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 $\pm$ 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

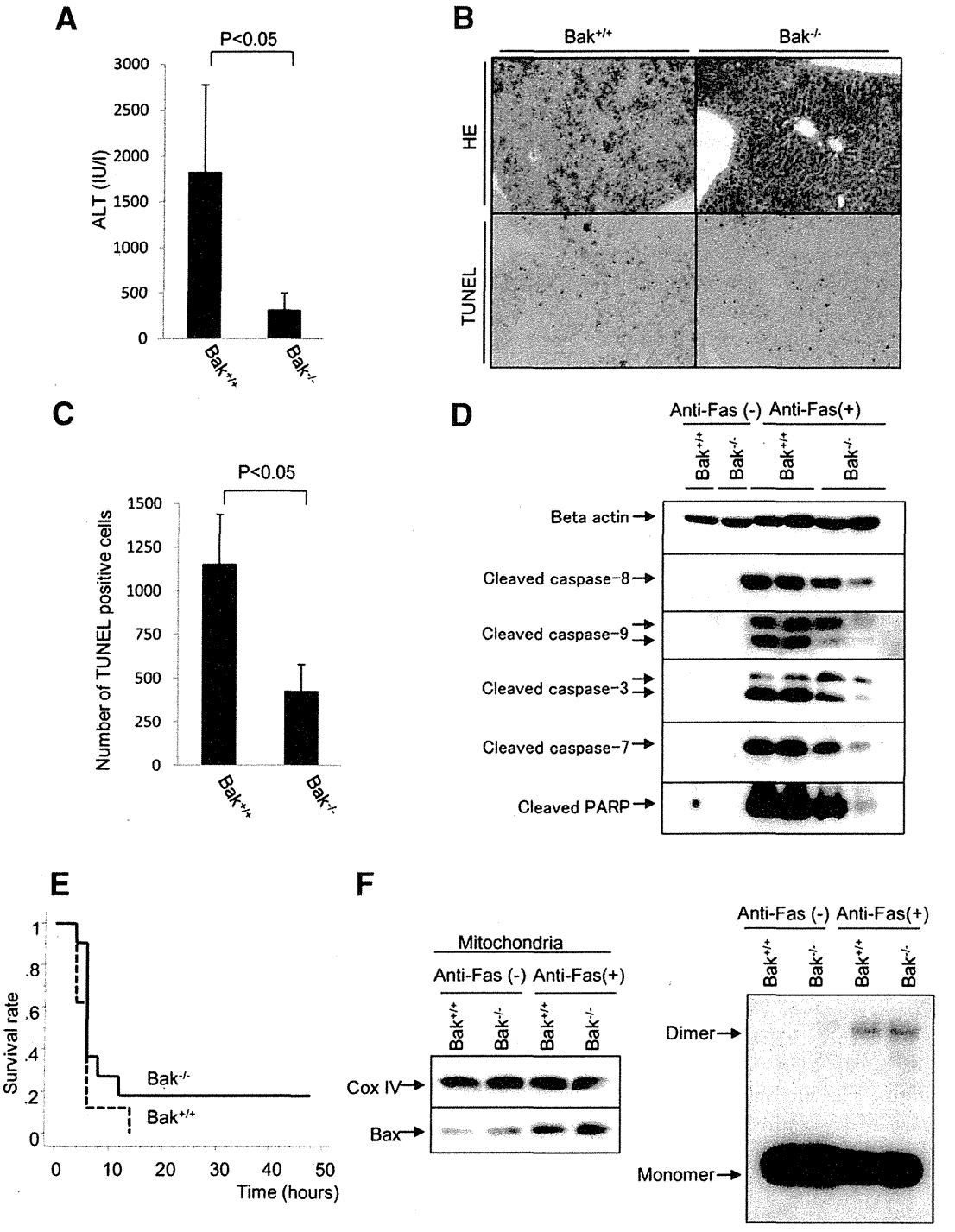

Fig. 1. Bak KO mice are partially resistant to Fas-induced hepatocellular apoptosis. Bak KO mice ($Bak^{-/-}$) or control WT littermates ($Bak^{+/+}$) were analyzed at 3 hours after intraperitoneal injection of 1.5 mg/kg Jo2 anti-Fas antibody. (A) Serum ALT levels (n = 10 or 11, respectively). (B) Hematoxylin 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.

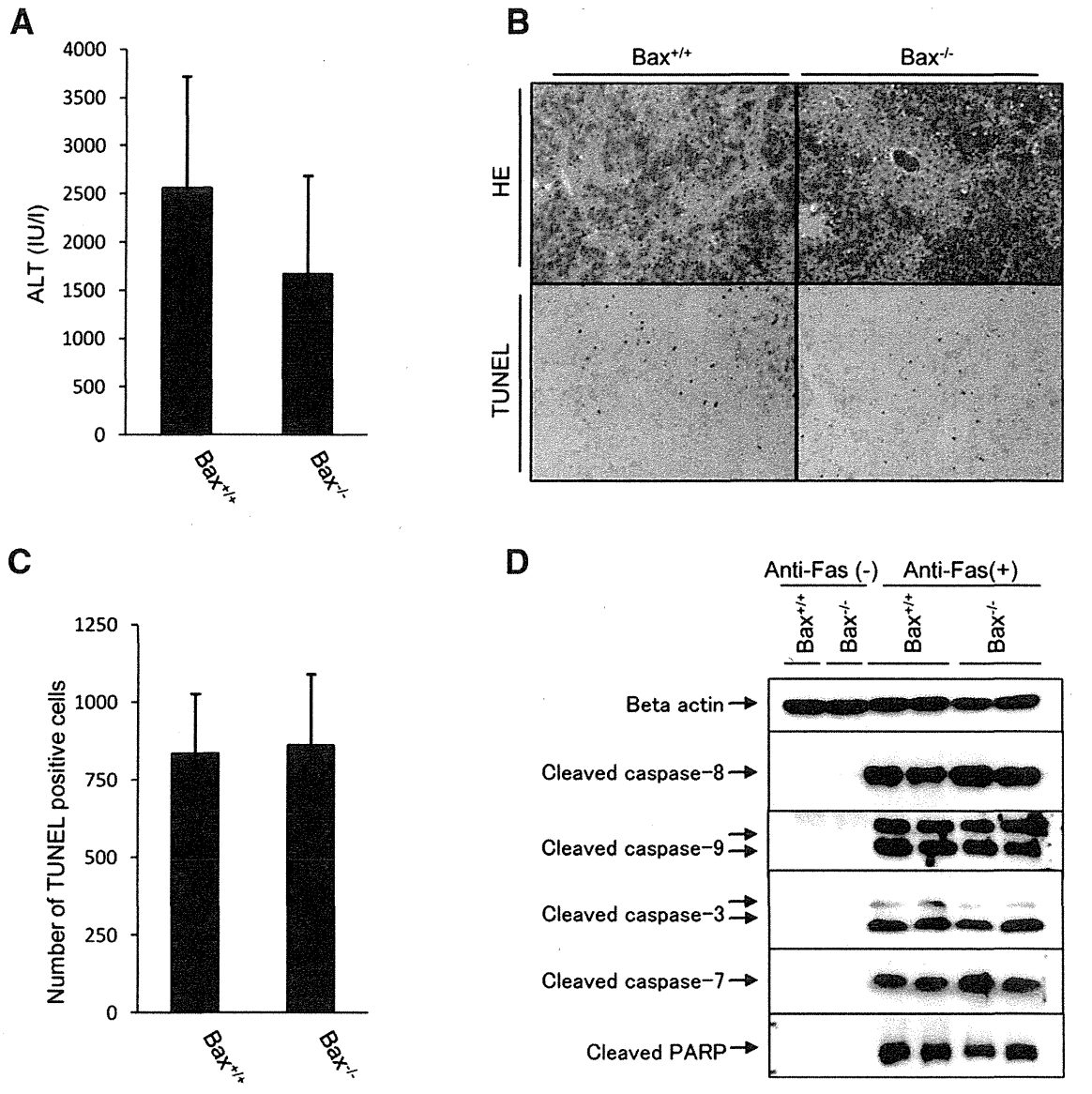

Fig. 2. Bax KO mice are not resistant to Fas-induced hepatocellular apoptosis. Bax KO mice ($Bax^{-/-}$) or control WT littermates ($Bax^{+/+}$) were analyzed 3 hours after intraperitoneal injection of Jo2 anti-Fas antibody (1.5 mg/kg). (A) Serum ALT levels (n = 11 per group). (B) Hematoxylin and eosin (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

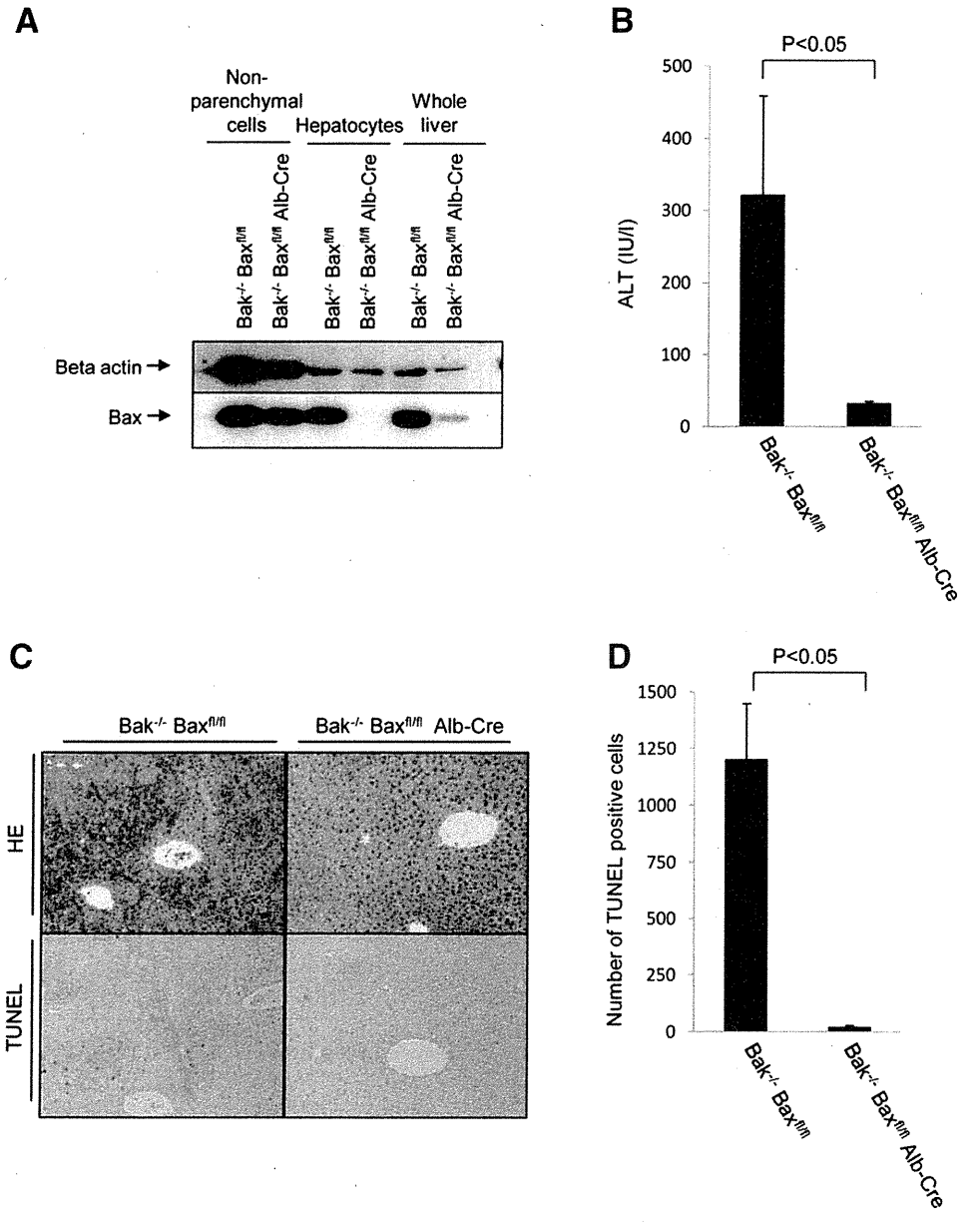

Fig. 3. Bak/Bax DKO mice are fully resistant to Fas-induced hepatocellular apoptosis in early phase. (A) Western blot analysis of the indicated fraction of the liver for the expressions of Bax. (B-D) Bak/Bax DKO mice ($Bak^{-/-}$ $Bax^{fl/fl}$ Alb-Cre) or control Bak KO littermates ($Bak^{-/-}$ $Bax^{fl/fl}$) 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

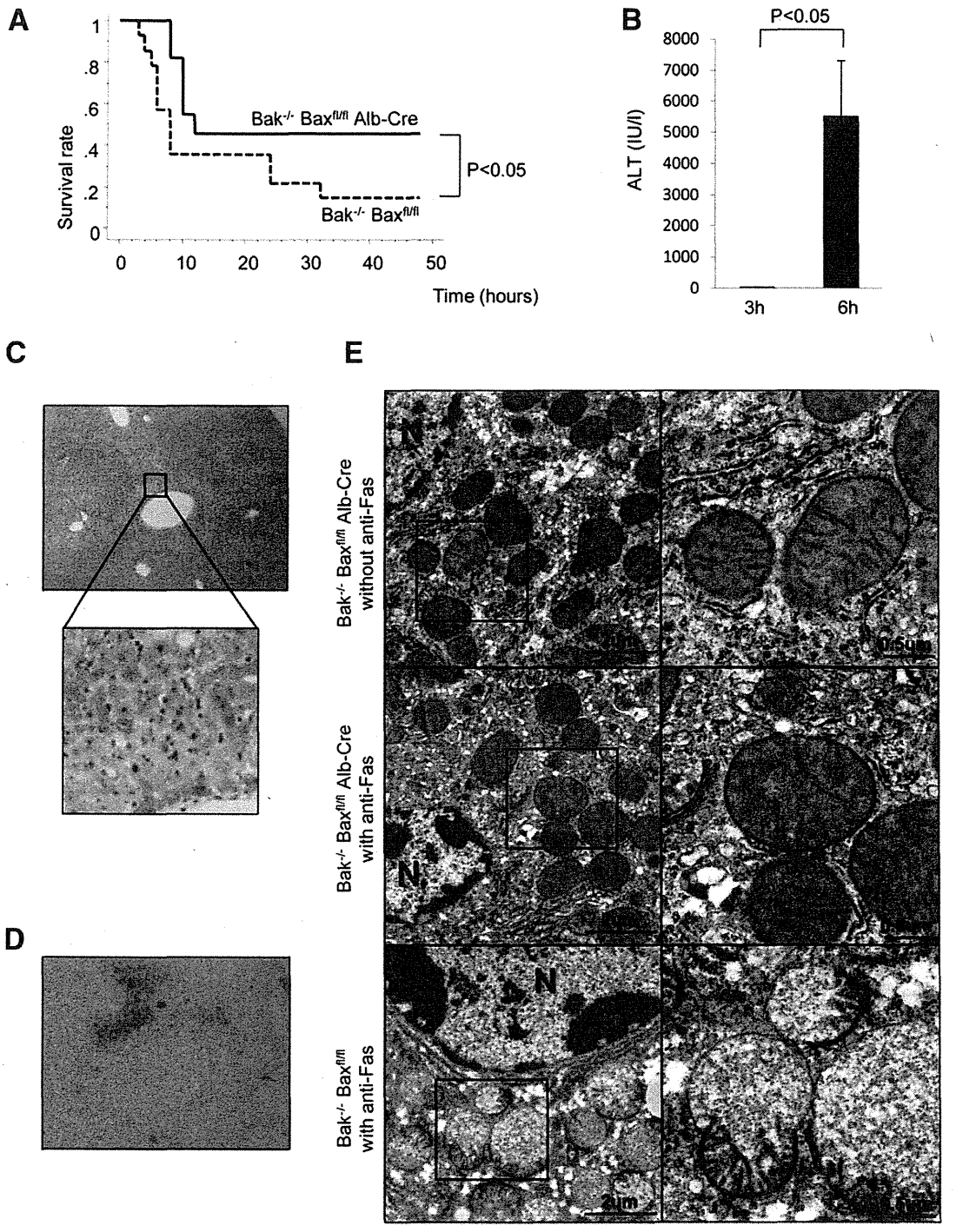

Fig. 4. Bak/Bax DKO mice develop late-onset severe liver injury upon Fas stimulation. Bak/Bax DKO mice ($Bak^{-/-}$ $Bax^{fl/fl}$ Alb-Cre) or control Bak KO littermates ($Bak^{-/-}$ $Bax^{fl/fl}$) were intraperitoneally injected with 1.5 mg/kg Jo2 anti-Fas antibody. (A) Survival rate after Jo2 injection (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.

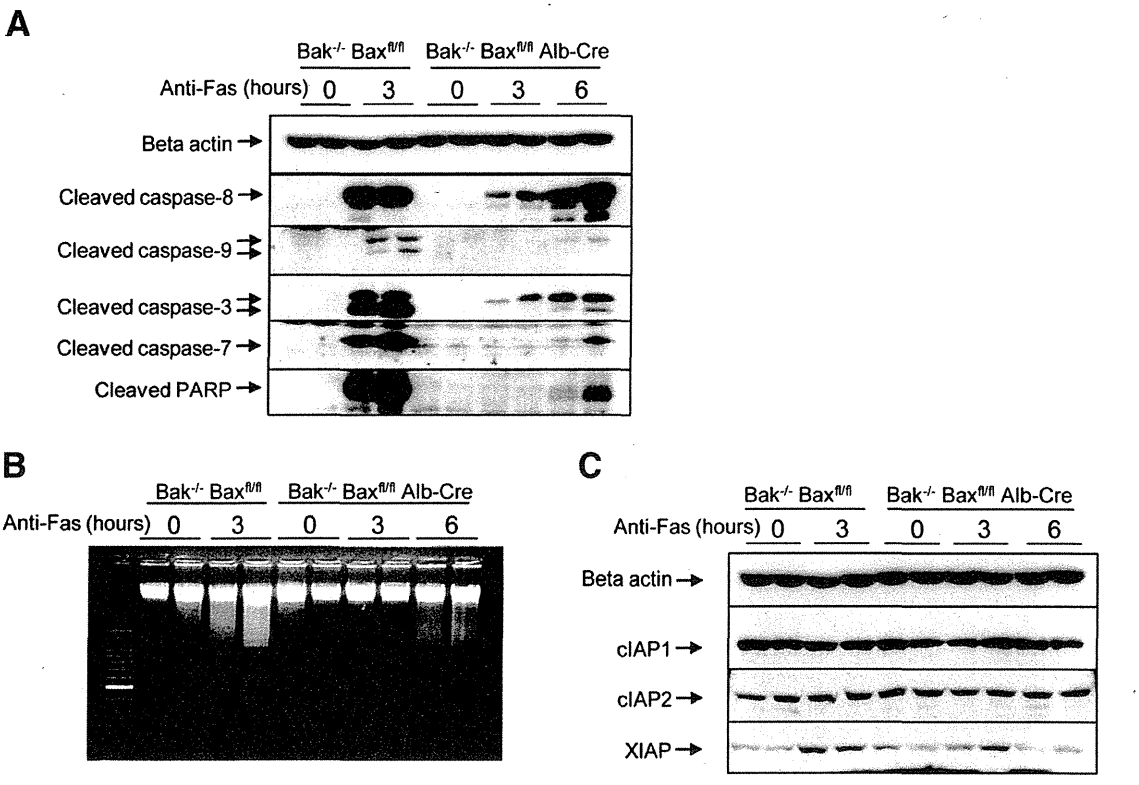

Fig. 5. Fas-mediated hepatocellular death in Bak/Bax DKO mice is associated with caspase-3/7 activation and oligonucleosomal DNA breaks. Bak/Bax DKO mice ($Bak^{-/-}$ $Bax^{fl/fl}$ Alb-Cre) or control Bak KO littermates ($Bak^{-/-}$ $Bax^{fl/fl}$) were intraperitoneally injected with Jo2 anti-Fas antibody (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.

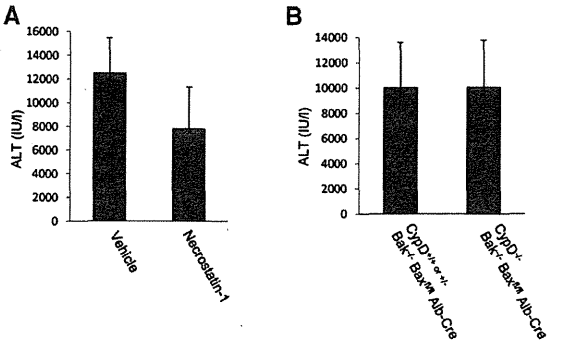

Fig. 6. Fas-induced hepatocellular death in Bak/Bax DKO mice is independent of RIP kinase and/or CypD. (A) Bak/Bax DKO mice ($Bak^{-/-}$ $Bax^{fl/fl}$ Cre) were intraperitoneally injected with 2 mg/kg necrostatin-1 in vehicle containing 0.2% dimethylsulfoxide or vehicle alone at 2 hours after injection 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

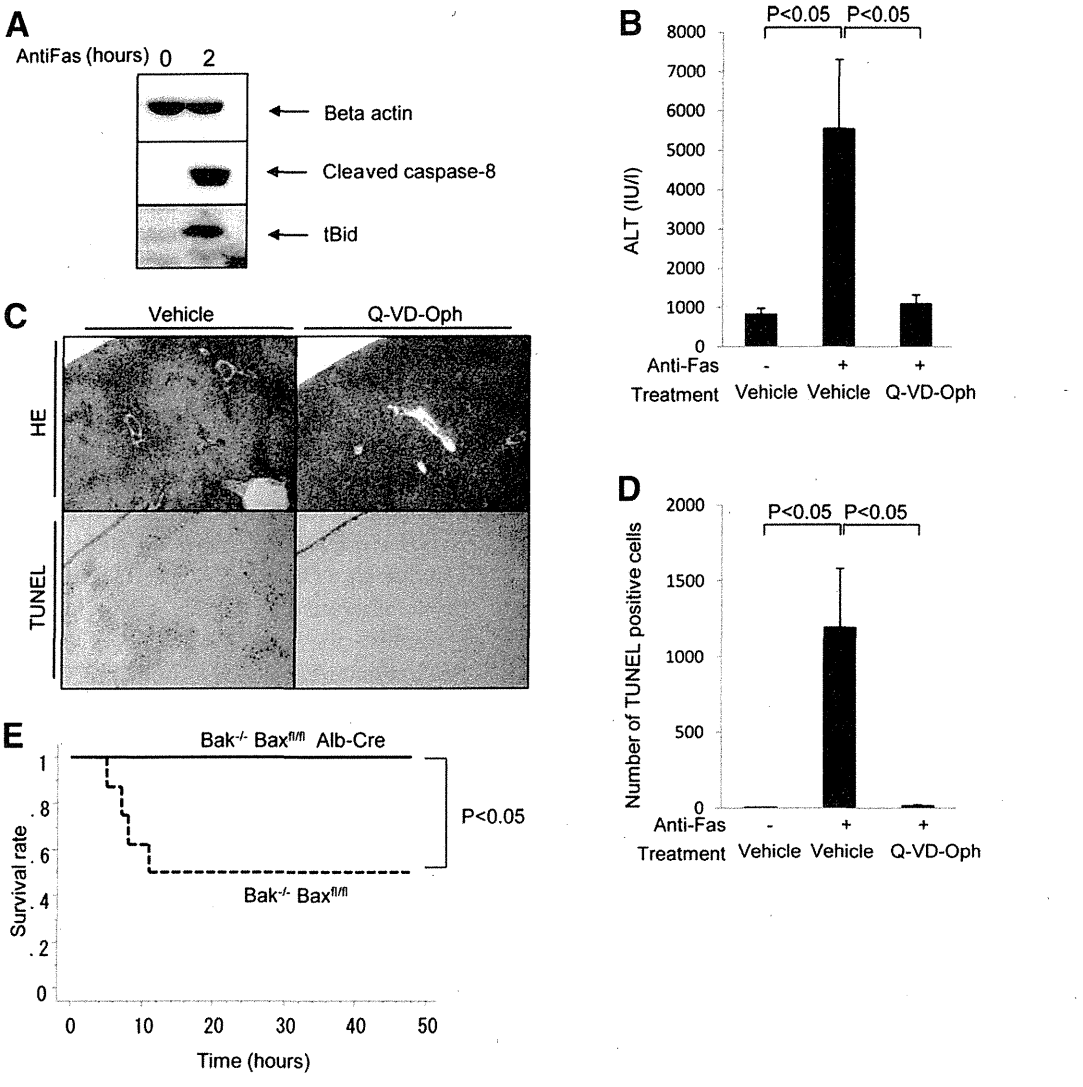

Fig. 7. Hepatocellular death in Bak/Bax DKO mice is dependent on caspase activation. (A) Bak/Bax DKO mice were analyzed before and 2 hours after intraperitoneal injection of Jo2 anti-Fas antibody (1.5 mg/kg). Western blot analysis of the liver for the expression of cleaved caspase-8 and truncated Bid (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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今月のテーマ●C型肝炎治療法の進歩

C型慢性肝炎の新規治療薬

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要旨：近年の分子ウイルス学の進歩により，C型肝炎ウイルスの増殖系が確立されウイルスのlife cycleが分子生物学的に明らかにされるにつれて，たくさんの治療薬の候補が多くの研究者により見つけられ続けている．NS3のプロテアーゼはウイルスのlife cycle上必須の酵素で，プロテアーゼ阻害薬の研究に全世界が取り組み，その結果Boceprevirが世界での承認薬第1号となった．プロテアーゼ阻害薬の導入により慢性C型肝炎初回治療例の70%は治癒することが期待される．また他のウイルス因子を標的としたポリメラーゼやNS5Aの阻害薬，またウイルス増殖に必須の宿主因子を標的としたサイクロフィリン阻害薬も治験中であり，近未来的には経口薬の組み合わせでウイルス排除が目標となる．

索引用語：慢性C型肝炎，プロテアーゼ，ポリメラーゼ，NS5A，サイクロフィリン

はじめに

C型肝炎ウイルス（hepatitis C virus；HCV）がカイロン社により発見されて，すでに20年以上が経過した[1]．現在に至るまでにわが国の標準治療もインターフェロン単独治療6カ月から，ペグインターフェロン・リバビリンの48週または72週に発展しており，それにともない治療成績も確実に向上してきている．世界的に見るとペグインターフェロンとリバビリンの併用療法は，ペグイントロン®・レベトール®の併用療法が2000年5月，ペガシス®・コペガス®の併用療法が2002年4月に認可されており，すでに10年以上の長きにわたり慢性C型肝炎の標準治療として君臨している．インターフェロンもリバビリンもHCVに対する特異的な治療薬として開発された薬剤ではない．インターフェロンは非特異的な抗ウイルス作用と免疫増強作用を持つ薬剤で，インフルエンザ様症状，間質性肺炎，鬱症状などの有害事象があり，リバビリンにも溶血による貧血といった避けがたい副作用がある．ペグインターフェロンとリバビリンの組み合わせは約半数の患者が治るといわれても，治療を受ける側にとっては有害事象を考えると一大決心のいる治療法である．そのため当然のことながらより特異的で効果が強く，有害事象の少ない治療薬の開発に全世界がしのぎを削っている[2]．HIVの治療においては，プロテアーゼ阻害薬を含んだ多剤併用療法が治療成績向上の大きなbreakthroughとなったが[3]，HCVもそのlife cycle上プロテアーゼが増殖に必須であることより，プロテアーゼ阻害薬の開発が全世界的な競争となった．世界ではじめてHCVのプロテアーゼ阻害薬を開発したのは，ベーリンガーインゲルハイム社で，基質ペプチドの構造アナログとしてデザインされた薬剤であった．効果はgenotype 1に限定されてはいたが，目を見張るような抗ウイルス効果を示した[4]．しかし臨床開

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New developed drugs for chronic hepatitis C
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Table 1. 現在開発中の direct-acting antivirals (DAA)

NS3 Protease	NS5B Polymerase		NS5A
	Nucleoside (NPOL)	Non-nucleoside (NNI)	
Telaprevir	Mericitabine	Tegobuvir	BMS-790052
Boceprevir	IDX184	Filibuvir	BMS-824393
Danoprevir	PSI-7977	ANA598	CF102
TMC435	PSI-938	BI207127	
BI201335		ABT-333	
BMS-650032		VX-222	
ABT-450		ABT-072	
GS-9451		BMS-791325	
GS-9256			
MK-5172			
ACH-1625			
VX-985			
CTS-1027			

発に進む前に心毒性があることが判明して，開発の中止を余儀なくされた．しかしこれが研究開発の嚆矢となりその後の世界の潮流を形作った．同時に分子ウイルス学的研究の進歩が治療薬の開発を促進した．HCV 治療薬の開発を阻んでいた一番の要因は，有効な増殖システムと小動物による実験系がなかったことであるが，HCV のレプリコンの開発[5]，Wakita らによる HCV の感染性クローンの確立[6]，人肝細胞を埋め込んだキメラマウスの開発[7]が進んだことにより HCV の創薬が飛躍的に進歩し，今日では Table 1，2 に示したごとく数多くの Direct-acting antivirals (DAA) が開発されている．

I プロテアーゼ阻害薬の開発の現状

1. 第1世代のプロテアーゼ阻害薬

第1世代のプロテアーゼ阻害薬は基質であるペプチド類似の非環状 (acyclic, linear) 構造をとっている．現在開発中の薬剤の中でも第1世代に属する VX-950 (Telaprevir；Vertex)[8]と SCH503034 (Boceprevir；MSD)[9]はアメリカの FDA でも first truck に指定され，認可を目指した Phase III の治験が行われ，その結果が 2010 年の AASLD と 2011 年の EASL で報告されている．Telaprevir の治験は，治療歴のない慢性C型肝炎を対象として ADVANCE study が[10][11]，同じく治療歴のない慢性C型肝炎を対象として Response-guided therapy を検討する目的で ILLUMINATE study が行われた[12]．またペグインターフェロンとリバビリンの併用療法の治療歴のある患者を対象として REALIZE study が行われた[13]．

ADVANCE study のプロトコールを Figure 1a に示すが，治療経験のない慢性C型肝炎患者 1088 名を3群に割り振り，Telaprevir の至適投与期間，つまりペグインターフェロンとリバビリンと Telaprevir の併用期間が8週と 12 週のどちらが有効であるか検討した study である．患者背景をまとめると 77% の患者でウイルス量が 800000 IU/ml 以上，58% が genotype 1a で，58% が男性，アフリカ系アメリカ人が 9% で，線維化の進展は 21% に見られていた．Telaprevir 12 週ペグインターフェロン，リバビリン併用群 (T12/PR 群)，Telaprevir 8 週ペグインターフェロン，リバビリン併用群 (T8/PR 群)，ペグインターフェロン・リバビリン併用群 (標準治療 standard of care；SOC 群) の3群で Rapid Virological Response (RVR，4週までにウイルスが検出限界以下になる)，End of Treatment Response (ETR，治療終了時にウイルスが検出されない)，Sustained virological response (SVR，治療終了6カ月後にウ

Table 2. プロテアーゼ阻害薬の耐性部位

	V36A/M	T54A	V55A	Q80R/K	R155K/T/Q	A156S	A156V/T	D168A/V/T/H	V170A
Telaprevir*	■	■			■	■	■		■
Boceprevir*	■	■	■		■	■	■		■
SCH900518*	■	■			■	■	■		■
BILN-2061					■		■	■	
ITMN191					■	■	■	■	
MK-7009		■			■	■	■	■	
TMC435				■	■		■	■	
BI201335*					■		■	■	■
MK-5172							■	■	
GS-9256					■		■	■	
ABT-450					■		■	■	
BMS-791325					■		■	■	

*Linear，他は Macrocyclic.

イルスが検出されない）を検討すると，RVR はそれぞれ68%，66%，9% で，ETR はそれぞれ87%，81%，63% で，SVR は75%，69%，44%であった（Figure 1b）．SVR については T12/PR群も T8/PR 群も，SOC 群より有意に高かった．Telaprevir 投与群において 25% 以上に見られた有害事象は倦怠感，掻痒感，悪心，嘔吐，頭痛，貧血，紅斑，インフルエンザ様症状，不眠，熱発，下痢であった．有害事象による中止率は T12/PR群で6.9%（うち皮疹1.4%，貧血0.8%），T8/PR群で7.7%（うち皮疹0.5%，貧血3.3%），SOC群で3.6%（皮疹0%，貧血0.6%）であった．安全性と忍容性は以前の study と同等であり，Telaprevir 12 週投与が8週に比べ，治療効果も，有害事象を勘案しても，よりよい治療であると考えられた．

ILLUMINATE study は，治療経験のない genotype 1 の 540 例の慢性C型肝炎症例を対象とした Response-Guided Therapy の検討である．3剤併用 12 週と SOC を 12 週行い，Extended RVR 症例（eRVR，4週と12週で HCV RNA が検出感度以下で20週でも治療を継続している症例）に対する治療を，24 週で打ち切るか，その後も 24週標準治療を継続して 48 週治療を行うべきかをランダム化して比較検討したものである（Figure 1c）．対象症例は男性が 60.2%，白人が 79.1%，黒人 13.5%，平均ウイルス量 6.5log IU/ml で，肝硬変が 11.3% を占めていた．最終的な SVR であるが，eRVR を達成した 24 週の治療群では 92.0%で，同じく 48 週の治療群では 87.5% であった．540 例全体で見ると，RVR は 72.0%，eRVR は65.2%，SVR は 71.9% で認められた（Figure 1d）．安全性の面では 17.4% の症例がすべての薬剤を中止した．そのうち 4% の患者が疲労を理由として，2.2% の患者が貧血を理由として，投与を中止した．eRVR を達成しランダム化後に投与を中止した例は，24 週投与群で 1 例，48 週投与群で20 例認められた．eRVR を達成した症例において 24 週投与群と 48 週投与群でほぼ同等の治療効果が認められたことより，初回治療の患者の約70% で治療期間の短縮が可能であることが示され，Telaprevir をベースにした治療では Response-guided therapy が適用できることが示された．

最後に，ペグインターフェロン・リバビリン既治療の慢性C型肝炎を対象とした REALIZE

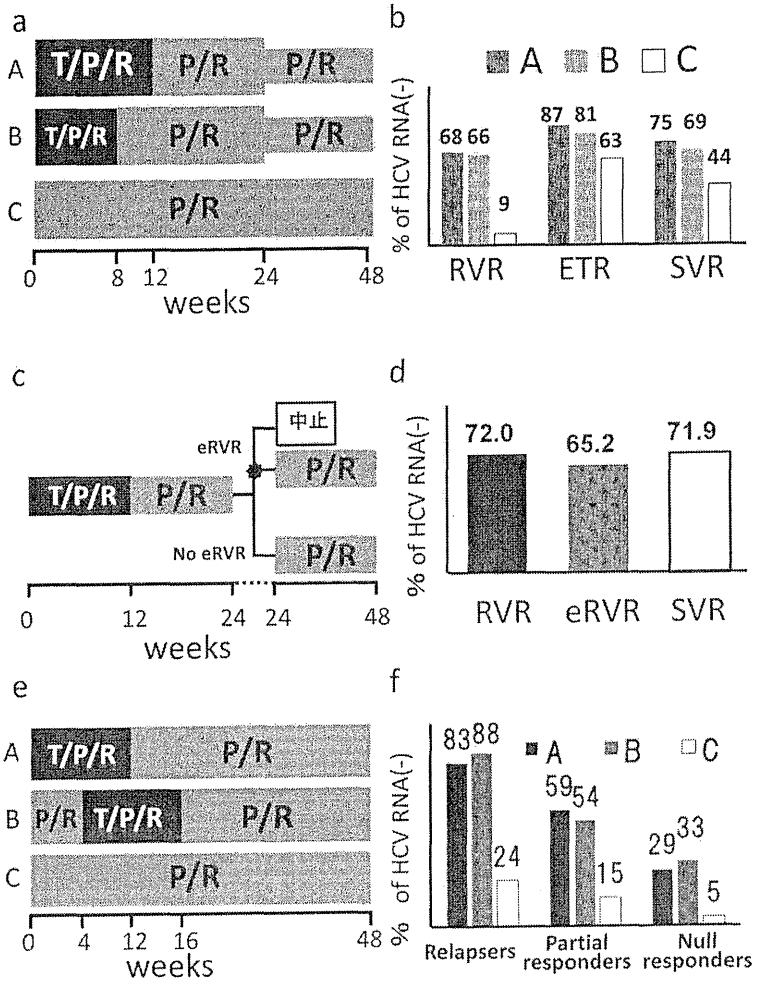

Figure 1. a) ADVANCE study のプロトコール．Genotype 1 の初回治療の症例．n＝1088．Genotype 1 のサブタイプと Baseline viral load を用いて症例を 3 群に割り付けた．Telaprevir 投与群（A，B）では 4 週と 12 週でウイルスが検出感度以下なら 24 週投与となる．P：ペグインターフェロン α2a 180μg/週，R：リバビリン，T：Telaprevir．b) ADVANCE study の治療成績．RVR：rapid virological response，ETR：End of treatment response，SVR：sustained virological response．c) ILLUMINATE study のプロトコール．Genotype 1 の初回治療例．n＝540．eRVR：extended RVR（4 週と 12 週でウイルスが測定感度以下）を達成しかつ 20 週で治療を継続している症例を対象に 20 週にランダム化割り付けを行い，24 週投与群と 48 週投与群に分ける．eRVR を達成できなかった症例は 48 週投与となる．d) ILLUMINATE study 全体の治療成績．e) REALIZE study のプロトコール．Genotype 1 で P/R 治療経験例．n＝662．A，B，C の 3 群に 2：2：1 の比率でランダム化割り付けを行う．f) 前治療に対する反応ごとの SVR．Relapser：治療終了時ウイルス陰性化にもかかわらず再燃した症例，Partial responders：前治療時に 12 週で 2log 以上ウイルスが減少したものの，治療期間中を通してウイルスが陽性であった症例，Null responders：12 週でウイルスが 2log 減少しなかった症例．

study のプロトコールを示す（Figure 1e）．対象は治療歴のある Genotype 1 の慢性C型肝炎662例である．Genotype 1a が50%，1b が50% で，relapser が53%，partial responder が19%，Null responder が28% で，89% でウイルス量が800000IU/ml 以上で，26% の患者は肝硬変であった．治療成績は T12/PR48 群で64%，T12/PR48 lead in 群で66%，SOC 群で17% であった．以前の治療に対する反応で層別解析すると，以前の治療での relapser における SVR は T12/PR48 群で83%，T12/PR48 lead in 群で88%，SOC 群で24% であった．以前の治療で partial responder（12週で HCV RNA が2log は低下するが，治療期間を通じて HCV RNA が陽性であったもの）においては，SVR は T12/PR48 群で59%，T12/PR48 lead in 群で54%，SOC 群で15% であった．以前の治療に Null responder であった症例（以前の治療で12週以降に2log 以上 HCV RNA が低下しなかった症例）では，SVR は T12/PR48 群で29%，T12/PR48 lead in 群で33%，SOC 群で5% であった（Figure 1f）．安全性に関してはこれまでの ADVANCE study や ILLUMINATE study と profile に変わりはなかった．また Lead in arm だけを対象にして4週で HCV RNA が1log 低下したか否かで SVR を層別解析すると Relapser では HCV RNA 1log 低下（+）で94%，同じく 1log 低下（−）で62%，Partial responder では HCV RNA 1log 低下（+）で59%，1log 低下（−）で56%，Null responder では 1log 低下（+）で54%，1log 低下（−）で15% であった[14]．Lead in の反応性で SVR を推定しうる可能性が示唆されたが，Lead in phase を置くことにより当初期待された Virological failure を減少させるという Evidence は得られなかったようである．

Boceprevir は2011年5月13日に FDA より承認された．Phase III study は，治療経験のない患者を対象に SPRINT-2 study[15]が，治療経験のある患者を対象に RESPOND-2 study が行われた[16]．SPRINT-2 study は genotype 1 の慢性C型肝炎1097例（非アフリカ系アメリカ人938例，アフリカ系アメリカ人159例）を対象に行われた．患者の92% はウイルス量が400000IU/ml であり，線維化の進展例（F3/4）は9% であった．これらの患者を Figure 2a にあるごとく3群に割り付けた．1st Arm は48週の標準治療である．2nd arm は Lead in 4週後 Boceprevir 投与開始から4週以降のアッセイで HCV RNA が連続的に陰性（治療開始8週から24週）の場合は28週で治療を打ち切る Response-guided therapy である．3rd arm は4週の lead in のあと44週間3剤併用療法を行う．SVR は SOC 群では38%，Response-guided therapy 施行群（Triple therapy-RGT 群）では63%，3剤併用を48週まで行った群（Triple therapy-48 週群）では66% であった（Figure 2b）．Lead in phase における反応で HCV RNA が1log 低下したか否かと，非アフリカ系アメリカ人（Non-AA）かアフリカ系アメリカ人（AA）かの2つの因子で層別解析を行うと，Non-AA での SVR は，lead in phase で1log 低下しなかった症例では SOC 群で5%，Triple therapy-RGT 群で29%，Triple therapy-48 週群で39% であった．Non-AA で lead in phase で1log 以上ウイルス量が低下した症例では，SVR は SOC 群で52%，Triple therapy-RGT 群で82%，Triple therapy-48 週群で82% であった．AA でウイルス量が lead in phase で1log 低下しなかったグループでの SVR は SOC 群で0%，Triple therapy-RGT 群で25%，Triple therapy-48 週群で31% であった．AA で lead in phase でウイルス量が1log 以上低下した症例での SVR は SOC 群で46%，Triple therapy-RGT 群で67%，Triple therapy-48 週群で61% であった．

SPRINT-2 study の結果より Triple therapy-RGT 群と Triple therapy-48 週群でほぼ同等の治療効果が得られた．Boceprevir の忍容性は高く，Boceprevir を併用することにより貧血の発生は増加するが，そのために治療の中断に繋がるケースはまれであった．

RESPOND-2 study は，SPRINT-2 と類似の3 arms のプロトコールを治療経験のある慢性C型肝炎403例に行った study である（Figure 2c）．これらの症例のうち前治療に対する反応が re-

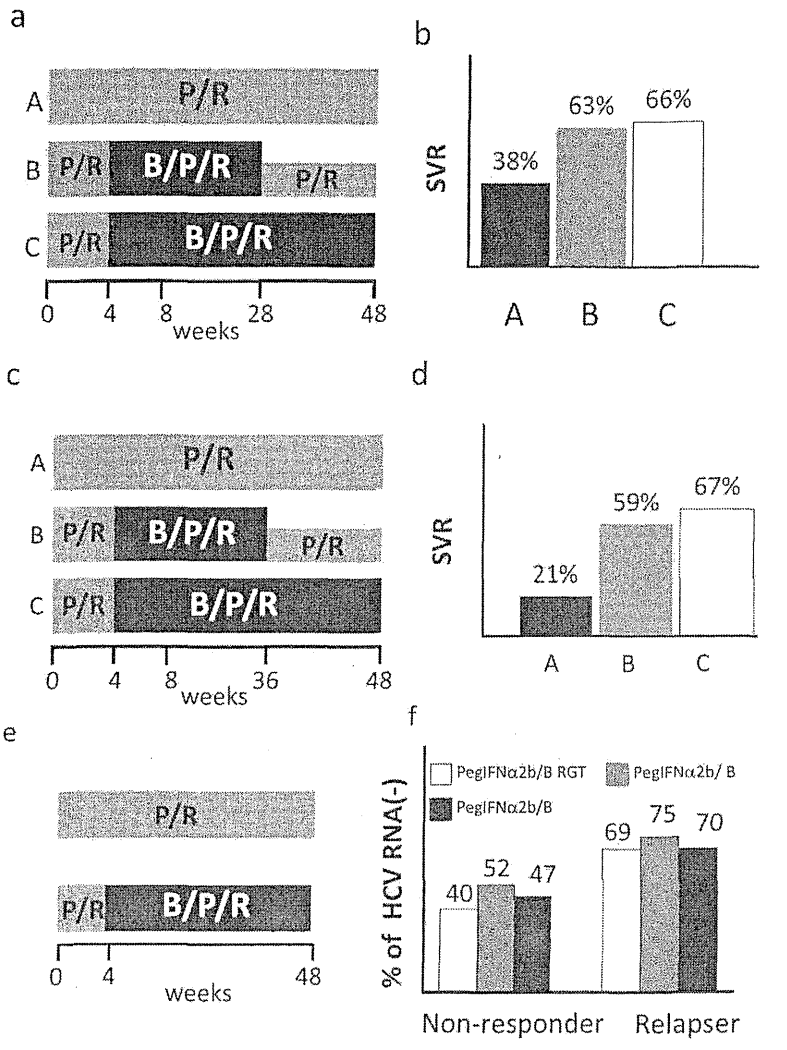

Figure 2. a) SPRINT-2 study のプロトコール．Genotype 1 初回治療例．n＝1097．P：ペグインターフェロン α2b，R：リバビリン，B：Boceprevir．Genotype 1 のサブタイプと baseline viral load を割り付け因子として，3 群に 1：1：1 に割り付ける．b) SPRINT-2 study の各群の SVR．c) RESPOND-2 study のプロトコール．Genotype 1 で P/R 治療経験例（Null responder を除く）．n＝403．前治療に対する反応と genotype 1 のサブタイプを割り付け因子として，A，B，C の 3 群に 1：2：2 にランダムに割り付ける．d) RESPOND-2 study の各群の SVR．e) ペグインターフェロン α2a とリバビリンと Boceprevir の併用療法．ペグインターフェロン α2a とリバビリン併用療法との比較検討プロトコール．Genotype 1 で前治療に対する反応が non-responder と relapser を対象とし null responder は含まれない．f) 前治療に対する反応性の non-responder と relapser における違いを，SVR を RESPOND-2 の成績に入れて比較．

lapser のものが 64% を占め，non-responder（12 週までに 2log 以上 HCV RNA が低下するものの経過中を通じて HCV RNA が陽性であったグループ）が残りの 36% である．今回の検討には対象症例としていわゆる null responder は含まれていない．SVR を 3 群で比較すると SOC 群で 21%，Triple therapy-RGT 群で 59%，Triple therapy-48 週群で 67% と，Boceprevir を投与し

た2群でSOC群より有意にSVRが高かった(Figure 2d). 前治療に対する反応で結果を層別すると, 前治療に対する反応がNon-responderであった症例ではSOC群で7%, Triple therapy-RGT群で40%, Triple therapy-48週群で52%であった. 前治療に対する反応がrelapserであった症例においては, SVRはSOC群で29%, Triple therapy-RGT群で69%, Triple therapy-48週群で75%であった. Lead in phaseに対する反応性で結果を層別すると, Lead inでウイルス量の減少が1log未満であった症例のSVRは, SOC群で0%, Triple therapy-RGT群で33%, Triple therapy-48週群で34%であった. Lead in phaseでウイルスの減少が1log以上ある症例ではSVRが, SOC群で26%, Triple therapy-RGT群で73%, Triple therapy-48週群で80%であった. 有害事象による治療の中断はSOC群で3.0%, Triple therapy-RGT群で8.0%, Triple therapy-48週群で12.0%であった. 有害事象のうちでBoceprevir併用により明らかに増加したのは貧血と味覚異常であった. SPRINT-2およびRESPOND-2のSVRをIL-28Bの解析結果に従って層別解析すると, まずSPRINT-2ではCC群でBoceprevir併用がSVRを向上させるのに何らの効果も示さなかったが, CT群とTT群では, Boceprevirの併用によりSVRの向上が認められた. RESPOND-2では3群ともBoceprevir併用によりSVRの向上が認められた[17].

RESPOND-2 studyと同様に慢性C型肝炎患者で前治療に対する反応がNon-responderとrelapserを対象としてペガシス®とBoceprevirの組み合わせを検討したstudyのプロトコールを示す(Figure 2e)[18]. ペガシス®・Boceprevir・リバビリン(P/B/R)の3剤併用群でSVRは64%で, SOC群では21%であった. 前治療の結果で層別するとRelapserではSVRはP/B/R群で70%であり, SOC群で28%であった. またNon-responderではSVRはP/B/R群では47%であり, SOC群では5%であった. この結果はペグイントロン®を用いたRESPOND-2とほぼ同等の結果であった(Figure 2f).

2. プロテアーゼインヒビター第2の波

現在治験が本邦でも進行中の新たなタイプのプロテアーゼ阻害薬としてはTMC435が挙げられる. この薬剤は第1世代の薬剤と異なり1日1回の投与が可能な非共有結合型のプロテアーゼ阻害薬である. この点は1日3回の投与が必要なTelaprevirやBoceprevirと比較するとコンプライアンスの点で非常に大きな利点である, 2010年のAASLDで治療経験のない慢性C型肝炎に対するPILLAR studyの中間報告と[19], 2011年のEASLで治療経験のある慢性C型肝炎に対するASPIRE studyの中間解析が発表された[20]. 投与プロトコールはFigure 3a, cに示したごとく多くのarmの比較検討であるが, 両studyとも治療成績がよく, PILLAR studyでは投与開始24週の途中経過(一部はETR)と24週で終了した症例の一部のSVR 12週までのデータであるが, 非常に高率にウイルスが消失している(Figure 3b). ASPIRE studyでは24週の段階でのTMC435 150mg投与群とSOC群の解析であるが, 前治療に対する反応がnull responderであっても71〜93%と高率にウイルスが消失している(Figure 3d).

その他の第2世代のプロテアーゼ阻害薬としては, 1日1回投与が可能なベーリンガーインゲルハイム社のBI201335とペガシス®・コペガス®との3剤併用の治験を, 治療経験のあるgenotype 1の慢性C型肝炎に対して行っている[21][22]. 治療終了後12週の結果(SVR12)を見ると, 前治療に対する反応がNull responderの患者でも最大で38.7%のSVRが達成されたものの, 全体ではbreakthroughが20%以上の高頻度で認められ, genotype 1aでは高頻度に耐性がおこりやすいのではないかと考えられる. 有害事象としては軽度のビリルビン上昇と紅斑光線過敏が報告されている. また9.3%の患者が有害事象によりBI201335の投与を中止している. その他の第2世代のプロテアーゼ阻害薬としてはDanoprevirと低用量のRitonavir・ペガシス®・コペガス®との4剤併用療法が少数例で検討されている(Figure 3e)[23][24]. RitonavirはHIVのプロテアーゼ阻害薬として

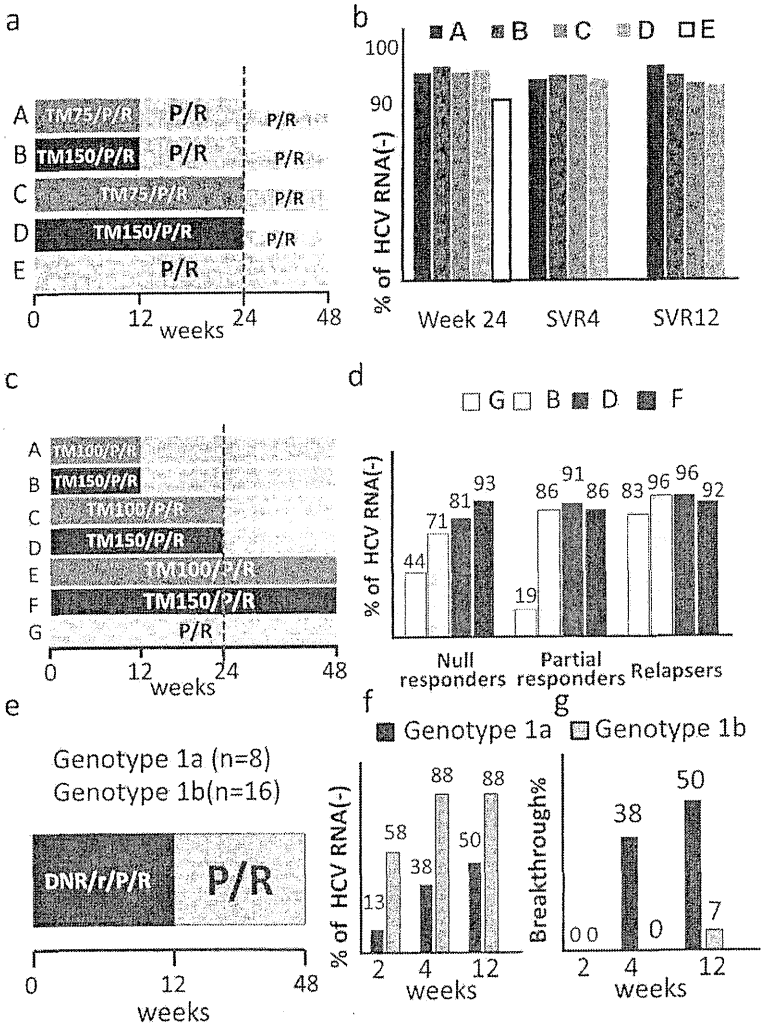

Figure 3. a) PILLAR study のプロトコール．TMC435 投与における用量（75 or 150mg）と投与期間（12 週，48 週）の設定試験．対象は Genotype 1 の初回治療例．n＝386．eRVR を達成した症例は治療期間が 24 週．b) PILLAR study の中間解析結果．24 週目でのウイルス陰性化率と，24 週で終了した患者の 4 週目と 12 週目の SVR．c) ASPIRE study のプロトコール．TMC435 投与における用量（100 or 150mg）と投与期間（12，24，48 週）の設定試験．対象は Genotype 1 の P/R 治療失敗例．n＝462．d) ASPIRE study の治療成績．TMC435 150mg 投与群の 24 週での成績．e) Danoprevir（DNR）と low dose ritonavir（r）とペグインターフェロン α2a とリバビリンの併用療法．対象は前治療に null responder の症例．Genotype 1a 8 例，genotype 1b 16 例．f) ITT（Intention to treat）での 2, 4, 12 週のウイルス陰性化率．g) ITT での 2, 4, 12 週のウイルスの Breakthrough 率．

元々開発された薬剤であるが，チトクローム P-450 CYP3A4 の阻害作用から，併用薬剤の PK profile を増強する働きがある．HIV 治療薬ではプロテアーゼ阻害薬に少量の Ritonavir を加えた合剤（カレトラ）が使用されている．前治療に対する反応が null responder に対する 4 剤併用療法を行うと，投与開始 12 週で genotype 1a では 50% の患者で血中のウイルスが測定感度以下になるが，残りの 50% で breakthrough が認められた（Figure 3f）．一方 genotype 1b では 88%

の患者で血中のウイルスが測定感度以下になりbreakthroughが認められたのは1例のみであった．Genotype 1aではR155Kのmutation（AGGからAAGへ1塩基の変化）がgenotype 1bより（CGGからAAGへ2塩基の変化）おきやすく[25]，この検討でもR155K変異が治療を中断後も数カ月持続することが確認され，genotype 1aの治療には新たなDAAを加える必要性が検討されている[26][27]．

3．ポリメラーゼ阻害薬

ポリメラーゼ阻害薬は基質のアナログとして作用する核酸系のポリメラーゼ阻害薬（NI）と，ポリメラーゼの酵素活性にアロステリックに阻害作用を及ぼす非核酸系のポリメラーゼ阻害薬（NNI）に大別される．前者はヌクレオシドとヌクレオチドがあり後者には少なくとも4カ所の作用部位（NNI1～4）がある．ポリメラーゼ阻害薬はこれまで，かなり長きにわたって研究されてきているが，副作用や耐性の問題などで開発が中止となったものが多くphase IIIに進んでいるものはない．

2009年のHEPDARTでは，IDX184は肝臓をターゲットにしたプロドラッグで，肝細胞内でのユニークなリン酸化メカニズムが注目されたが，2010年の9月に肝毒性のためにFDAに開発を止められている．そのような状況下で生き残ってきたいくつかのポリメラーゼ阻害薬の臨床治験が進められている．ヌクレオシドアナログのポリメラーゼ阻害薬では，未治療の慢性C型肝炎（genotype 1/4）166例を対象にして，RG7128（Mericitabine）とペグインターフェロン・リバビリン24週投与と，ペグインターフェロン・リバビリン48週投与（SOC群）の比較検討が行われた（Figure 4a）（JUMP-C study）[28]．24週の時点でRG7128併用群では91%（74/81）でウイルスが消失し，一方SOC群では62%（53/85）であった．eRVRはRG7128併用群では60%（49/81）で，SOC群では13%（11/85）であった（Figure 4b）．12週までのSVR（SVR12）については限定された検討であるが，eRVRを達成した患者の76%（37/49）であり，やや再燃が多いのが気がかりなところである．また24週の段階ではIL-28BがCCでも，non-CCでも，ウイルスの消失率はほぼ同じであった．今後他のDAAとの併用などさらなる検討を期待される薬剤である．核酸アナログ専門メーカーのPharmassetからも，PSI-7977（NI）（200mg/dayと400mg/day）とペグインターフェロン・リバビリンの3剤併用と，ペグインターフェロン・リバビリンの3群の比較検討が121例のgenotype 1の慢性C型肝炎を対象に行われている[29]．現在4週と12週の時点での中間解析のみであるが，PSI-7977投与群ではRVRは98%で，cEVRは100%である．現時点ではbreakthroughもなく重篤な副作用もないので，プロテアーゼ阻害薬なみの効果が期待されている．またPSI-7977は少数例の検討ではあるがgenotype 2/3にも同等の抗ウイルス活性を示すことが報告されている[30]．NNI製剤では*in vitro*では強力な抗ウイルス活性を示すTegobuvirが，未治療の252例の慢性C型肝炎を対象としてペガシス®，コペガス®の48週投与の標準治療群とペガシス®，コペガス®，Tegobuvirの3剤の48週投与と3剤併用のResponse-guided Therapyの3 armsのプロトコールで検討されたが，残念ながら3剤併用を行ってもSVRの向上は認められなかった[31]．今後は，Tegobuvirはmultidrug combination therapyの1つの薬剤として活路を見いだせればというところであろう．

4．NS5A阻害薬[32]

NS5AはHCVのreplication complexの中の必須の因子であり，また細胞側の機能調節，とりわけインターフェロンの抵抗性に関係する可能性が指摘されている．NS5Aは3つの機能ドメインに分かれるが，Domain Iは二量体形成やRNAとの結合，Znとの結合に関係し，NS5A阻害薬はこの領域に作用すると考えられているが，正確な機序は不明である．NS5A阻害薬は*in vitro*のstudyではピコモルオーダーで複製抑制効果のある薬剤である[33]．現在BMSのPhase IIaは2つ行われており，まずBMS-790052（3mg，10mg，60mg）とペグインターフェロン・リバビリン3剤を組み合わせた48週投与を，ペグインターフェ