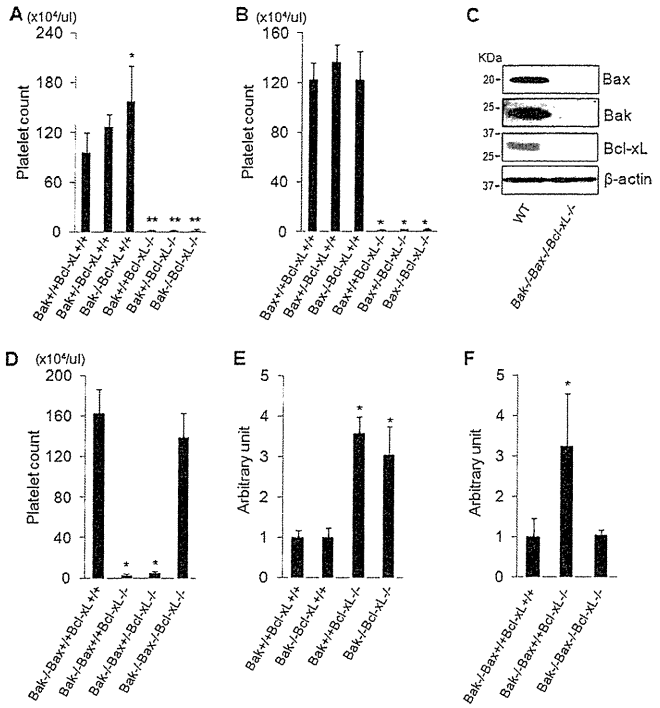

FIGURE 1. **Thrombocytopenia induced by Bcl-xL deficiency is dependent on Bcl-2 effector proteins Bak and Bax.** *Bcl-xL+/+* and *Bcl-xL−/−* stand for *bcl-x*$^{flox/flox}$ without *pf4-Cre* and *bcl-x*$^{flox/flox}$ with *pf4-Cre*, respectively. *Bak+/+*, *Bak+/−*, and *Bak−/−* stand for *bak*$^{+/+}$, *bak*$^{+/-}$, and *bak*$^{-/-}$, respectively. *WT* stands for wild type. *A*, platelet counts of the offspring from mating of *bak*$^{+/-}$*bcl-x*$^{flox/flox}$ *pf4-Cre* mice and *bak*$^{+/-}$*bcl-x*$^{flox/flox}$ mice (more than four mice per group; *, $p < 0.01$ *versus* all other groups; **, $p < 0.01$ *versus* Bcl-xL+/+ groups). *B*, platelet counts of the offspring from mating of *bax*$^{+/-}$*bcl-x*$^{flox/flox}$ *pf4-Cre* mice and *bax*$^{+/-}$*bcl-x*$^{flox/flox}$ mice (more than five mice per group; *, $p < 0.01$ *versus* Bcl-xL+/+ groups). *Bax+/+*, *Bax+/−*, and *Bax−/−* stand for *bax*$^{+/+}$, *bax*$^{+/-}$, and *bax*$^{-/-}$, respectively. *C*, Western blot of platelet lysates for the expression of Bcl-xL, Bak, and Bax. *D*, platelet counts of the offspring from mating of *bak*$^{-/-}$*bax*$^{flox/+}$*bcl-x*$^{flox/flox}$ *pf4-Cre* mice and *bak*$^{-/-}$*bax*$^{flox/+}$*bcl-x*$^{flox/flox}$ mice (more than eight mice per group; *, $p < 0.01$ *versus* Bak−/−Bax+/+Bcl-xL+/+ group and Bak−/−Bax−/−Bcl-xL−/− group. *Bax+/+*, *Bax+/−*, and *Bax−/−* stand for *bax*$^{+/+}$, *bax*$^{flox/+}$ with *pf4-Cre*, and *bax*$^{flox/flox}$ with *pf4-Cre*, respectively. *E*, serum caspase-3/7 activity of the offspring from mating of *bak*$^{+/-}$*bcl-x*$^{flox/flox}$ *pf4-Cre* mice and *bak*$^{+/-}$*bcl-x*$^{flox/flox}$ mice ($n = 5$ or 6/group; *, $p < 0.01$ *versus* Bcl-xL+/+ group). *F*, serum caspase-3/7 activity of the offspring from mating of *bak*$^{-/-}$*bax*$^{flox/+}$*bcl-x*$^{flox/flox}$ *pf4-Cre* mice and *bak*$^{-/-}$*bax*$^{flox/+}$*bcl-x*$^{flox/flox}$ mice ($n = 8$/group; *, $p < 0.01$ *versus* all). *Bax+/+*, *Bax+/−*, and *Bax−/−* stand for *bax*$^{+/+}$, *bax*$^{flox/+}$ with *pf4-Cre*, and *bax*$^{flox/flox}$ with *pf4-Cre*, respectively.

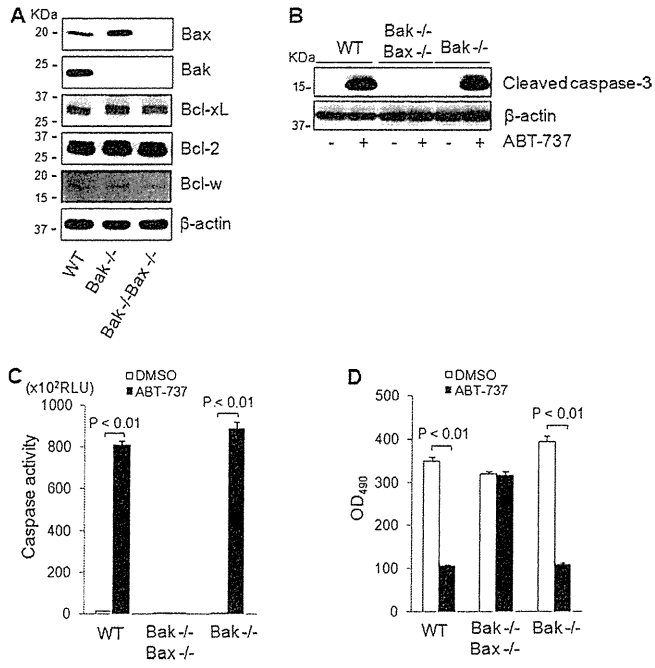

FIGURE 2. **ABT-737 treatment provokes Bak/Bax-dependent apoptosis in platelets.** *WT*, *Bak−/−Bax−/−*, and *Bak−/−* stand for wild type, *bak−/−bax*$^{flox/flox}$ with *pf4-Cre*, and *bak−/−*, respectively. *A*, Western blot of platelet lysates for the expression of Bak, Bax, Bcl-xL, Bcl-2, and Bcl-w. *B*, platelets (3.0×10^7) were incubated with 10 μM ABT-737 or vehicle for 2 h at room temperature. A Western blot of platelet lysates for the expression of cleaved caspase-3 is shown. *C* and *D*, platelets (2.0×10^6) were incubated with 10 μM ABT-737 or vehicle for 2 h at room temperature. *C*, caspase-3/7 activity of platelet supernatant ($n = 4$/group). *D*, MTS assay ($n = 5$/group). *RLU*, relative light units.

phobic groove of these proteins (31). Western blot revealed that these antiapoptotic Bcl-2 proteins existed in platelets (Fig. 2*A*), and ABT-737 has already been reported to cause apoptosis in platelets in both *in vivo* and *in vitro* settings (7, 8). We first examined whether ABT-737-induced platelet apoptosis was executed via the Bak/Bax-dependent mitochondrial pathway. In platelets isolated from wild-type mice, administration of ABT-737 caused cleavage of caspase-3 (Fig. 2*B*). Supernatants of ABT-737-treated platelets showed marked elevation of caspase-3/7 activity (Fig. 2*C*). In addition, platelet cellular viability, which can be assessed by MTS assay (3, 4), decreased upon ABT-737 treatment (Fig. 2*D*). On the other hand, although expression of targeted antiapoptotic Bcl-2 proteins was not different between platelets from wild-type mice and Bak/Bax double knock-out mice (Fig. 2*A*), ABT-737 treatment neither caused caspase activation nor impaired cellular integrity in Bak/Bax-deficient platelets (Fig. 2, *B–D*). These findings demonstrated that ABT-737 caused platelet apoptosis via the Bak/Bax-dependent mitochondrial pathway. Interestingly, unlike what was reported previously (8), Bak deficiency could alleviate neither caspase activation nor loss of cellular viability in ABT-737-treated platelets (Fig. 2, *B–D*), offering evidence of the redundancy of Bak and Bax proteins in executing apoptosis in platelets under inhibition of these antiapoptotic Bcl-2 proteins.

ABT-737 Treatment Causes Bax Activation in Platelets—After ABT-737 treatment of the platelets, we next examined the activation status of the Bax protein in these platelets. In general, Bax activation is divided into sequential steps. When subjected to a variety of apoptotic stimuli, the Bax protein first undergoes a conformational change such as exposure of the amino terminus. This active form is translocated from the cytosol to the mitochondria. Finally, mitochondrial Bax undergoes self-oligomerization, leading to permeabilization of the outer mitochondrial membrane (32). We found that upon addition of ABT-737 to platelets the Bax protein underwent a conformational change as demonstrated by Western blotting upon immunoprecipitation with an antibody that specifically recognizes the amino terminus of the Bax protein (33) (Fig. 3*A*). In addition, upon ABT-737 treatment, the Bax protein was translocated from the cytosol to the mitochondria (Fig. 3*B*) and then underwent homo-oligomerization (Fig. 3*C*). These findings indicated that inhibition of antiapoptotic Bcl-2 proteins in

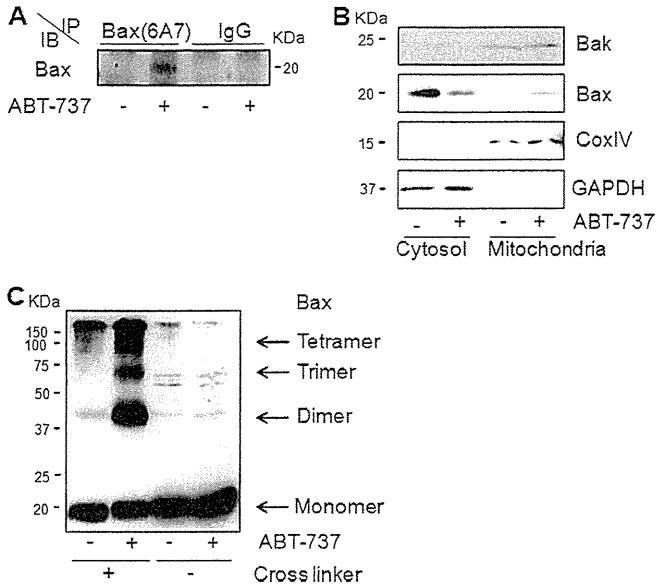

FIGURE 3. **ABT-737 treatment causes Bax activation in platelets.** *A–C*, platelets (1.0×10^8) isolated from C57BL/6J mice were incubated with 10 μM ABT-737 or vehicle for 2 h at room temperature. *A*, Western blot of platelet lysates for the expression of Bax after immunoprecipitation (*IP*) using mouse antibody that specifically recognizes activated Bax (6A7) or mouse control IgG (active Bax exposes an amino-terminal epitope (amino acids 12–24) that is recognized by 6A7). *B*, Western blot for the expression of Bak, Bax, *CoxIV* (cytochrome c oxidase IV), and GAPDH after cellular fractionation of the platelet lysates. *C*, Western blot for the expression of Bax after incubation of the platelet lysates with or without protein cross-linkers (5 mM bismaleimidohexane and 5 mM bis(sulfosuccinimidyl) suberate). *IB*, immunoblot.

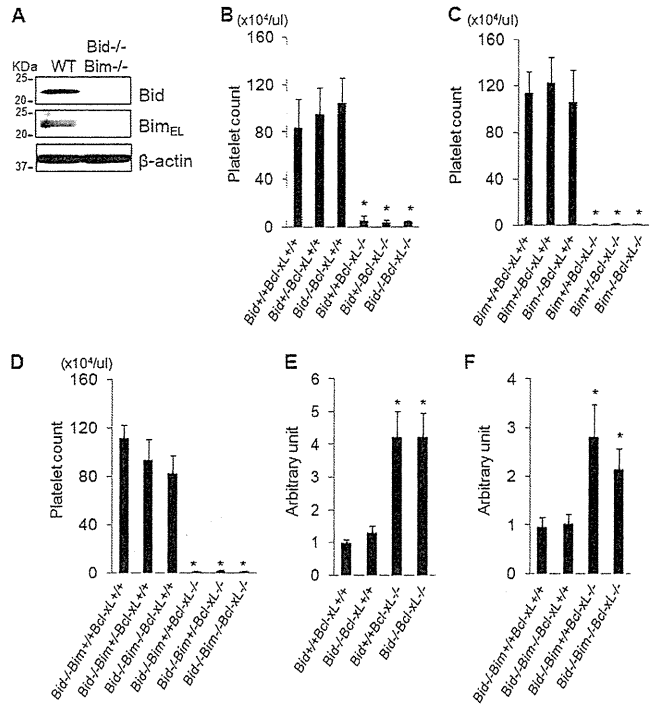

FIGURE 4. **Thrombocytopenia induced by Bcl-xL deficiency does not require BH3-only activator proteins Bid and Bim.** *Bcl-xL+/+* and *Bcl-xL−/−* stand for *bcl-x$^{flox/flox}$* without *pf4-Cre* and *bcl-x$^{flox/flox}$* with *pf4-Cre*, respectively. *Bid+/+*, *Bid+/−*, and *Bid−/−* stand for *bid$^{+/+}$*, *bid$^{+/-}$*, and *bid$^{-/-}$*, respectively. *Bim+/+*, *Bim+/−*, and *Bim−/−* stand for *bim$^{+/+}$*, *bim$^{+/-}$*, and *bim$^{-/-}$*, respectively. *WT* and *Bid−/−Bim−/−* stand for wild type and *bid$^{-/-}$bim$^{-/-}$*, respectively. *A*, Western blot of platelet lysates for the expression of Bid and Bim$_{EL}$. *B*, platelet counts of the offspring from mating of *bid$^{+/-}$bcl-x$^{flox/flox}$ pf4-Cre* mice and *bid$^{+/-}$bcl-x$^{flox/flox}$* mice (more than five mice per group; *, $p < 0.01$ *versus* Bcl-xL+/+ groups). *C*, platelet counts of the offspring from mating of *bim$^{+/-}$bcl-x$^{flox/flox}$ pf4-Cre* mice and *bim$^{+/-}$bcl-x$^{flox/flox}$* mice (more than seven mice per group; *, $p < 0.01$ *versus* Bcl-xL+/+ groups). *D*, platelet counts of the offspring from mating of *bid$^{-/-}$bim$^{+/-}$bcl-x$^{flox/flox}$ pf4-Cre* mice and *bid$^{-/-}$bim$^{+/-}$bcl-x$^{flox/flox}$* mice (more than five mice per group; *, $p < 0.01$ *versus* Bcl-xL+/+ groups). *E*, serum caspase-3/7 activity of the offspring from mating of *bid$^{+/-}$bcl-x$^{flox/flox}$ pf4-Cre* mice and *bid$^{+/-}$bcl-x$^{flox/flox}$* mice ($n = 4–6$/group; *, $p < 0.01$ *versus* Bcl-xL+/+ groups). *F*, serum caspase-3/7 activity of the offspring from mating of *bid$^{-/-}$bim$^{+/-}$bcl-x$^{flox/flox}$ pf4-Cre* mice and *bid$^{-/-}$bim$^{+/-}$bcl-x$^{flox/flox}$* mice ($n = 4–6$/group; *, $p < 0.01$ *versus* Bcl-xL+/+ groups).

platelets caused Bax activation, promoting Bak/Bax-dependent mitochondrial apoptosis followed by caspase activation.

Thrombocytopenia Induced by Bcl-xL Deficiency Does Not Require BH3-only Activator Proteins Bid and Bim—We explored whether Bak/Bax-dependent platelet apoptosis induced by Bcl-xL deficiency requires the direct activator proteins Bid and Bim. Western blot revealed that Bid and Bim were both present in platelets (Fig. 4*A*). We generated Bcl-xL/Bid double knock-out mice and Bcl-xL/Bim double knock-out mice by mating thrombocyte-specific Bcl-xL knock-out mice with systemic Bid knock-out mice or Bim knock-out mice, respectively. These double knock-out mice showed massive thrombocytopenia that was not alleviated at all compared with that of thrombocyte-specific Bcl-xL knock-out mice (Fig. 4, *B* and *C*). It was possible that, in Bcl-xL-deficient platelets, the existence of either Bid or Bim was sufficient to activate Bak/Bax directly, leading to platelet apoptosis in these double knock-out mice. We then generated Bcl-xL, Bid, and Bim triple knock-out mice by mating Bcl-xL/Bid double knock-out mice with Bcl-xL/Bim double knock-out mice. These triple knock-out mice still showed massive thrombocytopenia without any difference of platelet count compared with that of Bcl-xL/Bid double knock-out mice (Fig. 4*D*). These findings clearly demonstrated that BH3-only activator proteins Bid and Bim were dispensable for the severe thrombocytopenia induced by thrombocyte-specific Bcl-xL deletion *in vivo*. In addition, caspase activation in thrombocyte-specific Bcl-xL knock-out mice was not alleviated even in the Bid and Bim double knock-out background (Fig. 4, *E* and *F*), suggesting that the lack of Bcl-xL required neither Bid nor Bim to trigger Bak/Bax-dependent platelet apoptosis.

Bax Activation and Subsequent Apoptotic Cell Death Provoked by ABT-737 Can Proceed in Absence of Bid and Bim—To investigate whether Bax can be activated by inhibition of anti-apoptotic Bcl-2 proteins even in the absence of Bid and Bim, we isolated platelets from Bid and Bim double knock-out mice. A Western blot study confirmed that neither Bid nor Bim existed in platelets of the double knock-out mice (Fig. 4*A*) and showed that Puma protein, another putative direct activator (13), was not detected in platelets of either wild-type mice or Bid/Bim double knock-out mice (Fig. 5*A*). The expression of antiapoptotic Bcl-2 proteins including Bcl-xL, Bcl-2, and Bcl-w was unchanged between these mice (Fig. 5*A*). Upon ABT-737 treatment, the Bax protein in Bid/Bim-deficient platelets could undergo conformational change (Fig. 5*B*), translocation from the cytosol to the mitochondria (Fig. 5*C*), and homo-oligomerization (Fig. 5*D*). These results clearly demonstrated that ABT-

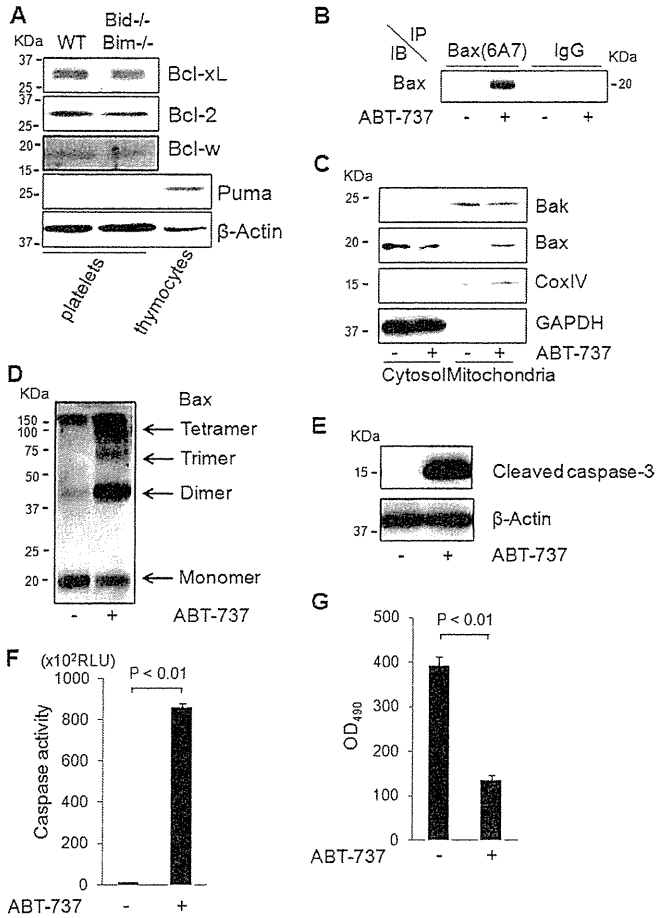

FIGURE 5. **Bax activation and subsequent apoptotic cell death provoked by Bcl-xL deficiency can proceed in absence of Bid and Bim.** *A*, Western blot of platelet lysates for the expression of Puma, Bcl-2, Bcl-w, and Bcl-xL. *WT* and *Bid−/−Bim−/−* stand for wild type and $bid^{-/-}bim^{-/-}$, respectively. *B–E*, platelets (1.0×10^8) isolated from Bid/Bim double knock-out mice were incubated with 10 μM ABT-737 or vehicle for 2 h at room temperature. *B*, Western blot for the expression of Bax after immunoprecipitation (*IP*) using mouse antibody that specifically recognizes activated Bax (6A7) or mouse control IgG. *C*, Western blot for the expression of Bak, Bax, *CoxIV* (cytochrome c oxidase IV), and GAPDH after cellular fractionation of the platelet lysates. *D*, Western blot for the expression of Bax after incubation of the platelet lysates with protein cross-linkers (5 mM bismaleimidohexane and 5 mM bis(sulfosuccinimidyl) suberate). *E*, Western blot of platelet lysates for the expression of cleaved caspase-3. *F* and *G*, platelets (2.0×10^6) isolated from Bid/Bim double knock-out mice were incubated with 10 μM ABT-737 or vehicle for 2 h at room temperature. *F*, caspase-3/7 activity of platelet supernatant ($n = 4$/group). *G*, MTS assay ($n = 5$/group). *IB*, immunoblot; *RLU*, relative light units.

737-induced Bax activation did not require the direct activator proteins Bid and Bim. Upon ABT-737 treatment of Bid/Bim-deficient platelets, cleavage of caspase-3 and elevation of caspase-3/7 activity were both observed (Fig. 5, *E* and *F*), and the MTS assay demonstrated that platelet cellular viability was also impaired (Fig. 5*G*). These findings indicated that Bid and Bim were dispensable for Bak/Bax-dependent platelet apoptosis provoked by inhibition of antiapoptotic Bcl-2 proteins.

Spontaneous Apoptotic Cell Death in Stored Human Platelets Occurs with Decline of Bcl-xL Despite Decrease in Bid and Bim—In stored human platelets, phosphatidylserine exposure increases with caspase-3 activation (4, 5), which leads to spontaneous platelet apoptosis, but the exact molecular mechanism of this process remains elusive. This led us to examine the profile of Bcl-2 family proteins in human platelets during the course of storage. In stored platelets, cleaved caspase-3 gradually increased (Fig. 6*A*) and caspase-3/7 activity rose simultaneously (Fig. 6*B*), indicating that the platelets steadily underwent apoptotic cell death with storage time. Regarding the Bcl-2 family protein profile, although expression of Bcl-xL and Bax proteins gradually decreased with time, the decrease in Bak expression occurred at a later time point (Fig. 6*A*). As for BH3-only direct activator proteins, Bid and Bim expression also decreased with time (Fig. 6*A*). To examine the involvement of Bcl-2 family proteins in spontaneous apoptosis in stored platelets, caspase-3/7 activity was measured in platelets from wild-type mice, Bak/Bax double knock-out mice, Bcl-xL transgenic mice, and Bid/Bim double knock-out mice upon storage. A Western blot revealed that Bcl-xL protein increased in platelets isolated from Bcl-xL transgenic mice compared with wild-type mice, whereas expression of effector proteins Bak and Bax did not differ between them (Fig. 6*C*). Although wild-type platelets showed elevation of the caspase-3/7 activity upon storage, it was significantly lower in Bak/Bax-deficient platelets than in

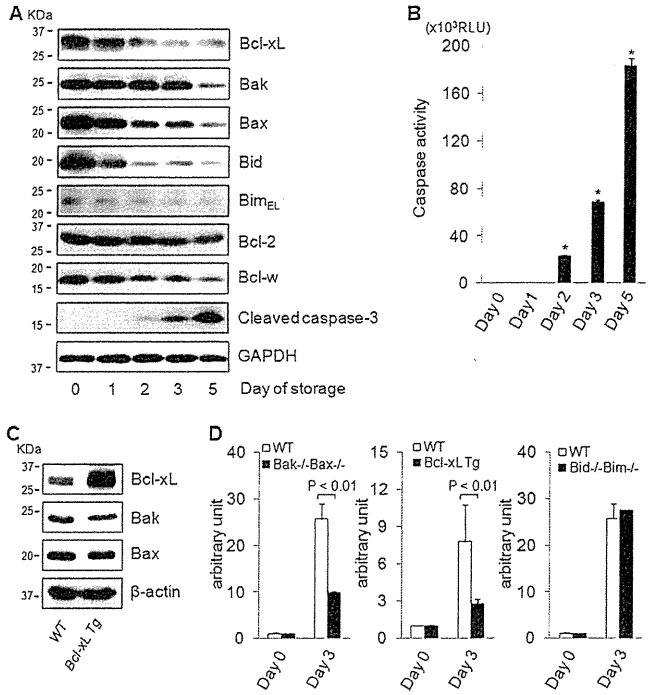

FIGURE 6. **Spontaneous apoptotic cell death in stored platelets occurs with decline of Bcl-xL despite decrease in Bid and Bim.** *A* and *B*, platelet-rich plasma derived from a healthy volunteer was stored for the indicated time course. *A*, Western blot of stored platelet lysates for the expression of Bcl-xL, Bak, Bax, Bid, Bim_{EL}, Bcl-w, Bcl-2, cleaved caspase-3, and GAPDH. Equal numbers of platelets were loaded per sample. *B*, caspase-3/7 activity of supernatant derived from platelet-rich plasma ($n = 4$/group; *, $p < 0.01$ *versus* all other groups). *C*, Western blot of platelet lysates derived from Bcl-xL transgenic mice for the expression of Bcl-xL, Bak, and Bax. *WT* and *Bcl-xL Tg* stand for wild-type mice and Bcl-xL transgenic mice, respectively. *D*, platelets derived from C57BL/6J mice, Bak/Bax double knock-out mice, Bcl-xL transgenic mice, and Bid/Bim double knock-out mice were stored for the indicated time course. Caspase-3/7 activity of stored platelet supernatant was assessed and is presented as the -fold induction compared with freshly isolated platelet supernatant ($n = 4$/group). *WT*, *Bak−/−Bax−/−*, and *Bid−/−Bim−/−* stand for wild-type, $bak^{-/-}bax^{flox/flox}$ with *pf4-Cre*, and $bid^{-/-}bim^{-/-}$ mice, respectively. *Bcl-xL Tg* stands for Bcl-xL transgenic mice. *RLU*, relative light units.

wild-type platelets (Fig. 6*D*). These findings indicated that Bak/Bax-dependent mitochondrial apoptosis played an important role in the execution of spontaneous apoptosis in stored platelets. Furthermore, caspase activation was alleviated in Bcl-xL-overexpressing platelets compared with wild-type platelets upon storage (Fig. 6*D*), suggesting an antiapoptotic function of Bcl-xL in stored platelets. On the other hand, caspase-3/7 activity increased in Bid/Bim-deficient platelets and was not different from that in wild-type platelets (Fig. 6*D*), suggesting that direct activator proteins Bid and Bim are dispensable for the spontaneous platelet apoptosis upon storage.

DISCUSSION

In the mitochondrial pathway, apoptotic cell death is dependent on activation of the proapoptotic effector proteins Bak and Bax. Cells lacking both Bak and Bax are resistant to multiple apoptotic stimuli (34). Genetic studies have revealed that Bax or Bak single knock-out mice have less pronounced phenotypes compared with Bak/Bax double knock-out mice, which display various severe defects during development, indicating the redundancy of their involvement in apoptosis (30, 35). With regard to the mitochondrial apoptosis machinery in platelets, the involvement of Bax seemed to be less critical because platelet numbers in Bax knock-out mice were normal in contrast to the thrombocytosis displayed in Bak knock-out mice (30, 35). However, our *in vitro* study revealed that ABT-737 could provoke apoptosis even in Bak-deficient platelets. Moreover, our *in vivo* studies have clearly demonstrated that either Bax or Bak was sufficient to cause platelet apoptosis in the absence of Bcl-xL, indicating that Bax and Bak are redundant and equivalently important for the mitochondrial apoptosis in platelets.

In support of the displacement model, co-immunoprecipitation studies revealed complexes of Bak with a variety of antiapoptotic proteins (36). However, the major concern with this model is that Bax is presumed to exist mainly in a cytosolic fraction as a monomer (37). Thus, Bax activation might not be controlled by displacement (38). Unlike Bak activation, sequential steps are necessary for Bax activation such as a conformational change, mitochondrial translocation, and homo-oligomerization. Recent reports have revealed the mechanism of how activator proteins Bid and Bim are directly involved in these steps and initiate Bax activation (39, 40). In the present study, we showed that all the serial steps of Bax activation can adequately proceed without the involvement of the activator proteins Bid and Bim *in vitro*. Moreover, Bak/Bax-dependent mitochondrial apoptosis could be fully executed by inhibition of antiapoptotic Bcl-2-like proteins even if the direct activator proteins Bid and Bim did not exist. Similar results have been presented by Willis *et al.* (15), who showed that embryonic fibroblasts from Bid and Bim double knock-out, when infected with retrovirus expressing BH3 sensitizer proteins, could undergo apoptosis *in vitro*. Based on their results, they claimed that the Bax protein may be constitutively active and inhibited through binding to antiapoptotic Bcl-2-like proteins for cells to survive. However, in our *in vitro* study, we could not detect physiological interaction between Bax and Bcl-xL in platelets. Therefore, it is difficult to evaluate whether Bak and/or Bax is active or inactive at the default state in platelets. On the other hand, genetically modified mice clearly showed that retrieval of direct activator proteins could not prevent caspase activation and thrombocytopenia induced by the lack of Bcl-xL. These findings demonstrated, for the first time, *in vivo* evidence that direct activator proteins Bid and Bim were dispensable for apoptosis execution provoked by the loss of antiapoptotic Bcl-2-family proteins.

Because ABT-737 can bind to and neutralize Bcl-2, Bcl-w, and Bcl-xL, all of which are present in platelets (Figs. 2*A* and 6*A*), it is difficult to directly conclude that the *in vitro* results from our ABT-737 study exactly reflect our *in vivo* results obtained from Bcl-xL deletion. However, in addition to reports that neither systemic Bcl-w knock-out nor Bcl-2 knock-out mice exhibit any phenotypes with respect to platelet counts (41–43), our *in vivo* results of massive thrombocytopenia seen in thrombocyte-specific Bcl-xL knock-out mice indicated that the antiapoptotic role of Bcl-2 and Bcl-w in platelets was apparently less important than that of Bcl-xL. Even if Bcl-2 and Bcl-w were involved in our *in vitro* results, our present results clearly demonstrated that neither Bid nor Bim is required for Bax activation and following mitochondrial apoptosis by inhibition of antiapoptotic Bcl-2 family proteins. Regarding the other antiapoptotic members of the Bcl-2 family, systemic A1a knock-out mice were not reported with any phenotype with respect to platelet counts (44). Mcl-1 is a rapid turnover protein and could not be detected in platelets (supplemental Fig. 1). Therefore, Bcl-xL may be the main antiapoptotic Bcl-2 family protein with functional significance in platelets. This simplicity may explain why Bid and Bim deficiency failed to ameliorate the phenotype of Bcl-xL knock-out in platelets in contrast to other scenarios in which Bid or Bim is apparently indispensable (19–21). Fatal polycystic kidney disease and lymphopenia observed in Bcl-2 knock-out mice are ameliorated in a Bim knock-out background (19). In this case, lymphocytes and other cell lineages may possess Bcl-2 and other antiapoptotic Bcl-2 proteins such as Mcl-1 (45). Hepatocyte apoptosis observed in hepatocyte-specific knock-out of Mcl-1 or Bcl-xL is ameliorated in a Bid knock-out background (20, 21). In this case, hepatocytes clearly have two critical antiapoptotic Bcl-2 family proteins, Bcl-xL and Mcl-1, and Bid may switch binding partners from one to the other in the case of deficiency of either protein. Bid and Bim could regulate the rheostat balance between antiapoptotic and proapoptotic Bcl-2 family proteins, which may become irrelevant if none of the antiapoptotic Bcl-2 family proteins are present.

Although among the BH3-only proteins Bid and Bim are recognized as the putative direct activators, Puma, one of the other BH3-only proteins, has been reported to have the ability to interact directly with effector proteins (13). However, a recent report has pointed out that Puma is a sensitizer protein, which indirectly activates Bak or Bax (46). Hence, its actual mechanism of action in apoptosis remains obscure and disputed. Importantly, in contrast to thymocyte tissue, a Western blot did not show a detectable amount of Puma protein in platelets (Fig. 5*A*), indicating that it might not be involved in the platelet apoptosis machinery. However, we could not exclude the possibility that other proteins may function as alternative direct acti-

vators in the absence of Bid and Bim, leading to Bax activation and mitochondrial apoptosis in platelets upon inactivation of antiapoptotic Bcl-2 family proteins.

In stored platelets, because of the lack of *de novo* protein synthesis, each protein may gradually decrease in relation to its half-life. Our current results showed that the decline of Bcl-xL and Bax protein was much faster than that of Bak protein, and the disruption of the balance between anti- and proapoptotic multidomain Bcl-2 proteins seemed to be associated with apoptosis in stored human platelets. In fact, upon storage, caspase activation was weakened in Bak/Bax-deficient or Bcl-xL-overexpressing platelets compared with wild-type platelets. Taken together with these findings, the balance between anti- and proapoptotic multidomain Bcl-2 family proteins seems to dictate the cellular fate of the life and death of stored platelets. Similar degradation of the Bcl-2 family proteins should occur in circulation, which may explain why Bak knock-out mice displayed mild thrombocytosis *in vivo* (Fig. 1*A*). On the other hand, spontaneous apoptosis occurred in stored platelets despite the absence of activator proteins Bid and Bim. Although in most physiological contexts cellular death is an active decision made by regulating BH3-only proteins, our present findings suggest that activator proteins Bid and Bim were dispensable for Bak/Bax-dependent spontaneous apoptosis in stored platelets.

How anti- and proapoptotic Bcl-2 family proteins interact to maintain cellular integrity and to command cellular survival and death is one of the most important issues that remain to be clearly determined. Although their networks seem to vary depending on the cellular context, our present findings provide an *in vivo* example indicating that the absence of antiapoptotic Bcl-2-like proteins can induce activation of the effector protein Bax, leading to apoptosis without the involvement of the activator proteins Bid and Bim.

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B型肝炎

B型肝炎の抗ウイルス療法の実際

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はじめに

わが国のB型肝炎の抗ウイルス療法は1986年にインターフェロン(IFN)βが保険適応となった以降はインターフェロン治療が主役であった．2000年にラミブジンが保険適応になってからは副作用の少ない経口薬であるという側面もあり，核酸アナログ製剤が抗ウイルス療法の中心となっている．当面の課題は作用機序の異なる二つの薬剤をいかに有効に用いて治療効果を向上させるかである．本稿ではわが国のB型肝炎の抗ウイルス療法の実際について解説する．

B型肝炎に対する抗ウイルス療法の適応と治療目標

B型慢性肝炎の治療目標はB型肝炎ウイルス(HBV)の持続的な増殖抑制であり，これが達成されると肝炎の鎮静化，肝予備能の改善が得られ，さらには肝発癌の抑制も期待される．

2009年度の厚生労働省班会議の「肝硬変を含めたウイルス性肝疾患の治療の標準化に関する研究」の結果を基にした治療ガイドラインでは，抗ウイルス療法の対象をALT 31 IU/l以上としている．治療目標は最終的にはHBs抗原陰性化を目指し，35歳未満ではdrug freeを念頭にIFN単独治療あるいは核酸アナログ・IFNのsequential療法を，35歳以上ではHBV-DNAの持続的陰性化およびALT値の持続正常化を目指して核酸アナログ製剤長期投与を推奨している．治療導入のタイミングとしてはF2以上の組織進展例においては可及的速やかに抗ウイルス療法開始が望まれる．肝組織が進展していない若年者においては，短期間の肝炎期を経て，HBe抗原・抗体のセロコンバーション(SC)の後，肝炎の鎮静化が得られる場合も多いため，経過観察した上で，抗ウイルス療法導入の可否を決定するべきである．またB型肝炎ではHBV genotypeにより自然予後が異なることが明らかになっている，すなわちわが国のB型肝炎の大部分を占めるgenotype BとCではgenotype Bで自然経過中にSCが起こり肝炎も鎮静化しやすく，また肝発癌も少ないことがわかっている．このため経過観察もHBV genotypeを意識して行うべきである．

インターフェロン療法の実際

1986年にIFN-β，1988年にIFN-αがHBe抗原陽性，DNAポリメラーゼ陽性のB型慢性肝炎に対して4週間の短期の投与期間で保険適応となったため，海外ではわが国とは遺伝子型，感染様式は異なるが約25～40%のHBe抗原・抗体のSC率に対して，わが国ではSC率は約10%に過ぎなかった．その後2002年から24週間の投与が保険適応となり，わが国でも24週間の長期投与が可能となりSC率も向上した．B型慢性肝炎に対するIFNの治療効果は人種差，水平感染と垂直感染の差，HBV genotypeによる差があり，海外のデータをそのままわが国のB型慢性肝炎症例に当てはめるのは困難である．わが国でのB型慢性肝炎に対するIFN長期投与のまとまった成績は少なく，治療効果の判定基準も定まっていないが，若年者，IFN開始時のHBV-DNA量低値，ALT高値例，genotype AまたはBに有効例が多いと報告されている．ガイドラインでもgenotype A，Bは，35歳以上でもIFNの効果が高率であることから，可能なかぎりIFNを第一選択にすることが望ましいとされている．

IFN長期投与の肝発癌を含めた長期的な有用性についての報告は少なく，今後の明らかにされて行くべき課題である．

HBe抗原陰性例に対するIFN治療効果は，IFN投与中の抗ウイルス効果，肝炎鎮静化は良

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B型肝炎の治療目標はHBVの持続的な増殖抑制である.
35歳未満のB型肝炎ではIFN治療が第一選択であり，長期投与が望ましい.
核酸アナログ製剤は副作用が軽微で，抗HBV効果は強力であるが，
長期投与が必要である.

好であるが，問題は治療後の肝炎再燃例が非常に多いことである．保険適応の枠を越えた長期投与が必要となる場合もある．

わが国ではまだ未承認であるが欧米からPEG-IFNα-2aの単独あるいはラミブジンとの併用の成績が報告されており，ラミブジン単独に優る効果が示されている[1]．わが国でもIFN-αを対照とした臨床試験が行われており，国内での成績が今後明らかにされる．

IFNは治療中にSCが得られれば，中止後も治療効果が持続する症例が多いが，HBe抗原陽性でHBV-DNA量の多い(＞7 log copies/ml)症例ではe抗原の陰性化やHBV-DNAの十分な低下が得られる確率が低く，これらの症例に対しては若年者であっても核酸アナログ＋sequential療法[2]を検討する必要があると思われる．

核酸アナログ治療の実際

2000年11月のゼフィックス®(ラミブジン)の承認以来，核酸アナログ製剤がB型慢性肝炎治療に広く用いられるようになっている．核酸アナログはDNA polymeraseの天然の基質と競合し酵素活性を阻害するという共通の作用を持っている．わが国ではラミブジンに加えアデホビル，エンテカビルが使用可能となっている．核酸アナログ製剤はIFNと異なり内服製剤であり，副作用が非常に軽微であり，抗ウイルス効果が非常に強力であるという特徴を有している．一方，核酸アナログ製剤の共通の問題点として① 耐性ウイルスの出現，② 投与中止後の肝炎の再燃，③ 胎児への安全性が確立されていない点があげられる．

1. 核酸アナログ治療の経過

核酸アナログ治療は一般的に長期間の治療となる．内服治療開始後，通常は速やかにHBV増殖が抑制され血中のHBV-DNAは減少する．抗ウイルス効果が順調に発揮されれば，やがてHBe抗原・抗体のSCが起こる．この段階で症例によっては核酸アナログ投与の中止が考慮される．さらに投与を継続することにより一部の症例ではHBs抗原の陰性化，さらにはHBs抗体が出現しウイルス学的治癒に至る(図1)．しかしながらHBs抗原陰性化まで至る症例は非常に少数であり，当科の3年以上核酸アナログ投与を投与した190例中5例にすぎない．

現在，国内では3種類の核酸アナログ製剤が使用可能であるが，耐性株の出現頻度がラミブジンでは1年17%，2年42%，3年53%，アデホビルでは1年0%，2年3%，3年11%であるのに対して，エンテカビルでは1年0%，2年0%，3年3.3%であり，ガイドラインではエンテカビルを第一選択の薬剤としている．エンテカビルのHBV増殖抑制作用は強力で当科のデータでは1年で約90%，2年，3年で約95%の症例で血中HBV-DNAが陰性化または2.1 log copies/ml未満(TaqMan HBV)に低下し，大部分の症例でALTの正常化も得られている(図2)．しかし少数ではあるがHBV-DNAの減衰は認めるが2.1 log copies/ml未満まで低下しない症例も存在し，これらの症例では将来的に耐性株が出現する可能性も考えられる．

2. 核酸アナログの投与中止

当初ラミブジンが主に使用されていた時期から核酸アナログ長期投与による抵抗株の出現問題，さらに若年症例のdrug freeの希望などにより，一定の治療効果のもと(HBV-DNA陰性かつHBe抗原陰性)に核酸アナログ製剤の中止が試みられている．しかしながら当科の成績でもラミブジン投与中止1年後には約70%の症例でHBV再増殖に伴い肝炎が再燃しており，核酸アナログの投与中止時期の設定は困難であった．HBV-DNA

核酸アナログ治療ではHBV-DNAの抑制，ついでHBe抗原・抗体のセロコンバージョン，さらに長期の投与でHBs抗原陰性化が治療目標となる．第一選択の核酸アナログであるエンテカビルでは2～3年の投与で，約95%の症例でHBV-DNAの陰性化が得られる．

図1　核酸アナログ治療の長期経過

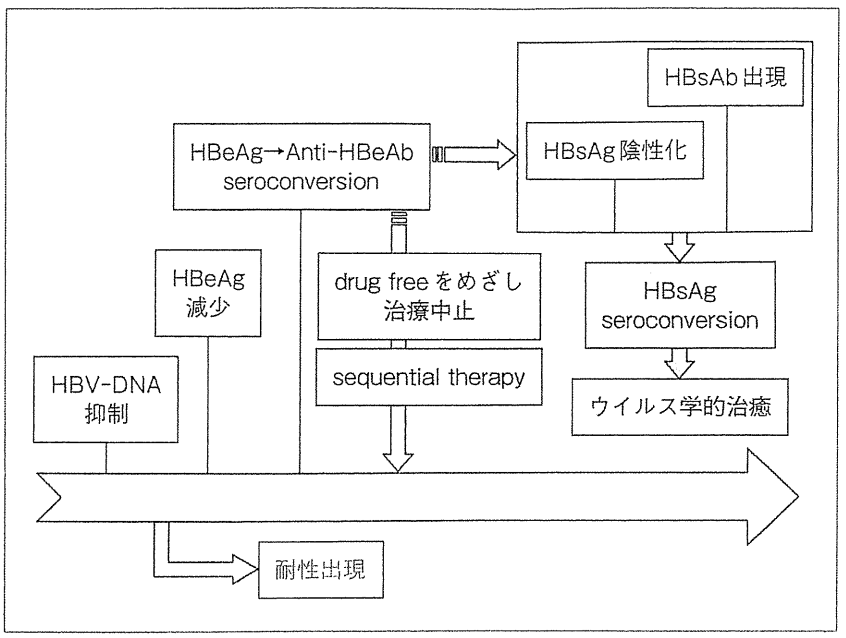
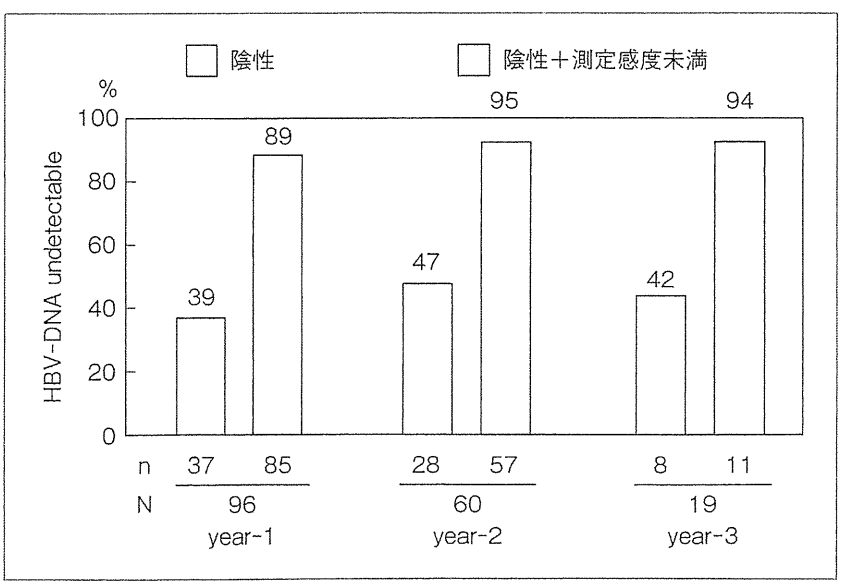


図2　HBV-DNA陰性化率(ETV)



とHBe抗原・抗体のみでは核酸アナログ投与中止時期決定のマーカーとしては十分ではなく，新たなマーカーとしてHBVコア関連抗原，HBs抗原が注目されている．核酸アナログ投与前はHBV-DNA，コア関連抗原，HBs抗原量は良好な相関を示すが，核酸アナログ投与後は減衰が乖離する(図3)．HBV-DNAは主に血液中のHBV量を反映し，コア関連抗原とHBs抗原は肝細胞中に存在するHBV量を反映していると考えられている．現在，厚生労働省の研究班ではこれらの

エンテカビル(naïve 例)では耐性ウイルスの出現はきわめて低率である．
核酸アナログ中止後の再燃を抑える目的で sequential therapy の効果が期待されている．

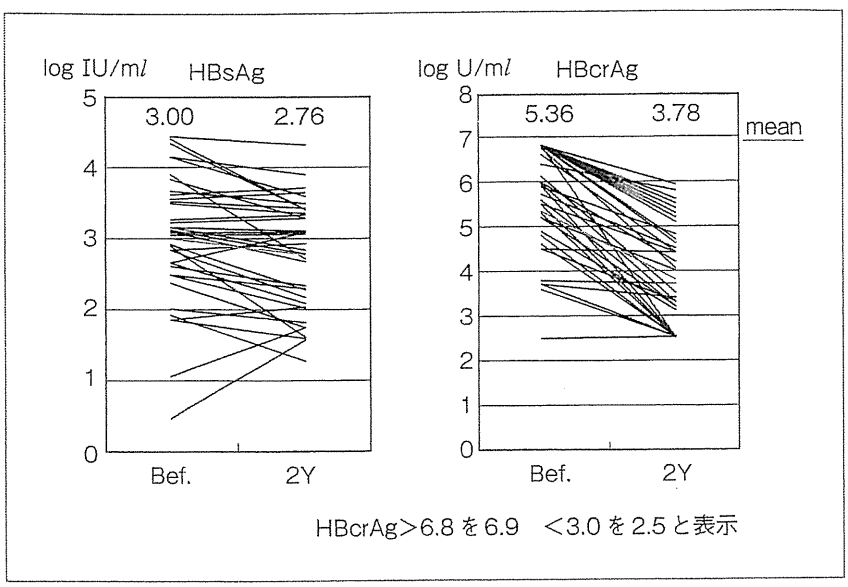


図3 ETV 投与2年後の HBsAg と HBcrAg の変化

マーカーを用いて核酸アナログ薬投与中止基準を策定中である．

3. 耐性ウイルス出現

エンテカビルが核酸アナログの第一選択薬となり，耐性株 HBV の出現による HBV 再増殖，さらに肝炎の再燃は激減した．しかしながら既存のラミブジン耐性ウイルス症例に対する対応が必要となっている．エンテカビルは rt180，204 のラミブジン変異に加えて rt184，202，250 のエンテカビルに特異的な変異が起こらなければ耐性ウイルスとはならず高い genetic barrier を有するが，ラミブジン耐性ウイルスではエンテカビルに特異的な変異が出現するだけでエンテカビル耐性(3年 36%)が出現する[3]．このためラミブジン耐性例に対してはラミブジン＋アデホビル併用療法が基本となる．ラミブジン＋アデホビル併用療法では HBV-DNA 陰性化は3年で 80% 程度にとどまり3年以降ではアデホビル耐性ウイルスの出現も認められる．当科ではこれらの症例に対してエンテカビル＋アデホビル併用療法を施行している(図4)．1年で平均約 1 log copies/ml の HBV-DNA 減衰を認めたが，効果不良な症例もありテノホビルなどの投与も検討する必要がある．今後は多剤耐性が疑われる症例に対しては耐性プロファイルを確認した上での薬剤選択が必要となる．

4. sequential 療法

Serfaty らが核酸アナログ療法に連続して IFN を投与後に治療を終了する sequential 療法の良好な効果を報告して以来，わが国でも同様の治療が行われているが核酸アナログ単独中止の成績を凌駕する成績は報告されていない．今年度のガイドラインでは，sequential 療法を行う場合は，核酸アナログ治療で HBe 抗原が陰性化(または陰性)症例で核酸アナログを十分投与し，HBV-DNA の陰性化期間が1年以上経過し，コア関連抗原も 4.0 logU/ml 以下の症例に行うのが望ましいと明記された．今後はガイドラインに沿った均一な集団でのわが国の sequential 療法の評価が望まれる．

5. pitfall

アデホビル投与時には定期的に腎機能(血清

アデホビル投与中は定期的に腎機能チェックが必要であり，
悪化例ではアデホビルの減量が必要である．

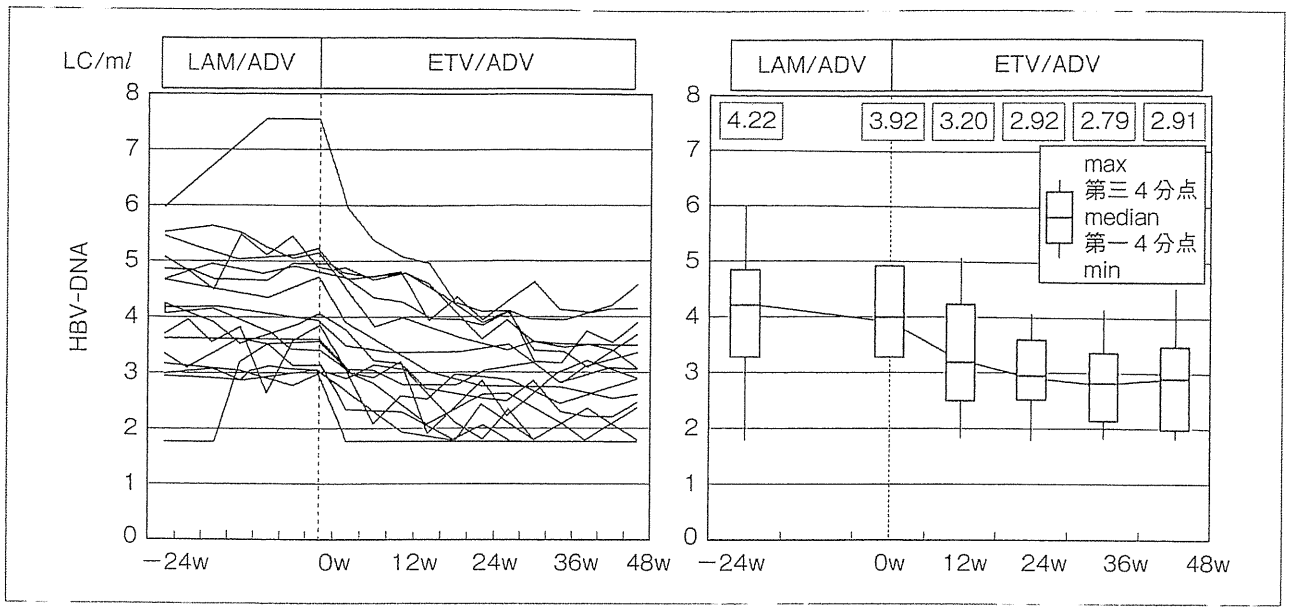


図４　LAM/ADV → ETV/ADV switch 例の HBV-DNA の推移

Cr，eGFR）の測定が必要である．腎機能低下例に対してはアデホビルを隔日投与，あるいは２日おきの投与とする．当科のアデホビル減量投与例では HBV-DNA の増加はほとんど認めていない．

HBV/HIV 重感染例は，エンテカビルの使用により HIV 耐性ウイルスが出現する可能性があるためエンテカビルは原則として使用すべきでない．したがってエンテカビル開始前にインフォームドコンセントを取得した上で HIV 抗体の測定を行うことが望ましい．

B 型肝硬変の抗ウイルス療法

肝硬変（代償性・非代償性）症例に対しては核酸アナログを投与する．ガイドラインでは適応 HBV-DNA 量 3 log copies/ml 以上と低い設定になっている．肝予備能の改善，肝発癌抑制が期待される．高度に肝予備能が低下した症例では回復が不良の症例もあり，可能な限り早期の導入が望ましい．

おわりに

B 型肝炎の自然経過には個人差が大きく，抗ウイルス療法導入後の HBV マーカーの変動も均一ではない．このため B 型肝炎の抗ウイルス療法に際しては個々の症例の治療適応と治療方法を十分吟味した上で治療にあたる必要がある．

文　献

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特集II　B型肝炎に対する新治療戦略

Entecavirによる抗ウイルス療法*

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Key Words：lamivudine, adefovir, entecavir, hepatitis B virus, resistance mutation

はじめに

Lamivudine(LAM)，adefovir dipivoxil(ADV)に続く3番目の核酸アナログ製剤として2006年に承認されたentecavir(ETV)は，既存の核酸アナログ製剤と比較して高い抗ウイルス効果を示し，さらには，耐性変異株の出現がきわめて低率であること[1)~3)]，また，ADVのような腎機能障害[4)5)]を認めないことから，現在，本邦の核酸アナログ製剤の中では第一選択薬剤となった．2011年度のB型慢性肝炎の治療ガイドラインにおいても，35歳以上の中高年に対しては第一選択薬剤とされた．

一方で，中止を考慮する際，肝炎再燃のリスクの予測が困難であること，生殖年齢に対する，特に挙児希望例に対する投与は実臨床では困難であること，ならびに低率であるがETV耐性が出現してしまった症例に対する十分な抗ウイルス活性を有する薬剤が本邦では存在しないこと，human immunodeficiency virus(HIV)合併例では禁忌であることなどが問題点としてあげられる．

本稿では当院におけるETVの抗ウイルス効果，hepatitis B virus(HBV)関連マーカーの推移，肝予備能改善効果，耐性出現率について基説する．

対象と方法

2011年4月までに当院においてETVを初回投与した314例中，少なくとも1年以上経過を追えた204例を対象とした．自己免疫性肝炎，アルコール性肝障害，うっ血性肝障害の併発例，C型肝炎ウイルスあるいはHIV合併例，HBV再活性化予防目的のETV投与例，過去に核酸アナログ製剤投与を受けた症例は除外した．

204例の背景因子の内訳を表1に示す．患者因子では年齢中央値は56歳，治療期間中央値31か月，男性137例(67.2%)，肝硬変例63例(30.1%)，interferon(IFN)投与歴あり20例(9.8%)，hepato-

表1　患者背景因子

年齢，歳	56(23～82)	HBeAg陽性	69(33.8%)
治療期間，月	31(13～55)	HBsAg, log IU/ml	3.2(−0.5～5.8)
性，男性	137(67.2%)	crAg, log U/ml	5.4(3.0～6.8)
肝病変，肝硬変	63(30.1%)	HBV DNA, log copies/ml	6.7(2.1～9.0)
IFN治療歴，あり	20(9.8%)	ALT, IU/l	77(17～1830)
HCC治療，あり	52(25.5%)	Genotype, A/B/C/H/ND	5/28/151/1/19

* Antiviral effect of entecavir.

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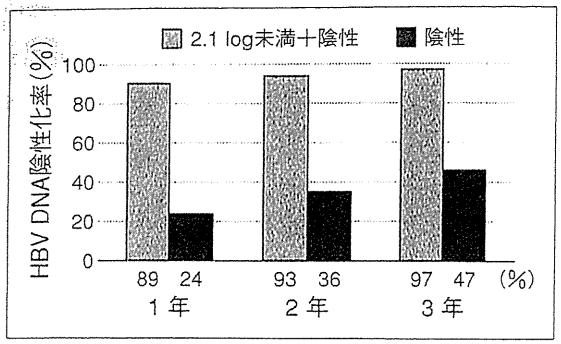


図1　HBV DNA陰性化率
TaqMan PCR法によるHBV DNA陰性化率の推移を示す．2.1 log未満と陰性例を加えた症例の割合は1年で89%，2年で93%，3年で97%，陰性例だけを抽出すると1年24%，2年36%，3年47%であった．

cellular carcinoma(HCC)治療歴あり52例(25.5%)であった．ウイルス側因子ではHBe抗原陽性69例(33.8%)，HBs抗原中央値3.2 logIU/m*l*(以下単位略)，コア関連抗原中央値5.4 logU/m*l*(以下単位略)，HBV DNA中央値 6.7 logcopies/m*l*(以下単位略)，ALT中央値77 IU/*l*，遺伝子型A型，B型，C型，H型がそれぞれ5例(2.7%)，28例(15.1%)，151例(81.6%)，1例(0.5%)であった．

HBV DNAはTaqMan PCR法(Roche Diagnostics, Tokyo, Japan)，遺伝子型はPCR-Invader法(BML, Tokyo, Japan)，HBs抗原はCLIA法，コア関連抗原はCLEIA法，耐性部位の検討はINNO-LiPA HBV DR version 2, version 3(Innogenetics Gent, Belgium)を用いた．なお，HBs抗原量は実数から対数に変換し検討を行った．

検討項目として①HBV DNA陰性化率，②ALT異常例の検討，③HBs抗原とコア関連抗原の推移，④肝予備能改善効果，⑤ETV耐性出現率を検討した．③の検討ではETV3年以上投与可能であった68例，④の検討ではETV3年以上投与可能で，かつ，HBs抗原とコア関連抗原が経時的に測定できた61例を対象とした．

抗ウイルス効果の検討

1．HBV DNA陰性化率

TaqMan PCR法にて測定した1年，2年，3年の3 pointsの成績を示す．陰性化率は2.1 log未満と陰性例を合わせると，1年89%，2年93%，3年97%，陰性例のみに限ると1年24%，2年36%，3年47%を示した(図1)．HBV DNA 2.6 log未満を陰性と定義した時の当院のLAM 1年以上投与例(187例)のHBV DNA陰性化率が1年64%，2年57%，3年44%であった．LAM投与時では，耐性株出現のためHBV DNA陰性化率は経年的に低くなるが，LAMと比較してもETVの優れた抗ウイルス効果は明らかである．

ETV投与2年時においてHBV DNAがTaqMan PCR法で陰性あるいは2.1未満を示した症例を陰性と定義すると，HBV DNA陰性例は127例，2.1 log以上のHBV DNA陽性例は13例であった．13例のうち，多剤耐性出現1例と明らかにコンプライアンスが不良であった1例を除く11例で解

表2　HBV DNA陽性例と陰性例の背景因子の比較

	陰性 n＝127	陽性 n＝11	*P*
年齢	55(23～78)	44(28～72)	0.089
男性	85(69)	8(73)	0.954
肝硬変	41(33)	3(27)	0.996
e抗原陽性	43(35)	5(46)	0.439
ALT	83(17～1478)	68(33～294)	0.877
HBV DNA	6.6(2.6～8.8)	7.6(4.6～8.8)	0.028
HBsAg	3.2(0.5～5.8)	3.9(1.2～5.0)	0.068
crAg	5.5(3.0～6.8)	5.7(3.8～6.8)	0.743
Genotype C	92/116(79)	7/10(70)	0.774

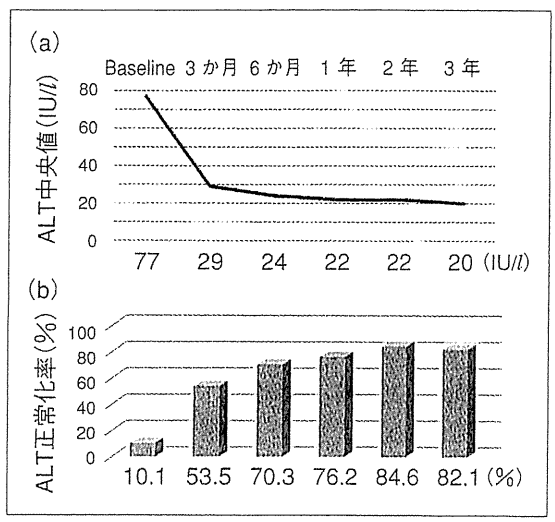


図2　ALT正常化率
(a)ALT中央値は治療開始時，3か月後，6か月後，1年後，2年後，3年後で，それぞれ，77 IU/*l*，29 IU/*l*，24 IU/*l*，22 IU/*l*，22 IU/*l*，20 IU/*l*であった．(b)ALT30 IU/*l*以下を正常として正常化率を算出した．治療開始時，3か月後，6か月後，1年後，2年後，3年後で，それぞれ，10.1%，53.5%，70.3%，76.2%，84.6%，82.1%であった．

表 3-a ALT正常例と異常例の比較

	正常 n＝111	異常 n＝29	*P*
年齢	55(26～78)	56(23～73)	0.902
男性	77(69)	20(69)	0.967
肝硬変	35(32)	10(35)	0.762
HCC合併あり	23(21)	12(41)	0.022
BMI 25以上	24(22)	16(55)	0.009
高血圧	21(19)	7(24)	0.532
糖尿病	19(17)	5(17)	0.987
高脂血症	19(17)	9(31)	0.095
脂肪肝	14(13)	6(21)	0.267
ALT	83(17～1487)	73(18～774)	0.765
HBV DNA	6.7(2.1～8.8)	6.6(2.6～8.8)	0.339
HBsAg	3.2(0.5～5.8)	3.2(0.9～5.0)	0.909
crAg	5.7(3.0～6.8)	5.2(3.0～6.8)	0.359
Genotype C	81(76)	23(96)	0.054
e抗原陽性	40(36)	9(31)	0.615
ETV 2年時DNA≧2.1	6(5)	4(14)	0.247

表 3-b ALT異常に寄与する因子ー多変量解析ー

因子	カテゴリー	オッズ比(95%信頼区間)	*P*値
HCC合併	1. No 2. Yes	1 2.617(1.047～6.538)	0.040
BMI	1. 25未満 2. 25以上	1 4.411(1.830～10.635)	0.001

析を行った．HBV DNA陽性例は陰性例と比較するとbaselineのHBV DNA量が有意に高値を示した(表 2)．

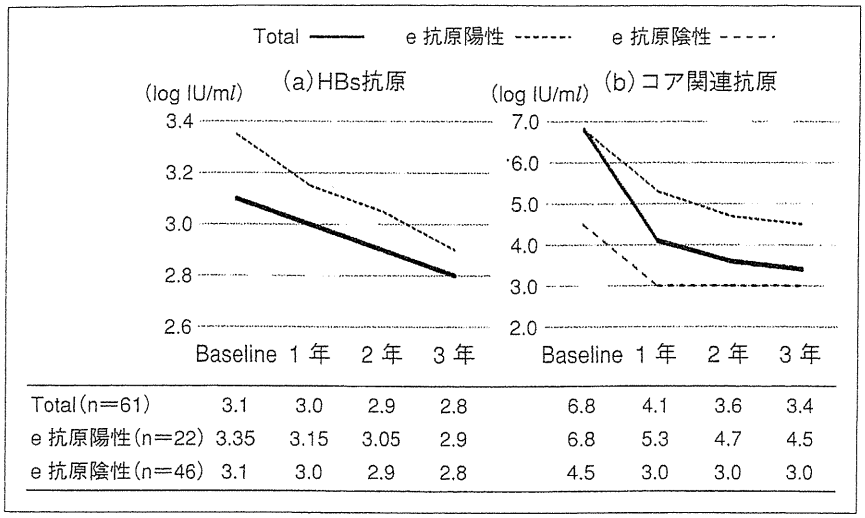
2．ALTの推移と陰性化率

ALT中央値は治療開始時，3か月後，6か月後，1年後，2年後，3年後で，それぞれ，77 IU/*l*，29 IU/*l*，24 IU/*l*，22 IU/*l*，22 IU/*l*，20 IU/*l*であった．ALT30 IU/*l*以下を正常として正常化率を算出すると，それぞれ，10.1%，53.5%，70.3%，76.2%，84.6%，82.1%であった(図 2)．ETV投与 2 年の時点におけるALT異常例と正常例を背景因子にETV投与 2 年時のHBV DNA量の治療後因子を加えて比較すると，HCC合併例とBMI 25以上の割合が異常例で有意に高率を示した(表 3-a)．多変量解析にてHCC合併はオッズ比2.6倍，BMI 25以上はオッズ比4.4倍でALT異常に寄与する因子として抽出された(表 3-b)．すなわち，ETV投与を行い，良好なウイルスコントロールが可能であってもALTが異常を示す症例では，HCCの治療中の症例，あるいは肥満を有する症例の可能性が高いと考えられた．

3．HBs抗原とコア関連抗原の推移

ETVは前述のように投与数年後にはほとんどの症例で血中のHBV DNAが陰性化するので，肝細胞内に残存するHBVウイルス(ccc DNA)量と乖離する現象が生じる．ETV投与例における抗ウイルス効果の類推やその症例の予後を推測する上で，また，ETV投与中止を判断する指標としてもHBs抗原量とコア関連抗原が期待されているが，これらの検査値の臨床的意義は不明な点も多い．

3年以上ETVを投与した68例中，HBs抗原とコア関連抗原の経時的推移を観察できた61例(e抗原陽性22例，陰性39例)を対象とした．HBs抗原はTotal61例ではbaseline 3.1 log，1 年3.0 log，2 年2.9 log，3 年2.8 logと緩徐に低下した．e抗原陽性例ではbaseline 3.35 log，1 年3.15 log，2 年3.05 log，3 年2.9 log，e抗原陰性例はbaseline 3.1 log，1 年3.0 log，2 年2.9 log，3 年2.8 logであった．e抗原の有無にかかわらず，HBs抗原の減衰は緩徐であった．一方で，コア関連抗原はbaseline 6.8 log，1 年4.1 log，2 年3.6 log，3 年3.4 log，e



	(a) HBs抗原				(b) コア関連抗原			
	Baseline	1年	2年	3年	Baseline	1年	2年	3年
Total (n=61)	3.1	3.0	2.9	2.8	6.8	4.1	3.6	3.4
e 抗原陽性 (n=22)	3.35	3.15	3.05	2.9	6.8	5.3	4.7	4.5
e 抗原陰性 (n=46)	3.1	3.0	2.9	2.8	4.5	3.0	3.0	3.0

図3　HBs抗原とコア関連抗原の推移

(a) HBs抗原はbaseline 3.1 log, 1年3.0 log, 2年2.9 log, 3年2.8 logと緩徐に低下した．e抗原の有無別の推移では陽性例ではbaseline 3.35 log, 1年3.15 log, 2年3.05 log, 3年2.9 log, 陰性例はbaseline 3.1 log, 1年3.0 log, 2年2.9 log, 3年2.8 logであった(Totalとe抗原陰性は同じ値を示した)．(b) コア関連抗原はbaseline 6.8 log, 1年4.1 log, 2年3.6 log, 3年3.4 log, e抗原陽性例では同様に6.8 log, 5.3 log, 4.7 log, 4.5 log, e抗原陰性例では4.5 log, 3.0 log, 3.0 log, 3.0 logを示した．

アルブミン

	開始	1年	2年	3年
Total	3.9	4.1	4.3	4.2
慢性肝炎	3.9	4.1	4.3	4.3
肝硬変	3.9	3.9	4.2	4.0

血小板

	開始	1年	2年	3年
Total	14.8	16.3	15.0	14.8
慢性肝炎	15.8	17.1	16.5	15.8
肝硬変	10.1	9.2	9.3	8.5

プロトロンビン時間

	開始	1年	2年	3年
Total	86	97	102	105
慢性肝炎	90	99	103	107
肝硬変	71	86	87	88

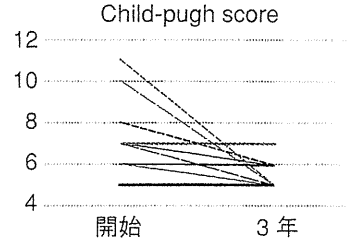


図4　肝予備能改善効果

アルブミンとプロトロンビン時間は背景肝病変が慢性肝炎，肝硬変にかかわらず投与3年の経過で改善を，血小板数はプラトーを示した．肝硬変例のChild-pugh scoreの推移をみると，8例でscore変化なし，9例でscoreの改善が得られた．

抗原陽性例では同様に6.8 log, 5.3 log, 4.7 log, 4.5 log, e抗原陰性例では4.5 log, 3.0 log, 3.0 log, 3.0 logであった．コア関連抗原はbaselineの時点でe抗原陽性例と陰性例ですでに2 log以上の乖離が認められた．また，e抗原陰性例では測定限界値である3 logを示す症例が多くなった．コア関連抗原の推移をみる場合は，e抗原陽性例と陰性例を分けて検討する必要があると考えられる(図3)．

4．肝予備能改善効果

3年以上ETVを投与できた68例(慢性肝炎51例，肝硬変17例)で，アルブミン，血小板，プロトロンビン時間の経時的推移を算出し，さらに肝硬変17例に対してChild-pugh scoreをETV開始時と

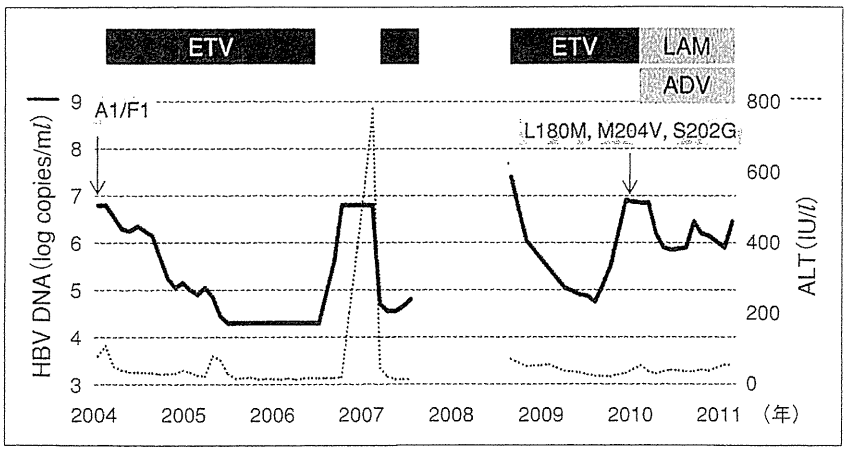


図5　ETV耐性が出現した1例
観察期間中央値31か月の経過でETV初回投与204例中1例(0.5%)でETV耐性(L180M, M204V, S202G)が出現した.

投与3年時で比較した.

アルブミンとプロトロンビン時間は背景肝病変が慢性肝炎，肝硬変にかかわらず投与3年の経過で改善を示した．一方，血小板は慢性肝炎ではプラトー，肝硬変ではわずかに低下した．肝硬変例のChild-pugh scoreの推移では，8例でscore変化なし，9例でscoreの低下をきたした(図4)．このようにETV投与例では明らかに肝予備能の改善が得られた．

5．ETV耐性出現率

観察期間中央値31か月の経過で204例中1例(0.5%)でETVのS202Gの変異が出現した(図5)．この症例は38歳男性，e抗原陽性で，遺伝子型はC型であった．2004年6月にETVの容量比較試験に参加し，ETVが開始された．なお，試験参加直前の肝生検では新犬山分類でA1F1であった．2006年9月試験終了に伴い，ETVが中止されるも，肝炎の再燃のため，2007年4月ETV再開．同年10月より受診されず，2008年10月に約1年の期間を空けて来院．HBV DNA 8.8 log, ALT70 IU/lにてETV再開も，2010年1月にviral breakthroughをきたし，前述のETV耐性が確認された．2010年4月からlamivudineとadefovir併用を行うも，HBV DNAは6log前後で推移している．この症例は2回にわたりETVを中断したという特殊な経緯を有していた．この1例を除くと，現在，ETV初回投与例からのETV耐性例は出現していない．

おわりに

ETVは抗ウイルス作用が強力で耐性出現はきわめて稀であり，初回治療例では第一選択の薬剤である．今後は長期投与による肝発癌抑止率を明らかにするとともに，HBs抗原消失すなわち臨床的治癒例の詳細な検討が必要である．

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BRIEF REPORT

Effects of Hepatitis B Virus Infection on the Interferon Response in Immunodeficient Human Hepatocyte Chimeric Mice

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Complementary DNA microarray analysis of human livers cannot exclude the influence of the immunological response. In this study, complementary DNA microarray analysis was performed under immunodeficient conditions with human hepatocyte chimeric mice, and gene expression profiles were analyzed by hepatitis B virus (HBV) infection and/or interferon treatment. The expression levels of 183 of 525 genes upregulated by interferon treatment were significantly suppressed in response to HBV infection. Suppressed genes were statistically significantly associated with the interferon signaling pathway and pattern recognition receptors in the bacteria/virus recognition pathway ($P = 1.0 \times 10^{-8}$ and $P = 1.2 \times 10^{-8}$, respectively). HBV infection attenuated virus recognition and interferon response in hepatocytes, which facilitated HBV escape from innate immunity.

Chronic hepatitis B virus (HBV) infection is associated with the development of virus-related liver diseases, including chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. Interferon α (IFN-α) has been used for the treatment of chronic hepatitis B, and many large clinical trials and meta-analyses have demonstrated the effectiveness of interferon [1–3]. However, the effect of IFN-α therapy is unsatisfactory, and the molecular basis for tolerance to IFN-α is not clearly defined.

DNA microarray technology has enabled genome-wide analysis of gene transcript levels with the use of clinical tissues and animal models, which has yielded insights into the molecular features of several liver diseases [4–6]. However, it has been difficult to determine whether the changes in gene expression were caused by viral interference or by the human immune response, because all of these studies that used clinical and experimental samples were analyzed under the influence of adaptive immune responses. Recently, Mercer and colleagues developed a human hepatocyte chimeric mouse model [7]. These mice were derived from severe combined immunodeficiency (SCID) mice, which are severely immunocompromised, and the mouse liver cells were extensively replaced with human hepatocytes [7, 8]. With the use of this chimeric mouse model, in which HBV can continuously infect human hepatocytes, the effect of drugs and the response of viral infection can be analyzed in human hepatocytes under immunodeficient conditions [9]. In this study, we performed microarray analysis with human hepatocyte chimeric mouse livers to assess the direct impacts of HBV infection and IFN treatments on gene expression profiles. We successfully demonstrated that HBV infection attenuated the expression of IFN-stimulating genes under immunodeficient conditions, which suggests that HBV proteins might afford escape mechanisms from cellular innate immunity.

METHODS

A serum sample was obtained from a HBV carrier after obtaining written informed consent for the donation and evaluation of the blood sample. The inoculum was positive for Hepatitis B surface and Hepatitis B e antigens, with slightly elevated levels of serum alanine aminotransferase and high-level viremia (HBV DNA load, 7.1 log copies/mL). The studied patient was infected with HBV genotype C. The experimental protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the Hiroshima University Hospital ethical committee (approval ID: D08-9).

The $uPA^{+/+}/SCID^{+/+}$ mice were prepared and the human hepatocytes were transplanted as described elsewhere [8]. The experiments were performed in accordance with the guidelines of the local committee for animal experiments at Hiroshima University.

Sixteen chimeric mice, in which >90% of the liver tissue was replaced with human hepatocytes, were divided into

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4 experimental groups. Group A contained 4 mice that were neither infected with HBV nor treated with IFN. Group B consisted of 3 mice that were treated with IFN-α for 6 h (7,000 IU per gram of body weight) just before being humanely killed but were not infected with HBV. Mice in groups C and D were inoculated via the mouse tail vein with human serum containing 6×10^6 copies of HBV. After inoculation, we collected mouse serum samples every 2 weeks and analyzed HBV DNA titers by real-time polymerase chain reaction (PCR) and human albumin levels by means of a human albumin enzyme-linked immunosorbent assay quantitation kit (Bethyl Laboratories), as described elsewhere [9]. Virus and human albumin titer levels are shown in Supplementary data 1. All 9 mice developed measurable viremia 4 weeks after inoculation. Eight weeks after inoculation, 4 of the 9 infected mice (group C) were humanely killed without IFN treatment and the remaining 5 mice (group D) were humanely killed after 6 h of IFN-α treatment (7,000 IU per gram of body weight). The mice were infected, had serum samples extracted, and were killed humanely under ether anesthesia, as described elsewhere [8].

All 16 chimeric mice were killed humanely, and human hepatocytes were finely dissected from the mouse livers and stored in liquid nitrogen after submerging in RNA later solution (Applied Biosystems). Total RNA was extracted with TRIzol reagent (Invitrogen) and labeled with cyanine 3 by use of a low RNA input linear amplification kit (Agilent Technologies) after amplification. Cyanine-3–labeled complimentary RNA was hybridized to a 44-K whole human genome oligo microarray (Agilent). Detailed protocols are described in Supplementary data 2.

Gene expression profiles were analyzed using GeneSpring GX software (version 10.0.2; Tomy Digital Biology). The detailed protocol is described in Supplementary data table 3. Complete linkage hierarchical clustering analysis was applied using Euclidean distance, and differentially expressed genes were annotated using information from the Gene Ontology (GO) Consortium. Global molecular networks and comparisons of canonical pathways were generated using Ingenuity Pathway Analysis (IPA) software (version 8.6; Ingenuity Systems).

Total RNA was extracted from the implanted human hepatocytes in the mouse livers by use of an RNeasy mini kit (Qiagen) and was reverse transcribed. The selected messenger RNA (mRNA) was quantified by real-time PCR using the 7300 real-time PCR system (Applied Biosystems), and the expression of glyceraldehyde-3-phosphate dehydrogenase served as a control. The amplification protocol and primer sequences are described in Supplementary data 4 and 5.

RESULTS

To analyze the direct effects of IFN in human hepatocytes, we compared the gene expression profiles between groups A (mice without IFN treatment) and B (mice with IFN treatment). Of the 1403 genes that remained after screening with the Welch *T* test, 685 genes showed a >3.0-fold change between groups. Of these 685 genes, 525 genes were up-regulated and the other 160 genes down-regulated by IFN. The top 20 IFN-regulated genes are listed in Supplementary data table 6. GO analysis revealed that 8 (40%) of the top 20 genes that were upregulated with IFN treatment were related to immune response.

To analyze the effect of HBV infection in human hepatocytes, we compared the gene expression profiles between groups A (mice without HBV infection) and C (mice with HBV infection). Among the 1,714 genes hat remained after screening, 373 genes showed a >3.0-fold change between groups. Of these 373 genes, 159 genes were up-regulated and the other 214 genes down-regulated by HBV. The top 20 HBV-regulated genes are listed in Supplementary data table 7. Several oncogenic genes such as growth differentiation factor 15 and glial cell derived neurotrophic factor were included in the top group. Most of the top 20 genes that were downregulated with HBV infection were associated with transcriptional regulation.

To examine whether HBV infection may alter the effect of IFN response in human hepatocytes, we compared gene expression profiles among all groups. As mentioned above, 525 genes were upregulated by >3.0-fold by IFN in the absence of HBV infection. A comparison of groups C (mice with HBV infection but no IFN treatment) and D (mice with both HBV infection and IFN treatment) revealed that 183 (34.9%) of the 525 genes showed statistically significantly reduced IFN response with HBV infection ($P < .01$) (Supplementary data 8A). The top 20 genes in which IFN response was significantly changed by HBV infection are shown in Table 1. The mRNA expression levels of 11 selected genes among the 183 genes with reduced IFN response were also analyzed by real-time PCR, and the reductions in IFN response by HBV infection were verified (Supplementary data 8B). Additionally, we used IPA software to analyze the influence of HBV infection on the IFN response of these 183 genes by means of a pathway-oriented approach. Pathway analysis revealed that several pathways were affected by HBV infection (Table 2). The IFN response was statistically significantly attenuated by HBV infection in the pathways related to IFN signaling and pattern recognition of bacteria and viruses ($P = 1.0 \times 10^{-8}$ and $P = 1.2 \times 10^{-8}$, respectively).

DISCUSSION

Elsewhere we have demonstrated a human hepatocyte chimeric mouse model that can be chronically infected with hepatitis B and C viruses [9–11]. This mouse model facilitates analysis of the effect of viral infection and the response to medication under immunodeficient conditions. In this study, we performed complementary DNA microarray analysis using the chimeric mouse model and obtained gene expression profiles to analyze

Table 1. Genes With Interferon Responsiveness Downregulated by Hepatitis B Virus (HBV) Infection

Gene symbol	GenBank accession no.	Function	Fold change in expression level		*P*
			Without HBV infection	With HBV infection	
ENST00000322831	None	Unknown	4.52	−1.45	4.15×10^{-7}
AA593970	AA593970	EST	9.70	1.61	5.58×10^{-7}
THC2533996	None	Unknown	3.74	−2.50	6.97×10^{-7}
LOC388532	None	Unknown	3.11	−2.48	1.61×10^{-6}
ZNF267	NM_003414	Transcription regulator	7.66	1.79	2.30×10^{-6}
ZNF217	NM_006526	Transcription regulator	3.69	1.03	3.62×10^{-6}
CRSP3	NM_015979	Transcription regulator	7.50	−1.02	4.06×10^{-6}
MGC39372	BC025340	Hypothetical protein	30.92	7.03	5.74×10^{-6}
BF972140	BF972140	EST	16.91	4.71	5.78×10^{-6}
LOC731599	XR_015536	Hypothetical protein	3.17	−4.18	8.58×10^{-6}
LOC645676	AK126559	Hypothetical protein	3.76	1.35	9.13×10^{-6}
THC2650457	None	Unknown	78.07	6.28	1.29×10^{-5}
ZNF24	NM_006965	Transcription regulator	3.69	1.36	1.64×10^{-5}
CCDC68	NM_025214	Unknown	5.88	−2.83	1.89×10^{-5}
SP110	NM_004510	Transcription regulator	5.00	10.77	2.00×10^{-5}
FLJ21272	AK024925	Hypothetical protein	14.70	2.49	3.18×10^{-5}
PLEKHF1	NM_024310	Unknown	6.65	1.84	4.70×10^{-5}
AK026418	AK026418	Unknown	9.50	2.58	5.02×10^{-5}
hCG_1790262	XM_001133847	Unknown	3.13	−2.94	6.25×10^{-5}
CEBPD	NM_005195	Transcription regulator	8.16	1.56	7.03×10^{-5}
FLJ20273	NM_019027	RNA binding	3.37	1.11	7.11×10^{-5}

NOTE. *P* values were analyzed by the Welch *T* test. *CEBPD,* CCAAT/enhancer binding protein (C/EBP) delta; *CCDC68,* coiled-coil domain containing 68; *CRSP3,* mediator complex subunit 23 (*MED23*); EST, expressed sequence tag; *FLJ20273,* RNA binding motif protein 47 (*RBM47*); *PLEKHF1,* pleckstrin homology domain containing, family F (with FYVE domain) member 1; *SP110,* SP110 nuclear body protein; *ZNF24,* zinc finger protein 24; *ZNF217,* zinc finger protein 217; *ZNF267,* zinc finger protein 267.

the direct influence of HBV infection and IFN-α treatment on human hepatocytes.

To avoid contamination with mouse tissue, human hepatocyte chimeric mice, in which liver tissue is largely (>90%) replaced by human hepatocytes, were used in the present study. However, a small amount of mouse-derived cells, such as interstitial cells, bile duct cells, and vascular cells, still remain in the chimeric mouse livers. Because of high homology between the human and mouse genomes, the signals from microarray analyses may be influenced by cross-hybridization with mouse mRNA. It is difficult to produce $uPA^{+/+}/SCID^{+/+}$ mice >10 weeks old without hepatocyte transplantation, and a previous study demonstrated that it is feasible to use microarray analysis in a functional genomics analysis of chimeric mice [12]. Therefore, to compensate for the contamination, the mice in group A, which were neither infected with HBV nor treated with IFN, were used as negative controls.

To analyze the effect of IFN treatment, we compared gene expression profiles between groups A (mice without IFN treatment) and B (mice with IFN treatment); 525 genes with >3.0-fold upregulation following IFN treatment were observed. Among them, chemokine (C-X-C motif) ligand 9, chemokine (C-X-C motif) ligand 10, and chemokine (C-X-C motif) ligand 11, which promote T cell adhesion, were remarkably highly induced with IFN treatment (Supplementary data table 6) [13]. These results suggest that the antiviral effects of IFN might involve not only direct activation of IFN-stimulated proteins such as myxovirus resistance protein A and double strand RNA-dependent protein kinase but also activation of immunity via chemokines.

Second, we compared the profiles between groups A (mice without HBV infection) and C (mice with HBV infection). As shown in Supplementary data table 7, more than half (12) of the top 20 genes upregulated by HBV infection localized to the cell membrane or the extracellular region, but 14 (70%) of the 20 downregulated genes localized to the nucleus. In addition, GO analysis demonstrated that genes related to cell cycle and DNA modification were affected by HBV infection. We speculate that HBV infection promotes cell growth and DNA damage in the hepatocyte nucleus and activates the immune response in the cytoplasm. From the clinical standpoint, some healthy HBV carriers develop hepatocellular carcinoma without chronic hepatitis or cirrhosis. The present results strongly support this observation, showing that most of the affected genes are known to be associated with carcinogenesis.

Clinically, HBV is known to develop tolerance to IFN treatment in patients with chronic hepatitis B, although the mechanism is not clear. We analyzed the IFN response with and

Table 2. Pathway Analysis of 183 Interferon-Induced Genes With Interferon Responsiveness Downregulated by Hepatitis B Virus Infection

Canonical pathways	*P*	Genes
Interferon signaling	1.00×10^{-8}	*IFIT3, SOCS1, IFIT1, MX1, IFNGR1, JAK2, STAT1, TAP1, IRF1*
Role of pattern recognition receptors in recognition of bacteria and viruses	1.20×10^{-8}	*IL12A, OAS2, OAS3*(includes EG:4940), *IFIH1, PIK3R3, TLR4, NOD2, TICAM1, DDX58, CASP1, NOD1, TLR3, RIPK2*
Type 1 diabetes mellitus signaling	2.00×10^{-4}	*SOCS1, IL12A, RIPK1, GAD1, SOCS6, SOCS2, IFNGR1, JAK2, STAT1, IRF1*
Prolactin signaling	2.70×10^{-4}	*PIK3R3, SOCS1, SOCS6, SOCS2, NMI, JAK2, STAT1, IRF1*
TREM1 signaling	3.50×10^{-4}	*TLR4, NOD2, ICAM1, CASP1, JAK2, TLR3, CASP5*
Production of nitric oxide and reactive oxygen species in macrophages	3.90×10^{-4}	*PIK3R3, TLR4, RND3, PPP2R2A, PPM1J, RHOU, IFNGR1, MAP3K8, IRF8, JAK2, STAT1, IRF1*
Pathogenesis of multiple sclerosis	1.10×10^{-3}	*CXCL10, CXCL9, CXCL11*
Activation of IRF by cytosolic pattern recognition receptors	2.60×10^{-3}	*IFIH1, RIPK1, DDX58, STAT1, IFIT2, ISG15*
Dendritic cell maturation	2.60×10^{-3}	*B2M, PIK3R3, TLR4, ICAM1, IL12A, IL1RN, IRF8, JAK2, TLR3, STAT1*
Interleukin 12 signaling and production in macrophages	3.60×10^{-3}	*PIK3R3, TLR4, IL12A, IFNGR1, MAP3K8, IRF8, STAT1, IRF1*
Sphingosine-1-phosphate signaling	3.60×10^{-3}	*PIK3R3, S1PR2, RND3, CASP1, RHOU, CASP4, CASP7, CASP5*
JAK-STAT signaling	4.00×10^{-3}	*PIK3R3, SOCS1, SOCS6, SOCS2, JAK2, STAT1*
Growth hormone signaling	4.70×10^{-3}	*PIK3R3, SOCS1, SOCS6, SOCS2, JAK2, STAT1*
Retinoic acid mediated apoptosis signaling	8.50×10^{-3}	*TNFRSF10B, PARP8, TNFSF10, TIPARP, IRF1*

NOTE. *B2M,* beta-2-microglobulin; *CASP1,* caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase); *CASP4,* caspase 4, apoptosis-related cysteine peptidase; *CASP5,* caspase 5, apoptosis-related cysteine peptidase; *CASP7,* caspase 7, apoptosis-related cysteine peptidase; *CXCL9,* chemokine (C-X-C motif) ligand 9; *CXCL10,* chemokine (C-X-C motif) ligand 10; *CXCL11,* chemokine (C-X-C motif) ligand 11; *DDX58,* DEAD (Asp-Glu-Ala-Asp) box polypeptide 58; *GAD1,* glutamate decarboxylase 1 (brain, 67kDa); *ICAM1,* intercellular adhesion molecule 1; *IFIH1,* interferon induced with helicase C domain 1; *IFIT1,* interferon-induced protein with tetratricopeptide repeats 1; *IFIT2,* interferon-induced protein with tetratricopeptide repeats 2; *IFIT3,* interferon-induced protein with tetratricopeptide repeats 3; *IFNGR1,* interferon gamma receptor 1; *IL1RN,* interleukin 1 receptor antagonist; *IL12A,* interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35); IRF, interferon regulatory factor; *IRF1,* interferon regulatory factor 1; *IRF8,* interferon regulatory factor 8; *ISG15,* ISG15 ubiquitin-like modifier; *JAK2,* Janus kinase 2; *MAP3K8,* mitogen-activated protein kinase kinase kinase 8; *MX1,* myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse); *NMI,* N-myc (and STAT) interactor; *NOD1,* nucleotide-binding oligomerization domain containing 1; *NOD2,* nucleotide-binding oligomerization domain containing 2; *OAS2,* 2′-5′-oligoadenylate synthetase 2, 69/71kDa; *OAS3,* 2′-5′-oligoadenylate synthetase 3, 100kDa; *PARP8,* poly (ADP-ribose) polymerase family, member 8; *PIK3R3,* phosphoinositide-3-kinase, regulatory subunit 3 (gamma); *PPM1J,* protein phosphatase, Mg2+/Mn2+ dependent, 1J; *PPP2R2A,* protein phosphatase 2, regulatory subunit B, alpha; *RHOU,* ras homolog gene family, member U; *RIPK1,* receptor (TNFRSF)-interacting serine-threonine kinase 1; *RIPK2,* receptor-interacting serine-threonine kinase 2; *RND3,* Rho family GTPase 3; *S1PR2,* sphingosine-1-phosphate receptor 2; *SOCS1,* suppressor of cytokine signaling 1; *SOCS2,* suppressor of cytokine signaling 2; *SOCS6,* suppressor of cytokine signaling 6; *STAT1,* signal transducer and activator of transcription 1, 91kDa; *TAP1,* transporter 1, ATP-binding cassette, sub-family B (MDR/TAP); *TICAM1,* Toll-like receptor adaptor molecule 1; *TIPARP,* TCDD-inducible poly(ADP-ribose) polymerase; *TLR3,* Toll-like receptor 3; *TLR4,* Toll-like receptor 4; *TNFRSF10B,* tumor necrosis factor receptor superfamily, member 10b; *TNFSF10,* tumor necrosis factor (ligand) superfamily, member 10; *TREM1,* triggering receptor expressed on myeloid cells 1.

without HBV infection, focusing on the 525 upregulated genes with IFN treatment and using all obtained gene expression profiles. Interestingly, 61.3% of the extracted genes maintained an IFN response, but in 34.9% of those genes, IFN responses were attenuated by HBV infection (Supplementary data 8A). Genes corresponding to interferon signaling, including suppressor of cytokine signaling 1 (*SOCS1*) and interferon regulatory factor 1, and those corresponding to pattern recognition of bacteria and viruses, including nucleotide-binding oligomerization domain containing 1 (*NOD1*) and receptor-interacting serine-threonine kinase 2 (*RIPK2*), were statistically significantly associated with HBV-mediated attenuation to IFN response ($P = 1.0 \times 10^{-8}$ and $P = 1.2 \times 10^{-8}$, respectively). According to these results, HBV infection significantly up-regulated *SOCS1* expression and reduced the IFN responsiveness of *SOCS1*. Thus, *SOCS1* might support chronic infection of HBV in escaping the effects of innate immunity or IFN therapy. On the other hand, genes involved in recognition of viral infection were also inhibited following HBV infection. Both *NOD1* and *RIPK2* are related to innate and adaptive immune responses [14, 15]. We speculated that inhibition of *NOD1* or *RIPK2* expression facilitates HBV survival. Although further study is needed, these results may have important implications for the mechanisms of viral escape from innate immunity.

In conclusion, we performed complementary DNA microarray analysis using human hepatocyte chimeric mice. With this system, we could analyze the direct effects of IFN treatment and HBV infection without the confounding effects of the lymphocyte immunological response and obtained evidence that HBV infection attenuated the virus recognition and IFN response in