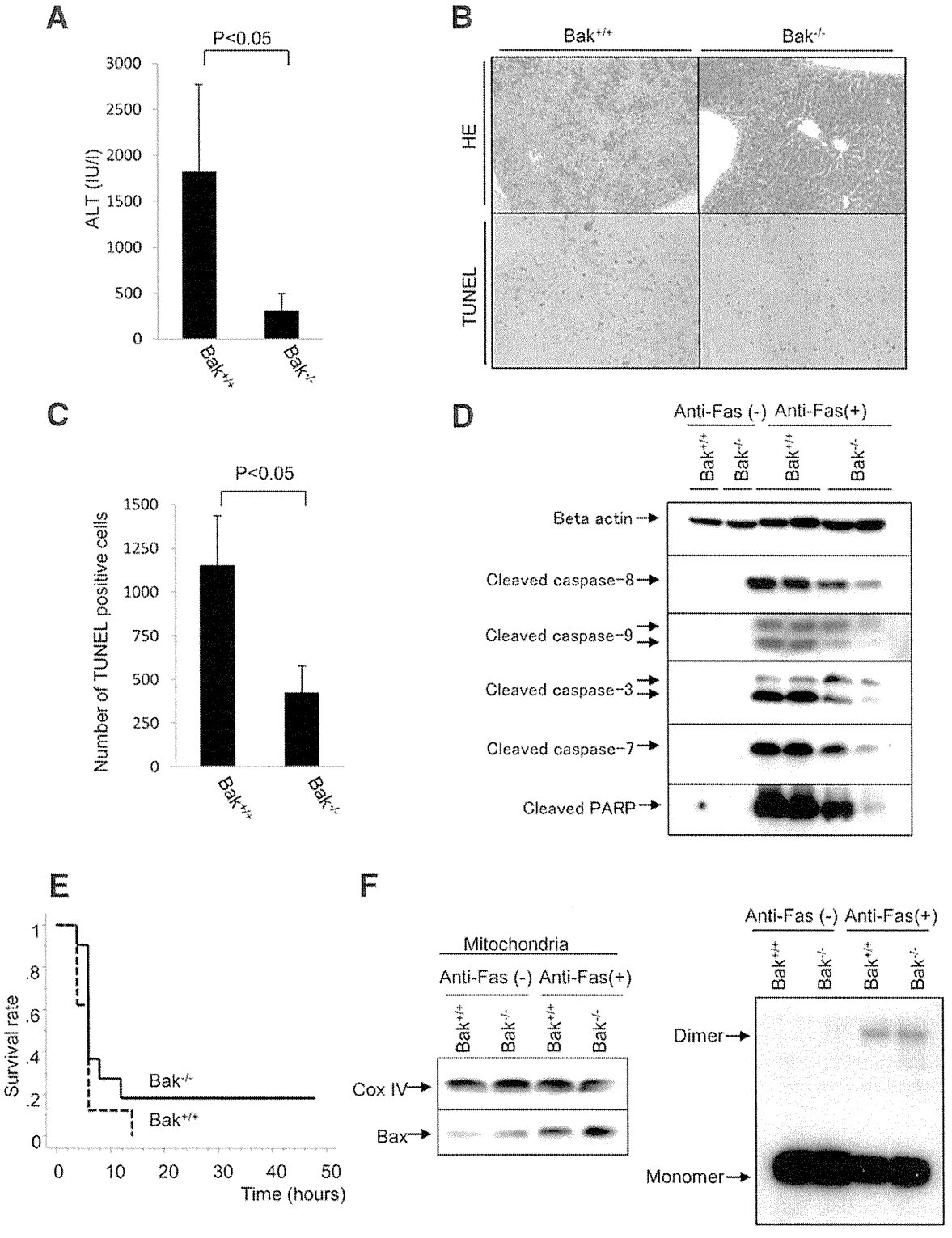

Fig. 1. Bak KO mice are partially resistant to Fas-induced hepatocellular apoptosis. Bak KO mice ($Bak^{-/-}$) or control WT littermates ($Bak^{+/+}$) were analyzed at 3 hours after intraperitoneal injection of 1.5 mg/kg Jo2 anti-Fas antibody. (A) Serum ALT levels (n = 10 or 11, respectively). (B) Hematoxylin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

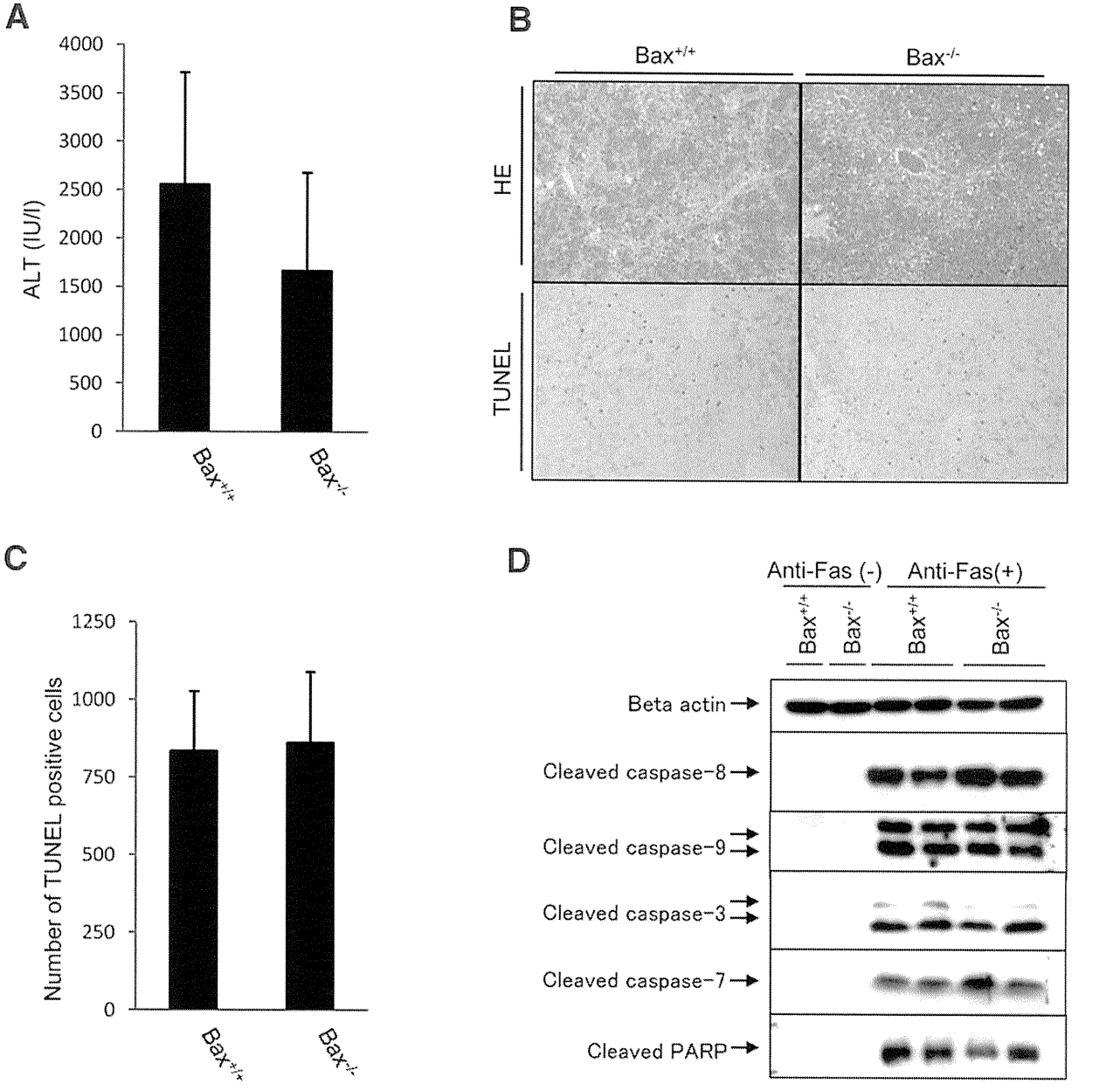

Fig. 2. Bax KO mice are not resistant to Fas-induced hepatocellular apoptosis. Bax KO mice ($Bax^{-/-}$) or control WT littermates ($Bax^{+/+}$) were analyzed 3 hours after intraperitoneal injection of Jo2 anti-Fas antibody (1.5 mg/kg). (A) Serum ALT levels (n = 11 per group). (B) Hematoxylin and eosin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

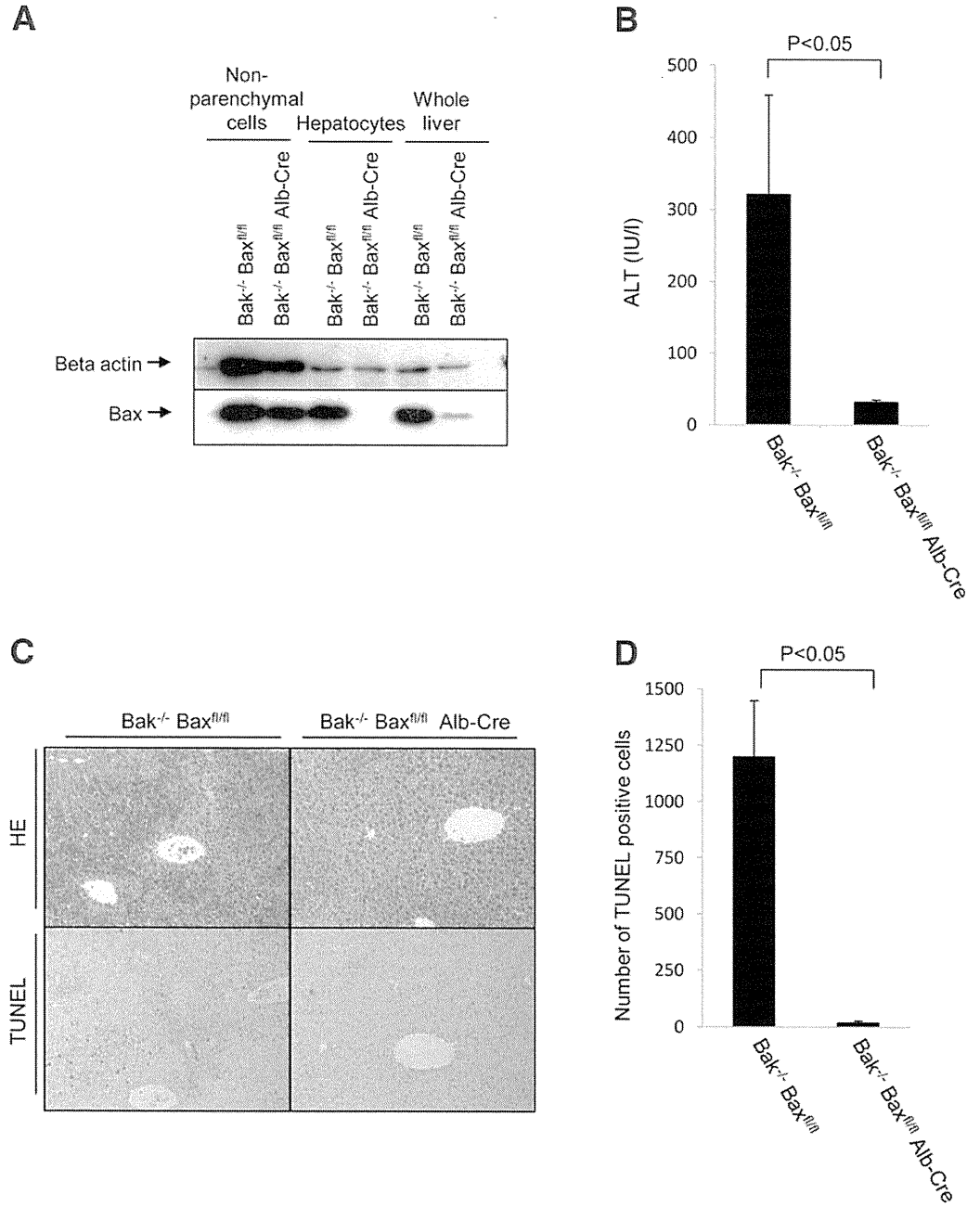

Fig. 3. Bak/Bax DKO mice are fully resistant to Fas-induced hepatocellular apoptosis in early phase. (A) Western blot analysis of the indicated fraction of the liver for the expressions of Bax. (B-D) Bak/Bax DKO mice ($Bak^{-/-}$ $Bax^{fl/fl}$ Alb-Cre) or control Bak KO littermates ($Bak^{-/-}$ $Bax^{fl/fl}$)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

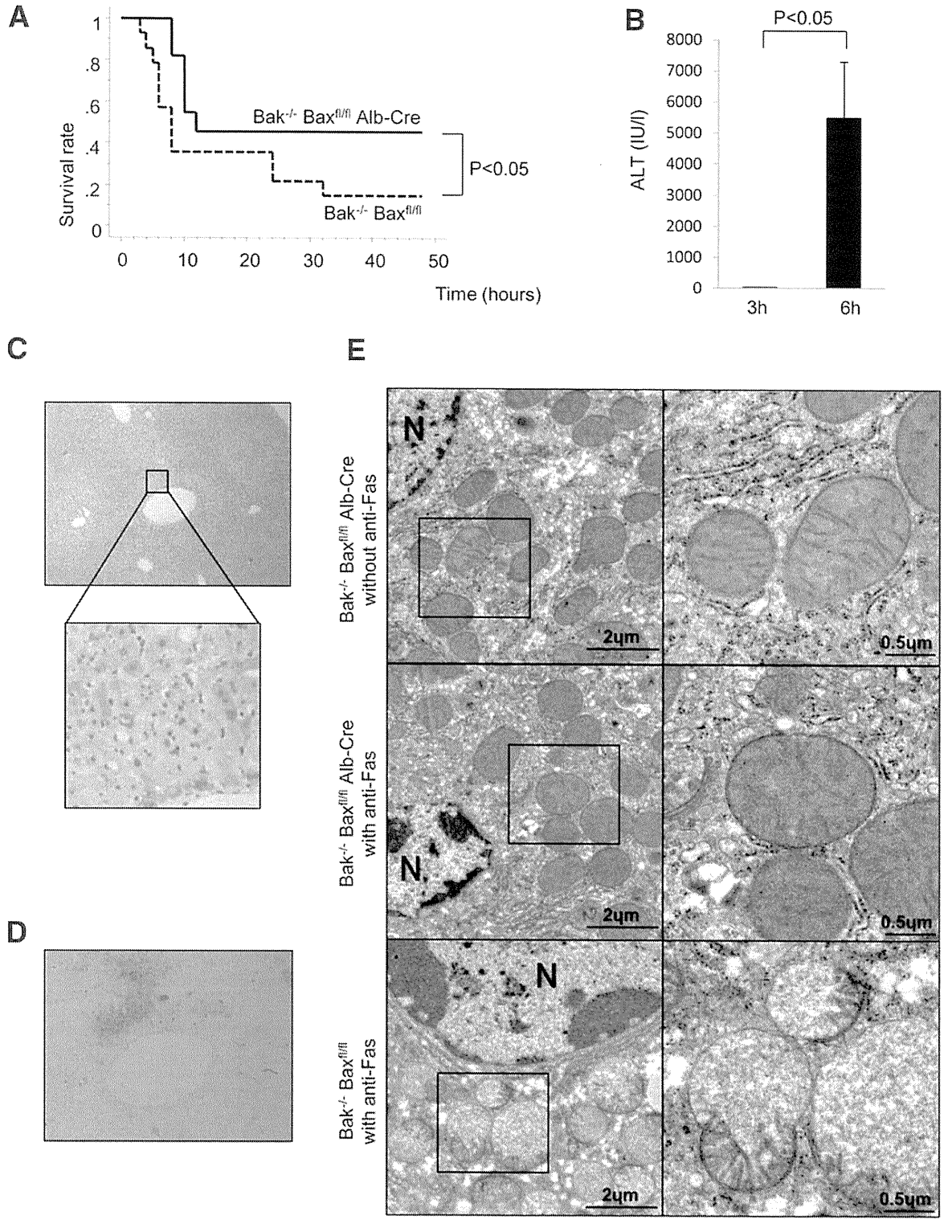

Fig. 4. Bak/Bax DKO mice develop late-onset severe liver injury upon Fas stimulation. Bak/Bax DKO mice ($Bak^{-/-}$ $Bax^{fl/fl}$ Alb-Cre) or control Bak KO littermates ($Bak^{-/-}$ $Bax^{fl/fl}$) were intraperitoneally injected with 1.5 mg/kg Jo2 anti-Fas antibody. (A) Survival rate after Jo2 injection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

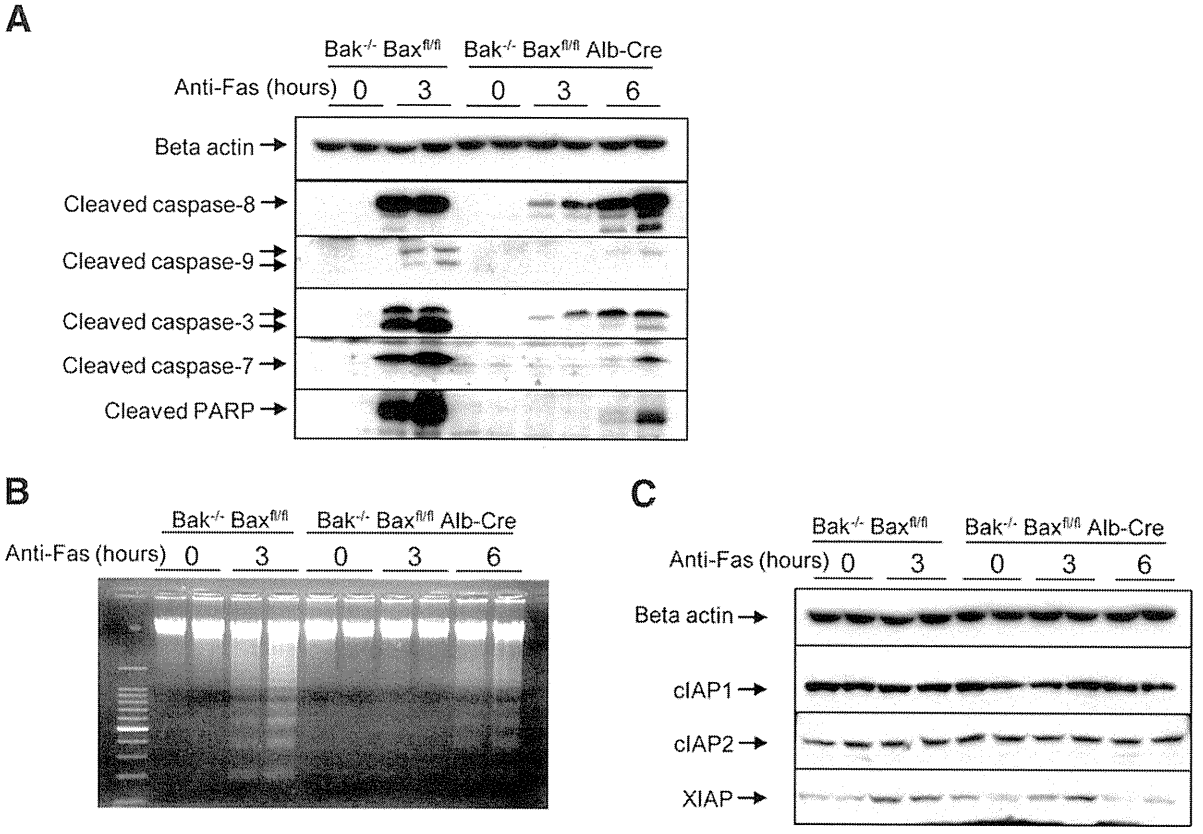

Fig. 5. Fas-mediated hepatocellular death in Bak/Bax DKO mice is associated with caspase-3/7 activation and oligonucleosomal DNA breaks. Bak/Bax DKO mice ($Bak^{-/-}$ $Bax^{fl/fl}$ Alb-Cre) or control Bak KO littermates ($Bak^{-/-}$ $Bax^{fl/fl}$) were intraperitoneally injected with Jo2 anti-Fas antibody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

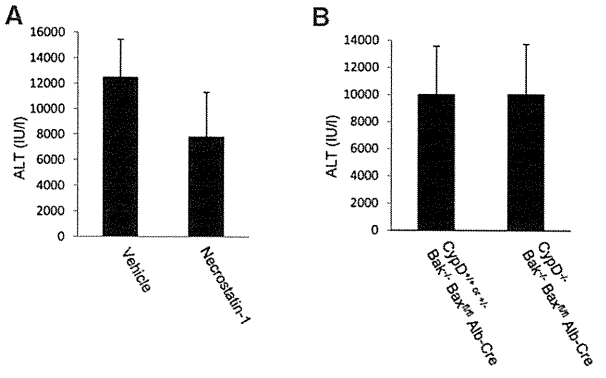

Fig. 6. Fas-induced hepatocellular death in Bak/Bax DKO mice is independent of RIP kinase and/or CypD. (A) Bak/Bax DKO mice ($Bak^{-/-}$ $Bax^{fl/fl}$ Cre) were intraperitoneally injected with 2 mg/kg necrostatin-1 in vehicle containing 0.2% dimethylsulfoxide or vehicle alone at 2 hours after injection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

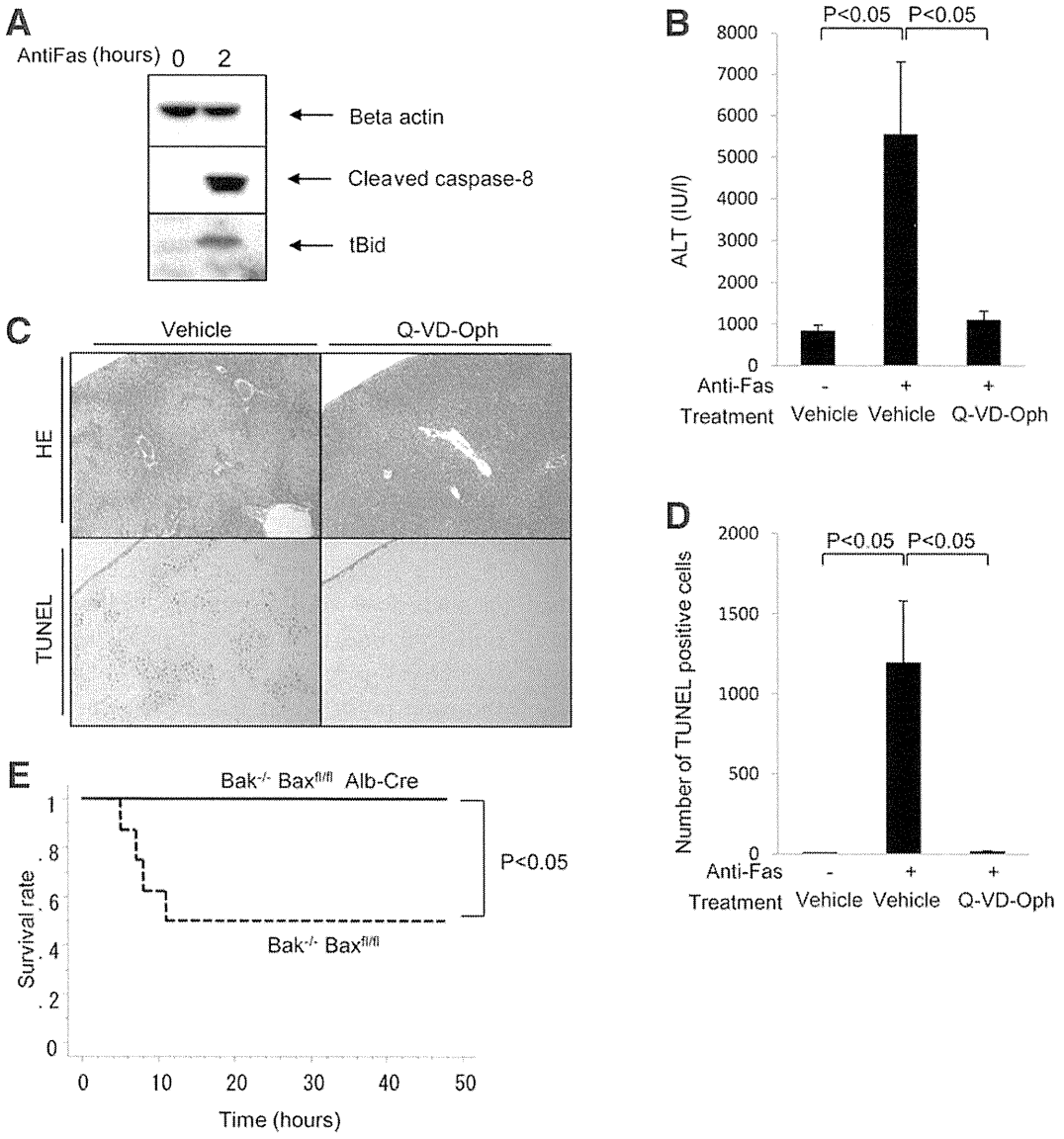

Fig. 7. Hepatocellular death in Bak/Bax DKO mice is dependent on caspase activation. (A) Bak/Bax DKO mice were analyzed before and 2 hours after intraperitoneal injection of Jo2 anti-Fas antibody (1.5 mg/kg). Western blot analysis of the liver for the expression of cleaved caspase-8 and truncated Bid (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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Original Article

α-Galactosylceramide activates antitumor immunity against liver tumor

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Aim: α-Galactosylceramide (α-GalCer) has been attracting attention as a novel approach to treat metastatic liver cancer. We investigated the detailed process of activating liver dendritic cells (DC) and immune cells after α-GalCer treatment in the mouse liver tumor model.

Methods: BALB/c mice bearing CMS4 liver tumor (p53 peptide-expressing tumor) were treated by α-GalCer. We evaluated the activation of liver DC and immune cells after α-GalCer treatment. Interferon (IFN)-γ enzyme-linked immunosorbent spot (ELISPOT) assay was performed to detect p53 peptide-specific cytotoxic T lymphocytes (CTL). To assess the impact of systemic acquired immunity by α-GalCer treatment, 28 days after liver tumor treatment, CMS4 cells or Colon26 cells were re-challenged s.c.

Results: The liver weights of α-GalCer-treated mice were significantly lighter than those of vehicle-treated mice. Depletion experiments revealed that natural killer (NK) cells were essential for the antitumor effect of α-GalCer. α-GalCer treatment significantly increased the population of DC and NK cells in the liver. The expressions of co-stimulatory molecules on liver DC significantly increased with the peak at 1 day after α-GalCer administration. IFN-γ ELISPOT assay demonstrated that p53 peptide-specific CTL was generated efficiently in α-GalCer-treated mice. ^{51}Cr-release assay revealed that $CD8^+$, not $CD4^+$, CTL against CMS4 cells were generated in α-GalCer-treated mice. The mice that had been protected from CMS4 liver tumor by α-GalCer injection became resistant against s.c. CMS4 re-challenge, but not against Colon26 re-challenge.

Conclusion: These results demonstrated the therapeutic potential of α-GalCer against liver cancer through activating liver DC and immune cells in the liver.

Key words: α-galactosylceramide, cytotoxic T lymphocytes, dendritic cells, liver, natural killer cells.

INTRODUCTION

THE GLYCOLIPID ANTIGEN α-galactosylceramide (α-GalCer) induced the activation of natural killer (NK) T cells in a CD1d-dependent manner.[1,2] Recently, α-GalCer has been attracting attention as a novel antitumor therapy. In *in vivo* animal studies, systemic administration of α-GalCer showed antitumor effects against various tumors (including melanoma, sarcoma, colon carcinoma and lymphoma) in hepatic and lung metastasis models.[3,4] Based on the promising results of preclinical studies demonstrating the antitumor potential of α-GalCer, several phase 1 clinical studies on cancer immunotherapy by the i.v. administration of α-GalCer has been carried out, but clinical responses of α-GalCer has been limited.[5] No clinical trial against liver cancer has been reported to date. In view of future α-GalCer treatment of liver cancer, the precise mechanism of activation of innate and acquired immunity in the liver by α-GalCer should be examined. However, these are still not fully understood.

The liver contains both a large compartment of innate immune cells (NK cells and NKT cells) and acquired immune cells (T cells).[6,7] Dendritic cells (DC) can induce the generation of both antigen-specific cytotoxic T lymphocytes (CTL) and T-helper (Th) cells.[8,9] Recent research of DC biology revealed that DC also contribute

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to innate immune responses by activating NK cells[10–14] and NKT cells[1,15,16] through interleukin (IL)-12 secretion and direct cellular interaction. Thus, DC can be expected to play critical roles in activating abundant T cells, NK cells and NKT cells in the liver after α-GalCer administration. We previously reported that administration of α-GalCer stimulated hepatic NKT cells and led to activation of hepatic NK cells,[4] and that α-GalCer activated liver DC have higher ability to generate acquired immunity.[17] However, the detailed process of activating liver DC and immune cells after α-GalCer treatment should be elucidated.

In this study, we evaluated the liver DC activation and antitumor effect mediated by both innate and acquired immunity against mouse liver tumor after administration of α-GalCer. Administration of α-GalCer induced early activation of liver DC with upregulation of antigen presenting-related molecules and resulted in complete rejection of local liver tumor by NK cells. Followed by early rejection of liver tumor, tumor antigen-specific CTL were generated and complete rejection in s.c. re-challenge of tumor cells was observed. Sequential activation of liver DC, innate and acquired immune cells in the liver may be an attractive strategy for treatment of local and distant tumor of liver cancer.

METHODS

Mice

SIX-TO-EIGHT-WEEK-OLD female BALB/c mice were purchased from Shizuoka Experimental Animal Laboratory (Shizuoka, Japan). All mice were maintained in micro-isolator cages. Procedures were performed according to approved protocol and in accordance with recommendations for the proper care and use of laboratory animals.

Cell lines and culture

CMS4 sarcomas (H-2^d) express mutated p53 and present the wild-type $p53_{232-240}$ epitope recognized by H-$2K^d$-restricted CTL.[18] Colon26, a mouse colon adenocarcinoma cell line, was kindly provided by Dr Takashi Tsuruo (Institute of Molecular and Cellular Bioscience, University of Tokyo, Tokyo, Japan). These cell lines were maintained as previously described.[19,20]

α-GalCer

α-Galactosylceramide was purchased from Funakoshi (Tokyo, Japan) and prepared as previously described by Kawano *et al.*[1]

Animal experiments

BALB/c mice were injected in the liver with 5×10^5 CMS4 cells on day 0. On day 1, BALB/c mice were injected i.p. with α-GalCer (2 μg/100 μL) or 100 μL of vehicle. Two weeks after the tumor injection, the livers of treated mice were removed, and the weight was measured to examine intrahepatic tumor growth. To assess the impact of systemic immunity from i.p. injection of α-GalCer, mice were injected intrahepatically with 5×10^5 CMS4 cells on day 0 and i.p. treated with α-GalCer on day 1. On day 28 after α-GalCer treatment, 1×10^6 CMS4 cells or Colon26 cells were injected in the right flank of treated mice. To confirm the involvement of $CD8^+$ T cells in this antitumor effect, we depleted $CD8^+$ T cells before re-challenge of CMS4 cells in α-GalCer-treated mice. On day 1 and day 3 of re-challenge of CMS4 cells, anti-CD8 antibody (53–6.72 hybridoma, ATCC) was injected i.p. as previously described.[19] Tumor size was assessed every 3 or 4 days and recorded in mm^2 by determining the product of the largest perpendicular diameters measured by vernier calipers. Data are reported as the average tumor area ± standard deviation (SD).

NK cell depletion experiments

For NK cell depletion, mice were injected with anti-asialo GM-1 (ASGM1) antibody (Ab) (Wako, Osaka, Japan) on day 1, 5, 10, 15 and 20 after tumor inoculation. The efficiency of NK cell depletion was validated by flow cytometry analysis of splenocytes using phycoerythrin (PE)-conjugated anti-DX5 monoclonal antibody (BD-Pharmingen, San Diego, CA, USA). In all cases, 99% of the targeted cell subset was specifically depleted (data not shown).

Preparation of hepatic mononuclear cells and liver DC

Hepatic mononuclear cells (MNC) were prepared as previously described.[4] $CD11c^+$ dendritic cells were isolated from hepatic MNC by magnetic cell sorting using MACS (Miltenyi Biotec, Gladbach, Germany) according to the manufacturer's protocol.

Flow cytometry

For phenotypic analysis of liver DC, NK cells, NKT cells and $CD4^+$ and $CD8^+$ T cells, PE- or fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies against mouse cell surface molecules (CD11c, CD40, CD80, CD86, T-cell receptor [TCR]-β, CD49b [DX5], CD4, CD8, CD69 [all from BD-Pharmingen], major his-

tocompatibility complex [MHC] class II [Miltenyi Biotec]), and appropriate isotype controls were used, and flow cytometric analysis was performed using a FACscan (Becton Dickinson, San Jose, CA, USA) flow cytometer. The results of flow cytometric analysis are reported in positive cell rates (%) determined by using isotype controls. DC were identified as $CD11c^+$/MHC class II^+ cells. NK cells were identified as $DX5^+$/TCR-β^- cells, NKT cells as $DX5^+$/TCR-β^+ cells, as previously described.[20]

IFN-γ enzyme-linked immunosorbent spot (ELISPOT) assays for $p53_{232-240}$ peptide-reactive CD8+ T-cell responses

Bone marrow derived DC (BMDC) were generated from normal mice as previously described[21] and were used as peptide-presenting cells. On day 14 after treatment of α-GalCer or vehicle, $CD8^+$ T cells were isolated from the spleen cells of treated mice by using magnetic beads (Miltenyi Biotec). We used a mouse IFN-γ ELISPOT kit (R&D systems, Minneapolis, MN, USA) to detect the $p53_{232-240}$ peptide-specific $CD8^+$ T-cell responses. To evaluate the $p53_{232-240}$ peptide, strongly expressing on CMS4 cells,[18] specific CTL induction, isolated $CD8^+$ T cells (1×10^5 cells/well) were co-cultured with syngeneic BMDC (2×10^4 cells/well) pulsed with $p53_{232-240}$ peptide in an ELISPOT culture plate. BMDC cells without $p53_{232-240}$ peptide served as the negative control, and this value was subtracted from all experimental determinations to establish $p53_{232-240}$ peptide-specific spot numbers. The data are represented as mean IFN-γ spots ± SD per 100 000 $CD8^+$ T cells analyzed.

Cytolytic assays

Splenocytes from α-GalCer or vehicle-treated mice were harvested 14 days after tumor inoculation. After 5 days of *in vitro* stimulation with mitomycin-C (Kyowa Hakko, Tokyo, Japan)-treated CMS4 cells, lymphocytes were analyzed for their ability to kill CMS4 cells in 4-h ^{51}Cr-release assays (effector cells/target cells ratio, 60:1), as previously described.[21] $CD4^+$ or $CD8^+$ T cells were depleted by magnetic sorting using CD4 or CD8 microbeads (Miltenyi Biotec), respectively.

Statistical analyses

All experiments with three or more groups in which treatment was applied with a completely random design were first analyzed by a one-way factorial ANOVA. If the resulting *P*-value was less than 0.05, specific pairwise

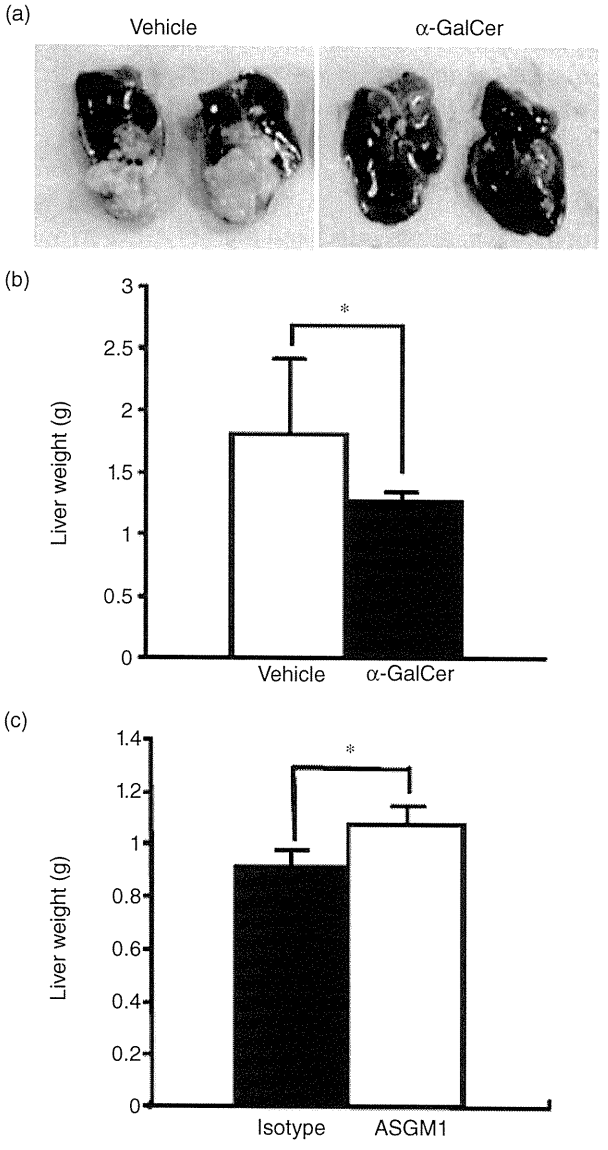


Figure 1 Therapeutic effectiveness of i.p. injection of α-galactosylceramide (α-GalCer) in CMS4 liver tumor model. BALB/c mice were injected intro-hepatically with 5×10^5 CMS4 cells. One day later, BALB/c mice were treated with i.p. injection of α-GalCer or vehicle (all treatment groups, $n = 7$). Two weeks after the CMS4 tumor injection, the livers of treated mice were removed. (a) Representative liver macroscopic view of each group. (b) Comparison of liver weight of each group. $^*P < 0.05$. (c) To prove that the therapeutic benefit of α-GalCer treatment in the CMS4 liver tumor model is natural killer (NK)-cell dependent, *in vivo* depletion of NK cells was performed (as described in Methods, ASGM1). In control mice, isotype antibody (Ab) was injected i.p. (isotype). Both mice were treated by α-GalCer. Ab-mediated *in situ* depletion of NK cells markedly reduces the therapeutic efficacy of α-GalCer treatment (all treatment groups $n = 5$). $^*P < 0.05$.

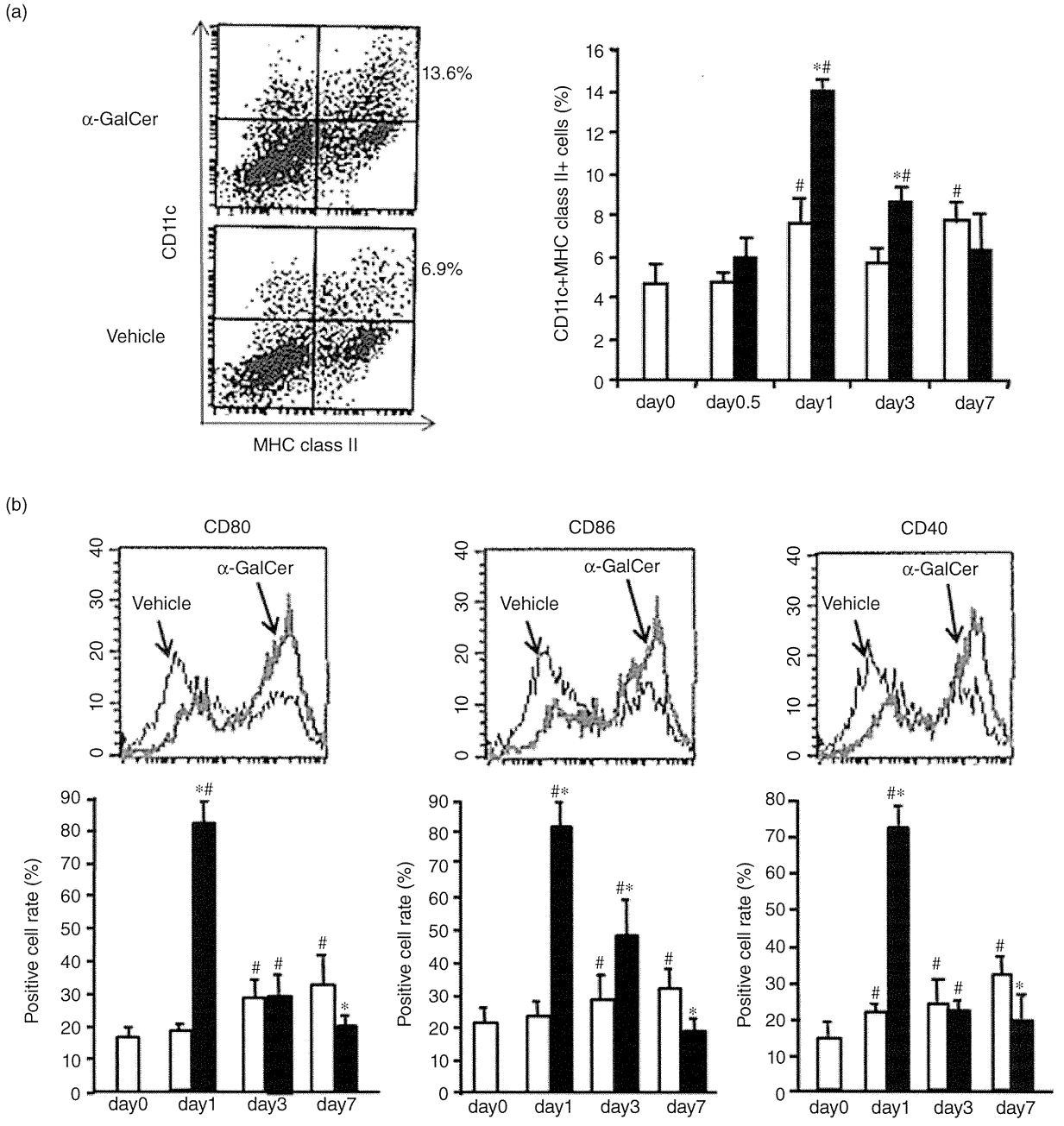


Figure 2 α-Galactosylceramide (α-GalCer) treatment increased dendritic cell (DC) population in the liver mononuclear cells (MNC) and activated DC functions. (a) BALB/c mice were treated with α-GalCer or vehicle. Hepatic MNC were prepared on days 0, 0.5, 1, 3 and 7. DC ($CD11c^+$ major histocompatibility complex [MHC] class II^+ cells) population was evaluated by flow cytometry. White bar, vehicle-treated mice; black bar, α-GalCer-treated mice. Representative dot plots of liver DC ($CD11c^+$ MHC class II^+ cells) at day 1 after α-GalCer or vehicle administration are shown in the left panels. The calculated percentages of liver DC are shown in the right. (b) BALB/c mice were treated with α-GalCer or vehicle. Hepatic MNC were prepared on day 0, 1, 3 and 7 and DC were isolated from liver MNC by a magnetic cell sorting system. For phenotypic analysis, liver DC were stained with phycoerythrin (PE)- or fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (CD11c, CD40, CD80, CD86), and the expressions of these molecules were analyzed by flow cytometry. White bar, vehicle-treated mice; black bar, α-GalCer-treated mice. $*P < 0.05$ vs vehicle-treated mice, $\#P < 0.05$ vs non-treated mice. Representative histograms of the expressions of CD80, CD86 and CD40 on liver DC at day 1 after α-GalCer or vehicle administration are shown in the upper panels.

contrasts were tested with a Student's *t*-test with Welch's correction for unequal variance as needed.

RESULTS

α-GalCer administration inhibited CMS4 liver tumor mediated by NK cells

WE INITIALLY EXAMINED whether α-GalCer administration could induce antitumor effect against CMS4 liver tumor. As shown in Figure 1(a), no tumors were observed in the livers of α-GalCer-treated mice whereas large tumors were observed in the livers of vehicle-treated mice. The liver weight of the α-GalCer treatment group was significantly lighter than that of the vehicle treatment group (Fig. 1b). Depletion of NK cells significantly inhibited the antitumor efficacy of α-GalCer treatment (Fig. 1c), whereas depletion of neither $CD4^+$ nor $CD8^+$ T cells was inhibited (data not shown). These results suggested that administration of α-GalCer was therapeutic against CMS4 liver tumor and NK cells were the main effector cells in this antitumor immunity.

Administration of α-GalCer increased DC population in the liver MNC and activated DC functions

We investigated the population changes of DC in the liver MNC after α-GalCer or vehicle treatment. On day 1 after α-GalCer administration, liver DC proportion in α-GalCer-treated mice was higher than that in vehicle-treated mice (Fig. 2a). Liver DC proportion increased with the peak at 1 day after α-GalCer administration and the liver DC proportion at 7 days decreased to the same level with that from non-treated mice (Fig. 2a). In contrast, liver DC proportion in vehicle-treated mice exhibited weaker change than those in α-GalCer-treated mice (Fig. 2a). The liver DC number also exhibited increase at the peak of 1 day after α-GalCer administration whereas that from vehicle-treated mice exhibited no change (data not shown). We examined the CD40, CD80 and CD86 expressions of liver DC after administration of α-GalCer, which is an indicator of DC activation. On day 1 after α-GalCer administration, CD40, CD80 and CD86 on liver DC from α-GalCer-treated mice expressed more strongly than those from vehicle-treated mice (Fig. 2b). The expressions of all these molecules on liver DC increased with the peak at 1 day after α-GalCer administration and the expression levels of these molecules at 7 days decreased to the same levels on liver DC from non-treated mice (Fig. 2b). In contrast, the expressions of these molecules on liver DC exhibited weaker change in vehicle-treated mice.

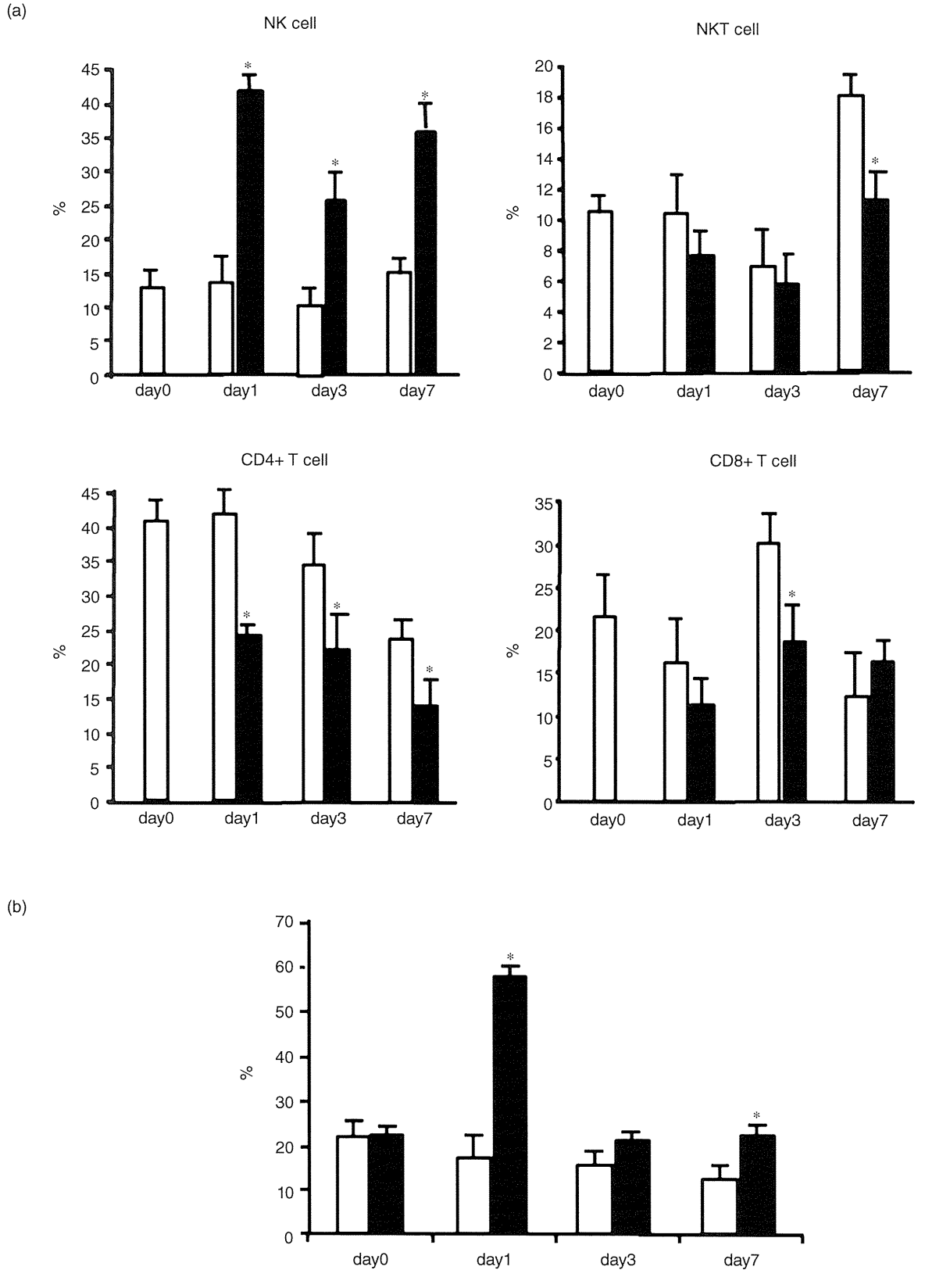
Activated NK cells composed the major subpopulation of hepatic MNC that increased after α-GalCer treatment

We examined the population change of MNC from the livers after α-GalCer or vehicle administration. It is notable that NK cells strikingly increased in proportion after α-GalCer administration, but not after vehicle administration (Fig. 3a). In contrast, the NKT cells decreased at 1 and 3 days after α-GalCer administration and recovered at day 7 after α-GalCer administration. Both $CD4^+$ and $CD8^+$ T cells decreased in proportion after α-GalCer administration, but not after vehicle administration. We also examined the CD69 expressions of NK cells, which is an indicator of lymphocyte activation. The CD69 expressions on liver NK cells increased with the peak at 1 day and gradually decreased at 7 days after α-GalCer administration (Fig. 3b). In contrast, those did not change after vehicle administration. These results demonstrated that the activated NK cells were the major subpopulation of MNC that increased in the liver after α-GalCer administration.

$p53_{232-240}$ peptide-specific CTL were generated after α-GalCer treatment of liver tumor

We evaluated whether $p53_{232-240}$ peptide-specific CTL were generated after α-GalCer treatment of liver tumor. $CD8^+$ T cells were isolated from the spleen cells of treated mice and then co-cultured with syngeneic DC pulsed with $p53_{232-240}$ peptide strongly expressed on CMS4 cells. As shown in Figure 4(a), the number of

Figure 3 Activated natural killer (NK) cells composed the major subpopulation of hepatic mononuclear cells (MNC) that increased after α-galactosylceramide (α-GalCer) treatment. (a) BALB/c mice were treated with α-GalCer or vehicle. Hepatic MNC were prepared on days 0, 1, 3 and 7. NK cells, NKT cells, $CD4^+$ T cells and $CD8^+$ T cells in liver MNC were evaluated by flow cytometry. (b) The expressions of CD69 on liver NK cells were also evaluated by flow cytometry. White bar, vehicle-treated mice; black bar, α-GalCer-treated mice. $^*P < 0.05$ vs vehicle-treated mice.



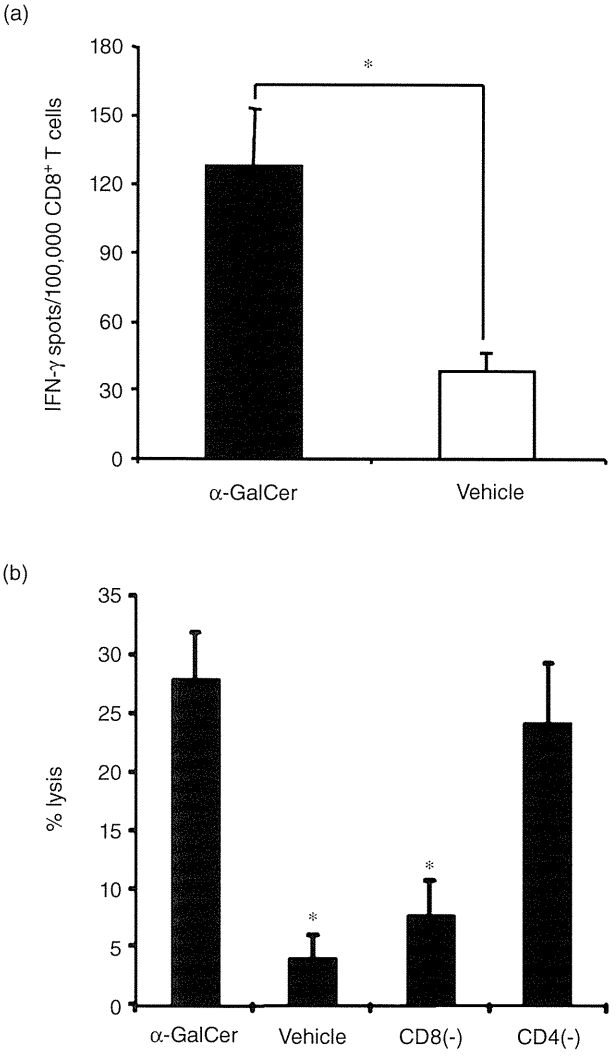


Figure 4 Evaluation of $p53_{232-240}$ peptide-specific $CD8^+$ cytotoxic T lymphocytes (CTL) in α-galactosylceramide (α-GalCer)-treated mice. (a) $CD8^+$ T cells were isolated from the spleen cells of treated mice 14 days after α-GalCer or vehicle treatment. The frequency of $p53_{232-240}$ peptide-specific $CD8^+$ CTL was evaluated by interferon (IFN)-γ enzyme-linked immunosorbent spot (ELISPOT) assay. The results are shown in spots/100 000 $CD8^+$ T cells; mean ± standard deviation of triplicate samples. $^*P < 0.05$. (b) Splenocytes from α-GalCer- or vehicle-treated mice were harvested 14 days after tumor inoculation and were analyzed for their ability to kill CMS4 cells in 4 h ^{51}Cr-release assays (effector cells/target cells ratio, 60:1). $CD4^+$ or $CD8^+$ T cells were depleted by magnetic sorting using CD4 or CD8 microbeads (Miltenyi Biotec), respectively. $CD8^-$, $CD8^+$ T-cell-depleted splenocytes. $CD4^-$, $CD4^+$ T-cell-depleted splenocytes. $^*P < 0.05$ vs the cytolytic activity of splenocytes from α-GalCer-treated mice.

IFN-γ spots (per 100 000 $CD8^+$ T cells) observed for T-cell responses against $p53_{232-240}$ peptide in α-GalCer-treated mice were significantly higher than that in vehicle-treated mice. These results suggested that strong $p53_{232-240}$ peptide-specific CTL were generated by α-GalCer treatment of liver tumor. Splenocytes from α-GalCer-treated mice displayed strong cytolytic activity against CMS4 cells, while those from vehicle-treated mice did not (Fig. 4b). $CD8^+$ T-cell-depleted splenocytes from α-GalCer-treated mice displayed significant weak cytolytic activity against CMS4 cells, but $CD4^+$ T-cell-depleted splenocytes did not. These results demonstrated that $CD8^+$ T cells (i.e. CTL) played essential roles in the cytolytic activity against CMS4 cells in α-GalCer-treated mice.

Systemic therapeutic antitumor immunity was induced by α-GalCer treatment of CMS4 liver tumor

Because strong $p53_{232-240}$ peptide-specific CTL were generated in α-GalCer-treated animals, we next chose to analyze whether the treatment of a CMS4 lesion in the liver would impact the progression of subcutaneous untreated CMS4 tumors. BALB/c mice were intrahepatically injected with CMS4 tumors and treated by administration of α-GalCer. Twenty-eight days later, 1×10^6 CMS4 cells or Colon26 cells were injected s.c. in the right flank. As shown in Figure 5(a), the non-treated CMS4 tumors in mice receiving α-GalCer treatment were completely rejected in all mice. The growth of non-treated Colon26 tumor in α-GalCer-treated mice was not inhibited (Fig. 5b). These results suggested that systemic CMS4-specific antitumor immunity could be induced by α-GalCer treatment. To confirm the involvement of CTL in this antitumor effect, we depleted $CD8^+$ T cells before re-challenge of CMS4 cells (s.c. injection of 1×10^6 CMS4 cells) in α-GalCer-treated mice bearing CMS4 liver tumor. On days 1 and 3 of re-challenge of CMS4 cells, anti-CD8 antibody (53–6.72 hybridoma, ATCC) was injected i.p. As shown in Figure 5(c), antitumor effect against re-challenged CMS4 subcutaneous tumor was diminished in $CD8^+$ T-cell-depleted mice. These results supported that $CD8^+$ T cells (i.e. CTL) play essential roles in the antitumor effect against re-challenge of CMS4 cells in α-GalCer-treated mice.

DISCUSSION

WE PREVIOUSLY DEMONSTRATED that administration of α-GalCer activated both NKT cells and NK cells in the liver, and that liver NK cells were the

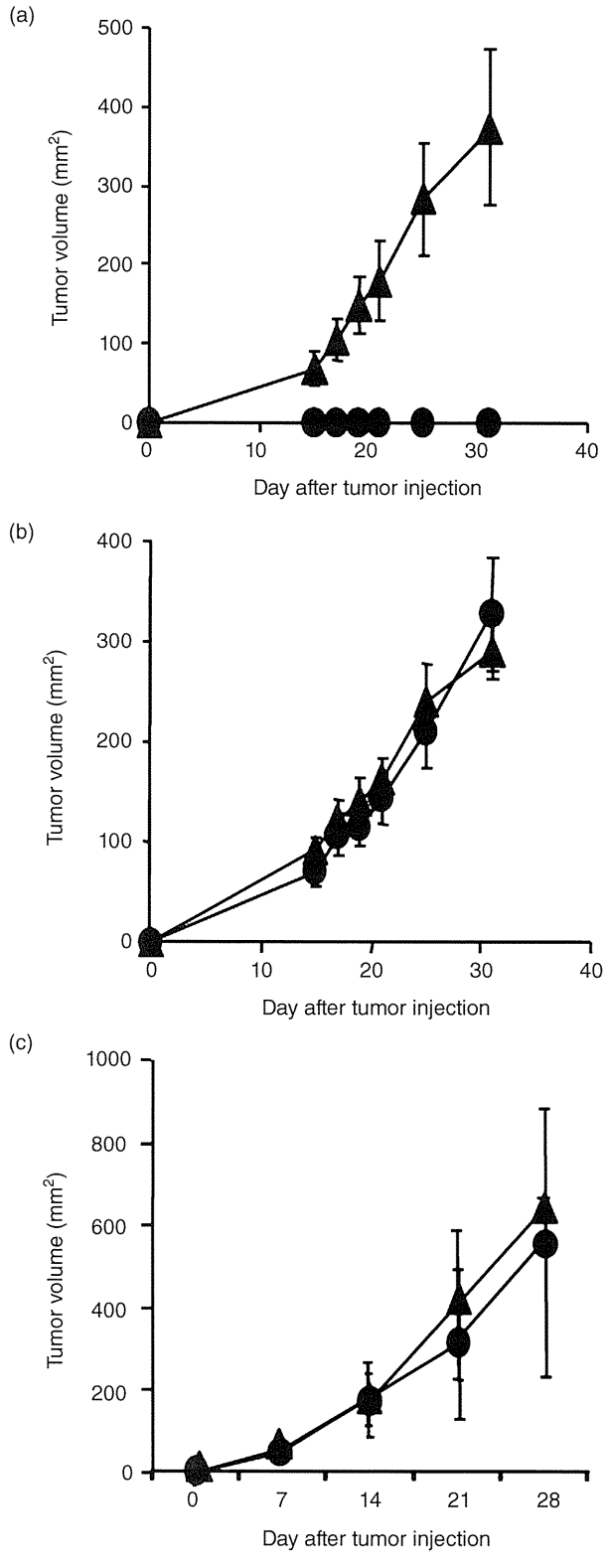


Figure 5 α-Galactosylceramide (α-GalCer) therapy results in the development of systemic antitumor immunity that protects distal tumor re-challenge. BALB/c mice were injected intrahepatically with CMS4 tumors. Twenty-four hours later, the mice were treated with α-GalCer. Twenty-eight days after treatment, α-GalCer-treated mice were re-challenged s.c. with 1×10^6 CMS4 cells (a) or Colon26 cells (b) in the right flank (all treatment groups, $n = 8$). To confirm the involvement of cytotoxic T lymphocytes (CTL) in this antitumor effect, we depleted $CD8^+$ T cells before re-challenge of CMS4 cells in α-GalCer-treated mice bearing CMS4 liver tumor (c). On days 1 and 3 of re-challenge of CMS4 cells, anti-CD8 antibody was injected i.p. Tumor size was assessed every 3 or 4 days after s.c. injection of tumor cells (on day 0). As control mice, naïve mice were injected s.c. with 1×10^6 CMS4 cells (a, $n = 8$; c, $n = 6$) or Colon26 cells (b) ($n = 8$) on day 0. (●) α-GalCer-treated mice, (▲) control mice. Each data point represents the mean tumor size ± standard deviations.

main effector cells to kill disseminated hepatoma cells injected from spleen in α-GalCer treatment.[4] In this study, we evaluated α-GalCer treatment in local injected liver tumor, and the α-GalCer treatment resulted in complete rejection of local liver tumor, which had a similar antitumor effect as α-GalCer in a previous metastatic liver tumor model. These findings suggested the ability of α-GalCer treatment to activate the liver NK cells efficiently, which may mainly contribute to eradication of local liver tumor cells. A normal liver contains lymphocytes that are usually enriched with NK and NKT cells; namely, 25% NK cells and 30% NKT cells in contrast to peripheral blood that contains only 10% NK and 5% NKT cells.[6,7] Thus, activation of innate immune cells, NK cells and NKT cells must be important to develop more effective immunotherapy against liver cancer. We believe that α-GalCer treatment must be a good candidate for human liver cancer treatment.

Recently, activated DC have been implicated in the activation of NKT and NK cells in both mice and humans,[1,5,8–12,22] suggesting that DC play crucial roles in the activation of abundant immune cells in the liver. To establish more efficient α-GalCer treatment in liver cancer, the precise mechanism of liver DC activation is needed. Our results demonstrated that the proportion of liver DC in liver MNC increased immediately and reached the peak 1 day after α-GalCer treatment. The infiltration of tumors by mature DC has been reported to correlate with a better prognosis in cancer patients.[23,24] Thus, the increase of liver DC by α-GalCer might contribute to generation of antitumor effect against liver cancer. The expressions of co-stimulatory

molecules on liver DC also increased early after administration of α-GalCer. IL-12 production from DC is key Th1-cytokine to enhance NK and CTL functionality,[25,26] IL-12 production from liver DC after α-GalCer treatment was significantly higher than that after vehicle treatment.[17] These results suggested that α-GalCer treatment resulted in rapid activation of liver DC, which might play important roles in activating liver NK cells and might contribute to the subsequent establishment of acquired immunity against liver cancer. Pillarisetty *et al.* identified new DC subsets, NK–DC, which presented in the liver of mice,[27] which may affect the interpretation of the activation of liver NK cells by α-GalCer. However, we previously demonstrated that α-GalCer had no direct effect on liver NK cells in mice.[4] These results supported the idea that α-GalCer activated liver DC, which activated the liver NK cells secondary.

Interferon-γ ELISPOT assay revealed that the frequency of $CD8^+$ T cells isolated from α-GalCer-treated mice in liver tumors in response to $p53_{232-240}$ peptide were much higher than that from vehicle-treated mice. Mayordomo *et al.* reported that immunization of $p53_{232-240}$ peptide-pulsed DC induced peptide-specific CTL in immunized mice that showed cytolytic activity against CMS4, p53 overexpressing cells.[18] In this study, ^{51}Cr-release assay demonstrated that $CD8^+$ T cell, not $CD4^+$ T cells, played essential roles in the cytolytic activity against CMS4 cells in α-GalCer-treated mice, which is consistent with the IFN-γ ELISPOT results. The detection of $p53_{232-240}$ peptide-specific CTL means the generation of CMS4 tumor-specific CTL after eradication of liver tumor by α-GalCer treatment. The activation of NKT cells was associated with an expansion of antigen-specific CTL, as might be expected if the DC that matured *in vivo* in response to NKT cells were capturing antigens.[28–31] Our results suggested that the activation of hepatic DC might be associated with the efficiency of generation of tumor antigen-specific CTL.

Additional experiments using an s.c. re-challenge with tumor demonstrated that α-GalCer treatment of liver tumors not only blocked treated CMS4 liver tumor progression but completely protected against consequent "recurrence" of that same tumor at a distant site. In contrast, Colon26 re-challenge tumor was not inhibited in treated mice, suggesting that CMS4-specific immunity was generated after liver tumor treatment. These results were consistent with the activation of acquired immunity evaluated by IFN-γ ELISPOT assay with increase of the frequency of $p53_{232-240}$ peptide-specific CTL. Taken together, we believe that α-GalCer treatment of liver tumors resulted in rejection of both local liver tumor and distant metastatic tumor.

In summary, we have shown that α-GalCer treatment activated both innate and acquired immune cells in the liver. These findings suggested that the use of α-GalCer might represent a particularly promising approach to suppress tumor growth and to promote regression of metastatic lesions in liver cancer patients.

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Research Article

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The *let-7* family of microRNAs inhibits Bcl-xL expression and potentiates sorafenib-induced apoptosis in human hepatocellular carcinoma

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Background & Aims: Bcl-xL, an anti-apoptotic member of the Bcl-2 family, is over-expressed in human hepatocellular carcinoma, conferring a survival advantage to tumour cells. The mechanisms underlying its dysregulation have not been clarified. In the present study, we explored the involvement of microRNAs that act as endogenous sequence-specific suppressors of gene expression.

Methods: The expression profiles of microRNAs in Huh7 hepatoma cells and primary human hepatocytes were compared by microarray analysis. The effect of *let-7* on Bcl-xL expression was examined by Western blot and a reporter assay. The involvement of *let-7* microRNAs in human tissues was analysed by western blot and reverse transcription-PCR.

Results: Microarray analysis, followed by *in silico* target prediction, identified *let-7* microRNAs as being downregulated in Huh7 hepatoma cells in comparison with primary human hepatocytes, as well as possessing a putative target site in the *bcl-xl* mRNA. Over-expression of *let-7c* or *let-7g* led to a clear decrease of Bcl-xL expression in Huh7 and HepG2 cell lines. Reporter assays revealed direct post-transcriptional regulation involving *let-7c* or *let-7g* and the 3′-untranslated region of *bcl-xl* mRNA. Human hepatocellular carcinoma tissues with low expression of *let-7c* displayed higher expression of Bcl-xL protein than those with high expression of *let-7c*, suggesting that low *let-7* microRNA expression contributes to Bcl-xL over-expression. Finally, expression of *let-7c* enhanced apoptosis of hepatoma cells upon exposure to sorafenib, which downregulates expression of another anti-apoptotic Bcl-2 protein, Mcl-1.

Conclusions: *let-7* microRNAs negatively regulate Bcl-xL expression in human hepatocellular carcinomas and induce apoptosis in cooperation with an anti-cancer drug targeting Mcl-1.

Keywords: Liver; Mcl-1; Bcl-2; Tumour; Epigenetic.
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Abbreviations: miRNA, microRNA; 3′UTR, 3′-untranslated region; mRNA, messenger RNA; HCC, hepatocellular carcinoma; CDK, cyclin-dependent kinase; DMEM, Dulbecco's modified Eagle medium; RT, reverse transcription; PCR, polymerase chain reaction; 7-AAD, 7-amino-actinomycin D; DMSO, dimethyl sulfoxide.

Introduction

MicroRNAs (miRNAs), a novel class of non-coding, small RNAs, repress gene expression by binding to the 3′-untranslated region (3′UTR) of target messenger RNAs (mRNAs) [1]. More than 500 miRNAs have been identified in humans. Each miRNA is capable of modulating the expression of many mRNAs to which it binds by imperfect sequence complementarity, although only a limited number of targeted genes has been identified. Through its activity of gene silencing, miRNA functions in a variety of cellular processes, such as development, organ homeostasis, and cancer development and progression [2]. In the context of cancer development and progression, miRNAs targeting oncogenes function as tumour suppressors, whereas those targeting tumour suppressor genes serve as oncogenes [3]. Accumulating evidence has revealed the aberrant expression of miRNAs in human hepatocellular carcinoma (HCC) [4–6]. *miR-122a* has been reported to be downregulated in HCC, in turn, leading to upregulation of cyclin G1 [7]. On the other hand, recent reports have demonstrated that *miR-21* [8], *miR-221* [9], and *miR-224* [10] are upregulated in HCC, leading to downregulation of PTEN, CDK inhibitors, and API-5, respectively. Furthermore, the miRNA expression signature was reported to be related to the clinical outcome of patients with HCC [11,12]. Thus, miRNAs may play an important role in HCC development and progression by modulating a variety of gene expression and cellular processes.

Apoptosis resistance is an important characteristic of tumour cells, in addition to dysregulated proliferation and aberrant differentiation. Apoptosis is regulated by a fine balance of Bcl-2 family proteins, such as anti-apoptotic Bcl-xL and Mcl-1 and pro-apoptotic Bak and Bax. We previously demonstrated that Bcl-xL