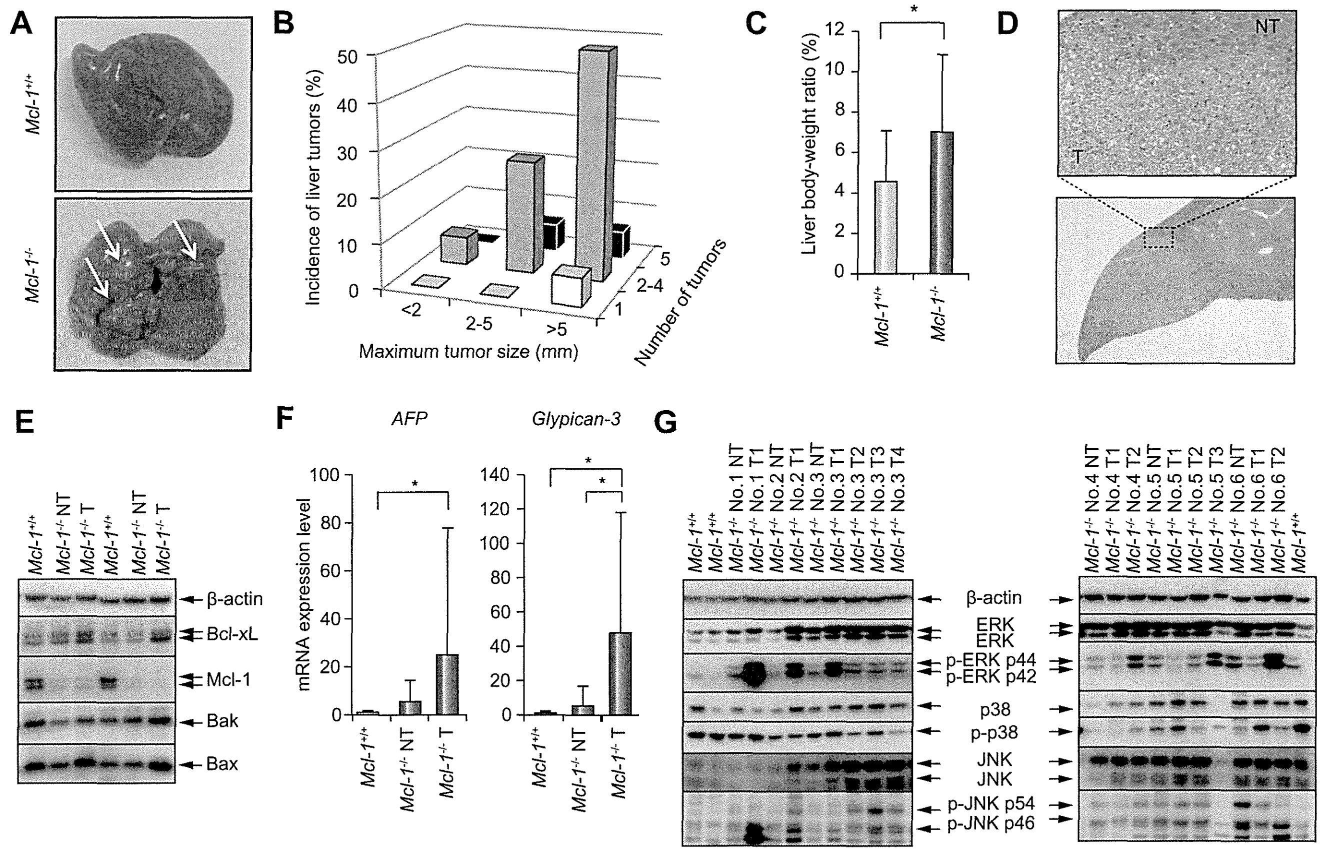

Fig. 2. Liver tumors in *Mcl-1* KO mice. (A–E) Hepatocyte-specific *Mcl-1*-deficient mice ($Mcl\text{-}1^{-/-}$) (N = 16) and their control littermates ($Mcl\text{-}1^{+/+}$) (N = 22) were sacrificed at 1.5 years of age. (A) Representative macroscopic view of the livers with arrows indicating tumors. (B) Incidence of liver tumors separated by maximum tumor size and number of tumors. (C) Liver body-weight ratio. (D) Representative histology of liver tumors in *Mcl-1* KO mice. (E) Western blot of the Bcl-2 family proteins in tumors (T) and surrounding non-cancerous livers (NT) of *Mcl-1* KO mice and livers of control mice. (F and G) Characteristics of liver tumors in *Mcl-1* KO mice. (F) Real-time RT-PCR analysis of the expression levels of α-fetoprotein (*AFP*) and *glypican-3* mRNA (N = 16 per group). (G) Expression and activation of mitogen-activated protein kinases. $^*p < 0.05$.

KO mice as evidenced by TUNEL staining of liver sections, serum ALT levels and caspase-3/7 activity at 6 weeks of age (Fig. 4A–C). Weber *et al.* [12] previously described hepatocyte regeneration in the *Mcl-1* KO liver. In agreement with this, *Mcl-1* KO livers showed higher expression of cell cycle markers PCNA and ki-67, than those from control littermates (Fig. 4A, B, and D and Supplementary Fig. 4). Importantly, the levels of PCNA and ki-67 expression decreased with a *Bak* KO background in *Mcl-1* KO mice. While *Mcl-1* KO livers show a mild fibrotic change [11], the levels of col1a1 expression at 6 weeks of age and Sirius red staining at 1 year of age decreased with a *Bak* KO background in *Mcl-1* KO livers (Fig. 4E and Supplementary Fig. 5). Bak deficiency also reduced expression levels of TNF-α, MCP-1, and CD68 at 6 weeks of age (Fig. 4F). Next, we examined the impact of apoptosis inhibition by Bak deficiency on oxidative stress markers, which were increased in *Mcl-1* KO livers. Real-time RT-PCR revealed that Bak deficiency reduced the levels of HO-1 and NQO1 expression at 6 weeks of age (Fig. 4G). Consistent with these observations, *Bak* KO significantly lowered the number of 8-OHdG-positive nuclei in *Mcl-1* KO livers at 1 year of age (Fig. 4H). These results suggested that inhibition of hepatocyte apoptosis reduced oxidative stress in the liver. Finally, to examine the impact of apoptosis inhibition on liver tumor development, we compared the carcinogenetic rates in *Mcl-1* KO mice with or without *Bak* KO background at 1 year of age and found that *Bak* KO significantly suppressed liver tumor development (Fig. 5A and B and Table 1).

Discussion

Mcl-1 was first identified as a gene induced during myeloid cell differentiation. Compared with other anti-apoptotic members such as Bcl-2, Bcl-xL, Bcl-w, and Bfl-1, Mcl-1 possesses a unique N-terminus containing two PEST domains, which are found in proteins displaying rapid turnover, and its expression is tightly regulated by growth factors and a variety of other stimuli. Mice systemically deficient for Bcl-xL suffered embryonic death due to massive apoptosis in hematopoietic organs and developing neurons [22]. On the other hand, systemic *Mcl-1* KO resulted in peri-implantation lethality, but *Mcl-1* KO embryos showed no alterations in the extent of apoptosis [23], suggesting that Mcl-1 may play a role early in development that is distinct from its anti-apoptotic functions. Indeed, *in vitro* studies have shown that Mcl-1 interacts with PCNA and Cdk1 in the nucleus and inhibits proliferation [13,14]. Recently, the early responding gene *IEX-1*

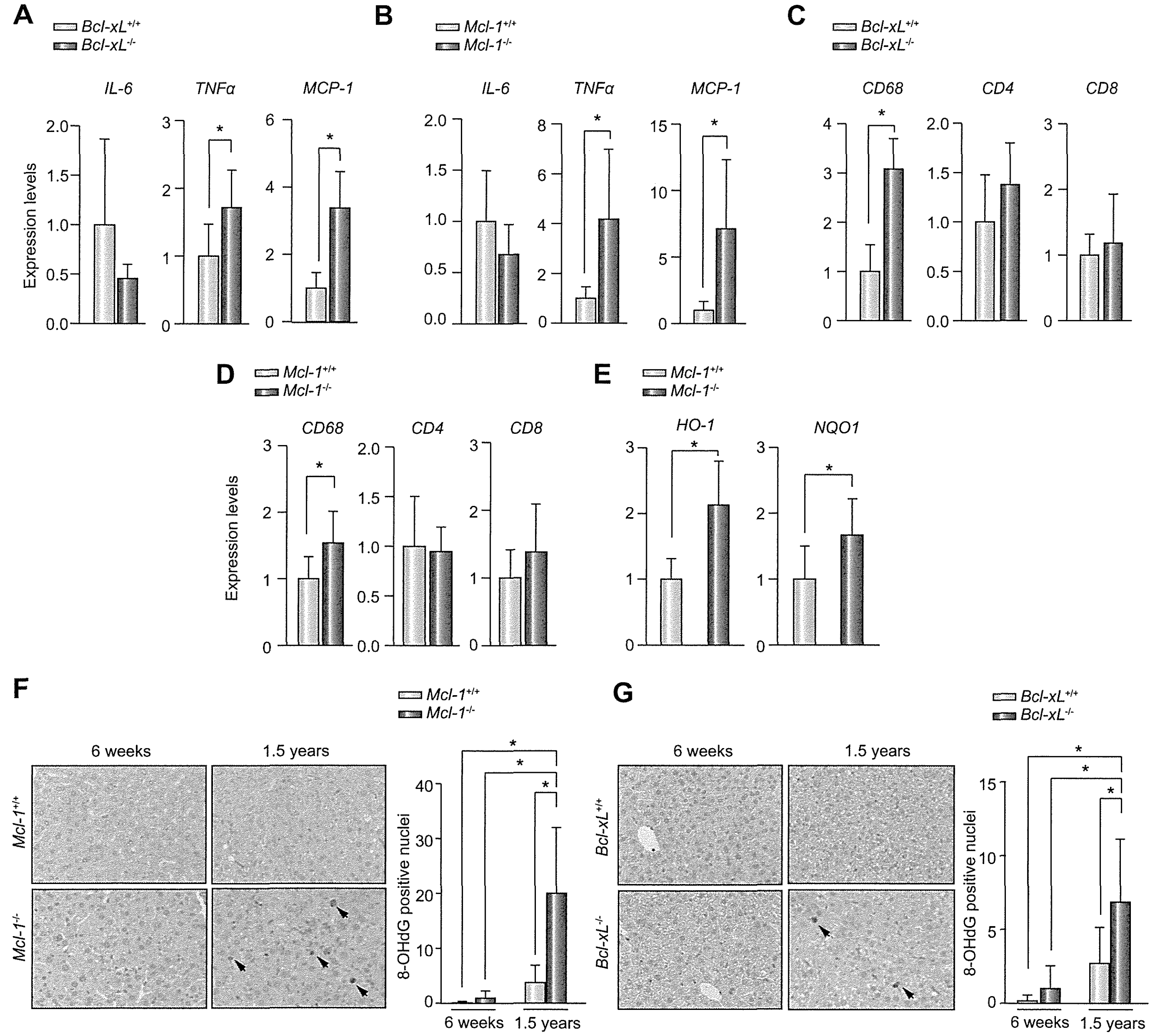

Fig. 3. Inflammatory response and oxidative stress in *Bcl-xL* or *Mcl-1* KO liver. (A–D) Inflammatory responses in KO livers. (A and C) Hepatocyte-specific *Bcl-xL* KO mice (*Bcl-xL*$^{-/-}$) and their control littermates (*Bcl-xL*$^{+/+}$) (N = 6 per group) as well as (B and D) hepatocyte-specific *Mcl-1* KO (*Mcl-1*$^{-/-}$) mice and their control littermates (*Mcl-1*$^{+/+}$) (N = 9 per group) were sacrificed at 6 weeks of age. Expression levels of (A and B) inflammatory molecules and (C and D) cell surface markers of immune cells were analyzed by real-time RT-PCR. (E–G) Oxidative injury in KO livers. (E) Real-time RT-PCR analysis of the expression levels of *HO-1* and *NQO1* of *Mcl-1* KO and control livers at 6 weeks of age (N = 9 per group). (F) Liver sections of *Mcl-1* KO or (G) *Bcl-xL* KO and the control liver at the indicated ages stained with anti-8-OHdG and statistics of the number of positive nuclei (N = 6 and more per group) (G). $^{*}p < 0.05$.

was found to be induced upon DNA damage and to be bound to and to transport Mcl-1 from the cytosol to the nucleus [15]. Mcl-1 was also reported to be induced upon DNA damage and to regulate the DNA damage response through activation of Chk1 [16]. These findings suggest that Mcl-1 possesses additional functions in cell cycle progression and the DNA damage response pathway. This raised concern as to whether the hepatocarcinogenesis observed in *Mcl-1* KO mice was actually related to increased apoptosis in the liver.

In the present study, we demonstrated that hepatocyte-specific destruction of Bcl-xL led to the development of liver cancer similarly to that in hepatocyte-specific *Mcl-1* KO mice. Although we could not completely exclude the possibility that Bcl-xL may have additional effects other than apoptosis, this finding clearly shows that hepatocarcinogenesis observed in the apoptosis-prone liver is not a specific finding of loss of Mcl-1 but is also observed with the knockout of other genes that are critically involved in hepatocyte integrity. Tumors observed in these murine livers frequently showed activation of ERK and JNK, similar to the activation observed in human HCC [18,19]. While 64% of *Mcl-1* KO mice (14/22) developed liver tumors within 1 year, only 27% of *Bcl-xL* KO mice (3/11) did so within 1 year (Table 1). These finding indicate that the incidence rate of carcinogenesis in *Bcl-xL* KO mice is lower than that of *Mcl-1* KO mice. This may be

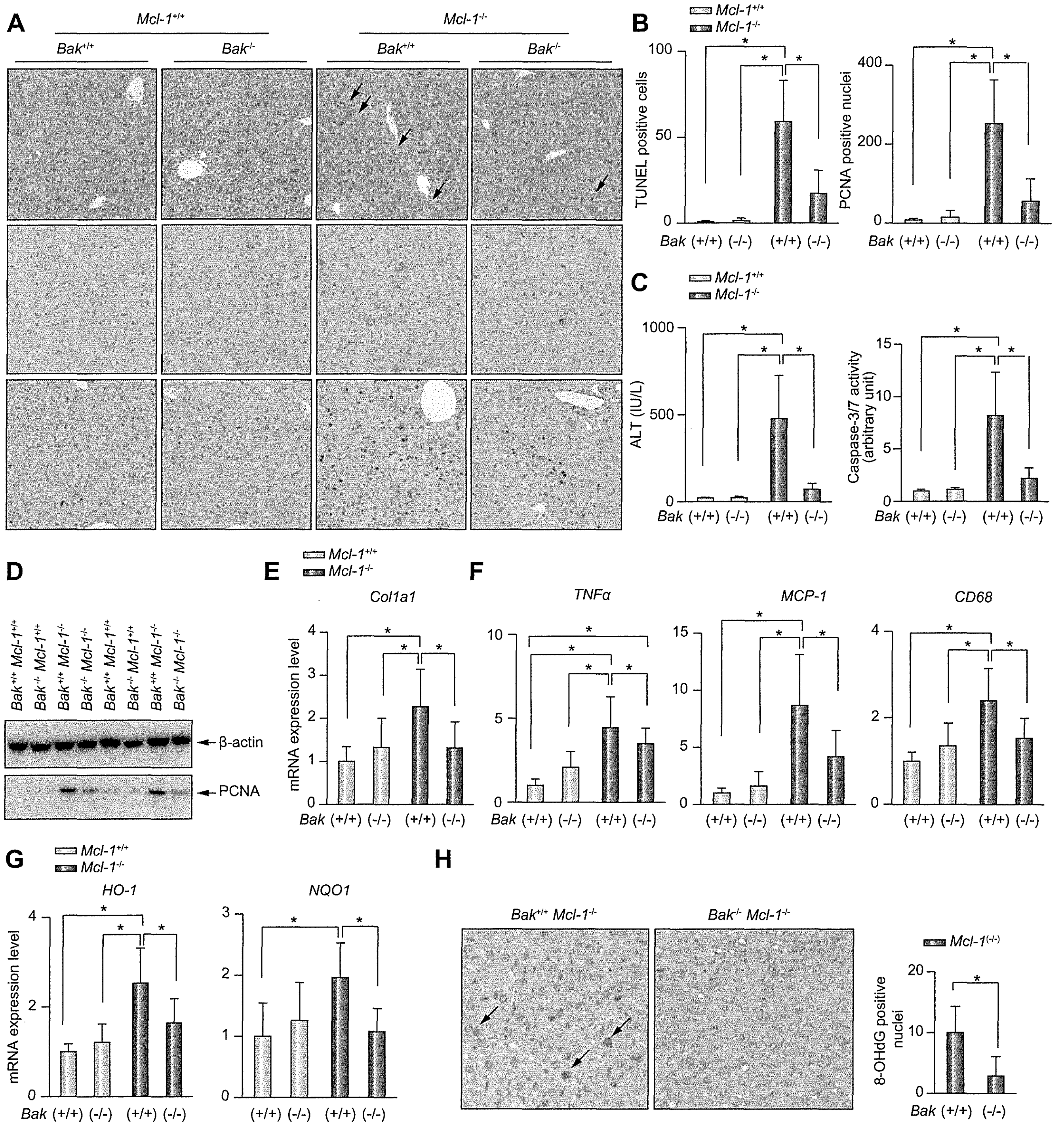

Fig. 4. Impact of Bak deficiency in *Mcl-1* KO mice. (A–G) *Bak*-deficient hepatocyte-specific *Mcl-1* KO mice (*Bak*$^{-/-}$ *Mcl-1*$^{-/-}$) were sacrificed at 6 weeks of age. (A) Representative pictures of hematoxylin–eosin with arrows indicating typical apoptotic cells (upper), TUNEL (middle) and PCNA staining (lower) and (B) statistics of TUNEL and PCNA staining of liver sections (N = 6 or 8 per group). (C) Serum levels of ALT and caspase-3/7 activity (N = 12 per group). (D) Western blot for PCNA expression. Real-time RT-PCR analysis for expression levels of (E) *Col1a1*, (F) *TNF-α*, *MCP-1*, *CD68*, (G) *HO-1* and *NQO1* in the livers at 6 weeks of age (N = 12 per group). (H) Liver sections of the *Bak*-deficient *Mcl-1* KO and control *Mcl-1* KO liver at 1 year of age stained with anti-8-OHdG. Representative images of liver sections stained with anti-8-OHdG (left) and statistics of the number of positive nuclei (N = 9 or 7 per group) (right). $^{*}p$ <0.05.

explained by the difference in levels of hepatocyte apoptosis and serum ALT, which are higher in *Mcl-1* KO mice than in *Bcl-xL* KO mice of the same age [10,11].

Mcl-1 executes its anti-apoptotic function by either directly or indirectly inhibiting the pro-apoptotic functions of Bak and/or Bax [24]. In the present study, we have shown that deletion of the *bak*

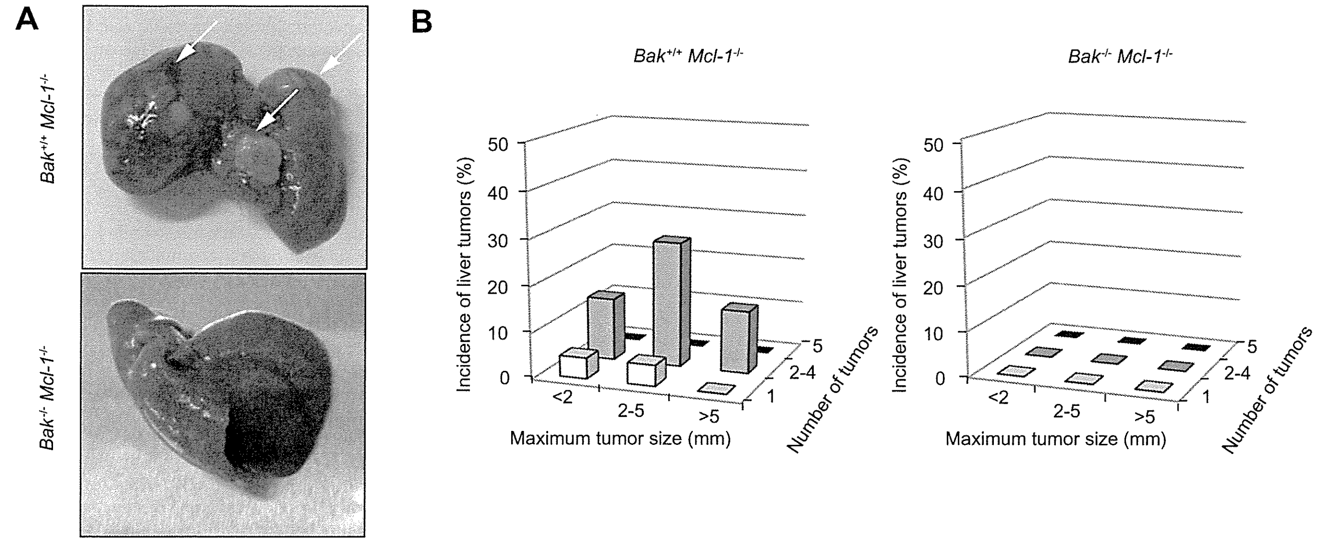

Fig. 5. Liver of aged *Bak/Mcl-1* double KO mice. (A and B) *Bak*-deficient *Mcl-1* KO mice (*Bak*$^{-/-}$ *Mcl-1*$^{-/-}$) (N = 7) and control *Mcl-1* KO mice (*Bak*$^{+/+}$ *Mcl-1*$^{-/-}$) (N = 22) were sacrificed at 1 year of age. (A) Representative macroscopic view of the livers with arrows indicating tumors. (B) Incidence of liver tumors separated by maximum tumor size and number of tumors.

gene resulted in a clear reduction in hepatocyte apoptosis in *Mcl-1* KO mice. Of importance is the finding that *bak* deletion leads to reduction of the liver regenerative response in *Mcl-1* KO mice. Bak is exclusively localized at the mitochondria in hepatocytes [25] and, upon exposure to apoptotic stimuli, undergoes oligomerization to form pores in the outer membrane of mitochondria, releasing cytochrome c, which in turn activates caspases. Since Bak is not involved in the activity of Mcl-1 in the nucleus, our present finding suggests that the regeneration observed in the *Mcl-1* KO liver is not due to loss of the Mcl-1 anti-proliferative effect but mainly to the compensatory regeneration of increased apoptosis. Most importantly, *bak* deletion clearly leads to reduced liver tumor incidence. This finding strongly suggests that the hepatocarcinogenesis observed in *Mcl-1* KO mice can be mostly ascribed to increased apoptosis in hepatocytes.

What does make hepatocytes undergo malignant transformation in the liver with increasing apoptosis? Regeneration is a physiological process in the liver like that in bone marrow or the intestine and compensatory liver regeneration itself is probably not sufficient to induce liver cancer [26]. The present study raised the possibility that TNF-α and oxidative stress are candidate factors responsible for the malignant transformation in the apoptosis-prone liver. TNF-α is reported to be a potent endogenous mutagen that promotes cellular transformation [20], and oxidative stress is reported to cause DNA damage leading to carcinogenesis [21]. Our results revealed that both TNF-α and oxidative stress were significantly increased in KO livers, and importantly, that inhibition of apoptosis by deletion of the *bak* gene reduced the levels of TNF-α and oxidative stress with a decrease in the tumorigenic rate. Some studies have shown that TNF-α induces oxidative stress in hepatocytes [27,28], while oxidative stress promotes production of inflammatory cytokines [29–31]. Taken together, oxidative stress and inflammatory cytokines may positively affect each other to turn healthy hepatocytes into malignant transformed hepatocytes in the liver of KO mice. Further studies are needed to examine the role of oxidative stress and inflammatory cytokines in apoptosis-induced hepatocarcinogenesis.

Apoptosis resistance has been established as a hallmark of cancer [32]. Indeed, accumulating evidence indicates that human HCC frequently overexpresses a variety of molecules which confer apoptosis resistance, such as anti-apoptotic Bcl-2 family proteins, Bcl-xL [33] and Mcl-1 [34,35]. Their overexpression was found to be associated with malignant phenotypes of tumors and poor prognosis of patients [36]. In the present study, tumors that developed in *Bcl-xL* or *Mcl-1* KO mice lacked expression of the respective proteins but reciprocally overexpressed Mcl-1 or Bcl-xL at high rates. We recently reported that conditional expression of Bcl-xL in tumor cells was translated into higher tumor growth in xenograft models [37], indicating that overexpression of anti-apoptotic Bcl-2 family proteins is important for tumor progression. Lack of Bcl-xL or Mcl-1 in hepatocytes generates persistent hepatocyte apoptosis leading to liver tumor development. On the other hand, reciprocal overexpression of Mcl-1 or Bcl-xL in the tumor of *Bcl-xL* or *Mcl-1* KO mice might be required for tumor progression.

Increasing evidence indicates that the serum level of ALT, a marker of hepatocyte apoptosis, is a risk factor for HCC in viral hepatitis [38] and non-alcoholic steatohepatitis [39]. A population-based study also revealed that elevated ALT levels raise the risk of liver cancer [40]. The present study provides evidence that spontaneous apoptosis in hepatocytes leads to liver cancer development and also offers genetic evidence that inhibition of apoptosis can help prevent liver cancer. Administration of caspase inhibitor was previously reported to lower serum ALT levels in patients with chronic hepatitis C [41]. It may be interesting and important, from a clinical point of view, to further determine whether pharmacological inhibition of apoptosis can be useful in preventing liver cancer development in *Bcl-xL* or *Mcl-1* KO mice.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2012.01.027.

References

[1] Malhi H, Gores G. Cellular and molecular mechanisms of liver injury. Gastroenterology 2008;134:1641–1654.

[2] Hiramatsu N, Hayashi N, Katayama K, Mochizuki K, Kawanishi Y, Kasahara A, et al. Immunohistochemical detection of Fas antigen in liver tissue of patients with chronic hepatitis C. Hepatology 1994;19:1354–1359.

[3] Mochizuki K, Hayashi N, Hiramatsu N, Katayama K, Kawanishi Y, Kasahara A, et al. Fas antigen expression in liver tissues of patients with chronic hepatitis B. J Hepatol 1996;24:1–7.

[4] Feldstein A, Canbay A, Angulo P, Taniai M, Burgart L, Lindor K, et al. Hepatocyte apoptosis and fas expression are prominent features of human nonalcoholic steatohepatitis. Gastroenterology 2003;125:437–443.

[5] Kronenberger B, Wagner M, Herrmann E, Mihm U, Piiper A, Sarrazin C, et al. Apoptotic cytokeratin 18 neoepitopes in serum of patients with chronic hepatitis C. J Viral Hepat 2005;12:307–314.

[6] Papatheodoridis GV, Hadziyannis E, Tsochatzis E, Chrysanthos N, Georgiou A, Kafiri G, et al. Serum apoptotic caspase activity as a marker of severity in HBeAg-negative chronic hepatitis B virus infection. Gut 2008;57:500–506.

[7] Wieckowska A, Zein NN, Yerian LM, Lopez AR, McCullough AJ, Feldstein AE. In vivo assessment of liver cell apoptosis as a novel biomarker of disease severity in nonalcoholic fatty liver disease. Hepatology 2006;44:27–33.

[8] Moriya K, Fujie H, Shintani Y, Yotsuyanagi H, Tsutsumi T, Ishibashi K, et al. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. Nat Med 1998;4:1065–1067.

[9] Pikarsky E, Porat RM, Stein I, Abramovitch R, Amit S, Kasem S, et al. NF-kappaB functions as a tumour promoter in inflammation-associated cancer. Nature 2004;431:461–466.

[10] Takehara T, Tatsumi T, Suzuki T, Rucker Er, Hennighausen L, Jinushi M, et al. Hepatocyte-specific disruption of Bcl-xL leads to continuous hepatocyte apoptosis and liver fibrotic responses. Gastroenterology 2004;127:1189–1197.

[11] Hikita H, Takehara T, Shimizu S, Kodama T, Li W, Miyagi T, et al. Mcl-1 and Bcl-xL cooperatively maintain integrity of hepatocytes in developing and adult murine liver. Hepatology 2009;50:1217–1226.

[12] Weber A, Boger R, Vick B, Urbanik T, Haybaeck J, Zoller S, et al. Hepatocyte-specific deletion of the antiapoptotic protein myeloid cell leukemia-1 triggers proliferation and hepatocarcinogenesis in mice. Hepatology 2010;51:1226–1236.

[13] Fujise K, Zhang D, Liu J, Yeh ET. Regulation of apoptosis and cell cycle progression by MCL1. Differential role of proliferating cell nuclear antigen. J Biol Chem 2000;275:39458–39465.

[14] Jamil S, Sobouti R, Hojabrpour P, Raj M, Kast J, Duronio V. A proteolytic fragment of Mcl-1 exhibits nuclear localization and regulates cell growth by interaction with Cdk1. Biochem J 2005;387:659–667.

[15] Pawlikowska P, Leray I, de Laval B, Guihard S, Kumar R, Rosselli F, et al. ATM-dependent expression of IEX-1 controls nuclear accumulation of Mcl-1 and the DNA damage response. Cell Death Differ 2010;17:1739–1750.

[16] Jamil S, Mojtabavi S, Hojabrpour P, Cheah S, Duronio V. An essential role for MCL-1 in ATR-mediated CHK1 phosphorylation. Mol Biol Cell 2008;19:3212–3220.

[17] Takehara T, Hayashi N, Tatsumi T, Kanto T, Mita E, Sasaki Y, et al. Interleukin 1beta protects mice from Fas-mediated hepatocyte apoptosis and death. Gastroenterology 1999;117:661–668.

[18] Ito Y, Sasaki Y, Horimoto M, Wada S, Tanaka Y, Kasahara A, et al. Activation of mitogen-activated protein kinases/extracellular signal-regulated kinases in human hepatocellular carcinoma. Hepatology 1998;27:951–958.

[19] Chen F, Beezhold K, Castranova V. JNK1, a potential therapeutic target for hepatocellular carcinoma. Biochim Biophys Acta 2009;1796:242–251.

[20] Yan B, Wang H, Rabbani ZN, Zhao Y, Li W, Yuan Y, et al. Tumor necrosis factor-alpha is a potent endogenous mutagen that promotes cellular transformation. Cancer Res 2006;66:11565–11570.

[21] Lonkar P, Dedon PC. Reactive species and DNA damage in chronic inflammation: reconciling chemical mechanisms and biological fates. Int J Cancer 2011;128:1999–2009.

[22] Motoyama N, Wang F, Roth KA, Sawa H, Nakayama K, Negishi I, et al. Massive cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice. Science 1995;267:1506–1510.

[23] Rinkenberger JL, Horning S, Klocke B, Roth K, Korsmeyer SJ. Mcl-1 deficiency results in peri-implantation embryonic lethality. Genes Dev 2000;14:23–27.

[24] Willis SN, Chen L, Dewson G, Wei A, Naik E, Fletcher JI, et al. Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. Genes Dev 2005;19:1294–1305.

[25] Hikita H, Takehara T, Kodama T, Shimizu S, Hosui A, Miyagi T, et al. BH3-only protein bid participates in the Bcl-2 network in healthy liver cells. Hepatology 2009;50:1972–1980.

[26] Aravalli RN, Steer CJ, Cressman EN. Molecular mechanisms of hepatocellular carcinoma. Hepatology 2008;48:2047–2063.

[27] Kamata H, Honda S, Maeda S, Chang L, Hirata H, Karin M. Reactive oxygen species promote TNFalpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. Cell 2005;120:649–661.

[28] Schwabe RF, Brenner DA. Mechanisms of Liver Injury. I. TNF-alpha-induced liver injury: role of IKK, JNK, and ROS pathways. Am J Physiol Gastrointest Liver Physiol 2006;290:G583–G589.

[29] Bulua AC, Simon A, Maddipati R, Pelletier M, Park H, Kim KY, et al. Mitochondrial reactive oxygen species promote production of proinflammatory cytokines and are elevated in TNFR1-associated periodic syndrome (TRAPS). J Exp Med 2011;208:519–533.

[30] Nakahira K, Haspel JA, Rathinam VA, Lee SJ, Dolinay T, Lam HC, et al. Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. Nat Immunol 2011;12:222–230.

[31] Zhou R, Yazdi AS, Menu P, Tschopp J. A role for mitochondria in NLRP3 inflammasome activation. Nature 2011;469:221–225.

[32] Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144:646–674.

[33] Takehara T, Liu X, Fujimoto J, Friedman S, Takahashi H. Expression and role of Bcl-xL in human hepatocellular carcinomas. Hepatology 2001;34:55–61.

[34] Fleischer B, Schulze-Bergkamen H, Schuchmann M, Weber A, Biesterfeld S, Müller M, et al. Mcl-1 is an anti-apoptotic factor for human hepatocellular carcinoma. Int J Oncol 2006;28:25–32.

[35] Sieghart W, Losert D, Strommer S, Cejka D, Schmid K, Rasoul-Rockenschaub S, et al. Mcl-1 overexpression in hepatocellular carcinoma: a potential target for antisense therapy. J Hepatol 2006;44:151–157.

[36] Watanabe J, Kushihata F, Honda K, Sugita A, Tateishi N, Mominoki K, et al. Prognostic significance of Bcl-xL in human hepatocellular carcinoma. Surgery 2004;135:604–612.

[37] Hikita H, Takehara T, Shimizu S, Kodama T, Shigekawa M, Iwase K, et al. The Bcl-xL inhibitor, ABT-737, efficiently induces apoptosis and suppresses

growth of hepatoma cells in combination with sorafenib. Hepatology 2010;52:1310–1321.

[38] Chen CF, Lee WC, Yang HI, Chang HC, Jen CL, Iloeje UH, et al. Changes in serum levels of HBV DNA and alanine aminotransferase determine risk for hepatocellular carcinoma. Gastroenterology 2011;141:1240–1248.

[39] Bhala N, Angulo P, van der Poorten D, Lee E, Hui JM, Saracco G, et al. The natural history of nonalcoholic fatty liver disease with advanced fibrosis or cirrhosis: an international collaborative study. Hepatology 2011;54:1208–1216.

[40] Ruhl CE, Everhart JE. Elevated serum alanine aminotransferase and gamma-glutamyltransferase and mortality in the United States population. Gastroenterology 2009;136:477–485, e411.

[41] Pockros P, Schiff E, Shiffman M, McHutchison J, Gish R, Afdhal N, et al. Oral IDN-6556, an antiapoptotic caspase inhibitor, may lower aminotransferase activity in patients with chronic hepatitis C. Hepatology 2007;46:324–329.

Delayed-Onset Caspase-Dependent Massive Hepatocyte Apoptosis upon Fas Activation in Bak/Bax-Deficient Mice

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The proapoptotic Bcl-2 family proteins Bak and Bax serve as an essential gateway to the mitochondrial pathway of apoptosis. When activated by BH3-only proteins, Bak/Bax triggers mitochondrial outer membrane permeabilization leading to release of cytochrome c followed by activation of initiator and then effector caspases to dismantle the cells. Hepatocytes are generally considered to be type II cells because, upon Fas stimulation, they are reported to require the BH3-only protein Bid to undergo apoptosis. However, the significance of Bak and Bax in the liver is unclear. To address this issue, we generated hepatocyte-specific Bak/Bax double knockout mice and administered Jo2 agonistic anti-Fas antibody or recombinant Fas ligand to them. Fas-induced rapid fulminant hepatocyte apoptosis was partially ameliorated in Bak knockout mice but not in Bax knockout mice, and was completely abolished in double knockout mice 3 hours after Jo2 injection. Importantly, at 6 hours, double knockout mice displayed severe liver injury associated with repression of XIAP, activation of caspase-3/7 and oligonucleosomal DNA breaks in the liver, without evidence of mitochondrial disruption or cytochrome c–dependent caspase-9 activation. This liver injury was not ameliorated in a cyclophilin D knockout background nor by administration of necrostatin-1, but was completely inhibited by administration of a caspase inhibitor after Bid cleavage. Conclusion: Whereas either Bak or Bax is critically required for rapid execution of Fas-mediated massive apoptosis in the liver, delayed onset of mitochondria-independent, caspase-dependent apoptosis develops even in the absence of both. The present study unveils an extrinsic pathway of apoptosis, like that in type I cells, which serves as a backup system even in type II cells. (HEPATOLOGY 2011;54:240-251)

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Fas, also called APO-1 and CD95, is one of the death receptors that are potent inducers of apoptosis and constitutively expressed by every cell type in the liver.[1] Dysregulation of Fas-mediated apoptosis is involved in several liver diseases.[2] In the liver of patients with chronic hepatitis C, Fas is overexpressed in correlation with the degree of hepatitis, and Fas ligand can be detected in liver-infiltrating mononuclear cells.[3,4] Fas is also strongly expressed in the livers of patients with chronic hepatitis B, autoimmune hepatitis, and nonalcoholic steatohepatitis.[4,5] Moreover, in the liver of patients with fulminant hepatitis, Fas is up-regulated with strong detection of Fas ligand.[6] In mice, injection of Jo2 agonistic anti-Fas antibody leads

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Abbreviations: ALT, alanine aminotransferase; CypD, cyclophilin D; DISC, death-inducing signaling complex; DKO, double knockout; DMSO, dimethylsulfoxide; IAP, inhibition of apoptosis protein; KO, knockout; PARP, poly(adenosine diphosphate ribose) polymerase; RIP, receptor-interacting protein; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling; WT, wild-type.

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**These authors contributed equally to this work.*

to massive hepatocyte apoptosis and lethality, suggesting that the hepatocyte is one of the most sensitive cell types to Fas stimulation.[7] This model is considered to at least partly mimic human fulminant liver failure.

Fas, upon ligation by Fas ligand, activates caspase-8 through the recruitment of Fas-associated protein with a death domain and formation of the death-inducing signaling complex (DISC).[1,2] Whereas activated caspase-8 directly activates effector caspases such as caspase-3 and caspase-7 through the so-called extrinsic pathway, leading to apoptosis in type I cells, it activates caspase-3/7 through the mitochondrial pathway in type II cells. In type II cells, activated caspase-8 cleaves the BH3-only protein Bid into its truncated form, which in turn directly or indirectly activates and homo-oligomerizes Bak and/or Bax to form pores at the mitochondrial outer membrane, leading to the release of cytochrome c. After being released, cytochrome c assembles with Apaf-1 to form apoptosomes which promote self-cleavage of procaspase-9 followed by activation of caspase-3/7 to cleave a variety of cellular substrates such as poly(adenosine diphosphate ribose) polymerase (PARP) and finally to execute apoptosis.[8,9] Hepatocytes are considered to be typical type II cells, because Bid knockout (KO) mice were reported to be resistant to hepatocyte apoptosis upon Fas activation.[10,11] Although Bak and Bax are crucial gateways to apoptosis of the mitochondrial pathway, little information is available about their significance in hepatocyte apoptosis because most traditional Bak/Bax double knockout (DKO) mice (*bak*$^{-/-}$ *bax*$^{-/-}$) die perinatally.[12]

In the present study, we tried to address this issue by generating hepatocyte-specific Bak/Bax DKO mice. We demonstrate that either Bak or Bax is required and sufficient to induce Fas-mediated early-onset hepatocyte apoptosis and lethal liver injury. Importantly, even if deficient in both Bak and Bax, Bak/Bax DKO mice still develop delayed-onset caspase-dependent massive hepatocyte apoptosis, suggesting that the mitochondria-independent pathway of apoptosis, as observed in type I cells, works as a backup system when the mitochondrial pathway of apoptosis in the liver is absent. This study is the first to demonstrate the significant but limited role of Bak and Bax in executing Fas-induced apoptosis in the liver.

Materials and Methods

Mice. Heterozygous Alb-Cre transgenic mice expressing Cre recombinase gene under the promoter of the albumin gene were described.[13] We purchased Bak KO mice (*bak*$^{-/-}$), Bax KO mice (*bax*$^{-/-}$), and Bak KO mice carrying the *bax* gene flanked by 2 *lox*P sites (*bak*$^{-/-}$ *bax*$^{flox/flox}$) from the Jackson Laboratory (Bar Harbor, ME). Traditional cyclophilin D (CypD) KO mice have been described.[14] All mice strains that we used were created from a mixed background (C57BL/6 and 129). We generated hepatocyte-specific Bak/Bax DKO mice (*bak*$^{-/-}$ *bax*$^{flox/flox}$ *Alb-Cre*) or hepatocyte-specific CypD/Bak/Bax triple KO mice (*cypd*$^{-/-}$ *bak*$^{-/-}$ *bax*$^{flox/flox}$ *Alb-Cre*) by mating the strains. Mice were injected intraperitoneally with 1.5 or 0.5 mg/kg Jo2 anti-Fas antibody (BD Bioscience, Franklin Lakes, NJ) or intravenously with 0.25 mg/kg recombinant Fas ligand (Alexis Biochemicals, San Diego, CA) cross-linked with 0.5 mg/kg anti-Flag M2 antibody (Sigma-Aldrich, St. Louis, MO) to induce apoptosis. In some experiments, mice were intraperitoneally injected with 2 mg/kg necrostatin-1 (Sigma-Aldrich) or 40 mg/kg Q-VD-Oph (R&D Systems, Minneapolis, MN). They were maintained in a specific pathogen-free facility and treated with humane care with approval from the Animal Care and Use Committee of Osaka University Medical School.

Apoptosis Assay. Measurement of serum alanine aminotransferase (ALT) levels, hematoxylin and eosin staining, and terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) of liver sections have been described.[15] Analysis of cytochrome c release from isolated mitochondria have also been described.[16] To detect DNA fragmentation, 1.5 μg DNA extracted from 30 mg liver tissue by Maxwell16 (Promega, Madison, WI) was incubated with 0.5 μg RNase A (Qiagen, Tokyo, Japan) and separated by way of electrophoresis on a 1.5% agarose gel.

Western Blot Analysis. For western immunoblotting, the following antibodies were used: anti–full-length Bid, anti–Cox IV, anti–cleaved caspase-3, anti–caspase-7, anti–caspase-8, anti–caspase-9, anti-PARP, anti-Bax, anti-cIAP1, and anti-XIAP antibodies were

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obtained from Cell Signaling Technology (Beverly, MA); anti-Bax and anti-cIAP2 antibodies were obtained from Millipore (Billerica, MA); anti-Bid antibody, which detects truncated Bid, was generously provided by Xiao-Ming Yin (Indiana University School of Medicine, Indianapolis, IN)[17]; and anti–β-actin antibody was obtained from Sigma-Aldrich. For isolation of the mitochondria-rich fraction, a Mitochondrial Isolation Kit (Thermo Scientific, Rockford, IL) was used. The isolation of hepatocytes from whole liver has been described.[13]

Detection of Bax Oligomerization. Liver tissue was lysed with HCN buffer (25 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid, 300 mM NaCl, 2% CHAPS, protease inhibitor cocktail, phosphatase inhibitor cocktail, 100 μM BOC-Asp(OMe)CH2F [MP Biomedicals, Solon, OH]; pH 7.5). After the liver lysate was sonicated and centrifuged, the supernatant was collected and the concentration was adjusted. For cross-linking, 100 μL of the lysate was incubated with 5 μL 100 mM bis(maleimido)hexane (Thermo Scientific) and 5 μL 100 mM BS^3 (Thermo Scientific) for 30 minutes at room temperature as described.[18] After quenching the cross-linkers by way of incubation with 12 μL 1 M Tris-HCl (pH 7.5) for 15 minutes at room temperature, the lysate was boiled with sample buffer followed by western blot analysis for Bax.

Electron Microscopy. Livers were fixed by perfusion of phosphate-buffered saline with 2.5% glutaraldehyde solution buffered at pH 7.4 with 0.1 M Millonig's phosphate, postfixed in 1% osmium tetroxide solution at 4°C for 1 hour, dehydrated in graded concentrations of ethanol, and embedded in Quetol 812 epoxy resin (Nisshin EM, Tokyo, Japan). Ultrathin sections (80 nm) cut on ultramicrotome were stained with uranyl acetate and lead citrate and examined with an H-7650 electron microscope (Hitachi Ltd., Tokyo, Japan) at 80 kV.

Statistical Analysis. Data are presented as the mean ± SE. Differences between two groups were determined using the Mann-Whitney U test for unpaired observations. The survival curves were estimated using the Kaplan-Meier method and were tested by way of log-rank test. $P < 0.05$ was considered statistically significant.

Results

Bak Deficiency Partially Ameliorates Fas-Induced Hepatocellular Apoptosis but Fails to Prevent Animal Death. First, to examine the significance of Bak in hepatocellular apoptosis induced by Fas stimulation, Bak KO mice ($bak^{-/-}$) and wild-type (WT) littermates ($bak^{+/+}$) were intraperitoneally injected with 1.5 mg/kg Jo2 anti-Fas antibody and analyzed 3 hours later. Consistent with previous reports,[10,19] WT mice showed severe elevation of serum ALT levels with massive hepatocellular apoptosis (Fig. 1A,B). Bak KO mice also developed liver injury, but the levels of serum ALT and the number of TUNEL-positive hepatocytes were significantly lower in Bak KO mice than in WT mice (Fig. 1A-C). Western blotting for cleaved caspase-3, caspase-7, and PARP revealed that activation of effector caspases were partially inhibited in KO livers compared with WT livers (Fig. 1D). Cleavage of procaspase-9, which is initiated by mitochondrial release of cytochrome c, was also suppressed in Bak KO livers compared with WT liver (Fig. 1D). The cleaved form of caspase-8, a direct downstream target of Fas activation, was detected in both mice, but its levels were reduced in Bak KO mice compared with WT mice (Fig. 1D). This reduction may be explained by the lesser activation of caspase-3/7, because it has been reported that caspase-3/7 could activate caspase-8 through an amplification loop during apoptosis.[20] Collectively, these findings demonstrated that Bak deficiency partially ameliorated Fas-induced hepatocellular apoptosis associated with reduced cleavage of caspase-9, caspase-3/7, and PARP. We then compared survival of mice after Jo2 injection but found that Bak KO mice also rapidly died with kinetics similar to those of WT mice, suggesting that partial amelioration of hepatocellular apoptosis induced by Bak deficiency did not lead to survival benefit under our experimental conditions (Fig. 1E). Because Bax residing in the cytosol moves to the mitochondria upon activation, where it undergoes oligomerization,[21] we analyzed its translocation and oligomerization in the liver at 3 hours after Jo2 injection. Western blot analysis revealed that the levels of Bax expression clearly increased in the mitochondrial fraction in both WT livers and Bak KO livers (Fig. 1F). Signals for the Bax dimer were also detected in both livers (Fig. 1F). These findings indicate that Bax is also activated after Fas stimulation, raising the possibility of its involvement in hepatocellular apoptosis.

Bax Deficiency Fails to Ameliorate Fas-Induced Hepatocellular Apoptosis. Next, to examine the significance of Bax in hepatocellular apoptosis induced by Fas stimulation, Bax KO mice ($bax^{-/-}$) and WT littermates ($bax^{+/+}$) were injected with Jo2 and examined 3 hours later. There was no significant difference in the levels of serum ALT or the number of TUNEL-positive hepatocytes between the two groups (Fig. 2A-C), which is consistent with a previous report.[22] The levels of the cleaved forms of caspase-8, -9, -3, -7, and

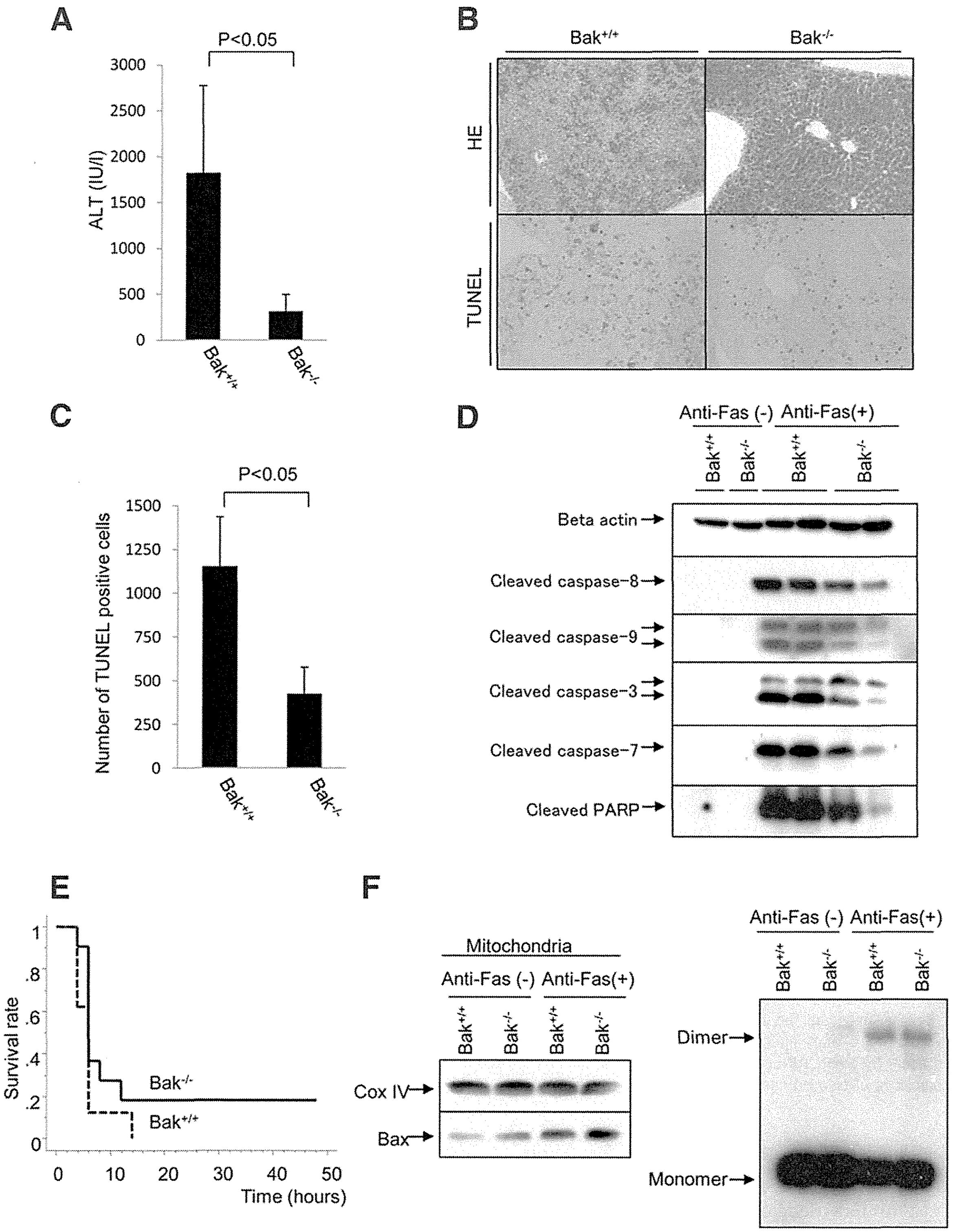

Fig. 1. Bak KO mice are partially resistant to Fas-induced hepatocellular apoptosis. Bak KO mice ($Bak^{-/-}$) or control WT littermates ($Bak^{+/+}$) were analyzed at 3 hours after intraperitoneal injection of 1.5 mg/kg Jo2 anti-Fas antibody. (A) Serum ALT levels ($n = 10$ or 11, respectively). (B) Hematoxylin and eosin (HE) and TUNEL staining of the liver sections. (C) Number of TUNEL-positive cells ($n = 8$ or 9, respectively). (D) Western blot analysis for the expressions of cleaved caspase-8, 9, -3, -7 and PARP. (E) Bak KO mice or control WT littermates were intraperitoneally injected with 1.5 mg/kg Jo2 anti-Fas antibody ($n = 8$ or 11, respectively). Survival rates after Jo2 injection are shown. (F) Bak KO mice or control WT littermates were analyzed 3 hours after intraperitoneal injection of Jo2 anti-Fas antibody (1.5 mg/kg) or vehicle. Left: Western blot analysis of the mitochondrial fraction of the liver for the expression of Bax. Right: Western blot analysis for the expression of Bax monomer and dimer in the liver.

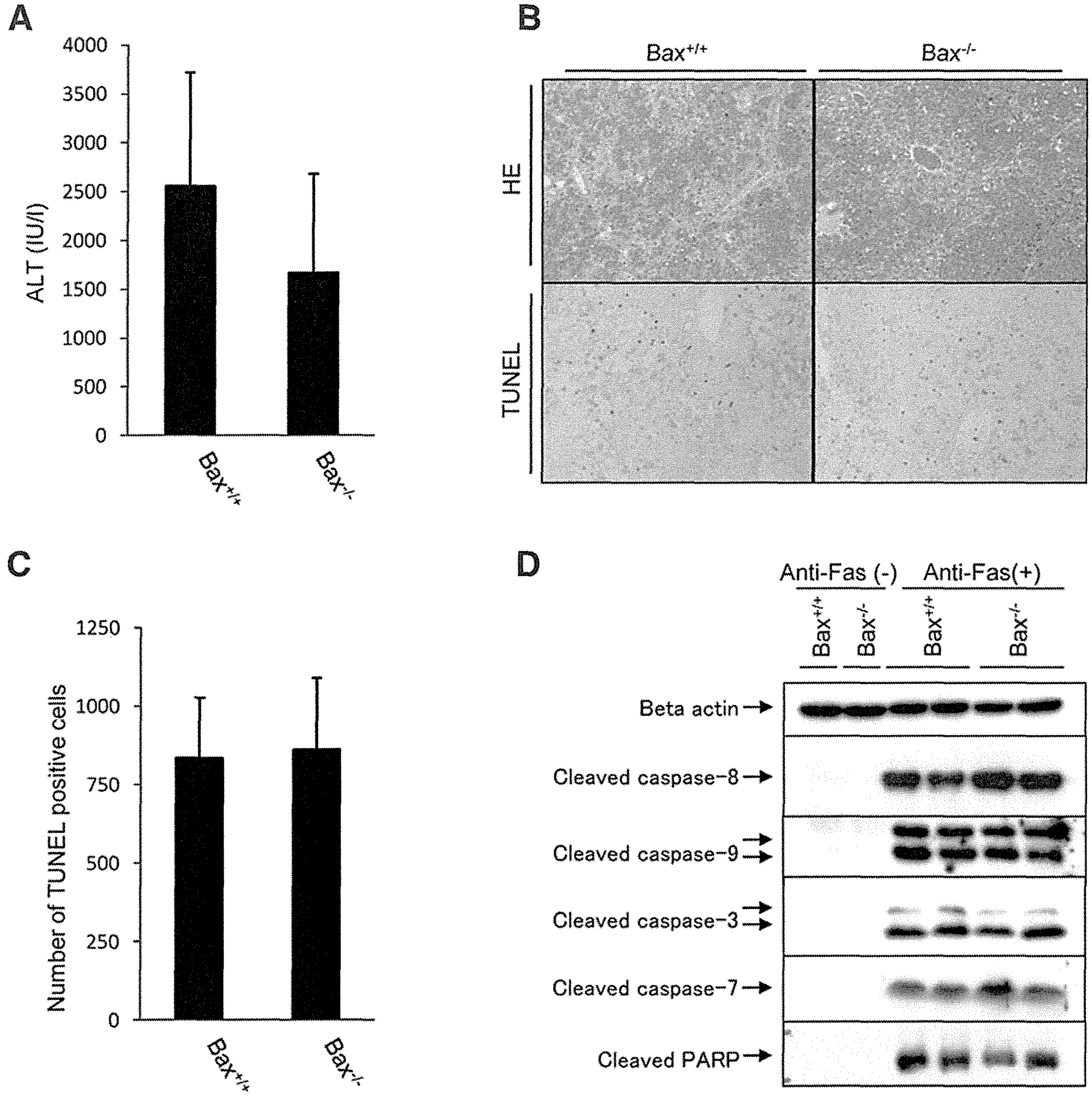

Fig. 2. Bax KO mice are not resistant to Fas-induced hepatocellular apoptosis. Bax KO mice (Bax$^{-/-}$) or control WT littermates (Bax$^{+/+}$) were analyzed 3 hours after intraperitoneal injection of Jo2 anti-Fas antibody (1.5 mg/kg). (A) Serum ALT levels (n = 11 per group). (B) Hematoxylin and eosin (HE) and TUNEL staining of the liver sections. (C) Number of TUNEL-positive cells (n = 8 per group). (D) Western blot analysis for the expressions of cleaved caspase-8, -9, -3, -7, and PARP.

PARP in Bax KO livers did not differ from those of WT livers (Fig. 2D). These findings demonstrate that, in contrast to Bak deficiency, Bax deficiency was not able to inhibit Fas-induced hepatocellular apoptosis.

Bax Deficiency Completely Blocks Fas-Induced Early-Onset Hepatocellular Apoptosis in a Bak-Deficient Background. To examine the impact of Bax in a Bak-deficient background, hepatocyte-specific Bak/Bax DKO mice ($bak^{-/-}$ $bax^{flox/flox}$ *Alb-Cre*) and Bak KO mice ($bak^{-/-}$ $bax^{flox/flox}$), which served as control littermates of this mating, were injected with Jo2 and analyzed 3 hours later. We confirmed the hepatocyte-specific defects of Bax protein in Bak/Bax DKO mice by way of western blot analysis (Fig. 3A). The serum ALT levels of Bak/Bax DKO mice were in the normal range and were significantly lower than those of Bak KO mice (Fig. 3B). Liver histology and TUNEL staining did not show evidence of hepatocyte apoptosis in Bak/Bax DKO livers, in contrast to Bak KO livers (Fig. 3C,D). Taken together, these results indicate that Bak and Bax are basically redundant molecules for execution of hepatocellular apoptosis induced by Fas

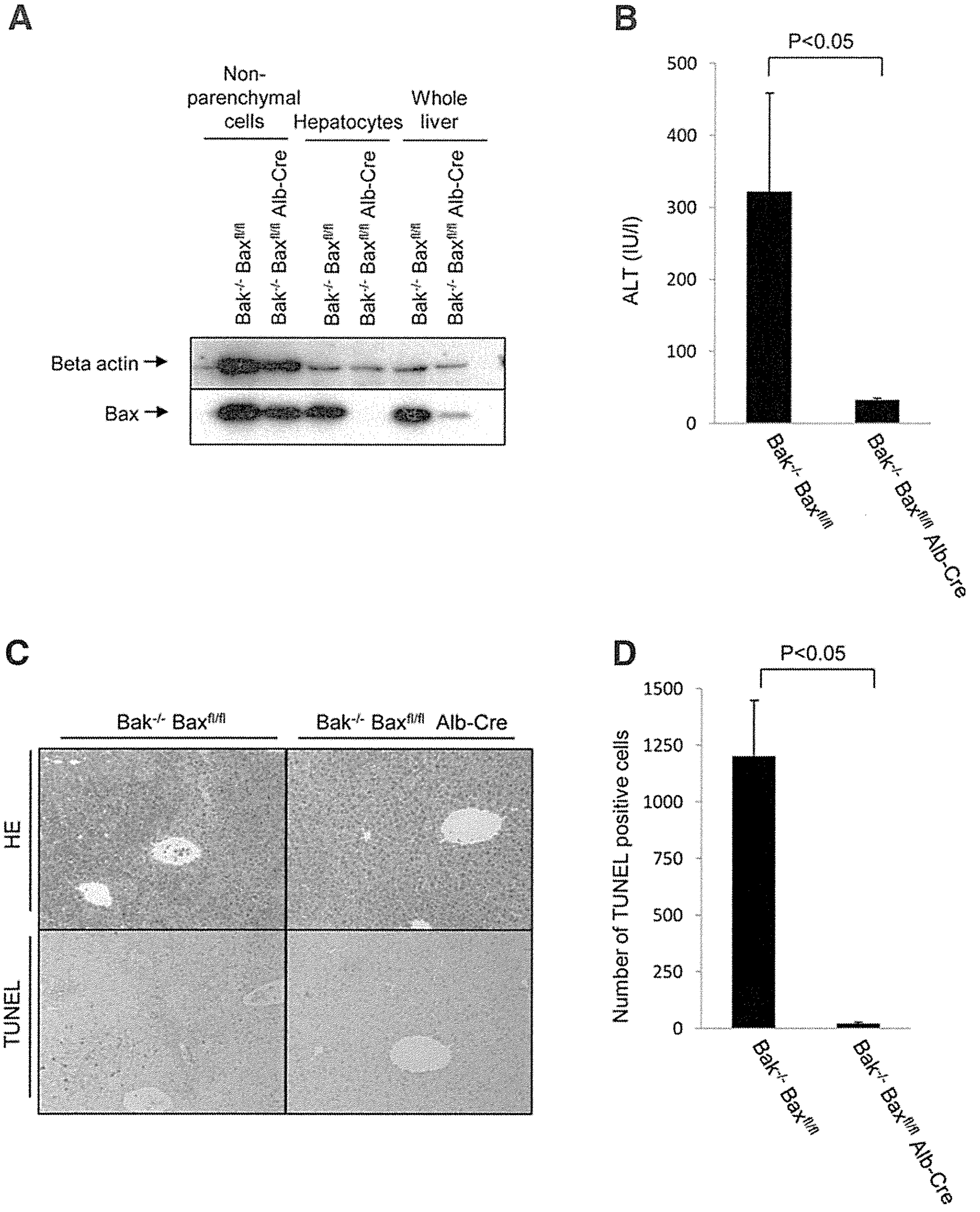

Fig. 3. Bak/Bax DKO mice are fully resistant to Fas-induced hepatocellular apoptosis in early phase. (A) Western blot analysis of the indicated fraction of the liver for the expressions of Bax. (B-D) Bak/Bax DKO mice (Bak$^{-/-}$ Bax$^{fl/fl}$ Alb-Cre) or control Bak KO littermates (Bak$^{-/-}$ Bax$^{fl/fl}$) were analyzed 3 hours after intraperitoneal injection of Jo2 anti-Fas antibody (1.5 mg/kg). (B) Serum ALT levels (n = 10 per group). (C) Hematoxylin and eosin (HE) and TUNEL staining of the liver sections. (D) Number of TUNEL-positive cells (n = 9 per group).

activation, although the former appears to be clearly required for full-blown apoptosis in vivo.

Fas Stimulation Leads to Late-Onset Hepatocellular Death Even in Bak/Bax Deficiency with Moderate Caspase-3/7 Activation Without Mitochondrial Disruption. To examine whether the inhibition of Fas-induced rapid liver injury in Bak/Bax deficiency is a durable effect, we analyzed the survival rate after Jo2 injection. The survival rate of Bak/Bax DKO mice was significantly higher than that of Bak KO mice, but approximately half of the Bak/Bax DKO mice died within 12 hours (Fig. 4A). To examine the cause of this late-onset lethality, we analyzed the serum ALT levels and liver tissue 6 hours after Jo2 injection. Unexpectedly, the serum ALT levels were highly elevated in Bak/Bax DKO mice (Fig. 4B). Liver histology revealed many hepatocytes with cellular shrinkage and scattered regions of sinusoidal hemorrhage (Fig. 4C), indicating that Bak/Bax DKO mice still developed severe liver injury at this time point. TUNEL staining

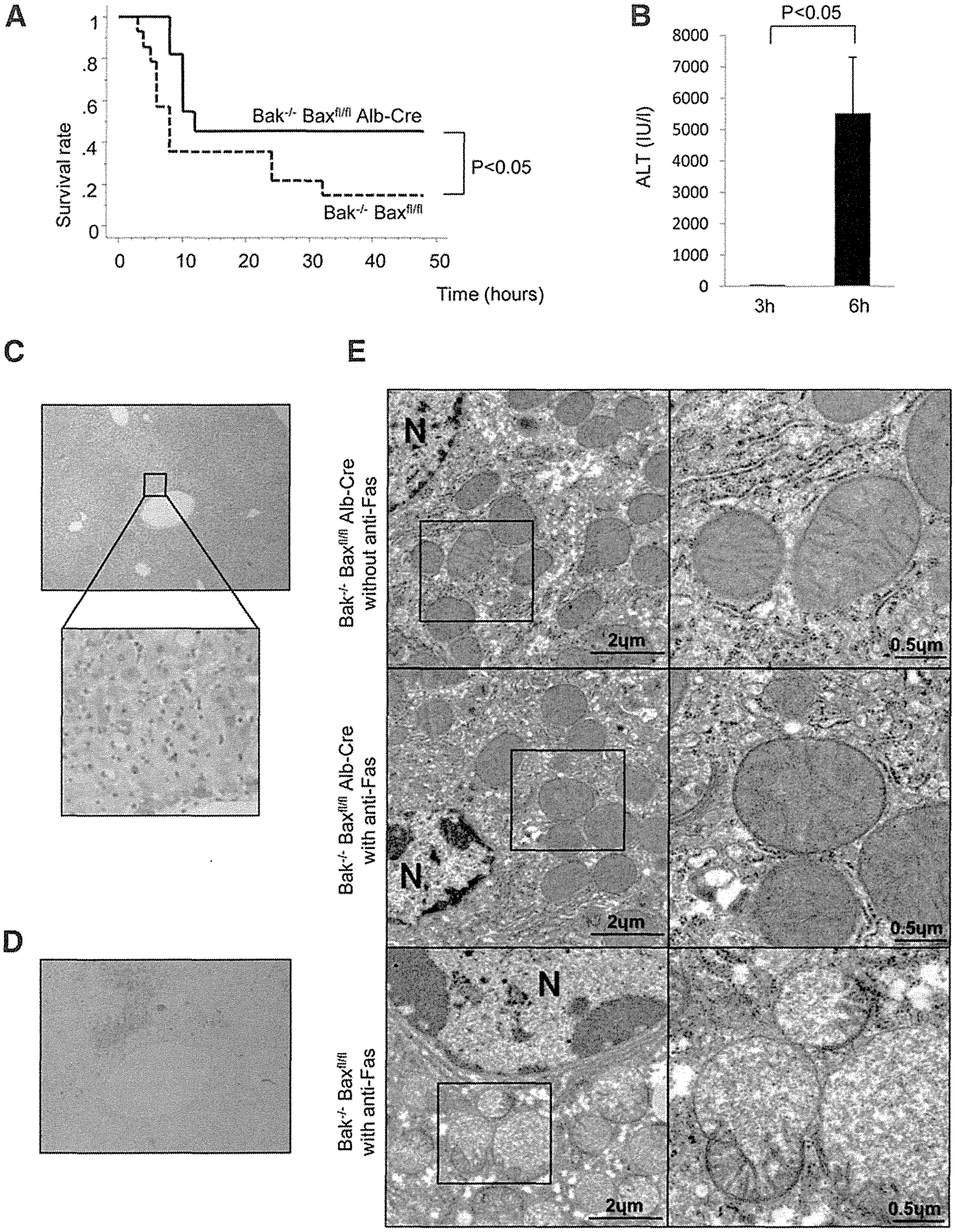

Fig. 4. Bak/Bax DKO mice develop late-onset severe liver injury upon Fas stimulation. Bak/Bax DKO mice (Bak$^{-/-}$ Bax$^{fl/fl}$ Alb-Cre) or control Bak KO littermates (Bak$^{-/-}$ Bax$^{fl/fl}$) were intraperitoneally injected with 1.5 mg/kg Jo2 anti-Fas antibody. (A) Survival rate after Jo2 injection (n = 11 per group). (B) Serum ALT levels of Bak/Bax DKO mice. (C, D) Hematoxylin and eosin (C) and TUNEL (D) staining of the liver sections of Bak/Bax DKO mice 6 hours after Jo2 injection. Representative photomicrographs are shown. (E) Representative electron microscopy photomicrographs of the livers of Bak/Bax DKO mice before and 6 hours after Jo2 anti-Fas injection (1.5 mg/kg) and control Bak KO mice 2 hours after Jo2 anti-Fas injection (1.5 mg/kg). Right panels are enlarged images of the square area of each left panel. N, nucleus.

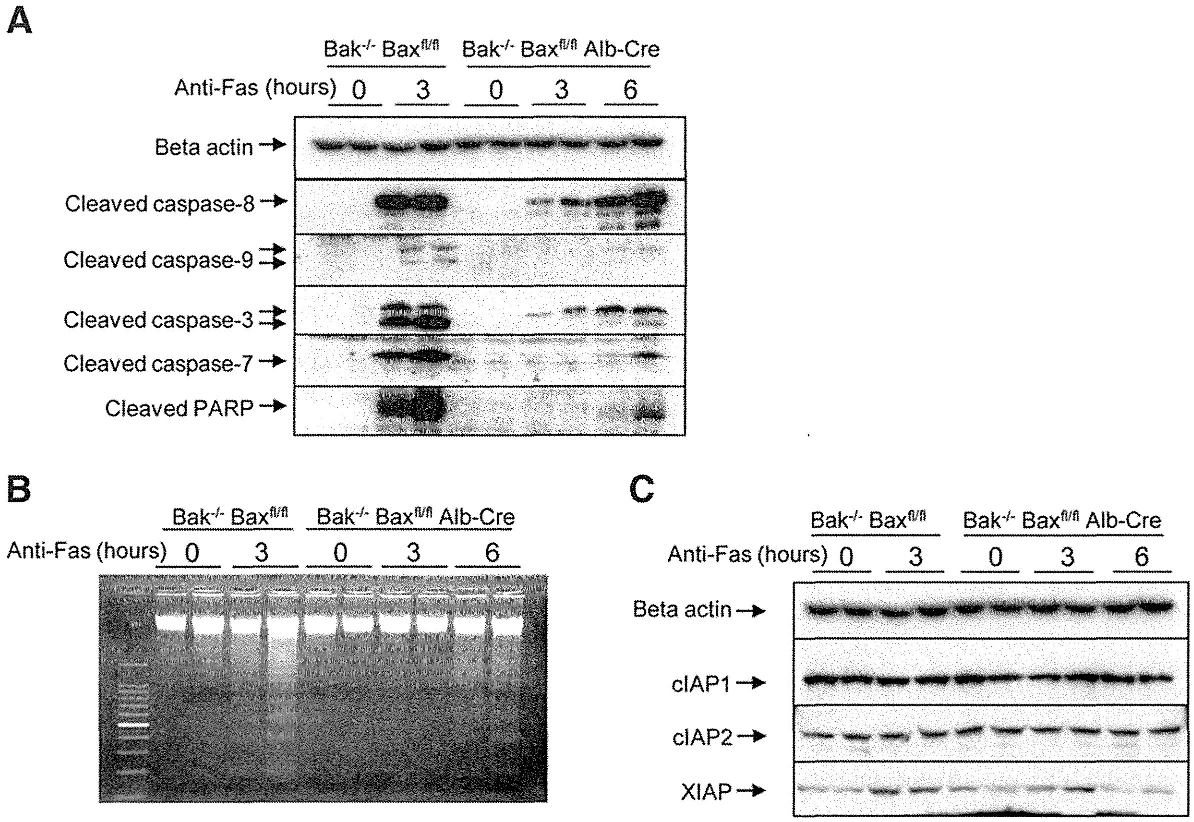

Fig. 5. Fas-mediated hepatocellular death in Bak/Bax DKO mice is associated with caspase-3/7 activation and oligonucleosomal DNA breaks. Bak/Bax DKO mice ($Bak^{-/-}$ $Bax^{fl/fl}$ Alb-Cre) or control Bak KO littermates ($Bak^{-/-}$ $Bax^{fl/fl}$) were intraperitoneally injected with Jo2 anti-Fas antibody (1.5 mg/kg). (A) Western blot analysis for expression of cleaved caspase-8, -9, -3, -7, and PARP. (B) DNA laddering of the liver. (C) Western blot analysis for expression of IAP family proteins.

revealed many TUNEL-positive hepatocytes in the liver sections. Of importance, electron microscopic analysis revealed mitochondrial alterations (such as disruption of the membrane and herniation of the matrix) in hepatocytes of Bak KO mice but not in hepatocytes of Bak/Bax DKO mice with chromatin condensation (Fig. 4E). Because some reports showed that hepatocytes act like type I cells with a high dose of Jo2 anti-Fas antibody and that anti-Fas antibody does not always reliably mimic the action of the physiological Fas ligand,[23,24] we also injected 0.5 mg/kg Jo2 or recombinant Fas ligand into Bak/Bax DKO mice. Similarly, both injected mice showed severe elevation of serum ALT levels and severe hepatitis with many TUNEL-positive cells at 6 hours (Supporting Figs. 1 and 2).

To examine the kinetics of caspase activation and apoptosis in the liver after Jo2 administration, we performed western blot analysis for caspase activation and agarose gel electrophoresis for DNA laddering. All signals for cleaved forms of caspase-3, caspase-7, and PARP in the liver were clearly detected at 6 hours in Bak/Bax DKO mice, although they were weaker than those at 3 hours in control Bak KO littermates (Fig. 5A). Regarding the cleaved form of caspase-9, two bands were detected at 3 hours in Bak KO liver, but not in Bak/Bax DKO liver. Previous research established that procaspase-9 has two sites for cleavage upon activation: both Asp353 and Asp368 sites are autoprocessed by caspase-9 activation after cytochrome c release, whereas the Asp368 site is preferentially processed over the Asp358 site by caspase-3.[25] In our western blot analysis, the slow migrating species corresponding to the fragment cleaved at Asp368, but not the rapid migrating species corresponding to that at Asp353, was weakly detected at 6 hours in Bak/Bax DKO liver. This indicated that caspase-3–mediated cleavage of procaspase-9 takes place without evidence of cytochrome c–induced autoprocessing of procaspase-9. Agarose gel electrophoresis clearly detected oligonucleosomal DNA laddering at 6 hours in Bak/Bax DKO livers, similar to our observation at 3 hours in control Bak KO livers (Fig. 5B). Collectively, these morphological and biochemical data support the idea that hepatocellular death occurring at 6 hours in the Bak/Bax DKO liver seems to involve apoptosis.

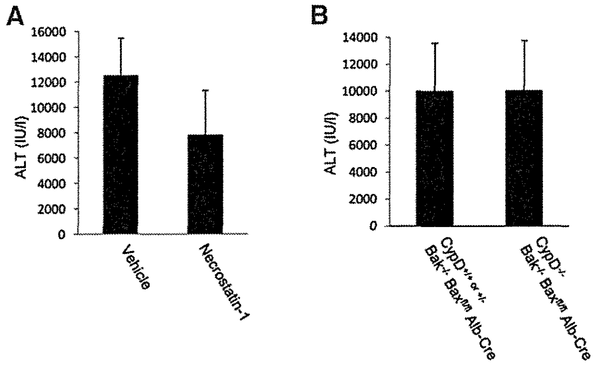

Fig. 6. Fas-induced hepatocellular death in Bak/Bax DKO mice is independent of RIP kinase and/or CypD. (A) Bak/Bax DKO mice (Bak$^{-/-}$ Bax$^{fl/fl}$ Cre) were intraperitoneally injected with 2 mg/kg necrostatin-1 in vehicle containing 0.2% dimethylsulfoxide or vehicle alone at 2 hours after injection of 1.5 mg/kg Jo2 anti-Fas antibody. Serum ALT levels at 6 hours after Jo2 injection are shown ($n = 8$ per group). (B) CypD$^{+/+\text{ or }+/-}$ mice in a Bak/Bax-deficient background (CypD$^{+/+\text{ or }+/-}$ Bak$^{-/-}$ Bax$^{fl/fl}$ Alb-Cre) or control CypD$^{-/-}$ littermates (CypD$^{-/-}$ Bak$^{-/-}$ Bax$^{fl/fl}$ Alb-Cre) were intraperitoneally injected with 1.5 mg/kg Jo2 anti-Fas antibody. Serum ALT levels at 6 hours after injection are shown ($n = 7$ per group or 8 per group, respectively).

To examine the underlying mechanisms by which caspase-3/7 was increasingly activated from 3 to 6 hours in Bak/Bax DKO mice, we analyzed the expression of inhibition of apoptosis proteins (IAPs), which can block cleavage of procaspase-3, -7, and -9.[26] The expression levels of cIAP1 and cIAP2 were not changed in the liver after Jo2 injection (Fig. 5C, Supporting Fig. 3). In contrast, the expression levels of XIAP were up-regulated in the livers of both Bak KO and Bak/Bax DKO mice at 3 hours after Jo2 injection, as in WT mice (Fig. 5C, Supporting Fig. 3), which is consistent with previous reports.[27] However, this up-regulation disappeared from the livers of Bak/Bax DKO mice at 6 hours. Repression of XIAP overexpression might explain why weak activation of capsase-3/7 gradually increased from 3 to 6 hours in the Bak/Bax DKO liver.

Cell Death with Bak/Bax Deficiency Is Not Dependent on a Necrotic Pathway. Fas activation was reported to induce not only caspase-dependent apoptosis but also caspase-independent necrosis, which is required for receptor-interacting protein (RIP) kinase.[28] To exclude the possibility of this necrotic cell death in the Bak/Bax DKO liver, we first examined the effect of necrostatin-1, which specifically inhibits RIP kinase to protect against necrotic cell death caused by death-domain receptor stimulation.[2,29] Bak/Bax DKO mice were injected with 2 mg/kg necrostatin-1 at 2 hours after or 1 hour before Jo2 injection. The ALT levels at 6 hours after Fas stimulation were clearly elevated without a significant difference between the necrostatin-1 injection group and the vehicle injection group (Fig. 6A and Supporting Fig. 4). We next examined the effect of CypD, which is a key molecule of mitochondrial permeability transition generated by Ca^{2+} overload and/or oxidative stress leading to necrotic cell death.[14,30] We injected Jo2 into CypD$^{-/-}$ mice with a Bak/Bax-deficient background (*cypd*$^{-/-}$ *bak*$^{-/-}$ *bax*$^{flox/flox}$ *Alb-Cre*) or control CypD$^{+/+\text{ or }+/-}$ littermates (*cypd*$^{+/+\text{ or }+/-}$ *bak*$^{-/-}$ *bax*$^{flox/flox}$ *Alb-Cre*). The ALT levels of CypD/Bak/Bax triple KO mice upon Fas stimulation were the same as those of control mice (Fig. 6B). These results indicate that liver injury in Bak/Bax deficiency induced by Fas stimulation was not dependent on the necrotic pathway, at least that mediated by RIP kinase and/or CypD.

Late-Onset Cell Death in Bak/Bax Deficiency Is Completely Dependent on Caspase. Although cell death observed in Bak/Bax DKO mice appears to be apoptosis, the question arose of whether relatively weak caspase-3/7 activity compared with that observed in Bak KO mice is sufficient for inducing liver injury 6 hours after Fas simulation. To this end, Bak/Bax DKO mice were given 40 mg/kg Q-VD-Oph, a potent broad spectrum caspase inhibitor,[31] 2 hours after injection of Jo2. Western blot analysis revealed the existence of truncated Bid and cleaved caspase-8 in the liver 2 hours after Jo2 injection, demonstrating that caspase-8 had already been activated by this point (Fig. 7A). Administration of the caspase inhibitor at 2 hours completely blocked the elevation of serum ALT levels and hepatocellular apoptosis, as evidenced by liver histology and TUNEL staining 6 hours after Jo2 injection (Fig. 7B-D). Finally, we tried to analyze the survival rate of Bak/Bax DKO mice and control Bak KO mice when therapeutically injected with the caspase inhibitor 2 hours after Jo2 injection. None of the Bak/Bax DKO mice showed lethal liver injury upon Jo2 injection, whereas half of the Bak KO mice died from severe liver injury (Fig. 7E). These findings suggest that Fas-induced liver injury in Bak/Bax deficiency was dependent on caspase activity, which could be fully negated by the caspase inhibitor. On the other hand, caspase activation in Bak KO mice was too high to be negated by the same dose of the caspase inhibitor.

Discussion

In the present study, we demonstrate that Bak KO, but not Bax KO, provides partial resistance to Fas-induced hepatocellular apoptosis in vivo. We demonstrated previously that Bak KO mice, but not Bax KO

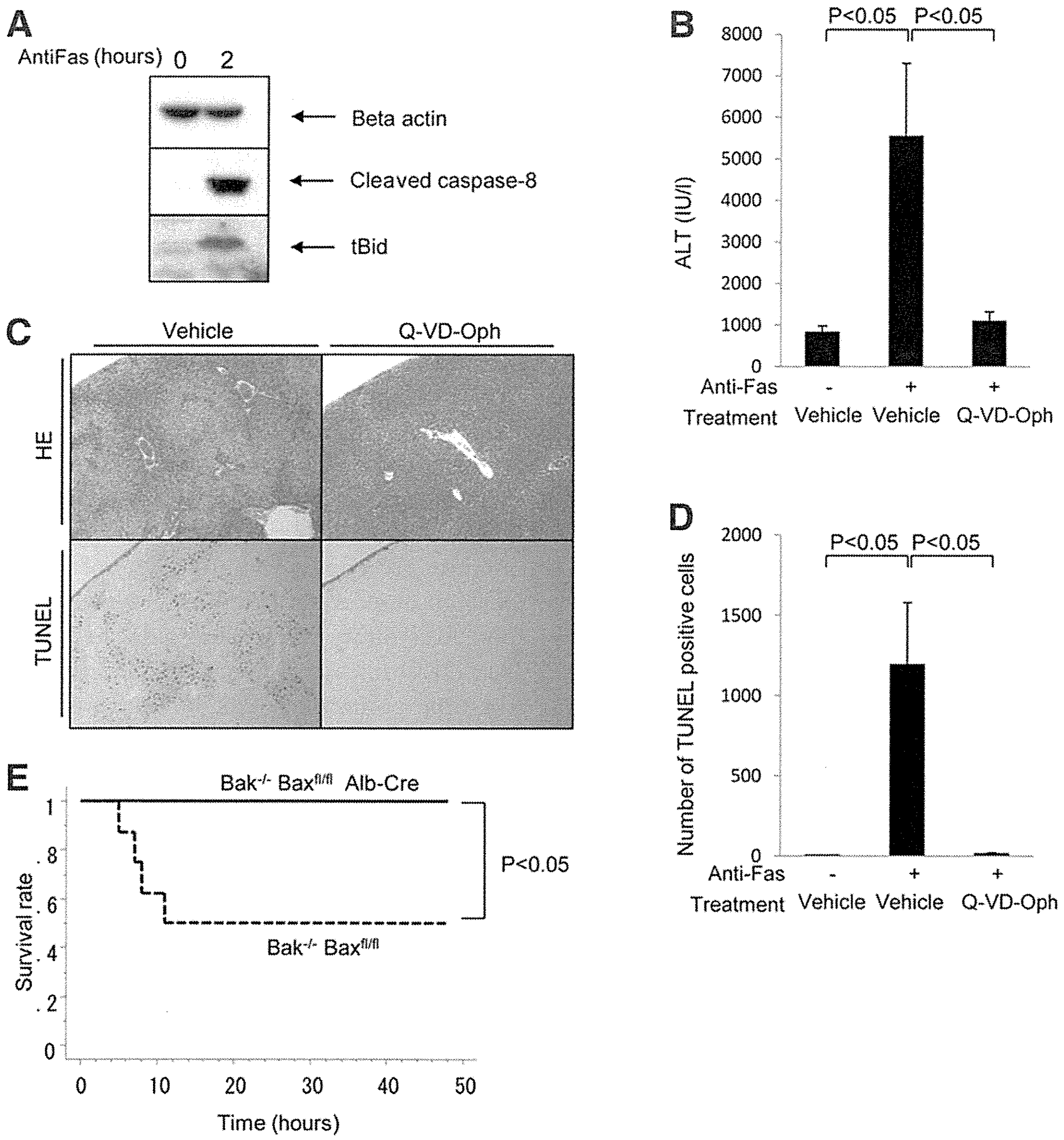

Fig. 7. Hepatocellular death in Bak/Bax DKO mice is dependent on caspase activation. (A) Bak/Bax DKO mice were analyzed before and 2 hours after intraperitoneal injection of Jo2 anti-Fas antibody (1.5 mg/kg). Western blot analysis of the liver for the expression of cleaved caspase-8 and truncated Bid (tBid). (B-D) Bak/Bax DKO mice were intraperitoneally administered 40 mg/kg Q-VD-Oph in 10 mL/kg dimethylsulfoxide (DMSO) or DMSO alone, as a vehicle, 2 hours after injection of 1.5 mg/kg Jo2 anti-Fas antibody and analyzed at 6 hours. (B) Serum ALT levels ($n = 6$ or 7 per group, respectively). (C) Hematoxylin and eosin (HE) and TUNEL staining of the liver sections. (D) Number of TUNEL-positive cells ($n = 6$ or 7 per group, respectively). Because intraperitoneal injection of DMSO leads to injury at the surface layer of the liver, TUNEL positivity close to the surface layer was excluded from the cell count. (E) Bak/Bax DKO mice (Bak$^{-/-}$ Bax$^{fl/fl}$ Alb-Cre) or control Bak KO littermates (Bak$^{-/-}$ Bax$^{fl/fl}$) were given 40 mg/kg Q-VD-Oph intraperitoneally in 10 mL/kg DMSO or DMSO alone at 2 hours after injection of 1.5 mg/kg anti-Fas antibody. The disease-free survival rate of lethal liver injury after Jo2 injection is shown ($n = 8$ per group).

mice, showed resistance to apoptosis induced by Bcl-xL deficiency, which depended mainly on Bid activation.[16] Research has shown that Fas induces apoptosis in hepatocytes through the Bid pathway,[10,11] and the present study also demonstrates that Bid becomes truncated in the liver upon anti-Fas injection. Therefore, truncated Bid may preferentially activate Bak rather than Bax in the liver. However, the present study also reveals that, in the absence of Bak, Bax plays an essential role in mediating the early onset of hepatocellular apoptosis. The most important finding of this study is that Bak/Bax deficiency failed to protect against the late onset of liver injury after Jo2 anti-Fas injection as well as Fas agonist injection. Wei et al.,[32] in their historical paper establishing the importance of Bak and Bax in the mitochondrial pathway of apoptosis, reported that hepatocytes were protected from Jo2-induced apoptosis in traditional Bak/Bax DKO mice (*bak*$^{-/-}$ *bax*$^{-/-}$). Because perinatal lethality occurs with most traditional Bak/Bax DKO mice, they could only analyze three animals, which did not enable detailed analysis of cell death due to Jo2 stimulation. The present study is the first to (1) thoroughly examine the impact of Bak and Bax in the liver using conditional KO mice and (2) demonstrate that Bak/Bax deficiency can protect against Fas-induced severe injury in the early phase but not in the late phase.

The late onset of liver injury observed in Bak/Bax DKO appeared to be apoptosis based on biochemical and morphological observations, including caspase activation, oligonucleosomal DNA breaks and, most importantly, identification of cell death with caspase dependency. In addition, the well-established necrotic pathway mediated by RIP kinase and/or CypD was not involved. However, the difference from apoptosis observed in Bak KO mice was the absence of mitochondrial alteration or cytochrome c–dependent caspase-9 processing in Bak/Bax DKO mice. We also confirmed that Bak/Bax-deficient mitochondria were not capable of releasing cytochrome c in the presence of truncated Bid (Supporting Fig. 5). These data support the idea that activation of the mitochondrial pathway of apoptosis is fully dependent on either Bak or Bax even in the late phase, indicating at the same time that late onset of apoptosis takes place through an extrinsic pathway rather than the mitochondrial pathway.

Although hepatocytes are generally considered to be type II cells, recent work has shown that the requirement of the mitochondrial pathway may be overcome through changes induced by in vitro culture conditions[33,34] or the strength of Fas stimulation.[23] Schüngel et al.[23] demonstrated that hepatocytes act as type II cells with a low-dose Jo2 injection (0.5 mg/kg) and act as type I cells with an extremely high-dose Jo2 injection (5 mg/kg). This agrees with the generally accepted idea that type I cells exhibit strong activation of DISC and caspase-8, which itself is sufficient to induce apoptosis, whereas type II cells exhibit weak activation and therefore require amplification of the apoptosis signal through the mitochondrial loop. In the present study, we used 1.5 mg/kg or 0.5 mg/kg Jo2 antibody, which could be considered relatively low doses, and found that hepatocytes act like type II cells in WT mice or Bak/Bax single KO mice but act like type I cells in Bak/Bax DKO mice. The present study therefore demonstrates that hepatocytes can act as type I cells in the absence of Bak and Bax independent of the strength of DISC formation or signals from microenvironments.

The question arises of why hepatocytes can act as type I cells where the levels of DISC formation or caspase-8 activation may be insufficient to induce activation of downstream caspases. Recently, Jost et al.[27] reported a discriminating role of XIAP between type I and type II cells; in type II cells, the levels of XIAP expression increased after Fas stimulation but decreased in type I cells. In agreement with this report, XIAP expression was up-regulated at 3 hours in both Bak KO and Bak/Bax DKO livers. Interestingly, this XIAP up-regulation disappeared at 6 hours after Jo2 injection in Bak/Bax DKO mice. Because XIAP is a potent inactivator of caspase-3, -7, and -9 processing, repression of XIAP may be one reason why hepatocytes can act as type I cells at this time point.

Previous studies have reported that liver endothelial cells express Fas receptor and have suggested that apoptosis of these cells may participate in the liver damage in mice receiving Jo2 antibody, especially in the case of high-dose administration.[35] However, we did not find liver injury in the sinusoidal hemorrhage in Bak/Bax DKO mice at 3 hours after Jo2 injection, which is the time point when Bak KO mice developed it (Fig. 3C). Together with the fact that Bax, but not Bak, was active in liver nonparenchymal cells in our Bak/Bax DKO mice, as was the case in Bak KO mice (Fig. 3A), we speculate that Bak-deficient sinusoidal cells could not contribute much to liver injury at 3 hours after Jo2 injection (1.5 or 0.5 mg/kg).

Recently, a pan-caspase inhibitor was reported to reduce hepatic damage in liver transplant recipients and patients with chronic hepatitis C in clinical trials.[36,37] For treatment of fulminant liver injury, caspase inhibitors seem to be attractive drugs. However, the present study demonstrates that Fas-induced apoptotic signals could be efficiently amplified through the mitochondrial pathway, leading to high lethality even if caspase inhibitor was administered 2 hours after Jo2 injection. In contrast, administration of the same dose of the caspase inhibitor was able to fully block hepatocyte apoptosis and lethality in Bak/Bax DKO mice. From a clinical point of view, when using caspase inhibitors to prevent fulminant liver failure, concomitant inactivation of the mitochondrial amplification loop may be required.

In conclusion, the extrinsic pathway of apoptosis exists in hepatocytes and causes late onset of lethal liver failure in the absence of Bak and Bax independent of the strength of Fas ligation. This pathway could be therapeutically intervened through the use of caspase inhibitors, presumably due to low levels of DISC formation and subsequent weak activation of effector caspases in hepatocytes. The present study unveils the entire framework of the Fas-mediated signaling pathway in hepatocytes, placing the mitochondrial pathway of apoptosis as a potent loop for amplifying activation of the caspase cascade to execute complete and rapid cell death in hepatocytes.

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References

1. Guicciardi M, Gores G. Life and death by death receptors. FASEB J 2009;23:1625-1637.
2. Malhi H, Guicciardi M, Gores G. Hepatocyte death: a clear and present danger. Physiol Rev 2010;90:1165-1194.
3. Hiramatsu N, Hayashi N, Katayama K, Mochizuki K, Kawanishi Y, Kasahara A, et al. Immunohistochemical detection of Fas antigen in liver tissue of patients with chronic hepatitis C. HEPATOLOGY 1994;19:1354-1359.
4. Hayashi N, Mita E. Involvement of Fas system-mediated apoptosis in pathogenesis of viral hepatitis. J Viral Hepat 1999;6:357-365.
5. Feldstein A, Canbay A, Angulo P, Taniai M, Burgart L, Lindor K, et al. Hepatocyte apoptosis and fas expression are prominent features of human nonalcoholic steatohepatitis. Gastroenterology 2003;125:437-443.
6. Ryo K, Kamogawa Y, Ikeda I, Yamauchi K, Yonehara S, Nagata S, et al. Significance of Fas antigen-mediated apoptosis in human fulminant hepatic failure. Am J Gastroenterol 2000;95:2047-2055.
7. Feldmann G, Lamboley C, Moreau A, Bringuier A. Fas-mediated apoptosis of hepatic cells. Biomed Pharmacother 1998;52:378-385.
8. Tsujimoto Y. Cell death regulation by the Bcl-2 protein family in the mitochondria. J Cell Physiol 2003;195:158-167.
9. Tait S, Green D. Mitochondria and cell death: outer membrane permeabilization and beyond. Nat Rev Mol Cell Biol 2010;11:621-632.
10. Yin X, Wang K, Gross A, Zhao Y, Zinkel S, Klocke B, et al. Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis. Nature 1999;400:886-891.
11. Kaufmann T, Tai L, Ekert PG, Huang DC, Norris F, Lindemann RK, et al. The BH3-only protein bid is dispensable for DNA damage- and replicative stress-induced apoptosis or cell-cycle arrest. Cell 2007;129:423-433.
12. Lindsten T, Ross A, King A, Zong W, Rathmell J, Shiels H, et al. The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. Mol Cell 2000;6:1389-1399.
13. Takehara T, Tatsumi T, Suzuki T, Rucker EB, 3rd, Hennighausen L, Jinushi M, et al. Hepatocyte-specific disruption of Bcl-xL leads to continuous hepatocyte apoptosis and liver fibrotic responses. Gastroenterology 2004;127:1189-1197.
14. Nakagawa T, Shimizu S, Watanabe T, Yamaguchi O, Otsu K, Yamagata H, et al. Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death. Nature 2005;434:652-658.
15. Hikita H, Takehara T, Shimizu S, Kodama T, Li W, Miyagi T, et al. Mcl-1 and Bcl-xL cooperatively maintain integrity of hepatocytes in developing and adult murine liver. HEPATOLOGY 2009;50:1217-1226.
16. Hikita H, Takehara T, Kodama T, Shimizu S, Hosui A, Miyagi T, et al. BH3-only protein bid participates in the Bcl-2 network in healthy liver cells. HEPATOLOGY 2009;50:1972-1980.
17. Wang K, Yin X, Chao D, Milliman C, Korsmeyer S. BID: a novel BH3 domain-only death agonist. Genes Dev 1996;10:2859-2869.
18. Yamagata H, Shimizu S, Nishida Y, Watanabe Y, Craigen WJ, Tsujimoto Y. Requirement of voltage-dependent anion channel 2 for pro-apoptotic activity of Bax. Oncogene 2009;28:3563-3572.
19. Ogasawara J, Watanabe-Fukunaga R, Adachi M, Matsuzawa A, Kasugai T, Kitamura Y, et al. Lethal effect of the anti-Fas antibody in mice. Nature 1993;364:806-809.
20. Wieder T, Essmann F, Prokop A, Schmelz K, Schulze-Osthoff K, Beyaert R, et al. Activation of caspase-8 in drug-induced apoptosis of B-lymphoid cells is independent of CD95/Fas receptor-ligand interaction and occurs downstream of caspase-3. Blood 2001;97:1378-1387.
21. Antonsson B, Montessuit S, Sanchez B, Martinou J. Bax is present as a high molecular weight oligomer/complex in the mitochondrial membrane of apoptotic cells. J Biol Chem 2001;276:11615-11623.
22. Kim T, Zhao Y, Barber M, Kuharsky D, Yin X. Bid-induced cytochrome c release is mediated by a pathway independent of mitochondrial permeability transition pore and Bax. J Biol Chem 2000;275:39474-39481.
23. Schüngel S, Buitrago-Molina L, Nalapareddy P, Lebofsky M, Manns M, Jaeschke H, et al. The strength of the Fas ligand signal determines whether hepatocytes act as type 1 or type 2 cells in murine livers. HEPATOLOGY 2009;50:1558-1566.
24. Huang DC, Hahne M, Schroeter M, Frei K, Fontana A, Villunger A, et al. Activation of Fas by FasL induces apoptosis by a mechanism that cannot be blocked by Bcl-2 or Bcl-x(L). Proc Natl Acad Sci U S A 1999;96:14871-14876.
25. Fujita E, Egashira J, Urase K, Kuida K, Momoi T. Caspase-9 processing by caspase-3 via a feedback amplification loop in vivo. Cell Death Differ 2001;8:335-344.
26. Deveraux Q, Reed J. IAP family proteins—suppressors of apoptosis. Genes Dev 1999;13:239-252.
27. Jost P, Grabow S, Gray D, McKenzie M, Nachbur U, Huang D, et al. XIAP discriminates between type I and type II FAS-induced apoptosis. Nature 2009;460:1035-1039.
28. Holler N, Zaru R, Micheau O, Thome M, Attinger A, Valitutti S, et al. Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. Nat Immunol 2000;1:489-495.
29. Degterev A, Huang Z, Boyce M, Li Y, Jagtap P, Mizushima N, et al. Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. Nat Chem Biol 2005;1:112-119.
30. Baines C, Kaiser R, Purcell N, Blair N, Osinska H, Hambleton M, et al. Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. Nature 2005;434:658-662.
31. Caserta T, Smith A, Gultice A, Reedy M, Brown T. Q-VD-OPh, a broad spectrum caspase inhibitor with potent antiapoptotic properties. Apoptosis 2003;8:345-352.
32. Wei M, Zong W, Cheng E, Lindsten T, Panoutsakopoulou V, Ross A, et al. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science 2001;292:727-730.
33. Walter D, Schmich K, Vogel S, Pick R, Kaufmann T, Hochmuth F, et al. Switch from type II to I Fas/CD95 death signaling on in vitro culturing of primary hepatocytes. HEPATOLOGY 2008;48:1942-1953.
34. Schmich K, Schlatter R, Corazza N, Ferreira KS, Ederer M, Brunner T, et al. Tumor necrosis factor α sensitizes primary murine hepatocytes to Fas/CD95-induced apoptosis in a Bim- and Bid-dependent manner. HEPATOLOGY 2011;53:282-292.
35. Cardier JE, Schulte T, Kammer H, Kwak J, Cardier M. Fas (CD95, APO-1) antigen expression and function in murine liver endothelial cells: implications for the regulation of apoptosis in liver endothelial cells. FASEB J 1999;13:1950-1960.
36. Baskin-Bey E, Washburn K, Feng S, Oltersdorf T, Shapiro D, Huyghe M, et al. Clinical Trial of the Pan-Caspase Inhibitor, IDN-6556, in Human Liver Preservation Injury. Am J Transplant 2007;7:218-225.
37. Pockros P, Schiff E, Shiffman M, McHutchison J, Gish R, Afdhal N, et al. Oral IDN-6556, an antiapoptotic caspase inhibitor, may lower aminotransferase activity in patients with chronic hepatitis C. HEPATOLOGY 2007;46:324-329.

The Bcl-xL Inhibitor, ABT-737, Efficiently Induces Apoptosis and Suppresses Growth of Hepatoma Cells in Combination with Sorafenib

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Tumor cells are characterized by uncontrolled proliferation, often driven by activation of oncogenes, and apoptosis resistance. The oncogenic kinase inhibitor sorafenib can significantly prolong median survival of patients with advanced hepatocellular carcinoma (HCC), although the response is disease-stabilizing and cytostatic rather than one of tumor regression. Bcl-xL (B cell lymphoma extra large), an antiapoptotic member of the B cell lymphoma-2 (Bcl-2) family, is frequently overexpressed in HCC. Here, we present *in vivo* evidence that Bcl-xL overexpression is directly linked to the rapid growth of solid tumors. We also examined whether ABT-737, a small molecule that specifically inhibits Bcl-xL but not myeloid cell leukemia-1 (Mcl-1), could control HCC progression, especially when used with sorafenib. Administration of ABT-737, even at an *in vivo* effective dose, failed to suppress Huh7 xenograft tumors in mice. ABT-737 caused the levels of Mcl-1 expression to rapidly increase by protein stabilization. This appeared to be related to resistance to ABT-737, because decreasing Mcl-1 expression levels to the baseline by a small interfering RNA–mediated strategy made hepatoma cells sensitive to this agent. Importantly, administration of ABT-737 to Mcl-1 knockout mice induced severe liver apoptosis, suggesting that tumor-specific inhibition of Mcl-1 is required for therapeutic purposes. Sorafenib transcriptionally down-regulated Mcl-1 expression specifically in tumor cells and abolished Mcl-1 up-regulation induced by ABT-737. Sorafenib, not alone but in combination with ABT-737, efficiently induced apoptosis in hepatoma cells. This combination also led to stronger suppression of xenograft tumors than sorafenib alone. *Conclusion:* Bcl-xL inactivation by ABT-737 in combination with sorafenib was found to be safe and effective for anti-HCC therapy in preclinical models. Direct activation of the apoptosis machinery seems to unlock the antitumor potential of oncogenic kinase inhibitors and may produce durable clinical responses against HCC. (HEPATOLOGY 2010;52:1310-1321)

The B cell lymphoma-2 (Bcl-2) family proteins regulate the mitochondrial pathway of apoptosis, a major form of cell death.[1] They include five antiapoptotic proteins, Bcl-2, B cell lymphoma extra large (Bcl-xL), myeloid cell leukemia-1 (Mcl-1), Bcl-2–related protien A1 (Bfl-1), and Bcl-2-like 2 (Bcl-w), and two structurally related proapoptotic proteins, Bcl-2–antagonist/killer (Bak) and Bcl-2–

Abbreviations: ALT, alanine aminotransferase; Bad, Bcl-2–associated agonist of cell death; Bak, Bcl-2–antagonist/killer; Bax, Bcl-2–associated X protein; Bcl-2, B cell lymphoma-2; BH3, Bcl-2 homology domain-3; Bid, BH3-interacting domain death agonist; cDNA, complementary DNA; HA, hemagglutinin; HCC, hepatocellular carcinoma; Mcl-1, myeloid cell leukemia-1; mRNA, messenger RNA; RT-PCR, reverse-transcription polymerase chain reaction; siRNA, small interfering RNA; USP9X, ubiquitin-specific peptidase 9 X-linked; WST, water-soluble tetrazolium.

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associated X protein (Bax), as well as several structurally diverse proapoptotic Bcl-2 homology domain-3 (BH3)-only proteins like Bcl-2–associated agonist of cell death (Bad), BH3-interacting domain death agonist (Bid), and Bcl-2-like 11 (Bim). Bak and Bax, effector molecules in this family, homo-oligomerize into proteolipid pores within the mitochondrial outer membrane, leading to release of cytochrome c followed by activation of downstream caspases, such as caspase-3/7, which dismantle a variety of cellular substrates, leading to cell death. Antiapoptotic Bcl-2 proteins function as regulators of apoptosis by directly or indirectly antagonizing Bak and Bax activity to maintain cellular integrity. BH3-only proteins, sensors of apoptosis, are activated by a variety of cellular stresses and either directly activate Bak and Bax or neutralize antiapoptotic Bcl-2 proteins, inducing cell death. Because tumor cells encounter a variety of cellular stresses, such as genotoxic and environmental factors, overexpression of antiapoptotic Bcl-2 family proteins is commonly observed and leads to survival of tumor cells.[2] We and others have reported that Bcl-xL is frequently overexpressed in human hepatocellular carcinomas (HCCs).[3–6] These reports point to the resistance of hepatoma cells to a wide variety of stress-inducing conditions. For example, Bcl-xL blocks p53-induced apoptosis in hepatoma cells, implying that Bcl-xL overexpression may be one of the mechanisms by which HCC survives under genotoxic conditions.[3] In addition, Bcl-xL overexpression was found to be associated with poor overall survival and disease-free survival after surgical resection for HCC.[7] These findings suggest that Bcl-xL may be a therapeutic target for HCC, although this possibility has not yet been addressed. Bcl-xL is also expressed in normal hepatocytes and plays a critical role in maintaining their integrity.[8] Thus, special caution is necessary when Bcl-xL inactivation is applied to therapeutic purposes.

Despite advances in understanding the mechanisms of cell death and the biology of Bcl-2 family proteins, therapeutic strategies for HCC targeting apoptotic molecules have been hampered due to a lack of specific inhibitors. ABT-737 is one of the first small-molecule inhibitors of Bcl-2 family proteins and opens the field for cancer treatment targeting the apoptosis machinery. ABT-737, designed as a Bad mimetic, binds and neutralizes Bcl-2, Bcl-xL, and Bcl-w, but not Mcl-1 or Bfl-1.[9-11] It has single-agent activity in a number of hematopoietic cancers and some solid tumors.[12,13] Its orally available derivative, ABT-263, is in early clinical trials against lymphoid malignancies, small-cell lung cancer, and chronic lymphocytic leukemia, with some promising results.[14] In this study, we investigated the impact of ABT-737 in treating human hepatoma in culture and using a xenograft model. We found that hepatoma cells are relatively resistant to ABT-737, presumably due to reciprocal up-regulation of Mcl-1 upon ABT-737 exposure. Although concomitant Mcl-1 inhibition appears to be effective for inducing apoptosis by ABT-737, it should be done in a tumor-specific manner, because administration of ABT-737 leads to liver deterioration in Mcl-1 knockout mice. Finally, sorafenib, an anti-HCC agent recently approved by the U.S. Food and Drug Administration, down-regulates Mcl-1 expression in a tumor-specific manner and induces apoptosis and tumor growth suppression in cooperation with ABT-737. Combination therapy with sorafenib and a Bcl-xL inhibitor seems to be an attractive strategy for controlling tumor progression in HCC.

Materials and Methods

Cell Lines and Reagents. Primary human hepatocytes were obtained from ScienCell Research Laboratories (Carlsbad, CA) and cultured with the provided medium. Human hepatoma cell lines were cultured with Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Sigma, St. Louis, MO). Cycloheximide was purchased from Nacalai Tesque (Kyoto, Japan), sorafenib tablets were purchased from Bayer HealthCare (Osaka, Japan), and ABT-737 was kindly provided by Abbott Laboratories (Abbott Park, IL). They were dissolved with dimethyl sulfoxide for *in vitro* use.

Hela Cells Expressed Bcl-xL with the Tet-on System. pcDNA3HABcl-xL, an expression vector coding human Bcl-xL tagged with hemagglutinin (HA), was provided by Dr. G. Nunez (University of Michigan Medical School, Ann Arbor, MI). The pcDNA4/TOHABcl-xL was constructed by inserting the complementary DNA (cDNA) for Bcl-xL gene with HA-tag from pcDNA3HABcl-xL into the EcoRI site of pcDNA4/TO (Invitrogen, Carlsbad, CA). TREx-Hela cells (Invitrogen) were transfected with pcDNA4/

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