

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*^{fllox/fllox} *Alb-Cre*) and Bak KO mice (*bak*^{-/-} *bax*^{fllox/fllox}), which served as control littermates of this mating, were injected with Jo2 and ana-

lyzed 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

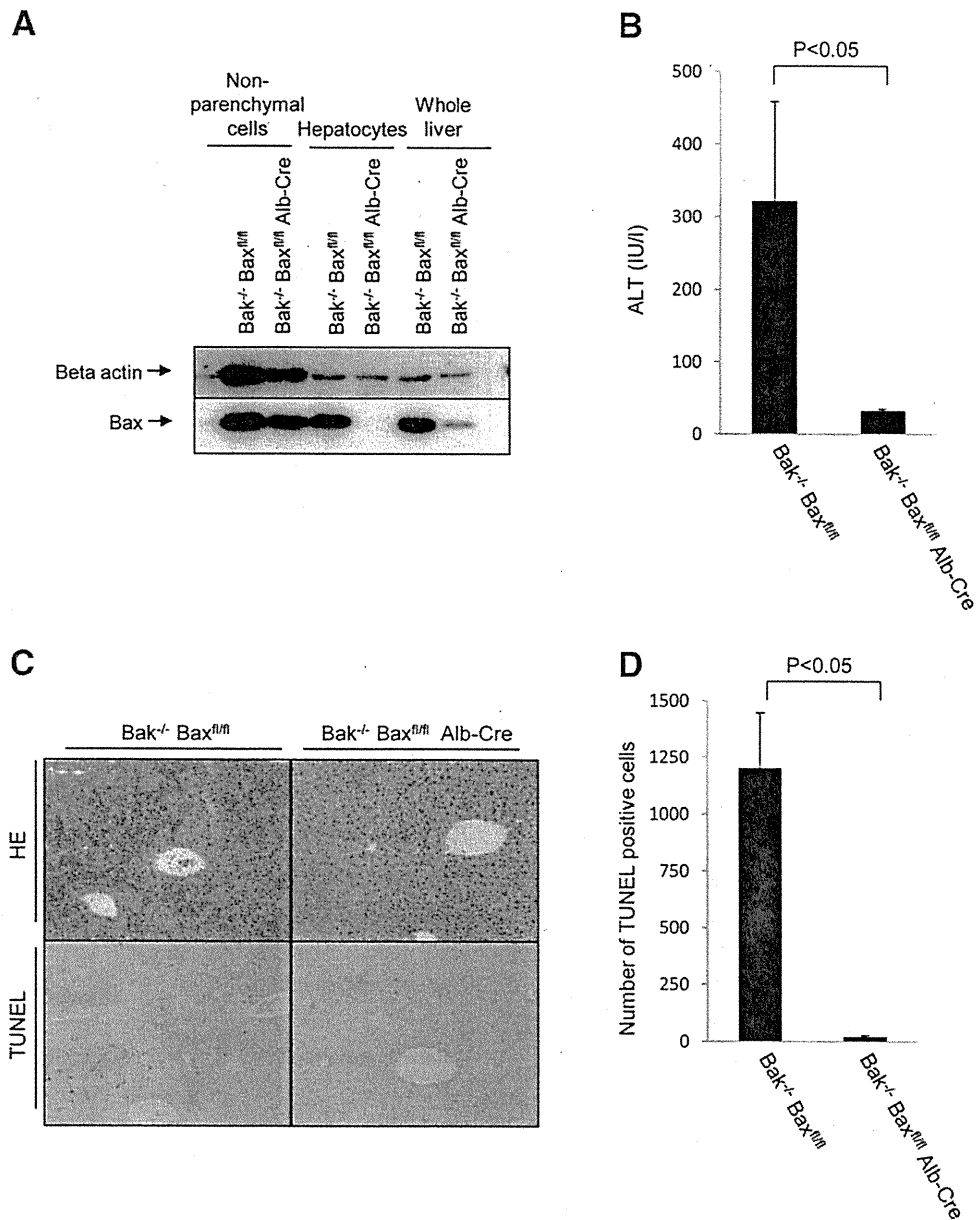


Fig. 3. Bak/Bax DKO mice are fully resistant to Fas-induced hepatocellular apoptosis in early phase. (A) Western blot analysis of the indicated fraction of the liver for the expressions of Bax. (B-D) Bak/Bax DKO mice (Bak^{-/-} Bax^{fl/fl} Alb-Cre) or control Bak KO littermates (Bak^{-/-} Bax^{fl/fl})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

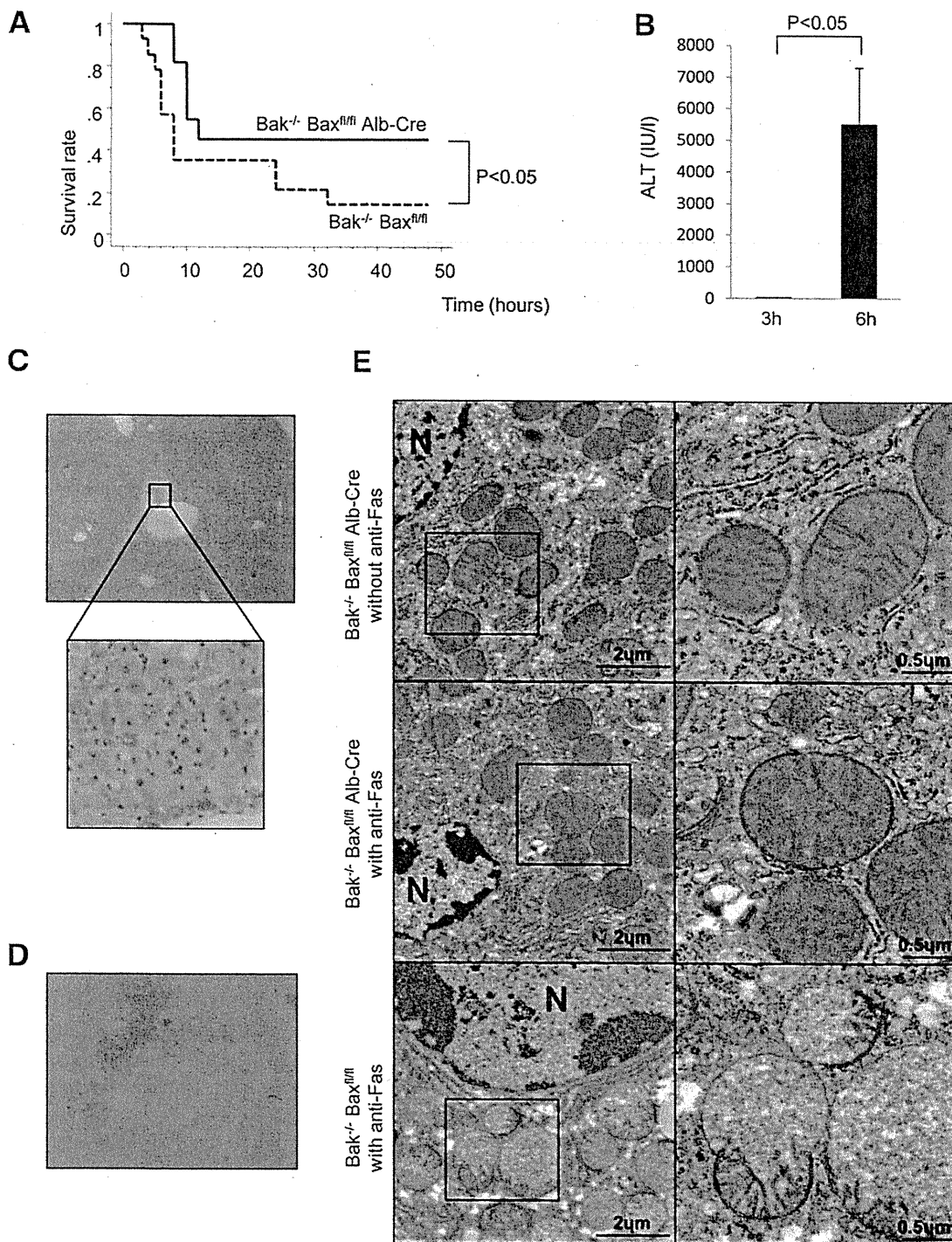


Fig. 4. Bak/Bax DKO mice develop late-onset severe liver injury upon Fas stimulation. Bak/Bax DKO mice (Bak^{-/-} Bax^{fl/fl} Alb-Cre) or control Bak KO littermates (Bak^{-/-} Bax^{fl/fl}) were intraperitoneally injected with 1.5 mg/kg Jo2 anti-Fas antibody. (A) Survival rate after Jo2 injection (n =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

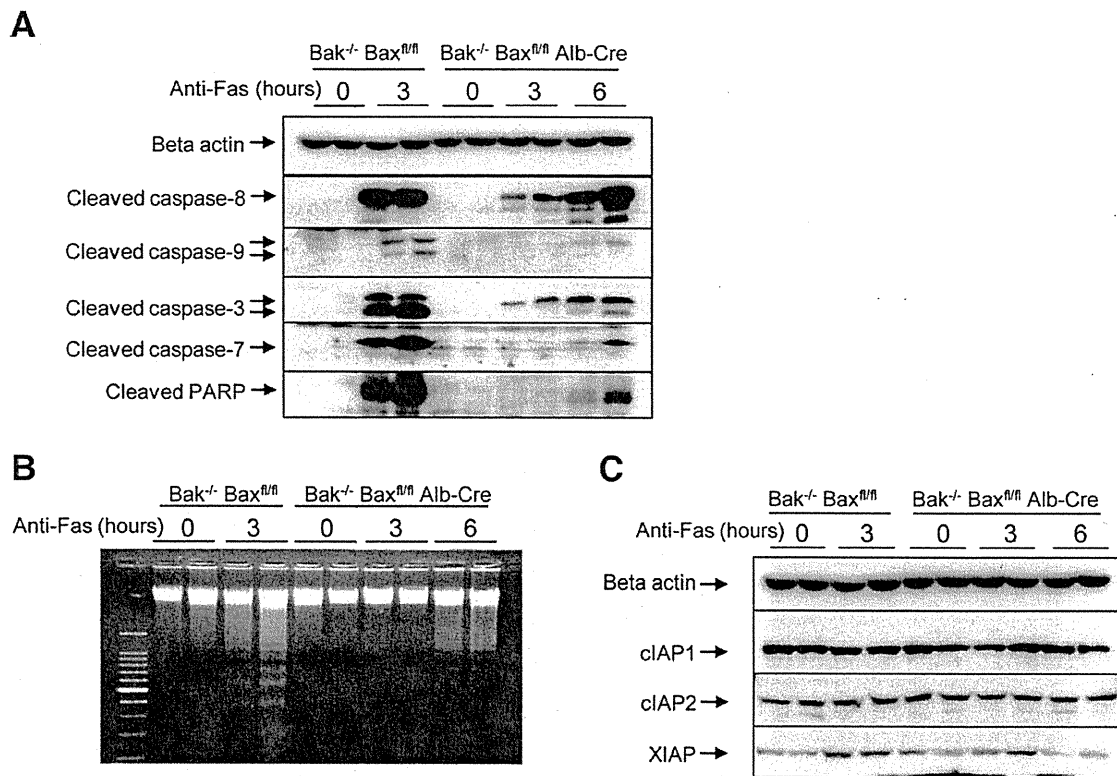


Fig. 5. Fas-mediated hepatocellular death in Bak/Bax DKO mice is associated with caspase-3/7 activation and oligonucleosomal DNA breaks. Bak/Bax DKO mice (Bak^{-/-} Bax^{fl/fl} Alb-Cre) or control Bak KO littermates (Bak^{-/-} Bax^{fl/fl}) were intraperitoneally injected with Jo2 anti-Fas antibody (1.5 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.

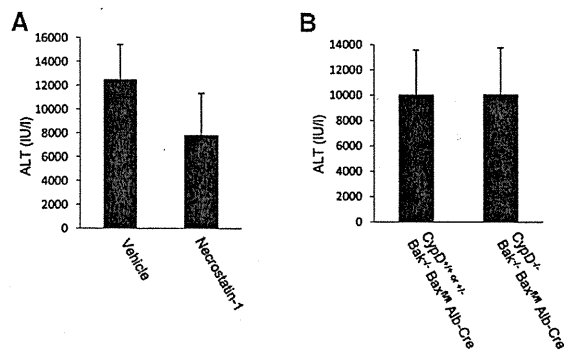


Fig. 6. Fas-induced hepatocellular death in Bak/Bax DKO mice is independent of RIP kinase and/or CypD. (A) Bak/Bax DKO mice (Bak^{-/-} Bax^{fl/fl} Cre) were intraperitoneally injected with 2 mg/kg necrostatin-1 in vehicle containing 0.2% dimethylsulfoxide or vehicle alone at 2 hours after injection of 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^{+/+} or ^{-/-} mice in a Bak/Bax-deficient background (CypD^{+/+} 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 caspase-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 Ca²⁺ 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^{fl/fl} Alb-Cre) or control CypD^{+/+} or ^{-/-} littermates (CypD^{+/+} or ^{-/-} Bak^{-/-} Bax^{fl/fl} 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 stimulation. 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

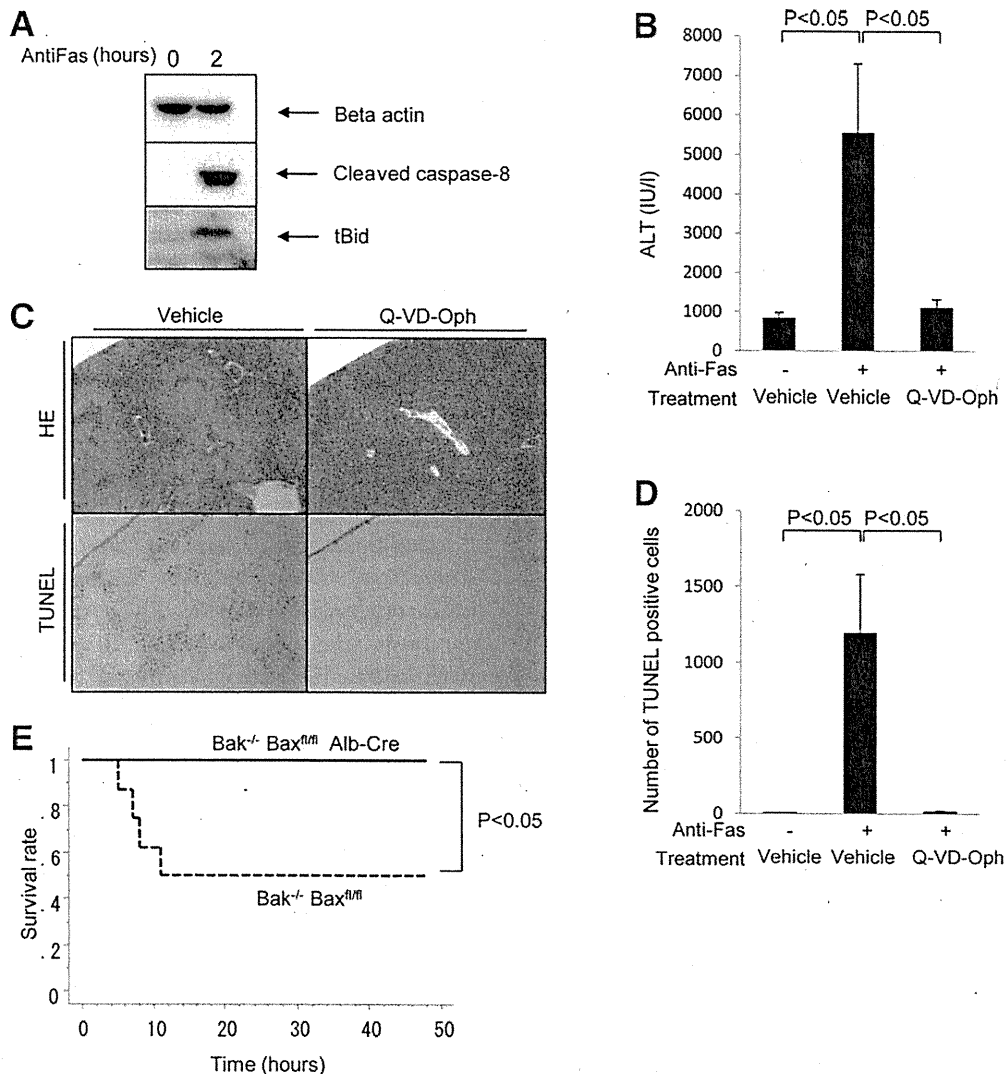


Fig. 7. Hepatocellular death in Bak/Bax DKO mice is dependent on caspase activation. (A) Bak/Bax DKO mice were analyzed before and 2 hours after intraperitoneal injection of Jo2 anti-Fas antibody (1.5 mg/kg). Western blot analysis of the liver for the expression of cleaved caspase-8 and truncated Bid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

pase-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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Indications and limitations for aged patients with chronic hepatitis C in pegylated interferon alfa-2b plus ribavirin combination therapy

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Background & Aims: This study investigated the efficacy and adverse effects of pegylated interferon (Peg-IFN) plus ribavirin therapy in aged patients with chronic hepatitis C (CH-C).

Methods: A total of 1040 naïve patients with CH-C (genotype 1, $n = 759$; genotype 2, $n = 281$), of whom 240 (23%) over 65 years old (y.o.), were treated with Peg-IFN alfa-2b plus ribavirin and assessed after being classified into five categories, according to age.

Results: The discontinuance rate was higher for patients over 70 y.o. (36%), the most common reason being anemia. In the presence of genotype 1, the SVR rate was similar (42–46%) among patients under 65 y.o. and declined (26–29%) among patients over 65 y.o. For patients over 65 y.o., being male (Odds ratio, OR, 3.5, $p = 0.035$) and EVR (OR, 83.3, $p < 0.001$) were significant factors for SVR, in multivariate analysis. The Peg-IFN dose was related to EVR, and when EVR was attained, 76–86% of patients over 65 y.o. achieved SVR. SVR was not achieved (0/35, 0/38, respectively) if a 1-log decrease and a 2-log decrease were not attained at week 4 and week 8, respectively. In the presence of genotype 2, the SVR rate was similar (70–71%) among patients under 70 y.o. and declined among patients over 70 y.o. (43%).

Conclusions: Aged patients up to 65 y.o. with genotype 1 and 70 y.o. with genotype 2 can be candidates for Peg-IFN plus ribavirin therapy. The response-guided therapy can be applied for aged patients with genotype 1.

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Introduction

Pegylated interferon (Peg-IFN) plus ribavirin combination therapy has led to a marked progress in the treatment of chronic hepatitis C (CH-C) [1–4]. However, in aged patients, problems remain with respect to its anti-viral effect and tolerability [5–9]. Recently, the addition of a protease inhibitor to Peg-IFN plus ribavirin combination therapy has been reported, on the one hand, to improve the anti-viral effect, and, on the other hand, to increase side effects, especially severe anemia [10–11].

Therefore, this new therapy does not solve the problems encountered when treating aged patients.

With aging, the progression of liver fibrosis and the occurrence of hepatocellular carcinoma (HCC) have been shown to be accelerated, especially in patients over 60 y.o. [12–14]. In general, the anti-viral therapy can lead to an improvement in liver fibrosis and thus diminish the risk of HCC and ameliorate the prognosis in patients with CH-C [15–21]. Among aged patients, those results are mainly achievable upon eradication of the hepatitis C virus (HCV) [18,21]. Accordingly, the first goal of treatment of aged patients with a high-risk of HCC should be HCV elimination.

Thus, a treatment strategy, aiming at the improvement of the anti-viral efficacy in aged patients, should be established based on detailed large-scale studies.

Some points need to be further elucidated when using the Peg-IFN plus ribavirin combination therapy for the treatment of aged patients with CH-C: (i) the characteristics before treatment

Keywords: Pegylated interferon plus ribavirin therapy; Chronic hepatitis C; Aged patients.

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Abbreviations: HCV, hepatitis C virus; CH-C, chronic hepatitis C; HCC, hepatocellular carcinoma; Peg-IFN, pegylated interferon; SVR, sustained virologic response; RVR, rapid virologic response; EVR, early virologic response; LVR, late virologic response; NR, non-response; WBC, white blood cell; RBC, red blood cell; Hb, hemoglobin; Plt, platelet; G-CSF, granulocyte-macrophage colony stimulating factor.



that would lead to the successful elimination of HCV, (ii) the prediction factors of treatment efficacy after the initiation of the therapy, and (iii) the utility of a response-guided therapy established in the treatment.

In the present study, using a large cohort, we aimed at clarifying these points taking into account the patients' age.

Patients and methods

Patients

This study was a retrospective, multicenter trial conducted by the Osaka University Hospital and other institutions participating in the Osaka Liver Forum. A total of 1040 naïve patients with CH-C were enrolled between December 2004 and June 2007. All patients were Japanese, infected with a viral load of more than 10^5 IU/ml, and treated with a combination of Peg-IFN alfa-2b plus ribavirin. Patients were excluded from the study if they had decompensated cirrhosis or other forms of liver disease (alcohol liver disease, autoimmune hepatitis), coinfection with hepatitis B or anti-human immunodeficiency virus. This study was conducted according to the ethical guidelines of the 1975 Declaration of Helsinki and informed consent was obtained from each patient.

Treatment

All patients received Peg-IFN alfa-2b (PEGINTRON; Schering-Plough, Kenilworth, NJ, USA) plus ribavirin (REBETOL; Schering-Plough). Treatment duration was 48 weeks for patients with genotype 1 and 24 weeks for those with genotype 2. As a starting dose, Peg-IFN alfa-2b was given once weekly, at a dosage of 1.5 µg/kg, and ribavirin was given at a total dose of 600–1000 mg/day based on body weight (body weight <60 kg, 600 mg; 60–80 kg, 800 mg; >80 kg, 1000 mg), according to a standard treatment protocol for Japanese patients.

Dose reduction and discontinuance

Dose modification followed, as a rule, the manufacturer's drug information on the intensity of the hematologic adverse effects. The Peg-IFN alfa-2b dose was reduced to 50% of the assigned dose when the white blood cell (WBC) count was below $1500/\text{mm}^3$, the neutrophil count below $750/\text{mm}^3$ or the platelet (Plt) count below $8 \times 10^4/\text{mm}^3$, and was discontinued when the WBC count was below $1000/\text{mm}^3$, the neutrophil count below $500/\text{mm}^3$ or the Plt count below $5 \times 10^4/\text{mm}^3$. Ribavirin was also reduced from 1000 to 600 mg, 800 to 600 mg, or 600 to 400 mg when the hemoglobin (Hb) was below 10 g/dl, and was discontinued when the Hb was below 8.5 g/dl. Peg-IFN alfa-2b and ribavirin had to be both discontinued if there was a need to discontinue either of them. No ferric medicine or hematopoietic growth factors, such as epoetin alpha, or granulocyte-macrophage colony stimulating factor (G-CSF), were administered.

Virologic assessment and definition of virologic response

Serum HCV RNA level was quantified using the COBAS AMPLICOR HCV MONITOR test, version 2.0 (detection range 6–5000 IU/ml; Roche Diagnostics, Branchburg, NJ) and qualitatively analyzed using the COBAS AMPLICOR HCV test, version 2.0 (lower limit of detection 50 IU/ml; Roche Diagnostics). The rapid virologic response (RVR) was defined as undetectable serum HCV RNA at week 4; the early virologic response (EVR) as undetectable serum HCV RNA at week 12; and the late virologic response (LVR) as detectable serum HCV RNA at week 12 and undetectable serum HCV RNA at week 24. Moreover, the sustained virologic response (SVR) was defined as undetectable serum HCV RNA, 24 weeks after treatment.

According to the protocol, genotype 1 patients, with less than a 2-log decrease in HCV RNA level at week 12 compared to the baseline, or with detectable serum HCV RNA at week 24, had to stop the treatment and were regarded as non-response (NR). Treatment discontinuance was evaluated except for those patients who had discontinued the treatment at up to 24 weeks, due to absence of response. Anti-viral efficacy was evaluated, for all study patients, using the intention-to-treat analysis (ITT analysis) and the per protocol analysis (PP analysis) for patients without treatment discontinuation due to side effects, and was assessed considering the definition of EVR or LVR for genotype 1, and RVR or non-RVR for genotype 2, as previously reported [1].

Assessment of drug exposure

The amounts of Peg-IFN alfa-2b and ribavirin, taken by each patient during the full treatment period, were evaluated by reviewing the medical records. The mean doses of Peg-IFN alfa-2b and ribavirin were calculated individually as averages, on the basis of the body weight at baseline: Peg-IFN alfa-2b expressed as µg/kg/week, ribavirin expressed as mg/kg/day.

Statistical analysis

Patients' baseline data are expressed as means ± SD or median values. To analyze the difference between baseline data, ANOVA or Mantel-Haenszel Chi-square test were performed. Factors associated with the viral response were assessed by univariate analysis using the Mann-Whitney *U* test or Chi-square test and multivariate analysis using logistic regression analysis. A two-tailed *p* value <0.05 was considered significant. The analysis was conducted with SPSS version 15.0J (SPSS Inc., Chicago, IL).

Results

Patient's profile

Baseline characteristics of the patients categorized by age are shown in Table 1.

Genotype 1 patients (*n* = 759) were distributed into five categories: 266 patients were under 55 y.o. (group 1A), 159 were 55–59 y.o. (group 1B), 149 were 60–64 y.o. (group 1C), 134 were 65–69 y.o. (group 1D), and 51 were 70 y.o. or older (group 1E). With advancing age, the male-to-female ratio and peripheral blood cell count (WBC, neutrophil count, Red blood cell (RBC), Hb, Plt) decreased significantly. Patients with a progression of liver fibrosis (METAVIR fibrosis score 3 or 4) significantly increased with age (Table 1A).

Genotype 2 patients (*n* = 281) were also distributed into five categories: 145 patients were under 55 y.o. (group 2A), 43 were 55–59 y.o. (group 2B), 38 were 60–64 y.o. (group 2C), 41 were 65–69 y.o. (group 2D), and 14 were 70 y.o. or older (group 2E). As observed in genotype 1 patients, the peripheral blood cell count decreased and the ratio of advanced fibrosis (score 3–4) increased significantly with age (Table 1B). For both genotypes, the initial doses of Peg-IFN in patients over 70 y.o. were lower than in those under 70 y.o., this was not the case for the ribavirin doses.

Dose reduction and discontinuance for adverse event

The overall discontinuance rate of treatment was 15% (140/919); 18% (112/639) for genotype 1 and 10% (28/280) for genotype 2, respectively. Table 2 shows the reason for and the rate of treatment discontinuance according to age. The discontinuance rate increased with age, being 10% (36/363) for patients under 55 y.o., 15% (27/182) for patients with 55–59 y.o., 17% (28/169) for patients with 60–64 y.o., 19% (28/147) for patients with 65–70 y.o., and significantly higher, 36%, (21/58) for patients over 70 y.o. The discontinuance of treatment due to hemolytic anemia was significantly higher for patients over 70 y.o. as compared to those under 70 y.o. (<70 y.o., 1% (9/861) vs. ≥70 y.o., 16% (9/58), *p* <0.0001).

The rate without dose reduction of both drugs decreased with age (<55 y.o., 41% (171/411); 55–59 y.o., 20% (40/202); 60–64 y.o., 26% (48/187); 65–69 y.o., 23% (41/175); ≥70 y.o., 18% (12/65)). In the presence of genotype 1, the mean dose of Peg-IFN

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Table 1. Baseline characteristics of patients.

Patients with genotype 1							
Factor	<55 y.o.	55 - 59 y.o.	60 - 64 y.o.	65 - 69 y.o.	≥70 y.o.	p value	
Number	266	159	149	134	51		
Age (y.o.)	44.4 ± 8.1	56.9 ± 1.4	62.0 ± 1.4	66.8 ± 1.4	71.4 ± 1.7	<0.001	
Sex: male / female	160 / 106	64 / 95	57 / 92	54 / 80	23 / 28	<0.001	
Body weight (kg)	64.6 ± 11.7	58.3 ± 9.4	58.1 ± 9.6	56.3 ± 9.3	56.3 ± 9.2	<0.001	
White blood cells (/mm ³)	5608 ± 1668	4901 ± 1664	4888 ± 1488	5113 ± 1426	4883 ± 1511	<0.001	
Neutrophils (/mm ³)	2923 ± 1214	2425 ± 1031	2559 ± 1155	2535 ± 1017	2599 ± 1149	<0.001	
Red blood cells (×10 ⁹ /mm ³)	454 ± 47	432 ± 38	427 ± 40	424 ± 37	424 ± 46	<0.001	
Hemoglobin (g/dl)	14.4 ± 1.5	13.8 ± 1.2	13.7 ± 1.3	13.6 ± 1.2	13.7 ± 1.4	<0.001	
Platelets (×10 ⁹ /mm ³)	18.6 ± 6.2	16.3 ± 5.7	15.4 ± 5.3	15.1 ± 5.0	14.4 ± 4.2	<0.001	
AST (IU/L)	62 ± 50	62 ± 45	64 ± 46	72 ± 45	64 ± 40	0.295	
ALT (IU/L)	79 ± 68	76 ± 64	73 ± 63	77 ± 58	65 ± 41	0.657	
Serum HCV RNA (KIU/ml)*	1800	1600	1700	1700	1700	0.691	
Histology (METAVIR)†	Fibrosis, 0 - 2 / 3 - 4	177 / 19	99 / 20	90 / 19	76 / 28	21 / 9	0.001
	Activity, 0 - 1 / 2 - 3	117 / 79	63 / 56	59 / 50	47 / 57	13 / 16	0.146
Peg-IFN dose (µg/kg/week)‡	1.47 ± 0.14	1.47 ± 0.16	1.46 ± 0.18	1.44 ± 0.18	1.36 ± 0.24	<0.001	
Ribavirin dose (mg/kg/day)¶	11.5 ± 1.1	11.5 ± 1.4	11.5 ± 1.4	11.5 ± 1.7	11.2 ± 2.2	0.65	

Patients with genotype 2							
Factor	<55 y.o.	55 - 59 y.o.	60 - 64 y.o.	65 - 69 y.o.	≥70 y.o.	p value	
Number	145	43	38	41	14		
Age (y.o.)	40.9 ± 8.9	56.7 ± 1.3	62.3 ± 1.4	66.7 ± 1.5	71.8 ± 1.8	<0.001	
Sex: male / female	78 / 67	17 / 26	17 / 21	18 / 23	6 / 8	0.441	
Body weight (kg)	63.4 ± 12.0	59.5 ± 11.5	58.6 ± 11.7	58.5 ± 9.8	55.9 ± 6.8	0.783	
White blood cells (/mm ³)	6011 ± 1965	4874 ± 1346	4982 ± 1210	5079 ± 1877	4414 ± 871	<0.001	
Neutrophils (/mm ³)	3214 ± 1511	2468 ± 971	2576 ± 950	2492 ± 1119	2521 ± 683	0.001	
Red blood cells (×10 ⁹ /mm ³)	454 ± 48	430 ± 42	432 ± 50	430 ± 43	408 ± 48	<0.001	
Hemoglobin (g/dl)	14.3 ± 1.6	13.5 ± 1.3	13.9 ± 1.4	13.9 ± 1.3	13.3 ± 1.2	0.001	
Platelets (×10 ⁹ /mm ³)	21.3 ± 5.4	18.3 ± 6.1	17.0 ± 5.2	15.8 ± 5.4	13.9 ± 4.7	<0.001	
AST (IU/L)	53 ± 59	57 ± 45	55 ± 38	83 ± 48	68 ± 29	0.029	
ALT (IU/L)	65 ± 59	73 ± 70	68 ± 62	105 ± 62	78 ± 43	0.008	
Serum HCV RNA (KIU/ml)*	1700	1100	900	1100	500	0.008	
Histology (METAVIR)†	Fibrosis, 0 - 2 / 3 - 4	102 / 0	25 / 3	29 / 2	21 / 9	7 / 1	<0.001
	Activity, 0 - 1 / 2 - 3	68 / 34	18 / 10	18 / 13	9 / 21	5 / 3	0.01
Peg-IFN dose (µg/kg/week)‡	1.48 ± 0.16	1.48 ± 0.14	1.45 ± 0.18	1.46 ± 0.15	1.28 ± 0.26	0.001	
Ribavirin dose (mg/kg/day)¶	11.5 ± 1.1	11.4 ± 1.2	11.5 ± 1.4	11.3 ± 1.6	11.0 ± 1.4	0.55	

*. Data shown are median values.

†. 201 Missing.

‡. 82 Missing.

¶. Initial doses.

during the whole treatment period was lower (1.1 ± 0.3 µg/kg/week) for patients over 70 y.o. than for those under 70 y.o. (1.3 ± 0.3 µg/kg/week) and that of ribavirin decreased with age (<55 y.o., 10.3 ± 1.9 mg/kg/day; 55–59 y.o., 9.8 ± 1.9 mg/kg/day; 60–64 y.o., 9.3 ± 2.3 mg/kg/day; 65–69 y.o., 9.2 ± 2.3 mg/kg/day; ≥70 y.o., 8.5 ± 2.5 mg/kg/day). The same tendency was observed with genotype 2.

Sustained virologic response

In genotype 1 patients, the overall SVR rate was 40% (305/759), being 46% (123/266) for group 1A, 44% (70/159) for group 1B, 42% (62/149) for group 1C, 26% (35/134) for group 1D, and 29% (15/51) for group 1E, following ITT analysis. The same tendency was observed using the PP analysis ($n = 647$). The SVR rates for patients over 65 y.o. were significantly lower than those for patients under 65 y.o. (ITT analysis: ≥65 y.o., 27% vs. <65 y.o.,

44%, $p < 0.0001$; PP analysis: ≥65 y.o., 31% vs. <65 y.o., 50%, $p < 0.0001$) (Fig. 1A). Among genotype 1 patients over 65 y.o., the SVR rate was significantly lower for female patients than for male patients (ITT analysis: male, 40% (31/77) vs. female, 18% (19/108), $p < 0.001$; PP analysis: male, 49% (27/55) vs. female, 20% (18/90), $p < 0.001$).

Moreover, for genotype 2 patients, the overall SVR rate was 78% (220/281), being 88% (128/145) for group 2A, 70% (30/43) for group 2B, 71% (27/38) for group 2C, 71% (29/41) for group 2D, and 43% (6/14) for group 2E, following ITT analysis. The same tendency was observed with the PP analysis ($n = 253$). The SVR rates for patients over 70 y.o. were significantly lower than those for patients under 70 y.o. (ITT analysis: ≥70 y.o., 43% vs. <70 y.o., 80%, $p < 0.0001$; PP analysis: ≥70 y.o., 56% vs. <70 y.o., 85%, $p < 0.05$) (Fig. 1B). Among patients over 70 y.o. with genotype 2, the difference according to gender was not clear because of the small sample.

Table 2. Reasons for treatment discontinuation.

Factor	<55 y.o. (n = 363)	55 - 59 y.o. (n = 182)	60 - 64 y.o. (n = 169)	65 - 69 y.o. (n = 147)	≥70 y.o. (n = 58)	Total (n = 919)
Neutropenia	2	3	0	0	0	5
Thrombopenia	1	0	1	1	0	3
Anemia	0	4	3	2	9	18
Fatigue	1	1	3	3	1	9
Gastrointestinal disorder	2	1	0	0	1	4
Cough, Dyspnea	1	0	3	0	0	4
Vertigo	1	0	0	0	3	4
Psychosis (depression)	7 (3)	7 (3)	4 (4)	3 (3)	2 (2)	23
Rash	5	2	5	7	1	20
Thyroid dysfunction	2	0	2	0	0	4
Fundal hemorrhage	0	2	0	2	0	4
Drug-induced hepatitis	3	1	0	0	0	4
Interstitial pneumonia	0	1	0	1	1	3
Cerebral hemorrhage, infarction	2	0	0	1	0	3
Others	9	5	7	8	3	32
Total	36 (10%)	27 (15%)	28 (17%)	28 (19%)	21 (36%)	140 (15%)

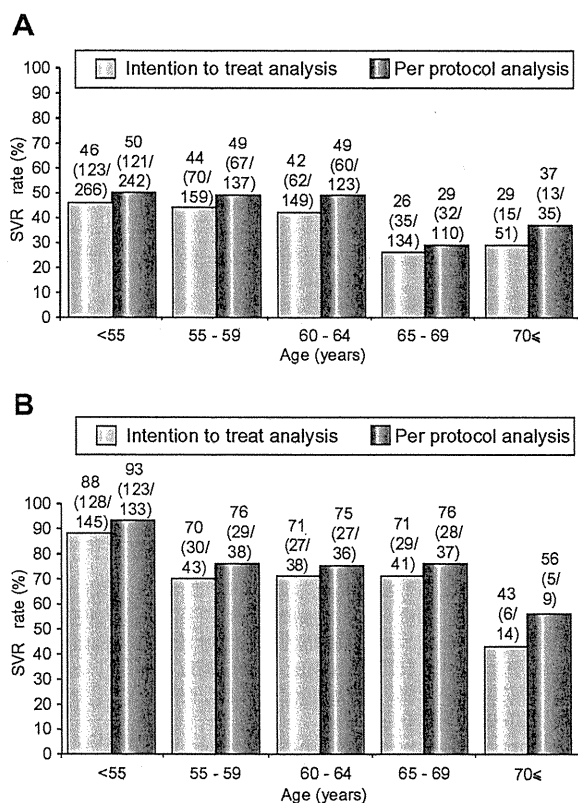


Fig. 1. SVR rate according to age. (A) Genotype 1. (B) Genotype 2.

Timing of HCV RNA negatigation for genotype 1, according to age

Treatment responses distributing EVR, LVR, and NR according to age are shown in Fig. 2. The rates of NR were similar in patient groups under 65 y.o. (30–36%), but increased in almost half of

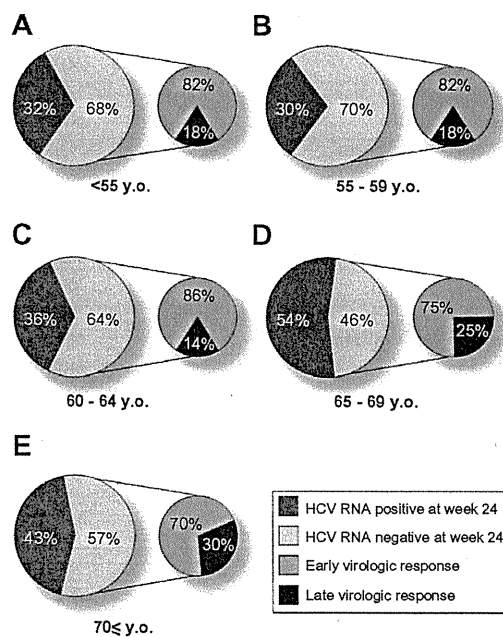


Fig. 2. Antiviral effect during treatment according to age. (A) <55 y.o. (B) 55–59 y.o. (C) 60–64 y.o. (D) 65–69 y.o. (E) ≥70 y.o.

the patients over 65 y.o. ($p < 0.0001$). Moreover, among the virologic responders, the proportion of LVR tended to increase in patients over 65 y.o. (25–30%) compared to patients under 65 y.o. (14–18%) ($p = 0.06$).

SVR rate according to the timing of HCV RNA negatigation

SVR rates according to EVR or LVR in genotype 1, and RVR or non-RVR in genotype 2 are summarized in Table 3. Genotype 1 patients with EVR achieved high SVR rates regardless of age; in particular, if EVR had been attained, 76% of patients with 65–69

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Table 3. SVR rate according to genotype and viral response in patients responding to PEG-IFN plus ribavirin combination therapy.

Factor	<55 y.o.	55 - 59 y.o.	60 - 64 y.o.	65 - 69 y.o.	≥70 y.o.
Genotype 1					
with EVR, % (n)	85 (114/134)	79 (62/79)	81 (55/68)	76 (29/38)	86 (12/14)
with LVR, % (n)	23 (7/30)	29 (5/17)	46 (5/11)	23 (3/13)	17 (1/6)
Genotype 2					
with RVR, % (n)	93 (57/61)	82 (14/17)	85 (17/20)	92 (11/12)	100 (4/4)
without RVR*, % (n)	96 (22/23)	60 (6/10)	57 (4/7)	50 (4/8)	0 (0/3)

RVR, rapid virologic response.

EVR, early virologic response.

LVR, late virologic response.

*, Serum HCV RNA was detectable at week 4, but undetectable at week 24.

Table 4. Multivariate analysis for the factors associated with SVR among all patients.

Factor	Category	Odds ratio	95% CI	p
Age (y.o.)	<65 / ≥65	0.485	0.295 - 0.799	0.005
Sex	male / female	0.524	0.353 - 0.777	0.001
Platelets ($\times 10^4/\text{mm}^3$)	<12 / ≥12	1.780	1.039 - 3.049	0.040
Serum HCV RNA (KIU/ml)	<2000 / ≥2000	0.599	0.401 - 0.896	0.010
Histology (METAVIR): Fibrosis	0 - 2 / 3 - 4	0.599	0.333 - 1.076	0.090

y.o. and 86% of patients over 70 y.o. achieved SVR, and these SVR rates compared favorably with those of younger patients. On the other hand, the SVR rates for patients with LVR ranged from 17% to 46%, which were lower than those for EVR patients in each age group, and no significant differences of SVR rates were found among LVR patients by age.

With genotype 2, patients with RVR achieved high SVR rates ranging from 82% to 100% regardless of age. Even for patients without RVR, 96% of those under 55 y.o. attained SVR, a rate that was significantly higher than that for patients over 55 y.o. (50%, 14/28) ($p < 0.001$).

Factors associated with SVR for genotype 1

The factors associated with SVR were assessed for the variables shown in Table 1. The factors selected as significant by the univariate analysis: age, gender, WBC, neutrophils, RBC, Hb, Plt, aspartate aminotransferase, serum HCV RNA level, the degree of liver fibrosis, and the initial dose of Peg-IFN, were evaluated by multivariate logistic regression analysis. The factor of age over 65 y.o. was the independent factor for SVR ($p = 0.005$), apart from the gender ($p = 0.001$), Plt value ($p < 0.05$), and serum HCV RNA level ($p = 0.01$) (Table 4).

Factors associated with EVR and SVR for patients over 65 y.o. with genotype 1

The results of univariate analysis for EVR among patients over 65 y.o. are shown in Table 5A. Gender, Plt value, and mean dose of Peg-IFN during the first 12 weeks were factors significantly associated with EVR. In multivariate analysis, the mean dose of Peg-IFN during the first 12 weeks was the independent factor for EVR ($p = 0.03$), apart from gender ($p = 0.002$) (Table 5B). The EVR rates were 41% (41/101) in patients who received $\geq 1.2 \mu\text{g}/\text{kg}/\text{week}$ on average during the first 12 weeks, and declined to 36% (8/22) in patients given 0.9–1.2 $\mu\text{g}/\text{kg}/\text{week}$ of Peg-IFN, and

to 14% (3/22) in patients administered with $< 0.9 \mu\text{g}/\text{kg}/\text{week}$ of Peg-IFN.

The baseline and on-treatment factors, which are correlated with the SVR among the patients over 65 y.o., were assessed by univariate and multivariate analyses. Univariate analysis showed that factors significantly associated with SVR were gender and virologic response (Table 6A), and they were also selected as significant independent factors in multivariate analysis ($p = 0.035$, $p < 0.001$) (Table 6B).

Negative prediction of SVR for patients over 65 y.o. with genotype 1

We tried positive and negative predictions of SVR for aged patients, focusing on the decrease of HCV RNA at treatment week 4 and 8. The SVR rate was 47% (29/62) for patients with more than a 1-log decrease in HCV RNA level at week 4, while no patients with less than a 1-log decrease at week 4 attained SVR (0/35) ($p < 0.0001$). Similarly, 55% (35/64) of patients with more than a 2-log decrease at week 8 attained SVR, whereas no patients with less than a 2-log decrease at week 8 attained SVR (0/38) ($p < 0.0001$).

Discussion

Peg-IFN plus ribavirin combination therapy can improve anti-viral efficacy and is presently recommended as first-line therapy [1–4]. However, with respect to aged patients with CH-C, there have been only a few small-scale cohort studies which reported poor anti-viral effect and poor tolerability in comparison with non-aged patients [5–9]. The problem in the treatment of aged patients with CH-C is most serious in Japan, because HCV carriers in Japan are 10–20 years older than those in the United States and European countries [22]. Therefore, in the present study, we examined the efficacy and prevalence of side effects with a focus on patient's age using a large-scale cohort.

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Table 5. Factors associated with EVR among patients over 65 y.o.

Univariate analysis				
Factor		EVR	Non-EVR	p value
Number		52	93	
Age (y.o.)		67.9 ± 2.3	67.8 ± 2.5	0.66
Sex: male / female		28 / 24	27 / 66	0.003
White blood cells (/mm ³)		5063 ± 1474	5001 ± 1422	0.76
Neutrophils (/mm ³)		2566 ± 1110	2551 ± 1071	0.87
Red blood cells (×10 ⁶ /mm ³)		426 ± 36	421 ± 38	0.64
Hemoglobin (g/dl)		13.7 ± 1.2	13.5 ± 1.2	0.21
Platelets (×10 ⁴ /mm ³)		16.5 ± 5.5	14.0 ± 4.6	0.009
AST (IU/L)		70 ± 51	70 ± 40	0.49
ALT (IU/L)		76 ± 58	70 ± 41	0.80
Serum HCV RNA (KIU/ml)*		1700	1900	0.62
Histology (METAVIR)†	Fibrosis, 0 - 2 / 3 - 4	25 / 10	47 / 20	0.54
	Activity, 0 - 1 / 2 - 3	16 / 19	29 / 37	0.52
Peg-IFN dose (µg/kg/week)‡		1.35 ± 0.24	1.25 ± 0.31	0.03
Ribavirin dose (mg/kg/day)‡		10.0 ± 2.2	9.6 ± 2.3	0.40

Multivariate analysis				
Factor	Category	Odds ratio	95% CI	p value
Sex	male / female	0.309	0.149 - 0.644	0.002
Platelets (×10 ⁴ /mm ³)	<12 / ≥12	-	-	N.S.
Peg-IFN dose (µg/kg/week)‡	<1.2 / ≥1.2	2.481	1.079 - 5.705	0.03

*, Data shown are median values.

†, 43 Missing.

‡, Mean doses during 0 to 12 weeks.

N.S., not statistically significant.

Table 6. Factors associated with SVR among patients over 65 y.o.

Univariate analysis				
Factor		SVR	Non-SVR	p value
Number		45	100	
Age (y.o.)		68.0 ± 2.4	67.7 ± 2.5	0.45
Sex: male / female		27 / 18	28 / 72	<0.001
White blood cells (/mm ³)		5006 ± 1516	5030 ± 1409	0.81
Neutrophils (/mm ³)		2575 ± 1130	2548 ± 1063	0.96
Red blood cells (×10 ⁶ /mm ³)		427 ± 40	421 ± 36	0.53
Hemoglobin (g/dl)		13.8 ± 1.3	13.5 ± 1.2	0.14
Platelets (×10 ⁴ /mm ³)		16.1 ± 5.6	14.3 ± 4.7	0.09
AST (IU/L)		71 ± 54	69 ± 40	0.47
ALT (IU/L)		76 ± 56	70 ± 43	0.77
Serum HCV RNA (KIU/ml)*		1700	2000	0.51
Histology (METAVIR)†	Fibrosis, 0 - 2 / 3 - 4	21 / 8	51 / 22	1.00
	Activity, 0 - 1 / 2 - 3	14 / 15	31 / 41	0.66
Peg-IFN dose (µg/kg/week)‡		1.27 ± 0.28	1.23 ± 0.33	0.31
Ribavirin dose (mg/kg/day)‡		8.8 ± 2.1	9.1 ± 2.5	0.38
Virologic response: EVR / non-EVR		41 / 4	11 / 89	<0.001

Multivariate analysis				
Factor	Category	Odds ratio	95% CI	p value
Sex	male / female	0.283	0.088 - 0.914	0.035
Virologic response	EVR / non-EVR	0.012	0.004 - 0.043	<0.001

*, Data shown are median values.

†, 43 Missing.

‡, Mean doses during treatment.

Research Article

With respect to the side effects and discontinuance rate of treatment in aged patients with CH-C, treated with Peg-IFN plus ribavirin combination therapy, Reddy et al. reported that there was no difference related to the incidence and reason for side effects between non-aged and aged patients [6]. Another paper reported that the incidence of side effects was more frequent in aged patients [5]. In our study, not only the continuance rate without reduction of both drug decreased with age, but also the discontinuance rate of treatment increased with age, with a third of the patients over 70 y.o. discontinuing the treatment. The discrepancy, existing between our results and those reported in the former study cited above, is due to the difference in the number of aged patients enrolled; Reddy's study analyzed a small cohort including only a few cases of patients over 65 y.o. and classified all those over 50 y.o. as aged patients.

Discontinuance of treatment due to progression of anemia was significantly higher in patients over 70 y.o., accounting for 43% (9/21) of the discontinuance in this group. Although the ratio of advanced fibrosis (score 3–4) increased with age, the high discontinuance rate due to anemia among patients over 70 y.o. was similar regardless of the progression of fibrosis (F0-2: <70 y.o., 1% (6/559) vs. ≥ 70 y.o., 21% (6/28), $p < 0.0001$; F3-4: <70 y.o., 0% (0/83) vs. ≥ 70 y.o., 22% (2/9), $p < 0.0001$). It is possible that poor hematopoietic function and renal function led to the progression of anemia in aged patients. For patients who develop severe anemia, using epoetin alpha or taribavirin, which are ribavirin prodrugs, has been shown to result in a lower incidence of anemia, although no significant increase of SVR has been reported so far, even with the addition of taribavirin to Peg-IFN [23–24].

With genotype 1 patients, the SVR rates were almost equal up to 65 y.o. (49–50%), but decreased to 31% (45/145) among the patients that were over 65 y.o., and even for those who completed the entire treatment schedule in this study. Since the degree of liver fibrosis and drug exposure have been shown to be associated with anti-viral efficacy, the progression of liver fibrosis or decrease of drug exposure with age could account for the reduction of SVR rate among the aged patients. However, the stratified analysis, according to the progression of liver fibrosis and drug exposure, revealed that older patients still yielded low a SVR rate (F0-2, Peg-IFN during the first 12 weeks ≥ 1.2 $\mu\text{g}/\text{kg}/\text{week}$: <65 y.o., 55% (143/261) vs. ≥ 65 y.o., 33% (15/46), $p < 0.0001$; F0-2, Peg-IFN during the first 12 weeks <1.2 $\mu\text{g}/\text{kg}/\text{week}$: <65 y.o., 43% (26/60) vs. ≥ 65 y.o., 23% (6/26), $p = 0.07$), which means that older patients would be difficult to treat. From our results showing a low SVR rate and a high discontinuance rate for patients over 65 y.o., the genotype 1 patients under 65 y.o. were those who benefited the most from Peg-IFN plus ribavirin combination therapy. The high prevalence of treatment failure (non-SVR) among the aged patients seems to be due to the high populations of NR and LVR (Fig. 2). A high population of LVR is considered to lead to a higher transient response rate among aged patients, since those over 65 y.o. with LVR showed a much higher relapse rate (79%, 15/19) than those with EVR (21%, 11/52) ($p < 0.0001$), as can be seen from Table 3.

In this study, multivariate analysis for SVR, in patients over 65 y.o., showed that the factors associated with SVR were EVR and gender. This indicates that better SVR can be expected even with older patients if EVR is attained and response-guided therapy guidelines can be useful for aged patients. A low SVR rate among aged female patients was as previously reported [7], although the

mechanism remains unclear. This finding suggests that female patients should be treated before 65 y.o.

The next question is how aged patients should be treated in order to attain EVR. We have examined the impact of drug exposure on treatment efficacy [25–26] and reported that Peg-IFN is dose-dependently correlated with EVR [25]. In this study, the dose-dependent efficacy of Peg-IFN for EVR was also revealed in aged patients over 65 y.o., with less than 0.9 $\mu\text{g}/\text{kg}/\text{week}$ of Peg-IFN leading to a low EVR rate for aged patients. If patients are difficult to treat with more than 1.2 $\mu\text{g}/\text{kg}/\text{week}$ of Peg-IFN, using as much Peg-IFN as possible is desirable, in order to attain higher EVR rates. Accordingly, a reduction of Peg-IFN to 80% may need to be considered, although the manufacturer's drug information recommends reducing the dose of Peg-IFN to 50% of the assigned one. Since reduction of Peg-IFN has been reported to not affect the SVR rate after HCV RNA disappearance [26], using G-CSF for aged patients who develop severe neutropenia can be beneficial, especially in the first 12 weeks.

We also examined the negative prediction of SVR, i.e. an HCV RNA decrease at an earlier point of treatment than the usual prediction at treatment week 12 of a 2-log decrease, among aged patients with CH-C treated by Peg-IFN plus ribavirin combination therapy. We found that none of the patients without a 1-log decrease at week 4 or a 2-log decrease at week 8 could attain SVR, even if the complete treatment duration was given, the negative predictive value (NPV) for SVR equaled 100%. This earlier prediction is applied just as well to aged patients as to non-aged patients in order to avoid additional adverse effects. Recently, a genetic polymorphism near the *IL28B* gene has been reported to be associated with non-response to Peg-IFN plus ribavirin combination therapy [27–29], which is beneficial to patients. Nevertheless, even in the presence of this genetic polymorphism, NPV for SVR remains at 57–87%; 100% accuracy is not guaranteed. Thus, in addition to the pretreatment prediction, an earlier negative prediction for SVR during treatment is also considered to be useful.

We have shown in this study that, in the presence of genotype 2, HCV was easily eliminated even among aged patients; the SVR rates were over 75% for patients who had completed the treatment, and these rates were similar up to 70 y.o. The SVR rate of genotype 2 patients over 70 y.o. was 43%, however, the age limitation of the treatment among patients over 70 y.o. remains unclear, because of the small number of patients enrolled in this study. We have reported that the reduction of treatment drugs had little effect on anti-viral efficacy for patients with genotype 2, meaning that SVR can be attained even with aged patients who are usually given lower drug doses than non-aged patients [30]. Patients under 70 y.o. with genotype 2 should, at least, benefit from this therapy. The SVR rate was maintained among genotype 2 patients being 65–69 y.o., compared to genotype 1 patients. The higher efficacy with shorter treatment duration in genotype 2 aged patients can account for it.

In conclusion, the strategy of a response-guided therapy and an earlier negative prediction for SVR may be beneficial for aged patients, especially those with genotype 1. At present, aged patients up to 65–70 y.o. with CH-C can be candidates for Peg-IFN plus ribavirin combination therapy, if its efficacy and adverse effects are fully taken into account. At the same time, there is an urgent need to establish new treatment procedures, such as combination therapy with protease inhibitor plus polymerase inhibitor without Peg-IFN or ribavirin, for non-responders or patients

with poor tolerability for Peg-IFN plus ribavirin combination therapy among aged patients.

Conflict of interest

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

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Differential alteration of CD56^{bright} and CD56^{dim} natural killer cells in frequency, phenotype, and cytokine response in chronic hepatitis C virus infection

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Abstract

Background Natural killer (NK) cells play an important role in immune responses to virus infection. The cell population consists of CD56^{bright} (bright-subset) and CD56^{dim} (dim-subset) subsets that possess armed functions of cytokine production and cytotoxicity, respectively. How these subsets are involved in chronic hepatitis C virus infection (CHC) remains obscure.

Methods We investigated the frequency, phenotype, and cytokine response of these subsets in blood from CHC patients and healthy subjects (HS).

Results Dim-subset, but not bright-subset, showed lower frequency in the patients than in HS. Bright-subset from the patients more frequently expressed the NKG2A/CD94 inhibitory receptor than that from HS, while both subsets from the patients expressed lower levels of the NKG2D activating receptor. Both subsets from the patients displayed a significantly higher level of the signal transducer and activator of transcription (STAT) 1, compared with the

HS. Upon stimulation with interferon- α , bright-subset activated less STAT4, required for interferon- γ production, and dim-subset activated more STAT1, required for cytotoxicity, in the patients than in HS.

Conclusions These results indicate alterations of NK cell subsets in frequency, phenotype, and cytokine response in CHC, which might be associated with the immune pathogenesis of CHC.

Keywords NK cells · CD56^{bright} · CD56^{dim} · HCV · Chronic hepatitis

Abbreviations

NK	Natural killer
IFN	Interferon
IL	Interleukin
HCV	Hepatitis C virus
CHC	Chronic hepatitis C virus infection
CHB	Chronic hepatitis B virus infection
PBMC	Peripheral blood mononuclear cell
STAT	Signal transducer and activator of transcription
pSTAT	Phosphorylated-signal transducer and activator of transcription
HS	Healthy subjects
ISG	Interferon-stimulated gene
MICA	Major histocompatibility complex class I-related chain A

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Introduction

Natural killer (NK) cells play an important role in innate immune responses to a variety of viral infections by directly killing infected cells with cytotoxic molecules such

as perforin and granzyme [1]. The cells also have great ability to secrete a key cytokine, interferon (IFN)- γ , which activates subsequent adaptive immune responses as well as inhibiting viral replication [1, 2]. The activation of NK cells is regulated by several cytokines, such as interleukin (IL)-12, IL-15, and IFN- α [1, 3]. It is also regulated by a balance of activating and inhibitory signals, respectively transmitted by activating and inhibitory receptors that recognize ligands on the cell surface of potential target cells [1, 3]. The NK cell population consists of two subsets in the context of the CD56 surface expression level: CD56^{bright} and CD56^{dim} subsets [4, 5]. It has been reported that CD56^{dim} NK cells are strongly cytolytic armed effector cells, and that CD56^{bright} NK cells are focused on the production of cytokines such as IFN- γ [4, 5]. However, how these subsets are distinguished in the context of immune responses to virus infections remains to be fully elucidated.

Hepatitis C virus (HCV) causes persistent infection in more than 70% of infected patients. Whereas some patients show a carrier-like state, most develop chronic liver diseases, including chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma, which is why HCV infection is a worldwide health problem [6]. The administration of IFN- α is a well-established anti-viral therapy for HCV infection. More than 90% of patients with acute HCV infection respond to IFN- α -based therapy, while the response rate falls to around 50% for patients with chronic HCV infection (CHC) [7–10], suggesting a mechanism by which persistent HCV infection leads to resistance to IFN- α -based therapy. The NK cell number has been demonstrated to decrease in patients with CHC [11–16], while it is controversial whether NK cell functions are impaired in patients with CHC [17–19]. It thus remains unclear whether the perturbation of NK cells, such as that of CD56^{bright} NK cells or CD56^{dim} NK cells, is involved in the persistence of CHC as well as the resistance to therapy.

In the present study, we investigated how the NK cell subsets differ in frequency, phenotype, and response to cytokine stimulation. We also examined how chronic HCV infection modifies those differences. We found clear differences between the NK cell subsets in the response to cytokine stimulation as well as in the frequency and phenotype, which were altered in the CHC patients. This alteration might be associated with the immune pathogenesis of CHC.

Subjects, materials, and methods

Subjects

Eleven patients with CHC (HCV RNA genotype 1) and eleven healthy volunteers were enrolled in this study from

October 2007 to March 2008. Flow cytometric analysis was carried out consecutively for these subjects. Since some of the flow cytometric data were lacking for two subjects in the patient group, they were excluded from the final analysis. The demographic data of these analyzed subjects are shown in Table 1. There was no significant difference in demographic variables (age/sex) between these two groups. The patients were histologically diagnosed as having mild or moderate chronic hepatitis and were evaluated for the degree of liver inflammation and fibrosis according to the METAVIR scoring system [20]. In addition, six patients with chronic hepatitis B virus infection (CHB) (male/female 4/2, age range (median) 31–63 years (38), alanine aminotransferase (ALT) range (median) 24–496 IU/l (70), hepatitis B virus (HBV)-DNA viral load range (median) 5.6 to >9.0 log copies/ml (7.3), hepatitis B envelope antigen (HBeAg)+/– 3/3) were enrolled in this study and consecutive flow cytometric analyses were performed. None of the subjects in the patient group displayed any evidence of other types of liver diseases. The study was approved by the Human Research Ethics Committee of Osaka University Hospital.

Isolation of peripheral blood mononuclear cell populations

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh heparinized peripheral blood by Ficoll–Hypaque density gradient centrifugation as described elsewhere [21].

Flow cytometric analysis

The staining of prepared cells was performed as described [21–26]. Briefly, for the NK receptor staining, cells were

Table 1 Characteristics of patients and subjects

	CHC	HS
Number	9	11
Sex: M/F	4/5	8/3
Age, years, range (median)	44–74 (60)	45–72 (50)
ALT, IU/l, range (median)	14–136 (43)	ND
Activity (A 0–1/2–3)	(7/2)	ND
Fibrosis (F 0–1/2–3)	(5/4)	ND
Viral load, kIU/ml, range (median)	130 to >5000 (3400)	ND
Viral genotype	1	ND

Serum hepatitis C virus (HCV) RNA levels were quantitatively determined using the COBAS AMPLICOR HCV MONITOR test, version 2.0 (detection range 6–5000 kIU/mL; Roche Diagnostics, Branchburg, NJ, USA)

CHC patients with chronic hepatitis C virus infection, HS healthy subjects, ALT alanine aminotransferase, ND not determined

stained with biotin-conjugated anti-CD56 antibody (B159), allophycocyanin-conjugated anti-CD3 antibody (UCHT1) and phycoerythrin-conjugated anti-NKG2D (1D11), phycoerythrin-conjugated anti-NKG2A (Z199) or fluorescein isothiocyanate-conjugated anti-CD94 antibody (HP-3D9), or the corresponding isotype control, followed by staining with peridinin chlorophyll protein-conjugated streptavidin (BD Biosciences, San Jose, CA, USA). For intracellular staining of the signal transducer and activator of transcription (STAT) or phosphorylated-STAT (pSTAT), cells were stained with biotin-conjugated anti-CD56 antibody (B159) and (1), (2) Alexa Fluor[®] 647-conjugated or (3), (4) fluorescein isothiocyanate-conjugated anti-CD3 antibody (UCHT1), fixed and permeabilized with Cytotfix/Cytoperm (BD Biosciences) and cold pure methanol, and then stained with (1) phycoerythrin-conjugated anti-pSTAT1 (pY701) antibody (4a) and Alexa Fluor[®] 488-conjugated anti-pSTAT4 (pY693) antibody (38/p-Stat4) or (2) Alexa Fluor[®] 488-conjugated anti-pSTAT4 (pY693) antibody (38/p-Stat4), or (3) Alexa Fluor[®] 647-conjugated anti-pSTAT1 (pY701) antibody (4a) or (4) phycoerythrin-conjugated anti-STAT1 antibody (1/Stat1), or the corresponding isotype control, followed by staining with peridinin chlorophyll protein-conjugated streptavidin (BD Biosciences). All antibodies except for anti-NKG2A antibody (Beckman Coulter, Brea, CA, USA) were purchased from BD Biosciences. The stained cells were analyzed with a FACSCalibur (BD Biosciences), and the data were processed using the FlowJo program (Tree Star, Ashland, OR, USA).

In vitro stimulation of PBMCs

Prepared PBMCs were resuspended at 2×10^7 cells/ml in RPMI 1640 (Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (Japan Bioserum, Hiroshima, Japan) for in vitro stimulation with cytokines. Natural human IFN- α , recombinant human IFN- γ , and IL-12 were used at concentrations of 1,000 IU/ml, 50 ng/ml, and 10 ng/ml, respectively. For cells left unstimulated, media were added. The cells were incubated at 37°C in 5% CO₂ for 90 min, and then collected for further analyses. Natural human IFN- α was a generous gift from Otsuka Pharma (Tokyo, Japan). Recombinant human IFN- γ and IL-12 were obtained from PBL Biomedical Laboratories (Piscataway, NJ, USA) and R&D Systems (Minneapolis, MN, USA), respectively.

Statistical analysis

The statistical significance of differences between two groups or that of changes in the indicated variables in response to IFN- α treatment was determined by applying

an unpaired or paired Student's *t*-test, respectively. The statistical significance was defined as $p < 0.05$.

Results

Frequency of CD56^{bright} NK cells or CD56^{dim} NK cells

The NK cell number has been demonstrated to decrease in patients with CHC [11–16]. We examined the proportion of CD56^{bright} NK cells or CD56^{dim} NK cells in PBMCs from the CHC patients and those from the healthy subjects (HS). We defined CD56^{bright} NK cells or CD56^{dim} NK cells as CD56 bright CD3-negative cells or CD56 dim CD3-negative cells, respectively, by flow cytometry (Fig. 1a). The proportion of CD56^{bright} NK cells was much lower than that of CD56^{dim} NK cells in both the HS and patient groups (Fig. 1b). The proportion of CD56^{dim} NK cells from the CHC patients was significantly lower than that from the HS, while that of CD56^{bright} NK cells did not show a significant difference between these groups.

Expression level of activating or inhibitory NK receptors on CD56^{bright} NK cells or CD56^{dim} NK cells

The activation of NK cells is partly regulated by the balance of signals transmitted by activating and inhibitory NK receptors [1, 3]. We therefore examined the expression level of activating or inhibitory NK receptors such as NKG2D or NKG2A/CD94 on CD56^{bright} NK cells or CD56^{dim} NK cells, by flow cytometry. We found lower expression of NKG2D, an activating receptor, on CD56^{bright} NK cells than on CD56^{dim} NK cells (Fig. 2). In contrast, the expression of NKG2A/CD94, an inhibitory receptor, on CD56^{bright} NK cells was higher than that on CD56^{dim} NK cells. The expression levels of NKG2D on both CD56^{bright} NK cells and CD56^{dim} NK cells from the CHC patients were significantly lower than those from the HS. However, the expression level of NKG2A/CD94 on CD56^{bright} NK cells from the CHC patients was significantly higher than that from the HS, while that on CD56^{dim} NK cells was not significantly different between these groups. In addition, no significant correlation was observed between the expression levels of the activating or inhibitory NK receptors and the level of viral load or the histological level of liver inflammation or fibrosis in the CHC patients (data not shown).

STAT1 expression levels in CD56^{bright} NK cells or CD56^{dim} NK cells

We have recently reported that NK cells displayed lower intracellular STAT1 expression than other immune cells

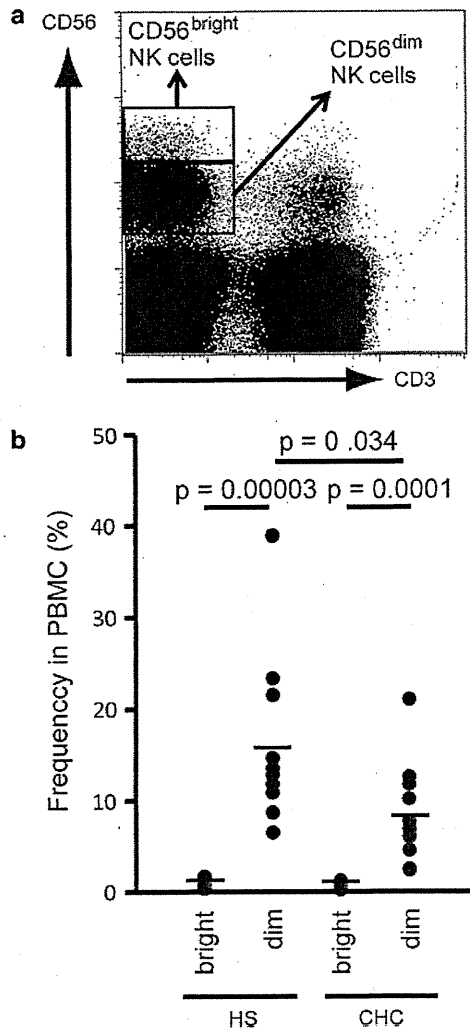


Fig. 1 Frequency of natural killer (NK) cell subsets in peripheral blood mononuclear cells (PBMCs). Frequencies of CD56^{bright} NK and CD56^{dim} NK cell subsets in PBMCs were evaluated by flow cytometry. PBMCs were obtained from patients with chronic hepatitis C virus (HCV) infection (CHC) ($n = 9$) and healthy subjects (HS) ($n = 11$). **a** CD56^{bright} NK cells or CD56^{dim} NK cells were defined as CD56 bright CD3⁺ cells or CD56 dim CD3⁺ cells, respectively, by flow cytometry. CD56 dim and CD56 bright were divided at a threshold where most CD3⁺ cells lost CD56 expression. **b** The frequency of CD56^{bright} subset (bright) or CD56^{dim} subset (dim) was evaluated by flow cytometry, electronically gating on CD56 bright CD3⁺ cells or CD56 dim CD3⁺ cells. Comparisons of the frequencies of those NK cell subsets in PBMCs between bright and dim subsets or between the CHC patients and the HS are shown with the statistically significant p values. Each circle represents data for an individual. Horizontal bars represent means. Statistical significance was analyzed using the unpaired Student's t -test

such as T cells or NKT cells, and that the level was higher in patients with CHC [24]. We therefore examined the expression level of STAT1 in the NK cell subsets, CD56^{bright} NK cells and CD56^{dim} NK cells. The expression level of STAT1 in CD56^{bright} NK cells was clearly higher

than that in CD56^{dim} NK cells in the HS (Fig. 3a, b). The expression level of STAT1 in either CD56^{bright} NK cells or CD56^{dim} NK cells from the CHC patients was significantly higher than that from the HS, and no significant difference was observed in the STAT1 expression levels between CD56^{bright} NK cells and CD56^{dim} NK cells in the CHC patients. In addition, no significant correlation was observed between the STAT1 expression level in these NK cell subsets and the level of viral load or the level of liver inflammation or fibrosis in the CHC patients (data not shown). We also examined the expression level of STAT1 in the NK cell subsets from patients with CHB, which is also capable of causing chronic hepatitis. The expression level of STAT1 in either CD56^{bright} NK cells or CD56^{dim} NK cells in the CHB patients was modestly, but not significantly, higher than that of the HS (positive cell rate, mean \pm SD; $35.0 \pm 26.8\%$ in CD56^{bright} subset and $28.7 \pm 18.1\%$ in CD56^{dim} subset in the CHB patients).

Activation of STAT1/4 occurring in response to cytokines in CD56^{bright} NK cells or CD56^{dim} NK cells

We have previously reported altered IFN- α -signaling in the total NK cell population from CHC patients [24]. We next examined the cytokine signaling in CD56^{bright} NK cells or CD56^{dim} NK cells. As IFN- α can phosphorylate both STAT1 and STAT4, IFN- γ can phosphorylate STAT1, and IL-12 can phosphorylate STAT4 in NK cells [1, 3], we evaluated the phosphorylation level of STAT1/4 that occurred in response to IFN- α , IFN- γ , or IL-12 in CD56^{bright} NK cells and CD56^{dim} NK cells.

In response to IL-12, the majority of CD56^{bright} NK cells phosphorylated STAT4, while only some of the CD56^{dim} NK cells did so (Fig. 4a). In response to IFN- γ , almost no CD56^{bright} NK cells phosphorylated STAT1, while some of the CD56^{dim} NK cells did so. These patterns were observed in both the HS and the patient group. Statistical analysis demonstrated that the phosphorylation level of STAT4 occurring in response to IL-12 in CD56^{bright} NK cells was significantly higher than that in CD56^{dim} NK cells in both the HS and the CHC patient group, while that of STAT1 occurring in response to IFN- γ in CD56^{bright} NK cells was significantly lower than that in CD56^{dim} NK cells in both groups (Fig. 4b). However, the phosphorylation level of STAT4 or STAT1, respectively, occurring in response to IL-12 or IFN- γ did not show any significant difference between these groups in either CD56^{bright} NK cells or CD56^{dim} NK cells.

In response to IFN- α , the whole population of CD56^{bright} NK cells or CD56^{dim} NK cells phosphorylated both STAT4 and STAT1 in both subject groups (Fig. 4a). Statistical analysis, however, demonstrated that