gores, G. J. (2006) Free fatty acids induce JNK-dependent hepatocyte lipoapoptosis. J. Biol. Chem. 281, 12093–12101

16. Dzhagalov, L., St John, A., and He, Y. W. (2007) The antiapoptotic protein Mcl-1 is essential for the survival of neutrophils but not macrophages. Blood 109, 1620–1626

17. Takehara, T., Tatsumi, T., Suzuki, T., Rucker, E. B., 3rd, Hennighausen, L., Jinushi, M., Miyagi, T., Kanazawa, Y., and Hayashi, N. (2004) Hepatocyte-specific disruption of Bcl-xL leads to continuous hepatocyte apoptosis and liver fibrotic responses. Gastroenterology 127, 1189–1197

18. Wagner, K. U., Claudio, E., Rucker, E. B., 3rd, Riedlinger, G., Broussard, C., Schwartzberg, P. L., Siebenlist, U., and Hennighausen, L. (2000) Conditional deletion of the Bcl-x gene from erythroid cells results in hemolytic anemia and profound splenomegaly. Development 127, 4949–4958

19. Maruyama, C., Sueimizu, H., Tamamushi, S., Kimoto, S., Tamaoki, N., and Ohnishi, Y. (2002) Genotyping the mouse severe combined immunodeficiency and therapeutic potential. J. Clin. Invest. 112, 3343–3356

20. Kodama, T., Takehara, T., Hikita, H., Shimizu, S., Shigekawa, M., Tsunematsu, H., Li, W., Miyagi, T., Hosui, A., Tatsumi, T., Ishida, H., Kanto, T., Hiramatsu, N., Kubota, S., Takigawa, M., Tomimaru, Y., Tomokuni, A., Nagano, H., Doki, Y., Mori, M., and Hayashi, N. (2011) Increases in p53 expression induce CTFG synthesis by mouse and human hepatocytes and result in liver fibrosis in mice. J. Clin. Pathol. 121, 3343–3356

21. Kodama, T., Takehara, T., Hikita, H., Shimizu, S., Li, W., Miyagi, T., Hosui, A., Tatsumi, T., Ishida, H., Tadokoro, S., Ido, A., Tsubouchi, H., and Hayashi, N. (2010) Thrombocytopenia exacerbates cholestasis-induced liver fibrosis in mice. Gastroenterology 138, 2487–2498, 2498.e2481–2487

22. Baskin-Bey, E. S., and Gores, G. J. (2005) Death by association. BH3 do-

23. Baskin-Bey, E. S., and Gores, G. J. (2005) Death by association. BH3 do-

24. Oltersdorf, T., Elmore, S. W., Shoemaker, A. R., Armstrong, R. C., Augeri,
The Novel Bcl-2 Network in Healthy Liver

D. J., Belli, B. A., Brunko, M., Deckwerth, T. L., Dinges, J., Hajduk, P. J., Joseph, M. K., Kitada, S., Korsmeyer, S. J., Kunzer, A., Letai, A., Li, C., Mitten, M. J., Nettlesheim, D. G., Ng, S., Nimmer, P. M., O’Connor, J. M., Oleksijew, A., Petros, A. M., Reed, J. C., Shen, W., Tahir, S. K., Thompson, C. B., Tomaselli, K. J., Wang, B., Wendt, M. D., Zhang, H., Fesik, S. W., and Rosenberg, S. H. (2005) An inhibitor of Bcl-2 family proteins induces regression of solid tumours. *Nature* **435**, 677–681

24. Kaufmann, T., Jost, P. J., Pellegrini, M., Puthalakath, H., Gugasyan, R., Gerondakis, S., Cretney, E., Smyth, M. J., Silke, J., Hakem, R., Bouillet, P., Mak, T. W., Dixit, V. M., and Strasser, A. (2009) Fatal hepatitis mediated by tumor necrosis factor TNFα requires caspase-8 and involves the BH3-only proteins Bid and Bim. *Immunity* **30**, 56–66

25. Higuchi, H., Miyoshi, H., Bronk, S. F., Zhang, H., Dean, N., and Gores, G. J. (2001) Bid antisense attenuates bile acid-induced apoptosis and cholestatic liver injury. *J. Pharmacol. Exp. Ther.* **299**, 866–873

26. Bouillet, P., Metcalf, D., Huang, D. C., Tarlinton, D. M., Kay, T. W., Köntgen, F., Adams, J. M., and Strasser, A. (1999) Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. *Science* **286**, 1735–1738

27. Blom, K. G., Qazi, M. R., Matos, J. B., Nelson, B. D., DePierre, J. W., and Abedi-Valugerdi, M. (2009) Isolation of murine intrahepatic immune cells employing a modified procedure for mechanical disruption and functional characterization of the B, T, and natural killer T cells obtained. *Clin. Exp. Immunol.* **155**, 320–329

28. Shultz, L. D., Schweitzer, P. A., Christianson, S. W., Gotte, B., Schweitzer, I. B., Tennent, B., McKenna, S., Mobraaten, L., Rajan, T. V., and Greiner, D. L. (1995) Multiple defects in innate and adaptive immunologic function in NOD/LtSz-scid mice. *J. Immunol.* **154**, 180–191

29. Kirkin, V., Joos, S., and Zörnig, M. (2004) The role of Bcl-2 family members in tumorigenesis. *Biochim. Biophys. Acta* **1644**, 229–249

30. García-Monzon, C., Lo Iacono, O., Mayoral, R., González-Rodriguez, A., Miñulena-Colina, M. E., Lozano-Rodríguez, T., García-Pozo, L., Vargas-Castrillón, J., Casado, M., Boscá, L., Valverde, A. M., and Martín-Sanz, P. (2011) Hepatic insulin resistance is associated with increased apoptosis and fibrogenesis in nonalcoholic steatohepatitis and chronic hepatitis C. *J. Hepatol.* **54**, 142–152

31. Iwata, M., Harada, K., Kono, N., Kaneko, S., Kobayashi, K., and Nakanuma, Y. (2000) Expression of Bcl-2 familial proteins is reduced in small bile duct lesions of primary biliary cirrhosis. *Hum. Pathol.* **31**, 179–184

32. Ibrahim, S. H., Kohli, R., and Gores, G. J. (2011) Mechanisms of lipotoxicity in NAFLD and clinical implications. *J. Pediatr. Gastroenterol. Nutr.* **53**, 131–140

33. Masuoka, H. C., Mott, J., Bronk, S. F., Werneburg, N. W., Akazawa, Y., Kaufmann, S. H., and Gores, G. J. (2009) Mcl-1 degradation during hepatocele lipoproteinosis. *J. Biol. Chem.* **284**, 30039–30048