BH3-Only Protein Bid Participates in the Bcl-2 Network in Healthy Liver Cells

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Bcl-2 homology domain 3 (BH3)-only protein Bid is posttranslationally cleaved by caspase-8 into its truncated form (tBid) and couples with stress signals to the mitochondrial cell death pathway. However, the physiological relevance of Bid is not clearly understood. Hepatocyte-specific knockout (KO) of Bcl-xL leads to naturally-occurring apoptosis despite co-expression of Mcl-1, which shares a similar anti-apoptotic function. We generated Bcl-xL KO, Bcl-xL/Bid double KO, Bcl-xL/Bak double KO, Bcl-xL/Bax double KO, and Bcl-xL/Bak/Bax triple KO mice and found that hepatocyte apoptosis caused by Bcl-xL deficiency was completely dependent on Bak and Bax, and surprisingly on Bid. This indicated that, in the absence of Bid, Bcl-xL is not required for the integrity of differentiated hepatocytes, suggesting a complicated interaction between core Bcl-2 family proteins and BH3-only proteins even in a physiological setting. Indeed, a small but significant level of tBid was present in wild-type liver under physiological conditions. tBid was capable of binding to Bcl-xL and displacing Bak and Bax from Bcl-xL, leading to release of cytochrome c from wild-type mitochondria. Bcl-xL–deficient mitochondria were more susceptible to tBid-induced cytochrome c release. Finally, administration of ABT-737, a pharmacological inhibitor of Bcl-2/Bcl-xL, caused Bak/Bax-dependent liver injury, but this was clearly ameliorated with a Bid KO background. *Conclusion:* Bid, originally considered to be a sensor for apoptotic stimuli, is constitutively active in healthy liver cells and is involved in the Bak/Bax-dependent mitochondrial cell death pathway. Healthy liver cells are addicted to a single Bcl-2–like molecule because of BH3 stresses, and therefore special caution may be required for the use of the Bcl-2 inhibitor for cancer therapy. (HEPATOLOGY 2009;50:1972-1980.)

Bcl-2 family proteins regulate the mitochondrial pathway of apoptosis in mammalian cells.[1] They are divided into two basic groups: core Bcl-2 family proteins and Bcl-2 homology domain 3 (BH3)-only proteins. Core Bcl-2 family proteins have three or four Bcl-2 homology domains (BH1-BH4 domains), referred to as multidomain members, and structural similarity. These proteins display opposing bioactivities from inhibition to promotion of apoptosis and can be further divided into two groups: anti-apoptotic members, including Bcl-2, Bcl-xL, Bcl-w, Mcl-1, and Bfl-1, and pro-apoptotic members, including Bax and Bak. Pro-apoptotic Bak and Bax are effector molecules of the Bcl-2 family and induce release of cytochrome c from mitochondria, presumably through their ability to form pores at the mitochondrial outer membrane. Anti-apoptotic members, which serve as regulators, inhibit Bak and Bax. The original rheostat model argues for a fine balance between Bax-like pro-apoptotic proteins and Bcl-2–like an-

Abbreviations: ALT, alanine aminotransferase; BH3, Bcl-2 homology domain 3; KO, knockout; tBid, truncated form of Bid; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine 5'-triphosphate nick-end labeling.

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Received May 20, 2009; accepted July 21, 2009.

Supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (to T. Takehara).

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Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.23207

Potential conflict of interest: Nothing to report.

Additional Supporting Information may be found in the online version of this article.

ti-apoptotic proteins in defining life and death, and this balance would be equal or favor survival in a healthy cell.[2]

BH3-only proteins consist of at least eight members and only share homology with each other and the core Bcl-2 family proteins through the short BH3 motif. They are transcriptionally induced or posttranslationally activated in response to a variety of apoptotic stimuli.[3] When they are induced or activated, they interact with core Bcl-2 family proteins and set the rheostat balance toward apoptosis by directly activating Bax-like molecules or neutralizing Bcl-2–like molecules.[4] Therefore, they serve as initial sensors of apoptotic signals that emanate from various cellular processes. Bid, a member of the BH3-only proteins, is activated via caspase-8–mediated cleavage in response to ligation of the death receptor, and its N-terminal truncated form (tBid) translocates to mitochondria and activates the mitochondrial death pathway.[5] In so-called type 1 cells, such as lymphoid cells, Fas activation leads to caspase-8 activation followed by direct activation of downstream caspases such as caspase-3 and caspase-7, where Bid dose not have significant roles.[6] In contrast, in type 2 cells, Fas-mediated activation of caspase-8 is not enough to activate downstream caspases. In those cells, tBid links the extrinsic or death-receptor pathway to the intrinsic or mitochondrial pathway to execute apoptosis. Hepatocytes are identified as a typical type 2 cell in which Bid plays a critical role in receptor-mediated cell death pathways.[7]

In our previous research, we found that genetic ablation of Bcl-xL in hepatocytes causes spontaneous apoptosis in mice.[8] This indicates that Bcl-xL is a critical apoptosis antagonist in adult healthy hepatocytes, although they possess other anti-apoptotic members of the Bcl-2 family such as Mcl-1. This might be simply explained by the fact that the absence of Bcl-xL affects the rheostat balance of core Bcl-2 family proteins by increasing the ratio of Bax and Bak to anti-apoptotic Bcl-2 proteins. Indeed, neuronal cell death during development caused by Bcl-xL deficiency is ameliorated by loss of Bax.[9] Platelet cell death caused by Bcl-xL deficiency is also ameliorated by loss of Bak.[10] These studies indicate that the stoichiometry between Bcl-xL and Bax or Bak dictates cellular fate. However, the possibility of BH3-only proteins being involved in the apoptosis rheostat in healthy cells has not been addressed. We generated Bcl-xL/Bid double-knockout (KO) mice and demonstrated that apoptosis caused by Bcl-xL deficiency is critically dependent on Bid. A small amount of Bid appears to be activated in the liver under physiological conditions and to be significant for inducing cytochrome c release from Bcl-xL–deficient mitochondria. This study shed light on the active participation of BH3-only proteins, which are generally considered to be sensors of apoptotic stimuli, in the Bcl-2 network regulating life and death of healthy differentiated hepatocytes.

Materials and Methods

Mice. Mice carrying a *bcl-x* gene with 2 *lox*P sequencers at the promoter region and a second intron (*bcl-x*$^{flox/flox}$) were described previously.[11] Heterozygous AlbCre transgenic mice expressing Cre recombinase gene under the promoter of the albumin gene[8] and traditional Bid KO mice[7] also have been described previously. We purchased from the Jackson Laboratory (Bar Harbor, ME) traditional Bak KO mice, traditional Bax KO mice, and conditional Bak/Bax KO mice (*bak*$^{-/-}$ *bax*$^{flox/flox}$).[12] We generated hepatocyte-specific Bcl-xL KO mice (*bcl-x*$^{flox/flox}$ *AlbCre*), Bcl-xL/Bid double-KO mice (*bid*$^{-/-}$ *bcl-x*$^{flox/flox}$ *AlbCre*), Bcl-xL/Bak double-KO mice (*bak*$^{-/-}$ *bcl-x*$^{flox/flox}$ *AlbCre*), Bcl-xL/Bax double-KO mice (*bax*$^{-/-}$ *bcl-x*$^{flox/flox}$ *AlbCre*), and Bcl-xL/Bak/Bax triple-KO mice (*bak*$^{-/-}$ *bax*$^{flox/flox}$ *bcl-x*$^{flox/flox}$ *AlbCre*) by mating the strains. They were maintained in a specific pathogen-free facility and treated with humane care under approval from the Animal Care and Use Committee of Osaka University Medical School.

Apoptosis Assay. The levels of serum alanine aminotransferase (ALT) were measured by a standard method, and serum caspase-3/7 activity was measured by a luminescent substrate assay for caspase-3 and caspase-7 (Caspase-Glo assay, Promega, Tokyo, Japan). The caspase-3/7 activity was normalized by each control group. For histological analysis, the liver sections were stained with hematoxylin-eosin. To detect cells with oligonucleosomal DNA breaks, the sections were also subjected to terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining, according to a previously reported procedure.[13]

Western Blot Analysis. Liver tissue was lysed with a lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 × protein inhibitor cocktail (Nacalai tesque, Kyoto, Japan), phosphate-buffered saline, pH 7.4). Equal amounts of protein were electrophoretically separated by sodium dodecyl sulfate polyacrylamide gels and transferred onto polyvinylidene fluoride membrane. For immunodetection, the following antibodies were used: anti-Bcl-xL antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Mcl-1 antibody (Rockland, Gilbertsville, PA), previously described anti-Bid antibody generated from glutation-S-transferase-Bid fusion protein,[14] anti-full-length Bid antibody, anti-cleaved caspase-7 antibody, anti-Bax antibody, anti-Cox IV antibody (Cell Signaling Technology, Beverly, MA),

anti-Bak antibody (Millipore, Billerica, MA), and anti–β-actin antibody (Sigma-Aldrich, St. Louis, MO).

Isolation of Mitochondria-Rich and Cytosolic Fraction. After liver tissue was homogenized using isolation buffer (225 mM mannitol, 75 mM sucrose, 0.1 mM ethylene glycol tetraacetic acid, 1 mg/mL fatty acid–free bovine serum albumin, 1 × protein inhibitor cocktail, 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid–potassium hydroxide, pH 7.4), the lysate was centrifuged at 600*g* for 10 minutes, and the supernatant was centrifuged at 15,000*g* for 10 minutes. The pellet was regarded as a mitochondria-rich fraction and the supernatant as a cytosolic fraction.

Immunoprecipitation of Bcl-xL. Approximately 30 mg liver tissue was lysed with a TNE buffer (1% Nonidet P-40, 1 mM ethylenediaminetetra-acetic acid, 1 × protein inhibitor cocktail, 0.15 M NaCl, 10 mM Tris-HCl, pH 7.8). Equal amounts of protein samples were rotated with protein G sepharose (GE Healthcare, Tokyo, Japan) and anti-Bcl-xL antibody (Abcam, Cambridge, MA) overnight at 4°C. After centrifugation, the pellet was collected as the immunoprecipitate protein.

Incubation of tBid or Bid for Immunoprecipitation. Liver tissue (90 mg) was lysed with 800 μL lysis buffer (2 mM ethylenediaminetetra-acetic acid, 10 mM ethylene glycol tetra-acetic acid, 50 mM NaF, 5 mM $Na_2P_4O_7$, 10 mM β-glycerophosphate, 0.1% 2-mercaptoethanol, 1% Triton X, 1 × protein inhibitor cocktail, 50 mM Tris-HCl, pH 7.5). Equal volumes of protein samples were incubated with or without recombinant mouse tBid or full-length Bid (R&D Systems, Minneapolis, MN).

Analysis of Cytochrome C Release. The mitochondria-rich fraction was diluted in a mitochondria dilution buffer (395 mM sucrose, 0.1 mM ethylene glycol tetra-acetic acid, 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid–potassium hydroxide, pH 7.4). The diluted mitochondria were incubated with recombinant mouse tBid or full-length Bid diluted with a reaction buffer (125 mM KCl, 0.5 mM $MgCl_2$, 3.0 mM succinic acid, 3.0 mM glutamic acid, 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid–potassium hydroxide, 1 × protein inhibitor cocktail, 2.5 mM ethylenediaminetetra-acetic acid and BOC-Asp (OMe) CH_2F 20 μM, pH 7.4) for 30 minutes at 37°C. The levels of cytochrome c in the buffer were determined using an enzyme-linked immunosorbent assay kit (R&D Systems). The maximum or spontaneous release of cytochrome c was defined as the level of samples incubated with 0.1% Triton X-100 or medium alone, respectively. The percentage release of cytochrome c was calculated using the following formula:

% release = (experimental release − spontaneous release) × 100/(maximum release − spontaneous release).

ABT-737 Injection Study. ABT-737 was provided by Abbott Laboratories (Abbott, Park, IL). ABT-737 was dissolved with a mixture of 30% propylene glycol, 5% Tween 80, and 65% D5W (5% dextrose in water), final pH 4 to 5. Mice were given a single intraperitoneal injection of ABT-737 at 100 mg/kg and sacrificed 16 hours later. Platelets were counted using an automated cell counter (Sysmex, Kobe, Japan).

Statistical Analysis. Data are presented as mean ± standard deviation. Multiple comparisons of TUNEL-positive cells were performed by analysis of variance followed by Fisher's *post hoc* correction. The other multiple comparisons were performed by analysis of variance followed by Scheffe *post hoc* correction. $P < 0.05$ was considered statistically significant.

Results

Hepatocyte Apoptosis Caused by Bcl-xL Deficiency Is Completely Lost with Bid-Deficient Background. To examine the possibility of whether Bid is involved in apoptosis caused by Bcl-xL deficiency, hepatocyte-specific Bcl-xL KO mice were crossed with traditional Bid KO mice. After mating *bid*$^{+/-}$ *bcl-x*$^{flox/flox}$ *AlbCre* mice with *bid*$^{+/-}$ *bcl-x*$^{flox/flox}$ mice, western blot analysis confirmed lack of Bcl-xL and Bid in the liver of Bcl-xL KO mice and Bid KO mice, respectively, and intermediate expression of Bid in the Bid+/- liver (Fig. 1A). Consistent with our previous findings,[8] Bcl-xL KO mice (*bid*$^{+/+}$ *bcl-x*$^{flox/flox}$ *AlbCre*) produced spontaneous hepatocyte apoptosis (Fig. 1B), which was associated with caspase-7 activation in the liver (Fig. 1C). Serum ALT levels (Fig. 1D), caspase-3/7 activity (Fig. 1E), and the frequency of TUNEL-positive hepatocytes (Fig. 1F) were significantly higher in Bcl-xL KO mice than in wild-type mice (*bid*$^{+/+}$ *bcl-x*$^{flox/flox}$). Bid KO mice (*bid*$^{-/-}$ *bcl-x*$^{flox/flox}$) did not produce any liver phenotypes under physiological conditions, in agreement with a previous report.[7] This was further confirmed by our additional analysis on Bid KO mice and control littermates, which showed no difference in serum ALT levels (Supporting Fig. 1A), caspase-3/7 activity (Supporting Fig. 1B), and the ratios of liver weight to body weight (Supporting Fig. 1C). Of importance is the finding that serum ALT levels were reduced to the normal levels in Bcl-xL/Bid double-KO mice (*bid*$^{-/-}$ *bcl-x*$^{flox/flox}$ *AlbCre*). Bcl-xL KO with Bid heterozygosity (*bid*$^{+/-}$ *bcl-x*$^{flox/flox}$ *AlbCre*) displayed intermediate ALT levels between Bcl-xL KO mice and double-KO mice. In agreement with this observation, the number of TUNEL-positive hepatocytes in Bcl-xL/Bid double-KO mice reached background levels. In addition, the levels of caspase-3/7 activity in serum were also normalized in Bcl-xL/Bid double-KO mice. Taken together, these observations indicated that

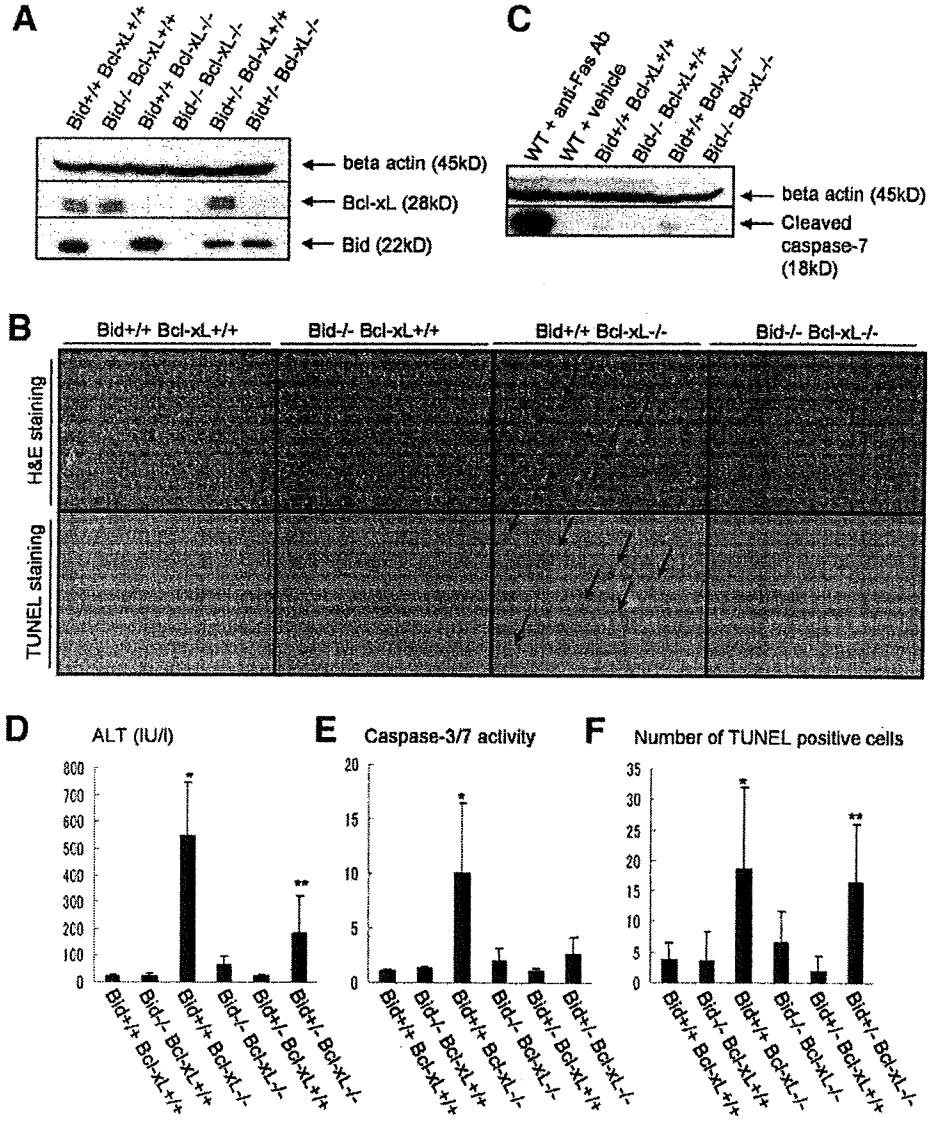

Fig. 1. Bcl-xL/Bid double-KO mice. Offspring from mating *bid*$^{+/-}$ *bcl-x*$^{flox/flox}$ *AlbCre* mice and *bid*$^{+/-}$ *bcl-x*$^{flox/flox}$ mice were sacrificed at 6 weeks after birth. Bcl-xL+/+ stands for *bcl-x*$^{flox/flox}$ without *AlbCre*, and Bcl-xL−/− stands for *bcl-x*$^{flox/flox}$ with *AlbCre*. (A) Western blot of whole liver lysate for the expression of Bcl-xL and Bid. (B) Representative pictures of liver histology stained with hematoxylin-eosin and TUNEL. Arrows indicate typical apoptotic cells. (C) Western blot of whole liver lysate for the expression of cleaved caspase-7. Live lysates from wild-type mice 3 hours after intraperitoneal injection of 30 μg anti-Fas antibody (clone Jo2) or vehicle were included as a positive and a negative control, respectively. (D) Serum ALT levels. N = 14 mice per group. *$P < 0.05$ versus the other five groups; **$P < 0.05$ versus the other four groups except Bid−/− Bcl-xL−/− group. (E) Serum caspase-3/7 activity. N = 13 mice per group. *$P < 0.05$ versus the other five groups. (F) Statistics of TUNEL- positive cells. N = 6 mice per group. * and **$P < 0.05$ versus all of Bcl-xL +/+ groups and Bid−/− Bcl-xL−/− group.

apoptosis caused by Bcl-xL deficiency is completely dependent on the BH3-only protein Bid. Bid is activated by tumor necrosis factor (TNF) receptor,[15] and TNF-α, which is a ligand of TNF receptor, is produced by Myd88 signal pathway.[16] To examine the possibility of involvement of Myd88 or TNF-α in this apoptosis, we generated Myd88 Bcl-xL double-KO mice by crossing myd88$^{-/-}$ mice with bcl-x$^{flox/flox}$ AlbCre mice and administered neutralizing anti–TNF-α antibody into Bcl-xL KO mice. Hepatocyte apoptosis caused by Bcl-xL deficiency was not ameliorated with Myd88 KO background or by administration of anti–TNF-α antibody (Supporting Fig. 2A, B).

Hepatocyte Apoptosis Caused by Bcl-xL Deficiency Requires Both Bak and Bax. To depict the precise relationships among core Bcl-2 family proteins in regulating liver homeostasis, hepatocyte-specific Bcl-xL–deficient mice were crossed with traditional Bak or Bax KO mice. The levels of serum ALT were slightly decreased with a Bak KO background (*bak*$^{-/-}$ *bcl-x*$^{flox/flox}$ *AlbCre*), whereas they did not change with a Bax KO background (*bax*$^{-/-}$ *bcl-x*$^{flox/flox}$ *AlbCre*) (Fig. 2A, B). To examine the contribution of both Bax and Bak, Bcl-xL KO mice were crossed with conditional Bak/Bax KO mice. The levels of serum ALT were completely normalized in Bak/Bax KO background (*bak*$^{-/-}$ *bax*$^{flox/flox}$ *bcl-x*$^{flox/flox}$ *AlbCre*) (Fig. 2C). Hepatocyte apoptosis determined by TUNEL staining of liver sections and caspase activation determined by caspase-3/7 activity in serum also returned to background levels (Fig. 2D, E). These observations clearly indicated that apoptosis caused by Bcl-xL deficiency was generated through the Bak/Bax-dependent mitochondrial cell death pathway. To clarify the background levels of hepatocyte apoptosis, we also analyzed the liver apoptosis in *bak*$^{-/-}$ and *bak*$^{-/-}$ *bax*$^{flox/flox}$ *AlbCre* mice. Similarly, in *bid*$^{-/-}$

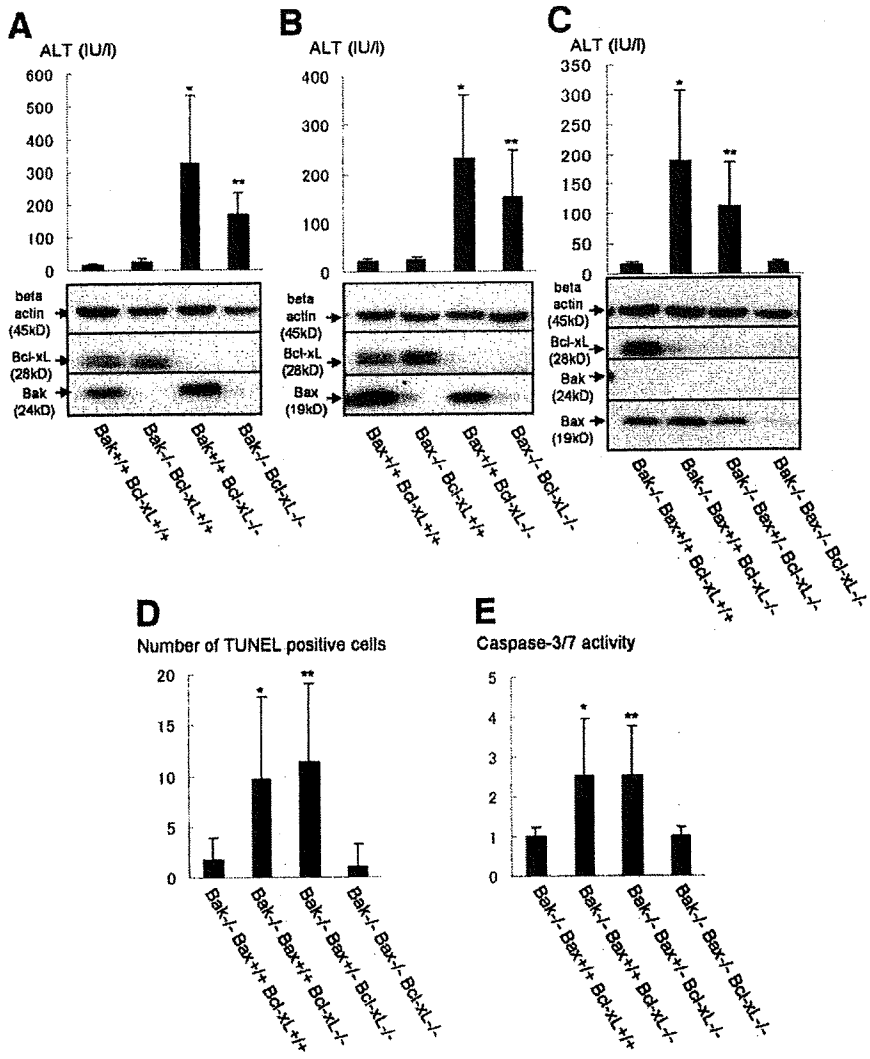

Fig. 2. Bcl-xL KO mice with Bak or Bax KO background. (A) Offspring from mating *bak*$^{+/-}$ *bcl-x*$^{flox/flox}$ *AlbCre* mice and *bak*$^{+/-}$ *bcl-x*$^{flox/flox}$ mice were sacrificed at 6 weeks after birth. Serum ALT levels and western blot of whole liver lysate for the expression of Bcl-xL and Bak are shown. N = 14 mice per group. * and **$P < 0.05$ versus the other three groups. (B) Offspring from mating *bax*$^{+/-}$ *bcl-x*$^{flox/flox}$ *AlbCre* mice and *bax*$^{+/-}$ *bcl-x*$^{flox/flox}$ mice were sacrificed at 6 weeks after birth. Serum ALT levels and western blot of whole liver lysate for the expression of Bcl-xL and Bax are shown. N = 15 mice per group. * and **$P < 0.05$ versus the other two Bcl-xL+/+ groups. (C, D, and E) Offspring from mating *bak*$^{-/-}$ *bax*$^{flox/+}$ *bcl-x*$^{flox/flox}$ *AlbCre* mice and *bak*$^{-/-}$ *bax*$^{flox/+}$ *bcl-x*$^{flox/flox}$ mice were sacrificed at 6 weeks after birth. Bax+/+ stands for *bax*$^{flox/flox}$ without *AlbCre*, and Bax−/− stands for *bax*$^{flox/flox}$ with *AlbCre*. N = 8 or 10 mice per group. Serum ALT levels and western blot of whole liver lysate for the expression of Bcl-xL, Bak, and Bax are shown (C). *$P < 0.05$ versus Bak−/− Bax+/+ Bcl-xL+/+ and Bak−/− Bax−/− Bcl-xL−/− groups; **$P < 0.05$ versus Bak−/− Bax−/− Bcl-xL−/− group. Statistics of TUNEL-positive cells (D). * and **$P < 0.05$ versus Bak−/− Bax+/+ Bcl-xL+/+ and Bak−/− Bax−/− Bcl-xL−/− groups. Serum caspase-3/7 activity (E). * and **$P < 0.05$ versus Bak−/− Bax+/+ Bcl-xL+/+ and Bak−/− Bax−/− Bcl-xL−/− groups.

mice, there was no difference between two groups in serum ALT levels (Supporting Fig. 3A), caspase-3/7 activity (Supporting Fig. 3B), and the ratios of liver weight to body weight (Supporting Fig. 3C), which suggests that healthy hepatocytes in wild-type mice are completely protected from Bid or Bak/Bax-mediated apoptosis by Bcl-xL.

Bcl-xL Interacts with Cytosolic Bax and Mitochondrial Bak in the Liver. To examine the expression of a variety of Bcl-2–related molecules in the liver, cytosolic and mitochondrial fractions from liver lysate were subjected to western blot analysis (Fig. 3A). Anti-apoptotic Bcl-2 proteins, Bcl-xL and Mcl-1, were expressed at both the mitochondria and the cytosol. In contrast, Bak and Bax were exclusively expressed at the mitochondria and the cytosol, respectively. Full-length Bid was expressed mainly in the cytosol. To examine whether Bcl-xL physically interacts with those Bcl-2–related proteins, liver lysate was immunoprecipitated with Bcl-xL and identified using corresponding antibodies (Fig. 3B). At least a part of Bcl-xL was bound to Bak and Bax, but not to Mcl-1 or full-length Bid.

tBid, But Not Full-Length Bid, Displaces Bak and Bax from Bcl-xL by Binding to Bcl-xL. Bcl-2–like molecules have been shown to be capable of binding Bak or Bax, and through this interaction, to neutralize each activity.[17] Conversely, other research showed that Bcl-xL does not have to bind Bax-like molecules to protect against cell death.[18] To examine the impact of tBid on the association between Bcl-xL and Bak or Bax, we added tBid to the liver lysate and examined the interaction of each Bcl-2–related protein with Bcl-xL by immunoprecipitation. Addition of 500 nM tBid abolished the association between Bcl-xL and Bak or Bax (Supporting Fig. 4). Simultaneously, Bcl-xL binding of tBid was observed. Addition of 20 nM tBid also abolished, if not completely, the association between Bcl-xL and Bak or Bax (Fig. 4). In contrast, adding the same concentration of full-length Bid had little effect on Bcl-xL binding of Bak or Bax (Fig. 4). These results indicated that tBid can bind to Bcl-xL

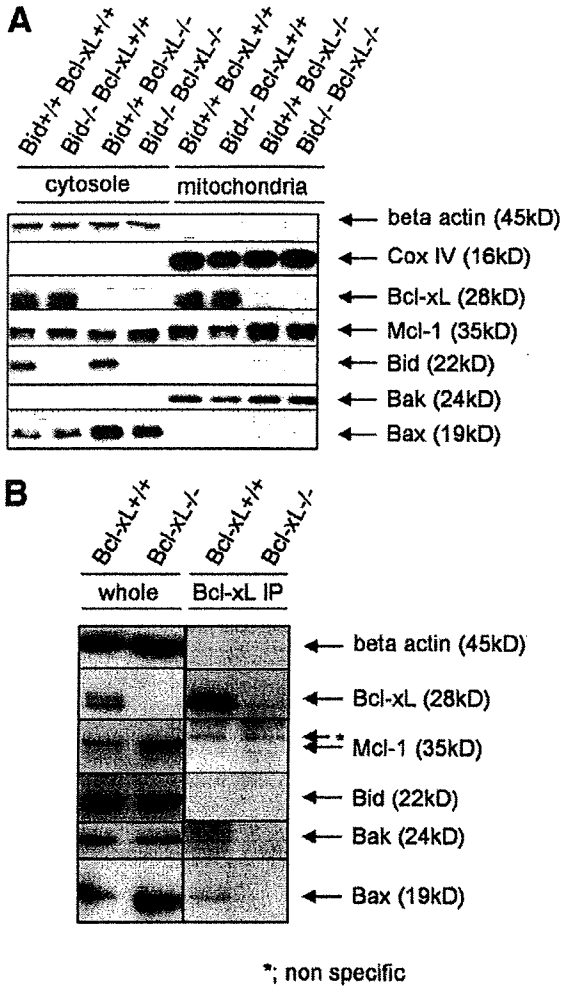

Fig. 3. Expression of Bcl-2-related molecules in the liver and their association with Bcl-xL. Bcl-xL+/+ stands for *bcl-x*$^{flox/flox}$ without *AlbCre*, and Bcl-xL−/− stands for *bcl-x*$^{flox/flox}$ with *AlbCre*. (A) Western blot after cellular fractionations of the liver lysate. Loading amounts of cytosolic and mitochondrial fractions were adjusted to be equivalent for the starting liver samples. (B) Western blot after anti-Bcl-xL immunoprecipitation. Whole cellular lysate and immunoprecipitates with anti-Bcl-xL were verified with the indicated antibodies. Samples from Bcl-xL−/− mice were included as a negative control.

and suggest that tBid binding of Bcl-xL unleashes Bak or Bax from Bcl-xL.

A Small But Significant Level of tBid Is Detected in the Healthy Liver. Genetic evidence that Bid is required for Bak/Bax-dependent apoptosis caused by Bcl-xL deficiency and biochemical evidence that full-length Bid is inactive for displaeing Bak or Bax from Bcl-xL together suggest that tBid is produced in wild-type liver. To confirm this, we performed western blot analysis using antibody that can detect tBid (Fig. 5A). Liver lysate from Bid KO mice served as a negative control, whereas that from wild-type mice injected with anti-Fas antibody served as a positive control. A significant level of tBid was detected in wild-type liver, although the amount was smaller than in Fas-stimulated mice, which displayed massive live cell apoptosis.

Bcl-xL–Deficient Mitochondria Are Susceptible to a Trace Amount of tBid. To examine the impact of a small amount of tBid on Bcl-xL–deficient mitochondria, tBid or full-length Bid at various concentrations was incubated with mitochondria isolated from Bcl-xL–deficient liver or wild-type liver (Fig. 5B). In agreement with previous reports,[19] wild-type mitochondria efficiently released cytochrome c on exposure to tBid. Full-length Bid was far less effective at releasing cytochrome c. Importantly, Bcl-xL–deficient mitochondria were capable of releasing cytochrome c on exposure to a smaller amount of tBid than wild-type mitochondria. This agrees with the in vivo findings that Bcl-xL–deficient hepatocytes, but not wild-type hepatocytes, underwent apoptosis with a trace amount of tBid.

Administration of ABT-737 Produces ALT Elevation in Wild-Type Mice But to a Lesser Extent in Bid KO Mice. Bcl-2–like molecules have been receiving attention as a target for inducing apoptosis, especially in cancer cells.[20] A variety of BH3 mimetics that interact with the hydrophobic groove of anti-apoptotic Bcl-2 proteins has been developed. They inhibit binding of anti-apoptotic Bcl-2–like molecules with BH3-only proteins and presumably with Bak and Bax. ABT-737, a prototype of this class of agents, was designed to mimic the BH3-only protein Bad and can inhibit the function of Bcl-2, Bcl-xL, or Bcl-w but not that of Mcl-1.[21] Our data on Bcl-xL KO mice raised the possibility that pharmacological inhibition of Bcl-xL may cause hepatocyte apoptosis. To examine this possibility, we injected ABT-737 and examined the liver injury. As expected, the levels of ALT

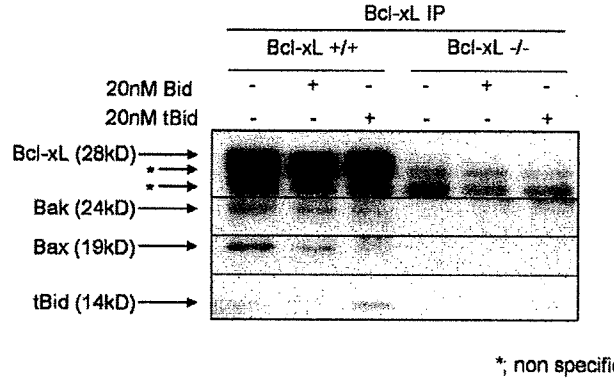

Fig. 4. tBid binds to Bcl-xL and displaces Bak or Bax from Bcl-xL. Liver lysate from *bcl-x*$^{flox/flox}$ without *AlbCre* (Bcl-xL+/+) and *bcl-x*$^{flox/flox}$ with *AlbCre* (Bcl-xL−/−) were incubated with or without 20 nM recombinant tBid or recombinant full-length Bid at 37°C for 20 minutes. After immunoprecipitation with Bcl-xL, immunoprecipitates are verified with indicated antibodies. Immunoprecipitated lysate from Bcl-xL−/− mice was loaded as a negative control.

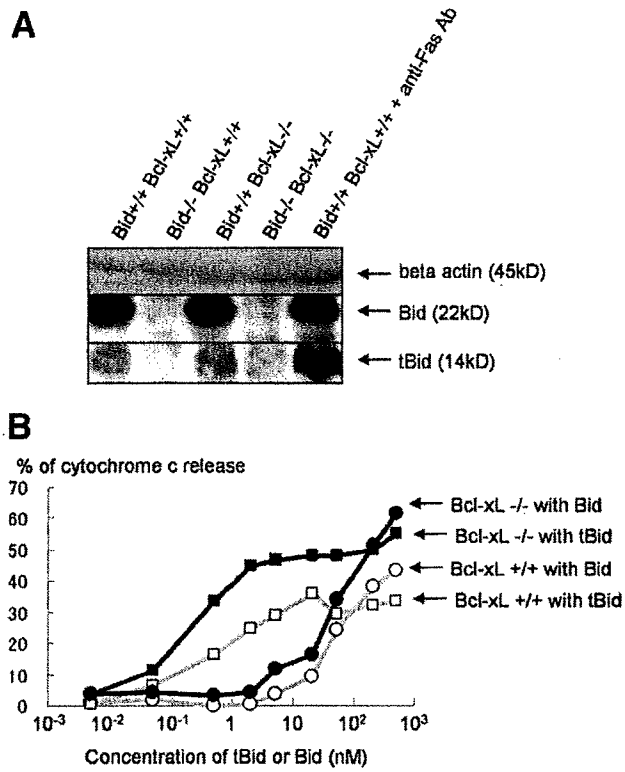

Fig. 5. A small amount of tBid is expressed in wild-type liver and is sufficient for producing cytochrome c release from Bcl-xL–deficient mitochondria. (A) Western blot of liver lysate for Bid and tBid expression. Lysate from wild-type (Bid+/+ Bcl-xL+/+) mice 1 hour after intravenous injection of 10 μg anti-Fas antibody (clone Jo2) and from Bid−/− mice were included as a positive and a negative control of tBid, respectively. (B) Mitochondrial release of cytochrome c to tBid. Mitochondria were isolated from Bcl-xL–deficient or wild-type liver and incubated with recombinant tBid or recombinant full-length Bid at various concentrations for 30 minutes. Similar results were obtained in three times repeated experiments.

were clearly elevated in wild-type mice (Fig. 6A). TUNEL staining of the liver section showed apoptosis in hepatocytes scattered in the liver lobule (Fig. 6B). Importantly, no significant elevation of serum ALT levels was observed with a Bak/Bax double-KO background. The data indicated that genetic and pharmacological ablation of Bcl-xL led to a similar apoptosis phenotype in the liver.

To examine the impact of Bid in ABT-737–induced hepatocyte apoptosis, ABT-737 was administered to wild-type mice and Bid KO mice. Elevation of serum ALT levels was ameliorated with a Bid KO background (Fig. 6C). It has been well established that administration of ABT-737 led to acute thrombocytopenia.[22] This was explained by the fact that Bcl-xL is a critical apoptosis antagonist in platelets.[10] In our experiment, the counts of circulating platelets declined significantly in the wild-type mice (Fig. 6D), which is in the agreement with previous studies.[10] Interestingly, a similar degree of thrombocytopenia was observed even in Bid KO mice, suggesting that Bid does not play a significant role in regulating platelet homeostasis, unlike in hepatocytes. The data imply that the impact of Bid in the Bcl-2 network in healthy cells is cell-type specific.

Discussion

One of the important findings of the current study is that the BH3-only protein Bid is an essential molecule for apoptosis of differentiated hepatocytes caused by Bcl-xL deficiency. This is surprising, because differentiated hepatocytes are generally considered to be quiescent cells. Organ homeostasis may be ensured in two ways: one is through turnover of cells, and the other is by the quies-

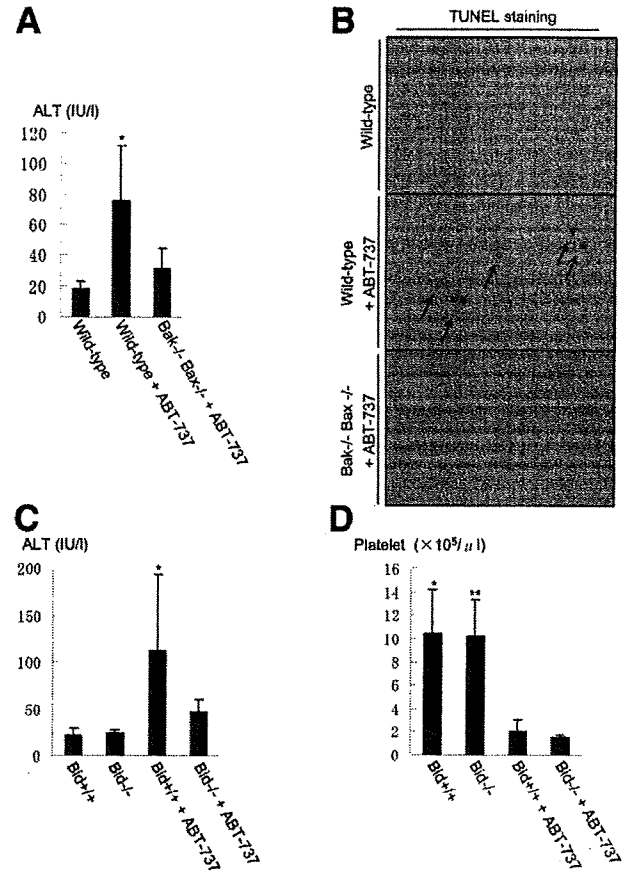

Fig. 6. ABT-737 administration in wild-type, Bak/Bax double-KO, and Bid KO mice. (A and B) Wild-type mice or hepatocyte-specific Bak/Bax double-KO mice were challenged with intraperitoneal injection of ABT-737 at 100 mg/kg or vehicle alone and sacrificed 16 hours later. Serum ALT levels (A) and representative pictures of TUNEL staining in the liver (B) are shown. N = 5 or more than 5 mice per group. *$P < 0.05$ versus the other two groups. (C and D) Wild-type mice or Bid KO mice were challenged with intraperitoneal injection of ABT-737 at 100 mg/kg or vehicle alone and sacrificed 16 hours later. Serum ALT levels (C) and circulating platelet counts (D) were determined. N = 5 or more than 5 mice per group. *$P < 0.05$ versus the other three groups for (C); * and **$P < 0.05$ versus the other two groups, with ABT-737 for (D).

cence of matured cells. Typical examples for the former are hematopoietic organs, intestine and skin, whereas those for the latter are a variety of solid organs, such as the liver, lung, pancreas, heart, and brain. Because hematopoietic cells die at particular time points to maintain host homeostasis, it would not be surprising that their life span may be controlled by a variety of death signals. Indeed, Bim KO mice have excess hematopoietic cells, particularly lymphocytes, suggesting that Bim strictly controls homeostasis of hematopoietic cells.[23] In contrast, healthy cells in the solid organs are usually considered to not suffer from apoptotic stimuli. Although interaction between core Bcl-2 proteins and BH3-only proteins is important for understanding apoptosis regulation, little work has been done by generating mice simultaneously deficient in molecules of both groups. To the best of our knowledge, the only example clearly using this approach is a study on Bim/Bcl-2 double-KO mice that showed that growth retardation, skin abnormality, and lymphoid cell reduction found in Bcl-2 KO mice were ameliorated with a Bim-deficient background.[24] This suggested that lymphoid cells constitutively sense Bim-mediated killing signals, and, without Bcl-2, decrease in number. The current study is the first demonstration that parenchymal cells in a solid organ such as differentiated hepatocytes also suffer from Bid-mediated BH3 stress.

Bid is ubiquitously expressed in many cell types. Generally, Bid is inactive for death induction and is activated on proteolytic cleavage by caspase-8 or other proteases. In the current study, we found that not only full-length Bid but also tBid could be detected in wild-type liver. Administration of tBid, but not that of full-length Bid, at 20 nM in wild-type liver lysate or mitochondria was sufficient for unleashing Bak or Bax from Bcl-xL and releasing cytochrome c. Conversely, a lesser amount of tBid (for example, at 2 nM) was sufficient for inducing cytochrome c release from Bcl-xL–deficient mitochondria. These results are consistent with the idea that a small amount of tBid produced in the liver could activate cytochrome c release and apoptosis in hepatocytes of the Bcl-xL–deficient mice. What mechanisms are involved in the production of tBid from full-length Bid in the healthy liver is not known yet. Our results suggest that Myd88 and TNF-α may not be involved in the activation of tBid under physiological conditions. However, other ligation of death receptors such as Fas, and TNF-related apoptosis-inducing ligand receptor, can cause caspase-8 activation followed by Bid cleavage.[15,25] Bile salts, which are consistently produced in and secreted from hepatocytes, are capable of inducing hepatocyte apoptosis through Fas activation.[26] Natural killer cells are predominant lymphocytes accumulating in the liver and constitutively express TNF-related apoptosis-inducing ligand.[27] Further study is needed to examine what kinds of stresses activate the Bid pathway in a physiological setting.

Adult differentiated hepatocytes express at least two anti-apoptotic Bcl-2 proteins, Bcl-xL and Mcl-1, but not prototype Bcl-2.[8] Recently, Vick et al.[28] reported that hepatocyte-specific Mcl-1 KO mice developed naturally occurring apoptosis in hepatocytes. We also independently generated hepatocyte-specific Mcl-1 KO mice and obtained an apoptosis phenotype that could not be distinguished from that of hepatocyte-specific Bcl-xL KO mice.[29] Thus, Mcl-1, like Bcl-xL, plays a critical role in maintaining integrity of differentiated hepatocytes. There are two major models regarding how BH3-only proteins mediate Bak/Bax-dependent apoptosis: a direct model and an indirect model.[30] From the viewpoint of the indirect model, our data would mean a small amount of tBid is sequestered by Bcl-xL and Mcl-1 and, without Bcl-xL, is sufficient for neutralizing Mcl-1 to promote apoptosis. Conversely, from the viewpoint of the direct model, both Bcl-xL and Mcl-1 are needed to completely sequester a small amount of tBid, and without Bcl-xL, unleashed tBid would directly activate Bak and Bax. In the current study, we observed that tBid when administered in liver lysate could bind to Bcl-xL. This observation seems to agree with the indirect model, although we could not exclude the possibility of the direct model. Further study will be needed by developing Bid/Bcl-xL/Mcl-1 KO mice to examine the underlying mechanisms of how activated Bid regulates the mitochondrial pathway of apoptosis in the liver.

Malignant tumors frequently overexpress one or more members of the anti-apoptotic Bcl-2 family, which confers the resistance of tumor cells to apoptosis.[31,32] Recently, small molecules targeting specific anti-apoptotic Bcl-2 family proteins have been developed for treatment of cancer therapy.[33,34] The underlying concept of this strategy is the difference in addiction to anti-apoptotic Bcl-2 family proteins between normal cells and transformed cells. In general, normal cells are not considered to suffer from apoptotic stimuli or to have activated BH3-only proteins. In contrast, transformed cells suffer from a variety of apoptotic stimuli such as genotoxic *p53* activation and environmental stresses, and possess activated BH3-only molecules. If a single anti-apoptotic Bcl-2 protein is neutralized by a small molecule, it could release BH3-only molecules, which then neutralize other anti-apoptotic Bcl-2 proteins or directly activate Bax-like molecules, leading to cell death. However, the current study clearly indicated that normal hepatocytes could be under activation of Bid, raising concern that hepatocyte injury may be produced if Bcl-xL function is completely knocked down. Indeed, we have shown that administra-

tion of a high dose of ABT-737, which is an antagonist for Bcl-xL/Bcl-2, not for Mcl-1,[21] induced Bak/Bax-dependent hepatocyte apoptosis in wild-type mice but to a lesser extent in Bid KO mice. Therefore, special caution should be paid to hepatotoxicity when systemically administering a high dose of Bcl-xL–targeting molecules, because hepatocytes are suffering from Bid-mediated stresses.

In conclusion, we have demonstrated here that the BH3-only protein Bid is activated and antagonized by anti-apoptotic Bcl-2 family proteins under physiological conditions. BH3 stress or Bcl-2 addiction is not a unique characteristic of tumor cells. Even in healthy cells, cellular integrity is not controlled by a simple rheostat between Bax-like molecules and Bcl-2–like molecules. The current study reveals a previously unrecognized complicated network of Bcl-2 family proteins controlling the integrity of healthy cells. Dissection of the Bcl-2 network will be important for further understanding of liver pathophysiology.

Acknowledgment: The authors thank Abbott Laboratories for providing ABT-737.

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Mcl-1 and Bcl-xL Cooperatively Maintain Integrity of Hepatocytes in Developing and Adult Murine Liver

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Anti-apoptotic members of the Bcl-2 family, including Bcl-2, Bcl-xL, Mcl-1, Bcl-w and Bfl-1, inhibit the mitochondrial pathway of apoptosis. Bcl-xL and Mcl-1 are constitutively expressed in the liver. Although previous research established Bcl-xL as a critical apoptosis antagonist in differentiated hepatocytes, the significance of Mcl-1 in the liver, especially in conjunction with Bcl-xL, has not been clear. To examine this question, we generated hepatocyte-specific Mcl-1–deficient mice by crossing *mcl-1*$^{flox/flox}$ mice and *AlbCre* mice and further crossed them with *bcl-x*$^{flox/flox}$ mice, giving Mcl-1/Bcl-xL–deficient mice. The *mcl-1*$^{flox/flox}$ *AlbCre* mice showed spontaneous apoptosis of hepatocytes after birth, as evidenced by elevated levels of serum alanine aminotransferase (ALT) and caspase-3/7 activity and an increased number of terminal deoxynucleotidyl transferase-mediated 2′-deoxyuridine 5′-triphosphate nick-end labeling (TUNEL)-positive cells in the liver; these phenotypes were very close to those previously found in hepatocyte-specific Bcl-xL–deficient mice. Although *mcl-1*$^{flox/+}$ *AlbCre* mice did not display apoptosis, their susceptibility to Fas-mediated liver injury significantly increased. Further crossing of Mcl-1 mice with Bcl-xL mice showed that *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/+}$ *AlbCre* mice also showed spontaneous hepatocyte apoptosis similar to Bcl-xL–deficient or Mcl-1–deficient mice. In contrast, *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/+}$ *AlbCre*, *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/flox}$ *AlbCre*, and *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/flox}$ *AlbCre* mice displayed a decreased number of hepatocytes and a reduced volume of the liver on day 18.5 of embryogenesis and rapidly died within 1 day after birth, developing hepatic failure evidenced by increased levels of blood ammonia and bilirubin. *Conclusion:* Mcl-1 is critical for blocking apoptosis in adult liver and, in the absence of Bcl-xL, is essential for normal liver development. Mcl-1 and Bcl-xL are two major anti-apoptotic Bcl-2 family proteins expressed in the liver and cooperatively control hepatic integrity during liver development and in adult liver homeostasis in a gene dose-dependent manner. (HEPATOLOGY 2009;50:1217-1226.)

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Abbreviations: ALT, alanine aminotransferase; PCR, polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction; TNF-α, tumor necrosis factor alpha; TUNEL, terminal deoxynucleotidyl transferase-mediated 2′-deoxyuridine 5′-triphosphate nick-end labeling.

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Received January 31, 2009; accepted June 8, 2009.

Supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (to T. Tak.).

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Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.23126

Potential conflict of interest: Nothing to report.

Additional Supporting Information may be found in the online version of this article.

The mitochondrial pathway of apoptosis is regulated by the Bcl-2 family proteins.[1,2] They are functionally divided into two basic groups: pro-apoptotic and anti-apoptotic members. Pro-apoptotic members are further divided into multi-domain members, such as Bax and Bak, and BH3-only proteins. Bax/Bak triggers release from mitochondria of cytochrome c, presumably by forming pores at the mitochondrial outer membrane. Cytochrome c released into the cytosol activates multiple caspases, which cut a variety of cellular substrates and dismantle the cell.[3] The release of Bax/Bak–mediated cytochrome c is considered to be a point of no return and a commitment to cell death.[4] Killing by BH3-only proteins, such as Bid, Bim, or Puma, requires Bax or Bak, placing them upstream of Bak/Bax activation. BH3-only proteins are transcriptionally or posttranslationally activated by a variety of cellular stresses. They are considered to be sensors that transmit apoptotic stimuli to mitochondria. Anti-apoptotic members, including Bcl-2, Bcl-xL, Mcl-1, Bcl-w, and Bfl-1, inhibit the mitochon-

drial pathway of apoptosis either by directly blocking Bak/Bax activation or by sequestering BH3-only proteins from Bak or Bax.

Mcl-1 has increasingly attracted attention because of its role in liver disease. Several reports have shown that Mcl-1 is overexpressed in a subset of human hepatocellular carcinomas and provides apoptosis resistance.[5-7] The multi-kinase inhibitor sorafenib, which was recently approved by the Food and Drug Administration as a chemotherapeutic agent for hepatocellular carcinoma,[8] is capable of down-regulating Mcl-1 expression and producing apoptosis in hepatoma cells.[9] Cycloxygenase 2 or hepatocyte growth factor up-regulates Mcl-1 expression in hepatocytes and improves Fas-mediated liver injury.[10,11] Recently, enforced expression of Mcl-1 was reported to reduce liver injury induced by anti-Fas injection in mice.[12] However, little is known about the physiologic significance of Mcl-1 in hepatocytes.

We previously reported that hepatocyte-specific Bcl-xL knockout mice were born and grew up but developed spontaneous hepatocyte apoptosis, identifying Bcl-xL as a critical apoptosis antagonist in hepatocytes.[13] This raises a question of whether other anti-apoptotic Bcl-2 family members, such as Mcl-1, have a significant role in regulating hepatocyte apoptosis and what the relationship is among those molecules. To this end, in the current study, we generated hepatocyte-specific Mcl-1 knockout as well as Bcl-xL/Mcl-1 double knockout mice and found that, like Bcl-xL, Mcl-1 is critical for maintaining hepatocyte integrity in adult liver, but not essential for liver development. However, both deficiencies cause a severe defect in liver development and lethality during the early neonatal period because of severe hepatic failure. The current study identifies Bcl-xL and Mcl-1 as two major anti-apoptotic Bcl-2 family proteins in the liver and demonstrates their gene dose–dependent effects for controlling hepatic integrity.

Materials and Methods

Mice. Mice carrying the *mcl-1* gene encoding amino acids 1 through 179 flanked by 2 *lox*P (*mcl-1*$^{flox/flox}$) were provided by Dr. You-Wen He of Duke University.[14] Mice carrying a *bcl-x* gene with two *lox*P sequencers at the promoter region and a second intron (*bcl-x*$^{flox/flox}$) were described previously.[15] Heterozygous AlbCre transgenic mice expressing Cre recombinase gene under the promoter of the albumin gene were described previously.[13] We generated hepatocyte-specific Mcl-1 knockout mice (*mcl-1*^{floxfloox} *AlbCre*) by mating *mcl-1*$^{flox/flox}$ and *AlbCre* mice. We then used these knockout mice to generate hepatocyte-specific Bcl-xL/Mcl-1 knockout mice (*bcl-x*$^{flox/flox}$ *mcl-1*^{floxflox} *AlbCre*) by mating them with *bcl-x*$^{flox/flox}$ mice. Traditional Bid knockout mice were described previously.[16] They were maintained in a specific pathogen–free facility and treated with humane care under approval from the Animal Care and Use Committee of Osaka University Medical School.

Genotyping. Genomic DNA was extracted from the tail and subjected for polymerase chain reaction (PCR) for genotyping mice. The primers used were as follows: 5′-GCCACCTCATCAGTCGGG-3′ and 5′-TCAGAAGCCGCAATATCCCC-3′ for the *bcl-x* allele; 5′-GGTTCCCTGTCTCCTTACTTACTGTAG-3′ and 5′-CTCCTAACCACTGTTCCTGACATCC-3′ for the *mcl-1* allele; 5′-GCGGTCTGGCAGTAAAAACTATC-3′, 5′-GTGAAACAGCATTGCTGTCACTT-3′, 5′CTAGGCCACAGAATTGAAAGATCT-3′ 5′-GTAGGTGGAAATTCTAGCATCATCC-3′ for the *AlbCre* allele; 5′-CCGAAA TGTCCCATAAGAG-3′, 5′-GAGATGGACCACAACATC-3′, and 5′ TGCTACTTCCATTTGTCACGTCCT-3′ for the *bid* allele. PCR products were electrophoretically separated using 2% agarose gels. The expected sizes of the PCR products were as follows: 165 bp for the wild-type *bcl-x* allele, 195 bp for the floxed *bcl-x* allele, 200 bp for the wild-type *mcl-1* allele, 300 bp for the floxed *mcl-1* allele, 130 bp for the wild-type *bid* allele, and 350 bp for the *bid* knockout allele. *AlbCre*-negative mice showed a 350-bp band, and heterozygous *AlbCre* mice showed 100-bp and 350-bp double bands.

Apoptosis Assay. To measure serum ALT level and caspase-3/7 activity, blood was collected from the inferior vena cava of mice and centrifuged. Serum was stored at −20°C until use. Serum ALT levels were measured by a standard method at Oriental Kobo Life Science Laboratory (Nagahama, Japan), and serum caspase-3/7 activity was measured by a luminescent substrate assay for caspase-3 and caspase-7 (Caspase-Glo assay, Promega, Tokyo, Japan). For histological analysis, livers were formalin-fixed, embedded in paraffin, and thin sliced. The liver sections were stained with hematoxylin-eosin. To detect cells with oligonucleosomal DNA breaks, the sections were also subjected to terminal deoxynucleotidyl transferase-mediated 2′-deoxyuridine 5′-triphosphate nick-end labeling (TUNEL) staining, according to a previously reported procedure.[17] For Fas-stimulating study, anti-Fas antibody (Jo2 clone) (PharMingen, San Diego, CA) was intraperitoneally injected into mice 3 hours before sacrifice.

Western Blot Analysis. Approximately 25 mg liver tissues was lysed with a lysis buffer (1% NP-40, 0.5%

sodium deoxycholate, 0.1% sodium dodecyl sulfate and 1× protein inhibitor cocktail (Nacalai tesque, Kyoto, Japan), phosphate-buffered saline; pH 7.4). After incubation on ice for 15 minutes, the lysate was centrifuged at 10,000*g* for 15 minutes at 4°C. The protein content of the supernatants was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Equal amounts of protein were electrophoretically separated by sodium dodecyl sulfate polyacrylamide gels (8% or 12%) and transferred onto polyvinylidene fluoride membrane. For immunodetection, the following antibodies were used: anti–Bcl-xL antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti–Mcl-1 antibody (Rockland, Gilbertsville, PA), anti-Bax antibody (Cell Signaling Technology, Beverly, MA), anti-Bid antibody (Cell Signaling Technology), anti-albumin antibody (Affinity Bioreagents, Golden, CO), and anti–beta actin antibody (Sigma-Aldrich, Saint Louis, MO). Detection of immunolabeled proteins was performed using a chemiluminescent substrate (Pierce).

Neonate Analysis. Neonatal mice delivered by cesarean section were suckled by a surrogate mother and sacrificed at 10 hours after birth. Blood from the neonatal mice was centrifuged, and the plasma was stored at −20°C until use. The levels of total bilirubin and ammonia were measured by Van den Bergh reaction and a standard enzymatic procedure, respectively, at Oriental Kobo Life Science Laboratory.

Real-Time Reverse-Transcription PCR. Total RNA was prepared from liver tissue using RNeasy kit (QIAGEN, Tokyo, Japan). For complementary DNA synthesis, 1 μg total RNA was reverse-transcribed using the High Capacity RNA-to-DNA Master Mix (Applied Biosystems, Foster City, CA). Complementary DNA, equivalent to 40 ng RNA, was used as a template for real-time reverse-transcription PCR (RT-PCR) using an Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems). The messenger RNA expressions of tumor necrosis factor alpha (TNF-α), collagen-alpha1(I), and transthyretin were measured using TaqMan Gene Expression Assays (Assay ID: Mm00443260_g1, Mm00801666_g1, and Mm00443267_m1, respectively), and were corrected with the quantified expression level of beta-actin messenger RNA measured using TaqMan Gene Expression Assays (Assay ID: Mm02619580_g1).

Statistical Analysis. Data are presented as mean ± standard deviation. Comparisons between two groups were performed by unpaired *t* test. Multiple comparisons were performed by analysis of variance followed by Scheffe *post hoc* correction. $P < 0.05$ was considered statistically significant.

Results

Hepatocyte-Specific Mcl-1 Deficiency Leads to Spontaneous Hepatocyte Apoptosis in the Adult Liver. To generate hepatocyte-specific Mcl-1–deficient mice, floxed *mcl-1* mice were crossed with heterozygous *AlbCre* mice. After *mcl-1*$^{flox/+}$ *AlbCre* mice were mated with *mcl-1*$^{flox/+}$ mice, and offspring were screened for genotyping and Mcl-1 expression. *mcl-1*$^{flox/flox}$ *AlbCre* mice were born and grew up. Their expression in the liver of Mcl-1 was greatly reduced compared with that of wild-type mice (Fig. 1A). The levels of Bcl-xL expression did not change in *mcl-1*$^{flox/flox}$ *AlbCre* liver. Bcl-xL and Mcl-1 proteins migrated as typical doublet bands of which the biochemical nature had been previously determined.[18] The trace amount of Mcl-1 expression found in the knockout liver may have been attributable to expression in nonparenchymal cells, as previously observed in hepatocyte-specific Bcl-xL–deficient mice.[13]

To investigate the significance of Mcl-1 in the liver, mice were sacrificed 6 weeks after birth and subjected to analysis of serum ALT levels and caspase-3/7 activity as well as liver histology and TUNEL staining. *mcl-1*$^{flox/flox}$ *AlbCre* mice displayed significantly higher levels of serum ALT than control mice (*AlbCre*-negative or *mcl-1*$^{+/+}$ *AlbCre* mice) (Fig. 1B). Hepatocytes with typical apoptosis morphology such as cellular shrinkage and nuclear condensation were frequently observed in the liver sections of *mcl-1*$^{flox/flox}$ *AlbCre* mice (Fig. 1C). Consistently, the number of cells with TUNEL positivity, a hallmark of apoptotic cell death, in the liver was significantly higher in *mcl-1*$^{flox/flox}$ *AlbCre* mice than in control mice (Fig. 1C). Activity of caspase-3/7, executioners of apoptosis, was significantly higher in circulation of *mcl-1*$^{flox/flox}$ *AlbCre* mice than in control mice, which might reflect activation of those proteases in the knockout liver (Fig. 1D). Bax expression was clearly increased in *mcl-1*$^{flox/flox}$ *AlbCre* mice, suggesting Bax activation being involved in the apoptosis in *mcl-1*$^{flox/flox}$ *AlbCre* mice (Fig. 1A). Furthermore, the expression of TNF-α and collagen-alpha1(I) was significantly increased in the *mcl-1*$^{flox/flox}$ *AlbCre* liver compared with the wild-type liver, as found in the Bcl-xL knockout liver (Fig. 1E). Taken together, hepatocyte-specific Mcl-1 knockout mice developed spontaneous apoptosis leading to sterile inflammation and fibrotic response in the liver, like hepatocyte-specific Bcl-xL knockout mice.[13]

Heterozygous Deletion of the mcl-1 Gene Does Not Produce Apoptosis But Increases the Susceptibility to Fas Stimulation. Although the levels of Mcl-1 expression were significantly decreased in *mcl-1*$^{flox/+}$ *AlbCre* liver (Fig. 1A, Supporting Fig. 1), *mcl-1*$^{flox/+}$ *AlbCre* mice did not have apoptosis phenotypes in the liver (Fig. 1B-D).

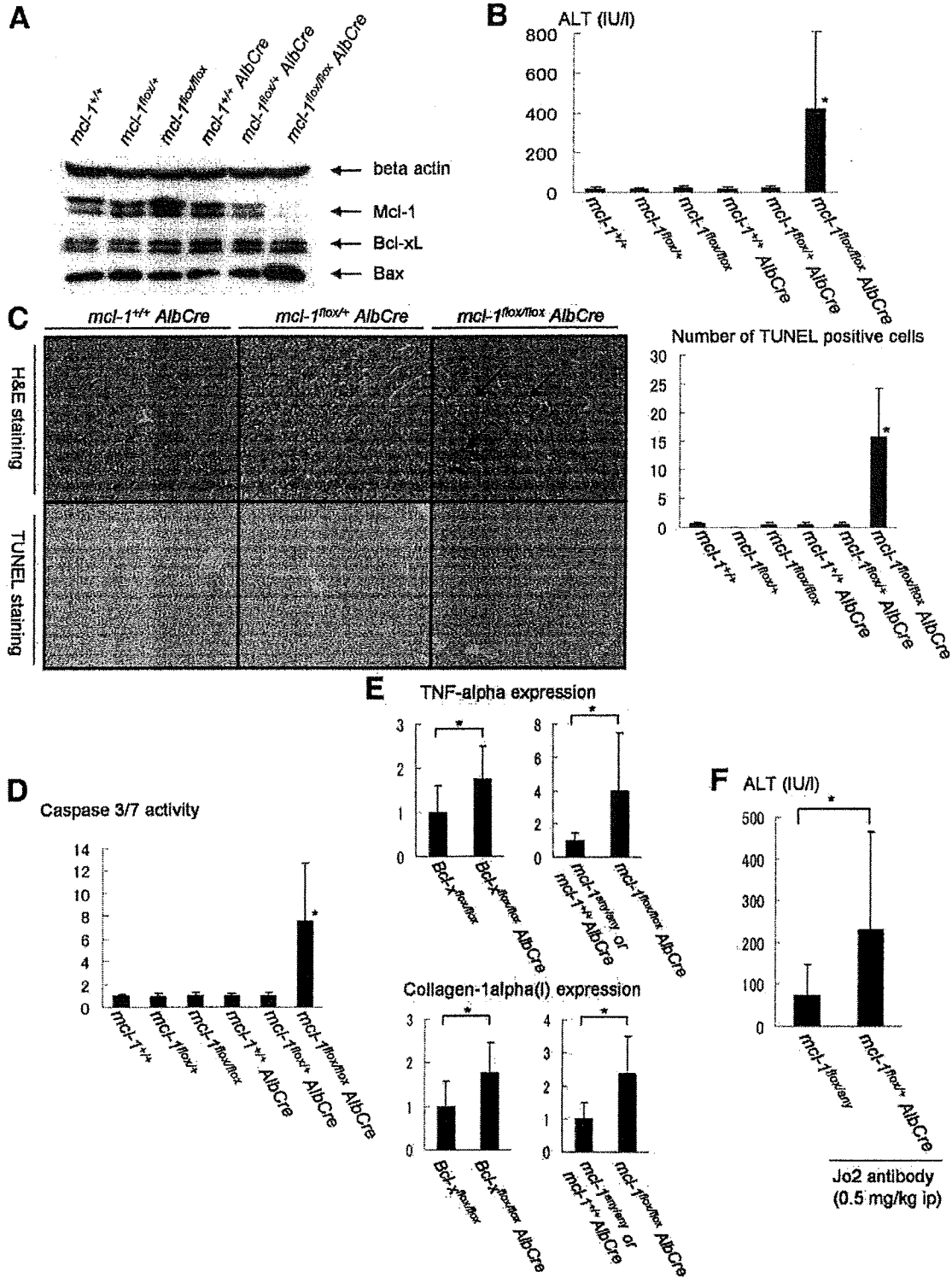

Fig. 1. Hepatocyte-specific Mcl-1 knockout mice. Offspring from mating of *mcl-1*$^{flox/+}$ *AlbCre* mice and *mcl-1*$^{flox/+}$ mice were sacrificed at the age of 6 weeks. (A) Western blot of whole liver lysate for the expression of Bcl-xL, Mcl-1, and Bax. (B) Serum ALT levels. N = 15 mice for each group. *$P < 0.05$ versus the other five groups. (C) Left panel shows hematoxylin-eosin and TUNEL staining of the liver section. Arrow indicates typical apoptotic cells. Right panel shows statistics of TUNEL-positive cells. The number of TUNEL-positive cells was determined in a defined area. N = 5 mice for each group. *$P < 0.05$ versus the other five groups. (D) Serum levels of caspase-3/7 activity. The levels were normalized to *mcl-1*$^{+/+}$ *AlbCre* (−) mice. N = 15 mice for each group. *$P < 0.05$ versus the other five groups. (E) Real-time RT-PCR analysis for TNF-α and collagen-1alpha(1) expression. *$P < 0.05$. N = 12 or 9. The levels were normalized to the wild-type mice. (F) Serum ALT levels of Fas-stimulated mice. The *mcl-1*$^{flox/+}$ *AlbCre* mice and *mcl-1*$^{flox/+ \text{ or } flox}$ mice were sacrificed 3 hours after intraperitoneal injection of 0.5 mg/kg Jo2 antibody. *$P < 0.05$. N = 13 or 7.

Therefore, we examined the susceptibility to Fas stimulation in these mice. We injected anti-Fas antibody into *mcl-1*$^{flox/+}$ *AlbCre* mice and *mcl-1*$^{flox/+ \text{ or } flox}$ mice and measured the levels of their serum ALT. *mcl-1*$^{flox/+}$ *AlbCre* mice displayed significantly higher levels of serum ALT than control mice (Fig. 1F). These findings suggest that haplo-deficiency of Mcl-1 does not produce apoptosis in a physiological setting but clearly reduces apoptosis resistance under pathological conditions.

Involvement of Bid in Apoptosis Caused by Mcl-1 Deficiency. BH3-only proteins regulate life and death balance by interacting with core Bcl-2 family members. The hepatocyte is a so-called type 2 cell, which requires Bid as a sensor for Fas-mediated apoptotic stresses.[19] In addition, it has been reported that the caspase-8/Bid pathway is involved in a variety of liver pathological conditions.[16,20] To examine the possibility of Bid being involved in hepatocyte apoptosis caused by Mcl-1 deficiency, we crossed hepatocyte-specific Mcl-1 knockout mice with Bid knockout mice. Offspring form mating of *bid*$^{+/-}$ *mcl-1*$^{flox/flox}$ *AlbCre* mice with *bid*$^{+/-}$ *mcl-1*$^{flox/flox}$ mice were sacrificed at 6 weeks after birth and subjected to analysis of apoptosis phenotypes. Mice with each genotype grew up, and, as expected, the levels of Bid and/or Mcl-1 expression in the liver were correspondingly reduced with their genotypes (Fig. 2A). The levels of serum ALT were significantly lower in *bid*$^{-/-}$ *mcl-1*$^{flox/flox}$ *AlbCre* mice than in *bid*$^{+/+}$ *mcl-1*$^{flox/flox}$ *AlbCre* mice (Fig. 2B). The results indicate that Bid was involved in hepatocyte apoptosis found in Mcl-1 knockout mice.

Combined Deficiency of Mcl-1 and Bcl-xL in Hepatocytes Causes Lethality. Phenotypes observed in hepatocyte-specific Mcl-1 knockout mice were very similar to those in hepatocyte-specific Bcl-xL knockout mice.[13] These results indicated that Bcl-xL and Mcl-1 share similar anti-apoptotic functions but do not compensate for the loss of each other. To examine whether their expression and function are completely nonredundant or just partially so, we generated hepatocyte-specific Bcl-xL/Mcl-1 double-knockout mice.

The *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/+}$ *AlbCre* mice were mated with *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/flox}$ mice, and genotypes of the offspring were screened at 3 weeks after birth. *AlbCre*-negative and *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/+}$ *AlbCre* mice were born and grew up, but not *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/+}$ *AlbCre*, *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/flox}$ *AlbCre*, and *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/flox}$ *AlbCre* mice (Table 1). The lack of Bcl-xL and Mcl-1 caused a more severe phenotype than either knockout, suggesting that they partially compensate for the loss of each other at least from the viewpoint of maintaining normal development.

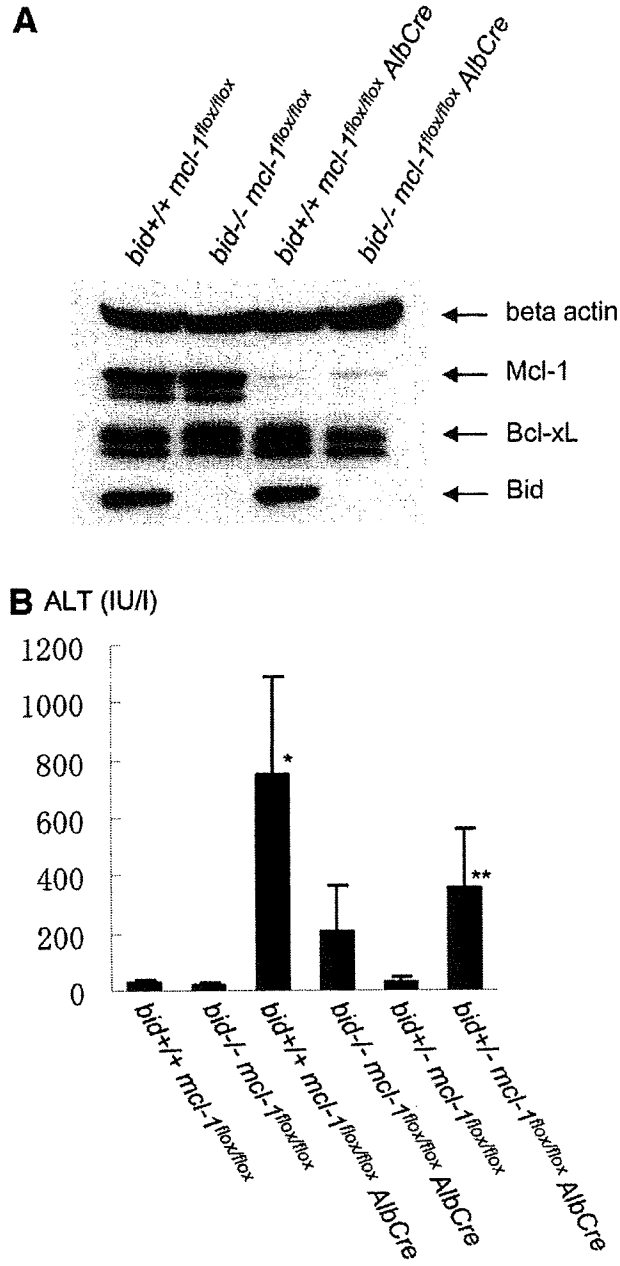

Fig. 2. Mcl-1/Bid double-knockout mice. Offspring from mating of *bid*$^{+/-}$ *mcl-1*$^{flox/flox}$ *AlbCre* mice with *bid*$^{+/-}$ *mcl-1*$^{flox/flox}$ mice were sacrificed at 6 weeks after birth. (A) Western blot of whole liver lysate for the expression of Mcl-1, Bcl-xL, and Bid. (B) Serum ALT levels. N = 12 mice for each group. *$P < 0.05$ versus the other five groups; **$P < 0.05$ versus the *AlbCre*-negative groups and the *bid*$^{+/+}$ *mcl-1*$^{flox/flox}$ *AlbCre* group.

Mice Lacking Single Alleles for Both Bcl-xL and Mcl-1 Develop Spontaneous Apoptosis in the Adult Liver Similar to Bcl-xL or Mcl-1 Knockout Mice. Offspring from mating of *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/+}$ *AlbCre and bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/flox}$ were sacrificed at 6 weeks after birth and subjected to analysis of Bcl-xL/Mcl-1 expression and

Table 1. Genotyping of Offspring Obtained by Crossing *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/+}$ *AlbCre* Mice and *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/flox}$ Mice

AlbCre	bcl-x	mcl-1	ED18.5	3 Weeks
−	flox/+	flox/+	4	14
−	flox/flox	flox/+	6	17
−	flox/+	flox/flox	12	17
−	flox/flox	flox/flox	7	17
+	flox/+	flox/+	11	22
+	flox/flox	flox/+	8	0
+	flox/+	flox/flox	9	0
+	flox/flox	flox/flox	10	0
	Total		67	87

ED, embryonic day.

Note that each genotype is expected to account for one-eighth of the offspring from this mating.

apoptosis phenotypes. As expected, *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/+}$ *AlbCre* liver expressed reduced levels of expression for both Bcl-xL and Mcl-1 (Fig. 3A). Interestingly, *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/+}$ *AlbCre* mice developed spontaneous hepatocyte apoptosis as evidenced by an increase in serum ALT levels and caspase-3/7 activity (Fig. 3B,C). In agreement with this, hepatocytes with typical apoptotic morphology and positive for TUNEL staining were found scattered in the liver lobules in these mice (Fig. 3D,E). Furthermore, *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/+}$ *AlbCre* mice showed higher expression of TNF-α than wild-type mice (Fig. 3F). The phenotypes were very similar to hepatocyte-specific Bcl-xL or Mcl-1knockout mice.

Hepatocyte-Specific Mcl-1/Bcl-xL-Deficient Mice Show Impaired Development of the Liver and Liver Failure During the Neonatal Period. To examine the impact of Bcl-xL/Mcl-1deficiency at an earlier time point, offspring obtained from crossing *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/+}$ *AlbCre* mice and *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/flox}$ mice were analyzed on gestational day 18.5. Live-obtained embryo followed expected Mendelian frequencies (Table 1). Overall, they looked normal, and their body weight did not differ among genotypes (Fig. 4A,B). However, the livers obtained from live pups with genotype of *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/+}$ *AlbCre*, *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/flox}$ *AlbCre*, or *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/flox}$ *AlbCre* were clearly smaller. The ratios of liver weight to body weight were significantly lower in those pups than in *AlbCre*-negative or *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/+}$ *AlbCre* pups (Fig. 4C). The ratios of liver weight to body weight were also examined in *mcl-1*$^{flox/flox}$ with *AlbCre* or without *AlbCre* mice, and there was no significant difference between the two (6.0 ± 0.8 versus 5.5 ± 0.9, $N = 5$, $P = 0.34$), excluding the possibility that Mcl-1 knockout itself affects the liver size at this time point. Histological analysis revealed that there were a number of hepatocytes with rectangular morphology and hematopoietic cells in the developing liver of the *AlbCre*-negative pups (Fig. 4D). Whereas the number of rectangular hepatocytes in *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/+}$ *AlbCre* livers was similar to that in the *AlbCre*-negative livers, it was lower in *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/+}$ *AlbCre*, *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/flox}$ *AlbCre*, and *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/flox}$ *AlbCre* livers. Rectangular cells were rarely observed in *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/flox}$ *AlbCre* livers. Furthermore, the expression of albumin and transthyretin was examined in the liver as a marker for hepatocyte differentiation.[21] Consistent with histological findings, both expressions were gradually reduced from the *AlbCre*-negative livers to the *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/flox}$ *AlbCre* livers (Fig. 4E,F).

We noticed that offspring obtained from crossing *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/+}$ *AlbCre* mice and *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/flox}$ mice frequently died within 1 day after birth. To examine the cause of the early neonatal death, offspring were sacrificed at 10 hours after birth. They were divided into two groups according to the data shown in Table 1: expected survivors including *AlbCre*-negative and *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/+}$ *AlbCre* pups, and expected nonsurvivors including *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/+}$ *AlbCre*, *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/flox}$ *AlbCre*, and *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/flox}$ *AlbCre* pups. The levels of total bilirubin and ammonia in circulation were determined and compared between the groups. Both blood bilirubin levels and ammonia levels were significantly higher in the expected nonsurvivors than in the expected survivors (Fig. 5A,B). These results suggested that *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/+}$ *AlbCre*, *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/flox}$ *AlbCre*, and *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/flox}$ *AlbCre* mice died quickly after birth because of hepatic failure, in agreement with the findings of impaired liver development.

Discussion

Five members of the anti-apoptotic Bcl-2 family have been found: Bcl-2, Bcl-xL, Bcl-w, Bfl-1, and Mcl-1. Traditional knockout of Bcl-2, a prototype of this family, displays growth retardation, hair color abnormality, lymphocyte decrease, and polycystic kidney.[22,23] In agreement with the finding that Bcl-2 is not expressed in hepatocytes,[13] these mice did not show any liver phenotypes. Similarly, Bcl-w[24,25] or Bfl-1 knockout mice[26] were generated but no liver phenotypes have been reported. Traditional knockout of Bcl-xL or Mcl-1 caused more severe phenotypes. Deletion of the *bcl-x* gene resulted in embryonic lethality because of abnormal neuronal development and hematopoiesis.[27] The *mcl-1* knockout embryo fails to be implanted *in utero*.[28] Thus, study of traditional knockout mice could not reveal the significance of Bcl-xL or Mcl-1 in the liver.

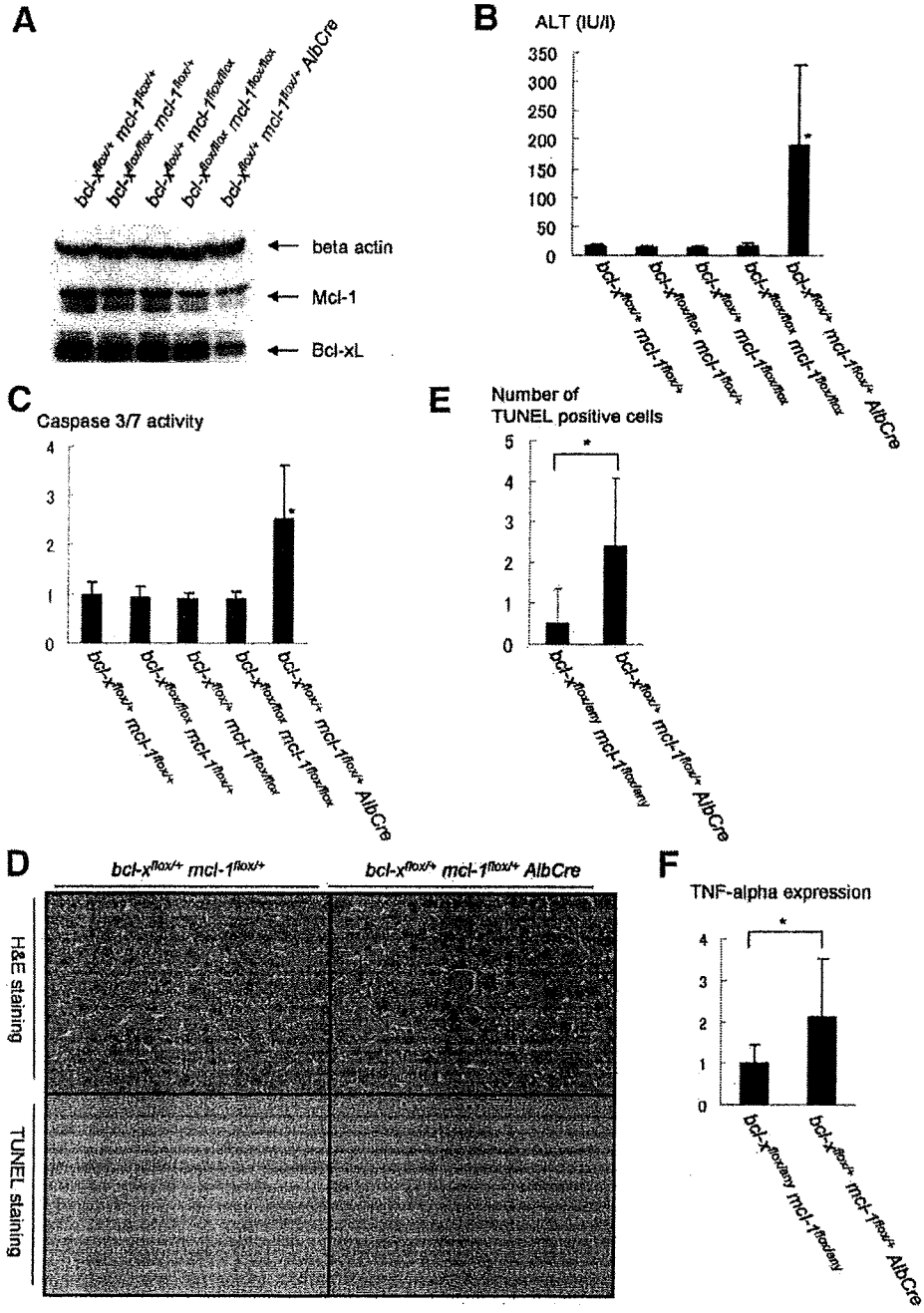

Fig. 3. Hepatocyte-specific Bcl-xL/Mcl-1-deficient mice. Offspring from mating *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/+}$ *AlbCre* mice and *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/flox}$ mice were sacrificed at the age of 6 weeks. (A) Western blot of whole liver lysate for the expression of Bcl-xL and Mcl-1. (B) Serum ALT levels. N = 9 mice for each group. *$P < 0.05$ versus the other five groups. (C) Serum levels of caspase-3/7 activity. The levels were normalized to *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/+}$ mice. N = 9 mice for each group. *$P < 0.05$ versus the other five groups. (D) Hematoxylin-eosin and TUNEL staining of the liver sections for *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/+}$ *AlbCre* mice. Findings for *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/+}$ mice are shown as a control. (E) Statistics of TUNEL-positive cells. The number of TUNEL-positive cells was determined in a defined area. N = 5 or 6. *$P < 0.05$. (F) RT-PCR analysis for TNF-α expression. The levels were normalized to the group of *bcl-x*$^{flox/+ \text{ or } flox}$ *mcl-1*$^{flox/+ \text{ or } flox}$. *$P < 0.05$. N = 9.

We previously reported that hepatocyte-specific knockout of Bcl-xL caused spontaneous apoptosis in hepatocytes after birth and established that Bcl-xL is critically important for the integrity of hepatocytes.[13] The current study demonstrated that Mcl-1 plays an anti-apoptotic role in differentiated hepatocytes similar to that of Bcl-xL. During the preparation of this manuscript, a report by Vick et al.[29] appeared on the Web, demonstrating a similar apoptosis phenotype in mice with specific knockout of the *mcl-1* gene in hepatocytes. Our findings

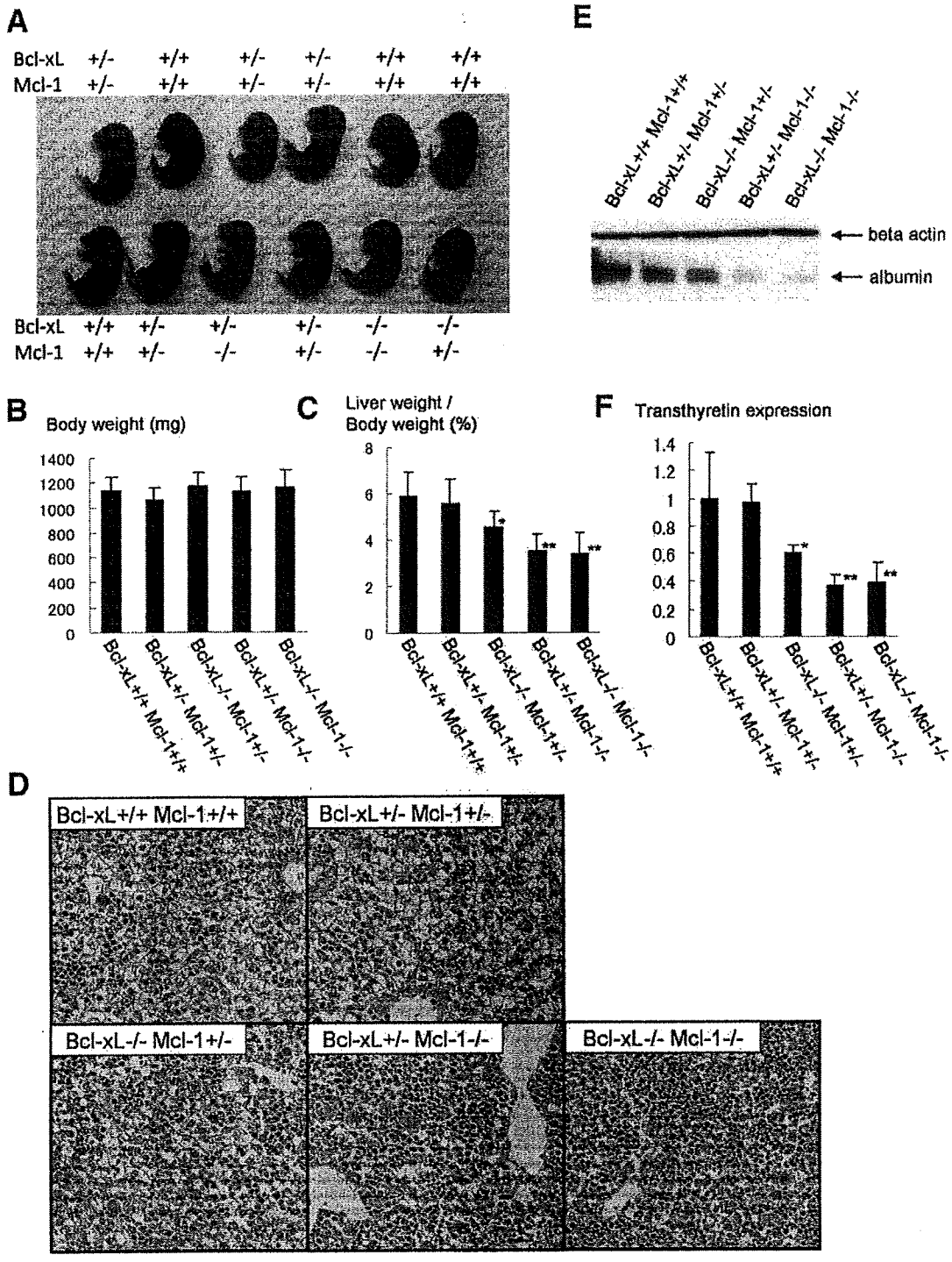

Fig. 4. Hepatocyte-specific Bcl-xL/Mcl-1-deficient embryos. Offspring from mating *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/+}$ *AlbCre* mice and *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/flox}$ mice were sacrificed on day 18.5 of gestation. Mice were classified into five groups. The *bcl-x*$^{flox/+ \text{ or } flox}$ *mcl-1*$^{flox/+ \text{ or } flox}$ are indicated by Bcl-xL +/+ Mcl-1 +/+; *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/+}$ *AlbCre* are indicated by Bcl-xL +/− Mcl-1 +/−; *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/+}$ *AlbCre* are indicated by Bcl-xL −/− Mcl-1 +/−; *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/flox}$ *AlbCre* are indicated by Bcl-xL +/− Mcl-1 −/−; *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/flox}$ *AlbCre* are indicated by Bcl-xL −/− Mcl-1 −/ −. The numbers of embryos analyzed were 30 for Bcl-xL +/+ Mcl-1 +/+, 11 for Bcl-xL +/− Mcl-1 +/−, 8 for Bcl-xL −/− Mcl-1 +/−, 9 for Bcl-xL +/− Mcl-1 −/−, and 10 for Bcl-xL −/− Mcl-1 −/−. (A) Gross appearance of embryos. Representative photo for a litter is shown. (B) Body weight. (C) The ratios of liver weight to body weight. *$P < 0.05$ versus Bcl-xL +/+ Mcl-1 +/+; **$P < 0.05$ versus Bcl-xL +/+ Mcl-1 +/+ and Bcl-xL +/− Mcl-1 +/−. (D) Hematoxylin-eosin staining of the liver sections. (E) Western blot of whole-liver lysate for albumin expression. (F) Real-time RT-PCR analysis for transthyretin expression. The levels were normalized to the group of Bcl-xL +/+ Mcl-1 +/+. *$P < 0.05$ versus Bcl-xL +/+ Mcl-1 +/+; **$P < 0.05$ versus Bcl-xL +/+ Mcl-1 +/+ and Bcl-xL +/− Mcl-1 +/−.

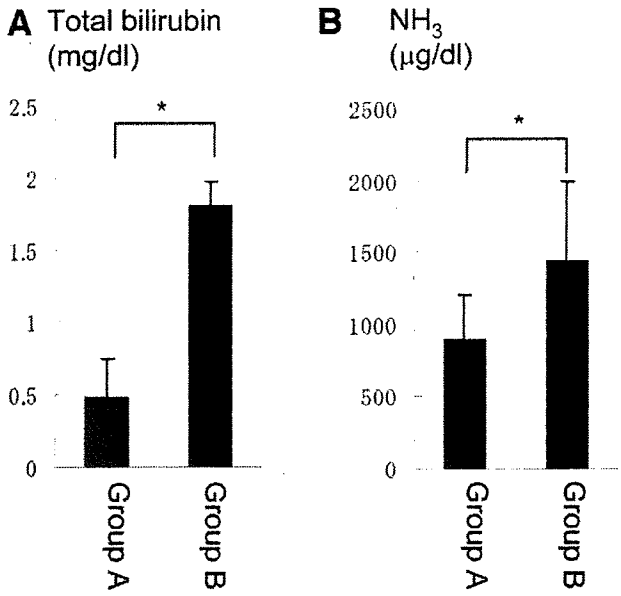

Fig. 5. Plasma biochemistry of hepatocyte-specific Bcl-xL/Mcl-1-deficient neonates 10 hours after birth. Group A (N = 13) was defined as expected survivors including *AlbCre*-negative mice and *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/+}$ *AlbCre* mice. Group B (N = 6) was defined as expected nonsurvivors including *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/+}$ *AlbCre*, *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/flox}$ *AlbCre*, *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/flox}$ *AlbCre*. (A) Plasma total bilirubin levels. *$P < 0.05$. (B) Plasma ammonia levels in both groups. *$P < 0.05$.

are in agreement with theirs and further provide evidence that deletion of a single allele for the *mcl-1* gene fails to produce apoptosis phenotypes under physiological conditions, as observed in knockout of the *bcl-x* gene.[13] Mcl-1 heterozygous disrupted mice did not produce apoptosis at least until 16 weeks of age (our unpublished data). It was demonstrated that hepatocyte-specific Mcl-1 knockout mice showed higher levels of liver injury than control mice on anti-Fas antibody injection.[29] However, because mice lacking both *mcl-1* alleles possess preexisting liver injury, it would be difficult to exactly compare liver injury after anti-Fas antibody injection and to conclude whether decreased Mcl-1 expression actually increases the susceptibility to Fas. In the current study, we took advantage of Mcl-1 heterozygous disrupted mice to address this point. They showed significantly higher levels of liver injury after Fas stimulation than wild-type mice, formally proving the significance of Mcl-1 expression under pathological conditions. Furthermore, our data on Mcl-1/Bid–deficient mice implies that the Bid pathway is involved in generating apoptosis found in Mcl-1 knockout mice. Because Bid mediates a variety of cellular stresses in hepatocytes upstream of Mcl-1,[30,31] it will be interesting in future study to determine what stresses generate hepatocyte apoptosis in Mcl-1 knockout mice.

Bcl-xL and Mcl-1 share similar structures and functions.[1] The observations that either deficiency similarly leads to spontaneous hepatocyte apoptosis imply that they play a non-redundant role in maintaining the integrity of hepatocytes in the adult liver. To further understand the relationship of both molecules, we generated hepatocyte-specific Bcl-xL/Mcl-1 knockout mice. Interestingly, mice lacking single alleles for both genes (*bcl-x*$^{+/-}$ *mcl-1*$^{+/-}$) induced spontaneous hepatocyte apoptosis that could not be distinguished from that found in Bcl-xL or Mcl-1 knockout mice. This indicates that, whereas knockout of a single allele of the *bcl-x* or *mcl-1* gene did not produce apoptosis, knockout of two alleles of any combination among both genes was sufficient to produce hepatocyte apoptosis. This finding suggests that both molecules are not independently but rather interdependently required for ensuring integrity of differentiated hepatocytes.

Bcl-xL/Mcl-1-deficient mice as well as mice only having a single allele of either *bcl-x* or *mcl-1* gene displayed a decreased number of hepatocytes and reduced liver size on day 18.5 of gestation and appeared to develop lethal liver failure within 1 day after birth. Because the liver contains a large number of hematopoietic cells during development (Fig. 4D), it is very difficult to determine the expression levels of Bcl-xL or Mcl-1 specifically in hepatocytes in each knockout mouse. Liver development begins on day 8.5 of gestation in the mouse when the liver primordium is delineated from the endoderm.[32] The albumin promoter, which is active in both hepatoblasts and hepatocytes, shows a 20-fold increase in transcriptional activity from day 9.5 to day 12.5 of gestation. The level of albumin then continues to increase as the liver develops simultaneously with the biliary tree and the hepatic bile duct being formed.[33] Thus, the target genes could probably be successfully deleted during embryogenesis in the *AlbCre* recombination system. The observation that Bcl-xL/Mcl-1–deficient mice developed severer phenotypes than Bcl-xL–deficient or Mcl-1–deficient mice supports the idea that Cre-mediated deletion of the target genes actually took place during embryogenesis in our model. In contrast to the knockout of two alleles, knockout of three alleles and more of the *bcl-x* and *mcl-1* genes induced lethal neonatal hepatic failure. Thus, hepatocyte integrity appeared to be strictly controlled by Bcl-xL and Mcl-1 in a gene dose–dependent manner.

Hepatocyte-specific deficiency of both Bcl-xL and Mcl-1 led to significant reduction of liver volume because of impaired hepatocyte development. However, overall, mice with these phenotypes were capable of developing normally until birth and rapidly developed liver failure and died within 1 day after birth. This finding suggests that differentiated hepatocytes are critically required for maintaining host homeostasis after birth but not during embryogenesis. The placenta

plays an important role in nutritional support and detoxification of the embryo. Our data imply that it could probably compensate for most functions of the liver cells during embryogenesis, whereas the liver would turn to the critical organ that is essential for maintaining host homeostasis after birth. Bcl-xL/Mcl-1 knockout mice provide interesting implications for the difference in the impact of differentiated hepatocytes between embryogenesis and the early neonatal period.

In conclusion, Mcl-1 and Bcl-xL are two major Bcl-2 family proteins inhibiting hepatocyte apoptosis. Together with previous work on traditional knockout mice, our data imply that other members, if any, could not compensate for their functions. Mcl-1 and Bcl-xL cooperatively maintain hepatocyte integrity during liver development and in adult liver homeostasis, and their effects are gene-dose dependent. Recent studies also have established that Mcl-1[5-7] and Bcl-xL[18,34] are frequently overexpressed and confer resistance to apoptosis in hepatocellular carcinoma. Therefore, Mcl-1 and Bcl-xL are important apoptosis antagonists in a variety of pathophysiological conditions of the liver.

Acknowledgment: We thank Dr. You-Wen He (Department of Immunology, Duke University Medical Center, Durham, NC) for providing the *mcl-1* floxed mice.

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J Gastroenterol 2008; 43:811–822
DOI 10.1007/s00535-008-2213-6

Journal of Gastroenterology

Review

Adipocytokines and liver disease

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Adipose tissue is a massive source of bioactive substances known as adipocytokines, including tumor necrosis factor (TNF)-α, resistin, leptin, and adiponectin. Recent advances in medical research view obesity as a chronic low-grade inflammatory state. Hypertrophied adipocytes in obesity release chemokines that induce macrophage accumulation in adipose tissue. Accumulated macrophages in obese adipose tissue produce proinflammatory cytokines and nitric oxide, and these inflammatory changes induce adipocytokine dysregulation. The latter is characterized by a decrease in insulin-sensitizing and anti-inflammatory adipocytokines, and an increase in proinflammatory adipocytokines. Adipocytokine dysregulation induces obesity-related metabolic disorders, the so-called metabolic syndrome. Metabolic syndrome is a cluster of metabolic abnormalities, including diabetes mellitus, hypertension, hyperlipidemia, and nonalcoholic steatohepatitis (NASH). Recent studies have revealed that obesity is an independent risk factor for chronic liver diseases, such as NASH, alcoholic liver disease, chronic hepatitis C, and hepatocellular carcinoma. A common mechanism underlying these hepatic clinical states is thought to be adipocytokine dysregulation. In this review, we discuss the association of adipocytokines, especially leptin, adiponectin, TNF-α, and resistin, with liver diseases.

Key words: nonalcoholic steatohepatitis (NASH), chronic hepatitis C, obesity, adipocytokine, adiponectin, leptin, TNF-α

Introduction

Adipose tissue is an energy-storing organ that produces and secretes several bioactive substances[1,2] known as adipocytokines,[3] such as adiponectin,[4] leptin,[5] resistin,[6] plasminogen activator inhibitor 1 (PAI-1),[3] and tumor necrosis factor α (TNF-α).[7] Recent studies have suggested that obesity is a state of chronic, low-grade inflammation that contributes to insulin resistance and type 2 diabetes.[8,9] Hypertrophied adipocytes in obesity release chemokines, which recruit macrophages, especially in visceral adipose tissue. Adipose tissue macrophages produce nitric oxide (NO) and inflammatory cytokines such as TNF-α, interleukin (IL)-6, and IL-1β. These inflammatory changes in adipose tissue induce adipocytokine dysregulation: a decrease in insulin-sensitizing and anti-inflammatory adipocytokines such as adiponectin, and an increase in proinflammatory adipocytokines involved in insulin resistance such as TNF-α, interleukins, and resistin[10,11] (Fig. 1). Furthermore, adipocytokine dysregulation is thought to play a crucial role in metabolic syndrome.[12]

Hepatic cirrhosis is six times more prevalent in obese individuals than in the general population,[13,14] and obesity is an independent risk factor for severity of liver fibrosis in nonalcoholic steatohepatitis (NASH), alcohol-induced liver disease, chronic hepatitis C (CHC), and hepatocellular carcinoma (HCC).[14–20] Recently, several studies have reported that adipocytokine dysregulation affects the pathological state of liver diseases.[21–34] For example, serum leptin and TNF-α levels were significantly higher, and adiponectin levels were significantly lower, in patients with NASH than in controls.[22] In this review, we describe the important roles of adipocytokines in liver diseases.

Leptin

Leptin is a 167-amino acid secreted protein encoded by the *ob* gene, and was identified by positional cloning in the *ob/ob* mouse as a key molecule in the regulation of body weight and energy balance.[35] Leptin is produced

Received / Accepted: May 1, 2008
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