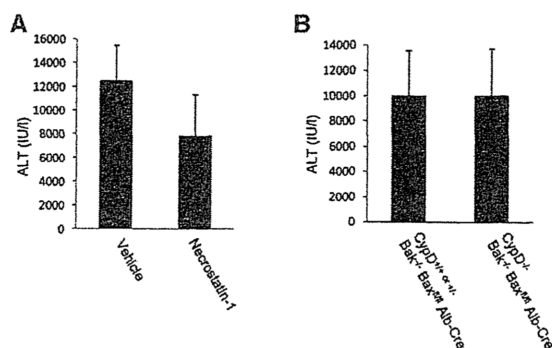

Fig. 6. Fas-induced hepatocellular death in Bak/Bax DKO mice is independent of RIP kinase and/or CypD. (A) Bak/Bax DKO mice ($Bak^{-/-}$ $Bax^{fl/fl}$ Cre) were intraperitoneally injected with 2 mg/kg necrostatin-1 in vehicle containing 0.2% dimethylsulfoxide or vehicle alone at 2 hours after injection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

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

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

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

Discussion

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

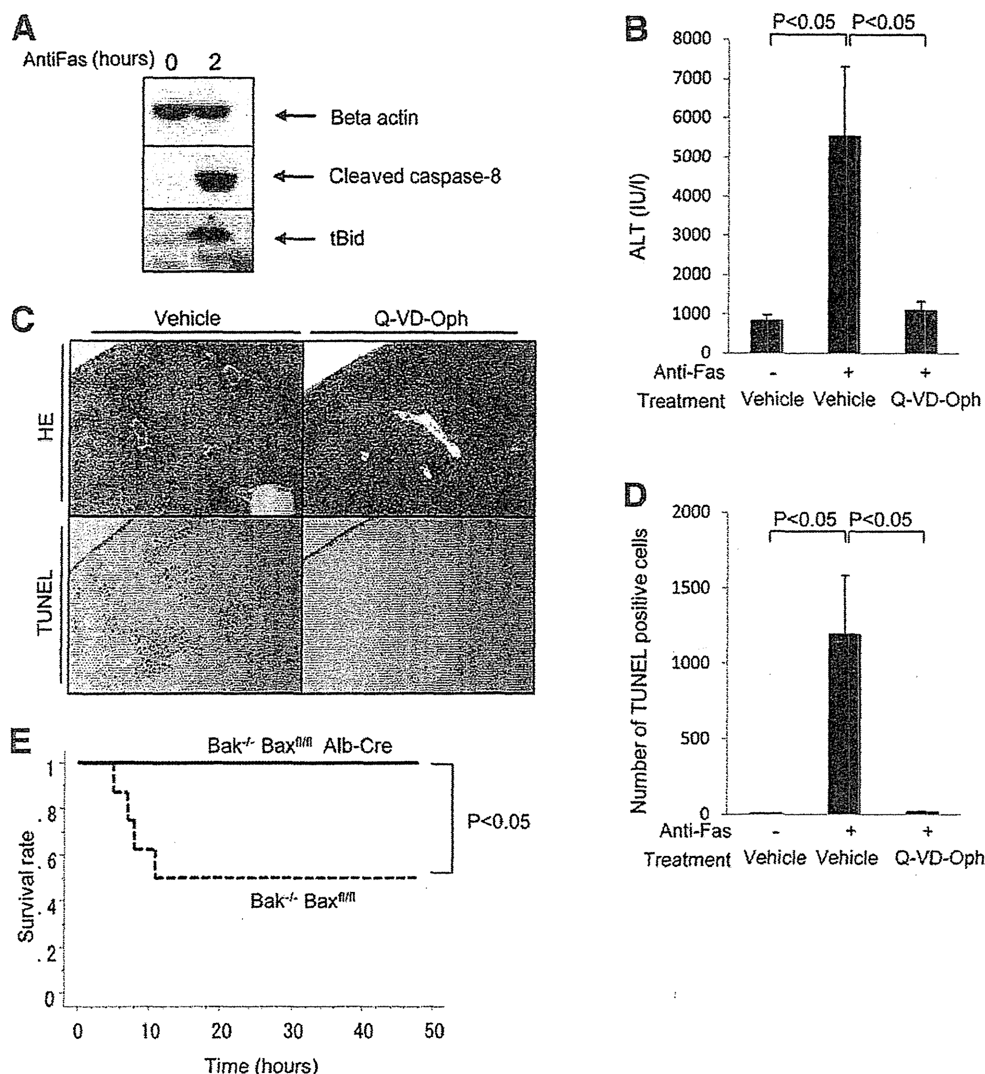

Fig. 7. Hepatocellular death in Bak/Bax DKO mice is dependent on caspase activation. (A) Bak/Bax DKO mice were analyzed before and 2 hours after intraperitoneal injection of Jo2 anti-Fas antibody (1.5 mg/kg). Western blot analysis of the liver for the expression of cleaved caspase-8 and truncated Bid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

mice, showed resistance to apoptosis induced by Bcl-xL deficiency, which depended mainly on Bid activation.[16] Research has shown that Fas induces apoptosis in hepatocytes through the Bid pathway,[10,11] and the present study also demonstrates that Bid becomes truncated in the liver upon anti-Fas injection. Therefore, truncated Bid may preferentially activate Bak rather than Bax in the liver. However, the present study also reveals that, in the absence of Bak, Bax plays an essential role in mediating the early onset of hepatocellular apoptosis. The most important finding of this study is that Bak/Bax deficiency failed to protect against the late onset of liver injury after Jo2 anti-Fas injection as well as Fas agonist injection. Wei et al.,[32] in their historical paper establishing the importance of Bak and Bax in the mitochondrial pathway of apoptosis, reported that hepatocytes were protected from Jo2-induced apoptosis in traditional Bak/Bax DKO mice (*bak*$^{-/-}$ *bax*$^{-/-}$). Because perinatal lethality occurs with most traditional Bak/Bax DKO mice,

they could only analyze three animals, which did not enable detailed analysis of cell death due to Jo2 stimulation. The present study is the first to (1) thoroughly examine the impact of Bak and Bax in the liver using conditional KO mice and (2) demonstrate that Bak/Bax deficiency can protect against Fas-induced severe injury in the early phase but not in the late phase.

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

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

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

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

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

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

pathway in hepatocytes, placing the mitochondrial pathway of apoptosis as a potent loop for amplifying activation of the caspase cascade to execute complete and rapid cell death in hepatocytes.

Acknowledgment: We thank Xiao-Ming Yin (Department of Pathology and Laboratory Medicine, Indiana University School of Medicine) for providing the anti-mouse Bid antibody.

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J Gastroenterol (2011) 46:944–952
DOI 10.1007/s00535-011-0403-0

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

The efficacy of extended treatment with pegylated interferon plus ribavirin in patients with HCV genotype 1 and slow virologic response in Japan

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Received: 27 December 2010 / Accepted: 23 March 2011 / Published online: 7 May 2011
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Abstract
Background Which patients with hepatitis C virus (HCV) genotype 1 can benefit from extended treatment with pegylated interferon (Peg-IFN) plus ribavirin is unknown, although the overall sustained virologic response (SVR) rate has been shown to improve in patients with a late virologic response (LVR), defined as detectable serum HCV RNA at week 12 and undetectable at week 24.

Methods Among 1163 chronic hepatitis C patients with genotype 1 treated with Peg-IFN plus ribavirin combination therapy, 213 patients with an LVR were examined in this study. In addition, we selected 81 patients of matched sex and age from each of the 48- and 72-week treatment groups, using the propensity score, to compare the efficacy of the two treatment durations.

Results With 72-week treatment, the timing of HCV RNA disappearance and the hemoglobin level at baseline

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showed a strong correlation with the SVR on multivariate analysis. Earlier HCV RNA disappearance was associated with a better SVR rate, regardless of the ribavirin dose (HCV RNA disappearance at week 16, 74%; at week 20, 52%; and at week 24, 31%, $p = 0.01$). The SVR rate with 72-week treatment was higher than that with 48-week treatment, irrespective of age, sex, or the platelet value, and, especially in aged patients ($\geq$65 years old), the SVR rate increased markedly with 72-week treatment (48 weeks, 25% vs. 72 weeks, 56%; $p < 0.05$).

Conclusions An earlier response predicts a higher SVR rate in patients with an LVR given 72-week treatment. Extended treatment with Peg-IFN plus ribavirin for patients with an LVR improved the treatment efficacy, even for aged patients.

Keywords Chronic hepatitis C · Pegylated interferon and ribavirin combination therapy · Extended treatment · Aged patients

Introduction

Long persistence of hepatitis C virus (HCV) infection can lead to the progression of liver fibrosis, causing liver cirrhosis and ultimately hepatocellular carcinoma (HCC) [1, 2]. Past studies have clearly shown alleviation of liver fibrosis, a reduced incidence of HCC, and markedly improved prognosis in patients in whom HCV has been successfully eradicated [3–9]. The currently recommended treatment for chronic hepatitis C is pegylated interferon (Peg-IFN) plus ribavirin therapy, which can improve antiviral efficacy for patients with chronic hepatitis C [10–16]. However, HCV still persists in approximately half of genotype 1 patients treated with Peg-IFN plus ribavirin [12–14, 16]. Accordingly, the treatment method needs to be well managed in order to maximize the virologic response.

For patients with HCV genotype 1, a high sustained virologic response (SVR) rate (73–81%) was found in patients who achieved an early virologic response (EVR), defined as undetectable serum HCV RNA at week 12. However, an SVR was attained at a low rate (14–44%) in patients with a late virologic response (LVR; defined as detectable serum HCV RNA at week 12 and undetectable at week 24), because of a high relapse rate [13, 16–24]. For the treatment strategy, drug dosages and durations of treatment can be modified by considering individual patient situations. We have reported a dose-dependent effect of ribavirin on reducing the relapse rate for patients responding to Peg-IFN plus ribavirin therapy [17, 18]. However, this effect was limited to patients with an EVR and sufficient efficacy was not observed in patients with an LVR, who should be treated not only with a high dose of ribavirin, but also for a longer duration.

For patients with an LVR, previous studies have verified that extended therapy (72-week treatment) can improve the SVR rate (38–60%) compared to standard 48-week therapy (18–36%) by reducing the relapse rate [19, 20]. However, which group of patients with an LVR can benefit from extended therapy remains obscure. In general, in order to clarify the relationship between treatment duration and anti-viral effect, a randomized control trial (RCT) should be conducted in which patients are distributed into standard and extended-therapy groups. However, it is impossible, from an ethical perspective, to conduct an RCT in Japan, because some previous studies have already revealed the usefulness of extended therapy [19–23].

In the present study, we tried to identify the factors associated with SVR in patients with an LVR infected with HCV genotype 1 who received extended treatment. Furthermore, a case-control matched study was conducted in order to compare the effectiveness of the extended treatment with that of the standard treatment of Peg-IFN plus ribavirin therapy.

Patients and methods

Patients

The present study was a retrospective, multicenter trial conducted by Osaka University Hospital and other institutions participating in the Osaka Liver Forum. Among 1163 chronic hepatitis C patients with genotype 1 treated with Peg-IFN plus ribavirin combination therapy between December 2004 and June 2007, 213 patients with an LVR who completed the therapy with undetectable HCV RNA at the end of the treatment were enrolled in this study. All patients were Japanese, infected with HCV genotype 1, and having a viral load of more than 10^5 IU/ml. The patients with an LVR continued combination therapy for 48 or 72 weeks according to the decision of the investigator at the participating clinical center. The patients treated for 46–52 weeks were classified as the 48-week treatment group and those who were treated for 68–78 weeks were classified as the 72-week treatment group. The baseline characteristics of all patients before matching are summarized in Table 1. In addition, we selected 81 patients of matched sex and age, using propensity scores, from each of the 48- and 72-week treatment groups.

Patients eligible for this study were negative for hepatitis B surface antigen and anti-human immunodeficiency virus. Patients were excluded from this study if they had decompensated cirrhosis or other forms of liver disease (alcoholic liver disease, autoimmune hepatitis). This study was conducted according to the ethical guidelines of the

Table 1 Baseline characteristics of patients with LVR according to treatment duration

Factor	48 weeks	72 weeks	p value
Number of patients	106	107	
Age (years)	56.6 ± 9.1	60.2 ± 7.8	0.002
Sex: male/female	51/55	38/69	0.07
Body weight (kg)	59.9 ± 11.5	59.2 ± 10.3	0.64
History of IFN treatment: naïve/experienced	64/42	69/38	0.57
White blood cells (/mm^3)	4908 ± 1389	4893 ± 1430	0.91
Neutrophils (/mm^3)	2455 ± 936	2503 ± 1042	0.91
Red blood cells ($\times 10^4$/mm^3)	438 ± 49	439 ± 38	0.48
Hemoglobin (g/dl)	13.9 ± 1.5	13.9 ± 1.4	0.76
Platelets ($\times 10^4$/mm^3)	17.0 ± 5.8	16.2 ± 5.7	0.21
AST (IU/l)	56 ± 34	56 ± 34	0.74
ALT (IU/l)	70 ± 50	68 ± 56	0.78
Serum HCV RNA (KIU/ml)[a]	1850	2400	0.03
Histology (METAVIR)[b]			
Fibrosis, 0–2/3–4	75/7	62/17	0.03
Activity, 0–1/2–3	49/33	45/34	0.75
Peg-IFN dose (μg/kg/week)[c]	1.47 ± 0.17	1.48 ± 0.7	0.21
Ribavirin dose (mg/kg/day)[c]	11.3 ± 1.7	11.5 ± 1.5	0.22
HCV RNA negativity: 16/20/24 weeks[d]	65/23/12	51/32/14	0.23

LVR late virologic response, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *IFN* interferon, *HCV* hepatitis C virus

[a] Data shown are median values

[b] 52 missing

[c] Initial dose

[d] The times of HCV RNA negativity were unknown in 6 patients with 48-week treatment and 10 patients with 72-week treatment

Declaration of Helsinki amended in 2008, 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) for the duration of the study of 48 or 72 weeks. Peg-IFN alfa-2b was given subcutaneously once weekly at a dosage of 60–150 μg/kg based on body weight (body weight 35–45 kg, 60 μg; 46–60 kg, 80 μg; 61–75 kg, 100 μg; 76–90 kg, 120 μg; 91–120 kg, 150 μg) and ribavirin was given orally twice a day 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

Dose modification followed, as a rule, the manufacturer's drug information according to the intensity of the hematologic adverse effects. The dose of Peg-IFN alfa-2b was reduced to 50% of the assigned dose if the white blood cell (WBC) count declined to <1500/mm^3, the neutrophil count declined to <750/mm^3 or the platelet (Plt) count declined to <8 × 10^4/mm^3, and the agent was discontinued if the WBC count declined to <1000/mm^3, the neutrophil count declined to <500/mm^3, or the Plt count declined to <5 × 10^4/mm^3. Ribavirin was also reduced, from 1000 mg to 600 mg, or from 800 mg to 600 mg, or from 600 mg to 400 mg, if the hemoglobin (Hb) level decreased to <10 g/dl, and it was discontinued if the Hb level decreased to <8.5 g/dl. Both Peg-IFN alfa-2b and ribavirin had to be discontinued if there was a need to discontinue one of the drugs. During this therapy, no iron supplements or hematopoetic growth factors, such as erythropoietin alfa or granulocyte–macrophage colony stimulating factor, were administered.

Virologic assessment and definition of virologic response

The serum HCV RNA level was quantified using the COBAS AMPLICOR HCV MONITOR test, version 2.0 (detection range 6–5000 KIU/ml; Roche Diagnostics, Branchburg, NJ, USA) and qualitatively analyzed using the

COBAS AMPLICOR HCV test, version 2.0 (lower limit of detection 50 IU/ml). LVR was defined as detectable serum HCV RNA at treatment week 12 and undetectable at treatment week 24; SVR was defined as the absence of detectable serum HCV RNA at 24 weeks after the the end of the treatment, and relapse was defined as the absence of detectable serum HCV RNA at the end of the treatment but detectable serum HCV RNA at 24 weeks after the end of the treatment.

Statistical analysis

Baseline data for various demographic, biochemical, and virologic characteristics of the patients were expressed as means ± standard deviation or median values. To analyze the relationship between baseline data and SVR, univariate analysis using the Mann–Whitney *U*-test or the χ^2test, and multivariate analysis using logistic regression analysis were performed. The significance of trends in values was determined with the Mantel–Haenszel χ^2test. A two-tailed p value of <0.05 was considered significant. Statistical analysis was conducted with SPSS version 15.0J (SPSS, Chicago, IL, USA).

Results

Baseline characteristics and efficacy of treatment in patients with LVR according to treatment duration

Table 1 shows the baseline characteristics of the patients with LVR stratified according to treatment duration before matching. The patients given 72-week treatment were significantly older ($p = 0.002$), had higher HCV-RNA ($p = 0.03$), and included many with advanced liver fibrosis (METAVIR fibrosis score 3 or 4) ($p = 0.03$). Those with 72-week treatment tended to include many female patients compared to the patients given 48-week treatment ($p = 0.07$). Drug reductions due to side effects occurred with a higher frequency in the 72-week treatment group than in the 48-week treatment group; Peg-IFN, 48-weeks, 40% (42/106) versus 72-weeks, 55% (59/107); ribavirin, 48-weeks, 53% (56/106) versus 63% (67/107). However, the main reasons for reductions of both drugs were almost the same; Peg-IFN, 48-weeks, neutropenia ($n = 23$), thrombocytopenia ($n = 14$); 72-weeks, neutropenia ($n = 24$), thrombocytopenia ($n = 19$), general fatigue ($n = 4$); and ribavirin, 48-weeks, anemia ($n = 47$), general fatigue ($n = 3$); 72-weeks, anemia ($n = 51$), general fatigue ($n = 4$). The SVR rate with 72-week treatment was significantly higher than that with 48-week treatment (59%, 63/107 vs. 37%, 39/106, $p = 0.002$), due to less relapse after treatment.

Factors associated with SVR for patients with LVR treated for 72 weeks

The baseline factors, including the timing of the HCV RNA disappearance, were assessed for association with SVR by univariate and multivariate logistic regression analyses in the 107 patients with 72-week treatment. Univariate analysis showed that factors significantly associated with SVR were age, sex, red blood cell count, Hb, and the timing of HCV RNA disappearance (Table 2A). The factors selected as significant by univariate analysis were evaluated by multivariate logistic regression analysis. The timing of HCV RNA disappearance and Hb at baseline were independent factors for SVR ($p = 0.002$, $p = 0.002$, respectively) (Table 2B).

Baseline characteristics of matched patients with LVR

In order to reduce the selection bias among the LVR patients with 48- and 72-week treatment, a matched case–control study was performed; 81 patients were selected from each of the two treatment duration groups, by matching sex and age, using propensity scores. Baseline characteristics were about the same for the two groups, except for the red blood cell count and the progression stage of liver fibrosis (Table 3). In terms of age and sex, the mean age of the male patients was 57.2 ± 8.3 years in the 48-week treatment group and 58.5 ± 8.2 years in the 72-week treatment group, and the mean ages of the female patients were 59.9 ± 7.6 and 60.0 ± 8.5 years, respectively. The male–female ratio of patients more than 65 years old was similar for the two treatment duration groups (male/female, 8/16; 48-week treatment, 10/17; 72-week treatment). Those less than 65 years old were of the same proportion (54%, male/female, 26/31; 48-week treatment, 25/29; 72-week treatment).

SVR rate among patients with LVR in relation to the factors at baseline and treatment duration

We analyzed the association between the SVR rate and baseline characteristics using the matched population. The SVR rate with 72-week treatment was significantly higher than that with 48-week treatment regardless of age (<65 years, 72 weeks, 63%, 34/54 vs. 48 weeks, 39%, 22/57, $p = 0.01$; ≥65 years, 72 weeks, 56%, 15/27 vs. 48 weeks, 25%, 6/24, $p < 0.05$) (Fig. 1a). For males, the SVR rate with 72-week treatment was 77% (27/35), which was significantly higher than that with 48-week treatment (38%, 13/34, $p = 0.001$). For females, the SVR rate with 72-week treatment tended to be higher than that with 48-week treatment (72 weeks, 48%, 22/46 vs. 48 weeks, 32%, 15/47, $p = 0.14$) (Fig. 1b). Among female patients

Table 2 Factors associated with SVR among patients with 72-week treatment before matching

Factor	SVR	Relapser	p value
A. Univariate analysis			
Number of patients	63	44	
Age (years)	58.8 ± 8.0	62.3 ± 7.2	0.02
Sex: male/female	28/35	10/34	0.03
Body weight (kg)	60.0 ± 10.0	58.2 ± 11.1	0.19
History of IFN treatment: naïve/experienced	38/25	31/13	0.31
White blood cells (/mm^3)	5021 ± 1474	4709 ± 1361	0.22
Neutrophils (/mm^3)	2621 ± 1046	2343 ± 1026	0.15
Red blood cells (×10^4/mm^3)	448 ± 39	426 ± 32	0.005
Hemoglobin (g/dl)	14.3 ± 1.3	13.3 ± 1.2	0.001
Platelets (×10^4/mm^3)	15.8 ± 5.3	16.7 ± 6.3	0.63
AST (IU/l)	56 ± 36	54 ± 32	0.68
ALT (IU/l)	71 ± 62	64 ± 45	0.33
Serum HCV RNA (KIU/ml)[a]	2400	2500	0.88
Histology (METAVIR)[b]			
Fibrosis, 0–2/3–4	34/9	28/8	1.00
Activity, 0–1/2–3	25/18	20/16	0.82
Peg-IFN dose (μg/kg/week)[c]	1.29 ± 0.30	1.28 ± 0.32	0.80
Ribavirin dose (mg/kg/day)[c]	9.7 ± 1.8	9.4 ± 2.1	0.57
HCV RNA negativity: 16/20/24 weeks[d]	39/15/4	12/17/10	0.001

Factor	Category	Odds ratio	95% CI	p value
B. Multivariate analysis				
Age	1 year old	–	–	NS
Sex	male/female	–	–	NS
Red blood cells	1 × 10^4/mm^3	–	–	NS
Hemoglobin	1 g/dl	2.030	1.289–3.197	0.002
HCV RNA negativity	16/20/24 weeks	0.751	0.633–0.890	0.001

SVR sustained virologic response, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *CI* confidence interval, *NS* not significant

[a] Data shown are median values

[b] 23 missing

[c] Mean doses throughout the treatment

[d] The times of HCV RNA negativity were unknown in 5 patients with 48-week treatment and 5 patients with 72-week treatment

more than 65 years old, the SVR rate with 72-week treatment increased with marginal significance (72 weeks, 53%, 9/17 vs. 48 weeks, 19%, 3/16, $p = 0.07$).

The SVR rate in patients with no to moderate fibrosis (METAVIR fibrosis score 0–2) was 58% (26/45) among patients with 72-week treatment, and this rate was significantly higher than that among patients with 48-week treatment (35%, 19/55) ($p = 0.03$). On the other hand, for patients with more advanced liver fibrosis (METAVIR fibrosis score 3 or 4), the SVR rate was 54% (7/13) among the patients with 72-week treatment and 33% (1/3) among those with 48-week treatment; the difference was not significant due to the small number of subjects. However, the SVR rate among the patients with a lower Plt value (<12 × 10^4/mm^3 at baseline), which is indicative of advanced fibrosis, was significantly higher among the patients given 72-week treatment (61%, 14/23) than that among those given 48-week treatment (24%, 4/17) ($p = 0.03$) (Fig. 1c).

SVR rate among patients with LVR in relation to the timing of HCV disappearance and treatment duration

We analyzed the association of the SVR rate with the timing of HCV RNA disappearance. The SVR rate among the patients with 72-week treatment was 74% (32/43) in patients with undetectable HCV RNA at week 16, 52% (13/25) at week 20, and 31% (4/13) at week 24, and the rates were higher than those among the patients with 48-week

Table 3 Baseline characteristics of matched patients with LVR

Factor	48 weeks	72 weeks	p value
Number of patients	81	81	
Age (years)	58.8 ± 8.0	59.4 ± 8.4	0.52
Sex: male/female	34/47	35/46	1.00
Body weight (kg)	58.9 ± 11.7	60.1 ± 11.0	0.46
History of IFN treatment: naïve/experienced	50/31	48/33	0.87
White blood cells (/mm^3)	4717 ± 1286	5020 ± 1516	0.19
Neutrophils (/mm^3)	2332 ± 926	2611 ± 1133	0.13
Red blood cells (×10^4/mm^3)	433 ± 44	445 ± 35	0.03
Hemoglobin (g/dl)	13.8 ± 1.3	14.1 ± 1.3	0.13
Platelets (×10^4/mm^3)	16.2 ± 5.3	16.2 ± 5.9	0.64
AST (IU/l)	56 ± 35	51 ± 27	0.63
ALT (IU/l)	68 ± 52	61 ± 37	0.88
Serum HCV RNA (KIU/ml)[a]	1900	2400	0.10
Histology (METAVIR)[b]			
Fibrosis, 0–2/3–4	55/5	45/13	0.04
Activity, 0–1/2–3	37/23	41/17	0.34
Peg-IFN dose (μg/kg/week)[c]	1.47 ± 0.19	1.48 ± 0.17	0.31
Ribavirin dose (mg/kg/day)[c]	11.4 ± 1.9	11.5 ± 1.5	0.57
HCV RNA negativity: 16/20/24 weeks	52/18/11	43/25/13	0.34

AST aspartate aminotransferase, *ALT* alanine aminotransferase

[a] Data shown are median values

[b] 44 missing

[c] Initial dose

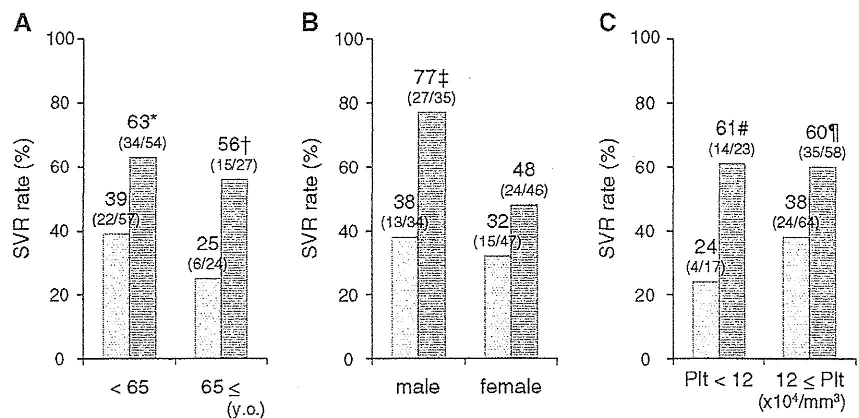

Fig. 1 Sustained virologic response (*SVR*) rate according to baseline characteristics and treatment duration. **a** SVR rate according to age. **b** SVR rate according to sex. **c** SVR rate according to platelet counts. *Light gray shade bars* indicate 48-week treatment. *Dark gray shade bars* indicate 72-week treatment. *y.o.* Years old, *Plt* platelets. $^{*}p = 0.014$, $^{\dagger}p = 0.045$, $^{\ddagger}p = 0.001$, $^{\#}p = 0.027$, $^{\P}p = 0.018$ compared to 48-week treatment

treatment (48, 11, and 9%, respectively) (Fig. 2). Regardless of the timing of the HCV disappearance, the SVR rate was raised among the patients with 72-week treatment, and the timing of the HCV RNA disappearance showed a strong correlation with SVR among the patients with 72-week treatment ($p = 0.01$). We also assessed the association of the SVR rate according to ribavirin adherence and the timing of HCV RNA disappearance in LVR patients with each treatment duration (Table 4). Ribavirin adherence was distributed in two categories by mean value (ribavirin throughout the treatment, 9.5 mg/kg/day). Among the patients with 48-week treatment, the SVR rates of patients with higher doses of ribavirin (more than 9.5 mg/kg/day) was slightly higher than that of patients with lower doses of ribavirin (less than 9.5 mg/kg/day) in each of categories of timing of HCV RNA disappearance, but the difference was not significant. However, among the patients given less than 9.5 mg/kg/day, the SVR rate increased significantly in patients with 72-week treatment, compared with 48-week treatment, in patients with

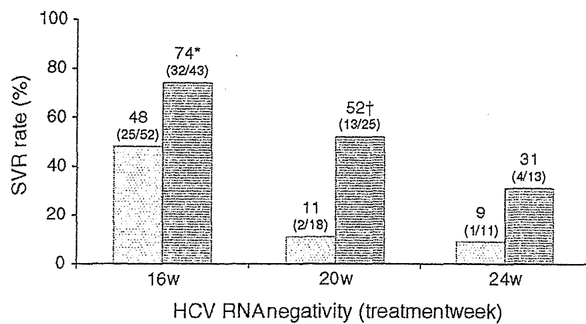

Fig. 2 SVR rate according to timing of hepatitis C virus (*HCV*) RNA negativity. *Light gray shade bars* indicate 48-week treatment. *Dark gray shade bars* indicate 72-week treatment. $*p = 0.012$, $^{\dagger}p = 0.009$, compared to 48-week treatment

Table 4 SVR rate according to timing of HCV RNA negativity and ribavirin adherence

	The timing of HCV RNA disappearance		
	16 weeks	20 weeks	24 weeks
Ribavirin <9.5 mg/kg/day			
48-week treatment	43% (9/21)	0% (0/7)	0% (0/6)
72-week treatment	75% (15/20)	47% (7/15)	25% (1/4)
p value	<0.05	0.05	0.40
Ribavirin ≥9.5 mg/kg/day			
48-week treatment	52% (16/31)	18% (2/11)	20% (1/5)
72-week treatment	74% (17/23)	60% (6/10)	33% (3/9)
p value	0.10	0.08	1.00

undetectable HCV RNA at week 16 (72 weeks, 75% vs. 48 weeks, 43%, $p < 0.05$), and increased with marginal significance in patients with undetectable HCV RNA at week 20 (72 weeks, 47% vs. 48 weeks, 0%, $p = 0.05$). Among the patients with undetectable HCV RNA at week 24, a significant difference was not observed because of the small number of patients in this category. For the patients given more than 9.5 mg/kg/day, the SVR rate with 72-week treatment tended to be higher than that with 48-week treatment, although the patient number was too small to reveal a benefit of extended treatment. As indicated above, the efficacy in patients with LVR given lower doses of ribavirin (less than 9.5 mg/kg/day) could be improved not by an increase in ribavirin dosage, but only by a longer treatment duration, irrespective of the category of timing of HCV RNA disappearance.

Discussion

In order to raise the SVR in patients with HCV genotype 1 treated with Peg-IFN plus ribavirin combination therapy, two strategies are possible: one is the use of a higher dose of drugs and the other is a longer duration of therapy. With respect to drug dose, we have reported that Peg-IFN is dose-dependently correlated with EVR, and ribavirin is dose-dependently correlated with relapse in patients with an EVR [17, 18]. On the other hand, among patients with an LVR, maintaining a high dose of ribavirin (>12 mg/kg/day average dose) did not lead to sufficient reduction of the relapse rate [18]. Thus, the SVR rate in patients with an LVR cannot be improved by a dose-increase strategy, and another treatment strategy, a longer duration of therapy, needs to be devised for patients with LVR in order to reduce the relapse rate.

Past studies have reported that extended therapy reduced the relapse rate. However, more consideration is needed to determine which group of patients can attain the desired effect by extended therapy. Eradication of serum HCV RNA is difficult in female or aged patients or patients with advanced liver fibrosis or a lower Plt count [17, 25], and these patients are considered to be mostly those with an LVR. Previously, we reported that patients more than 65 years old with an LVR showed a low SVR rate [25]. Therefore, in the present study, we tried to identify the group of patients for whom the SVR rate could be improved by extended therapy.

The factors associated with SVR in patients with extended therapy were evaluated by univariate and multivariate logistic regression analyses in the present study. As a result, the timing of HCV RNA disappearance was found to be a significant factor affecting SVR. This suggests that the earlier HCV RNA disappeared, the greater the SVR rate for 72-week treatment as well as 48-week treatment. Examination of the impact of ribavirin exposure on the SVR rate in patients with an LVR showed that, even if a high dose of ribavirin were given, the SVR rate did not show a significant increase among the patients with 48-week treatment, as previously reported [18]. However, the present study showed that an increase in the SVR rate was attained among the patients with 72-week treatment in each category of the timing of HCV RNA disappearance, especially in patients with lower doses of ribavirin. A similar result was found on stratified analysis for the timing of HCV RNA disappearance and Peg-IFN adherence (data not shown). That finding indicated that extend treatment is an effective strategy for LVR patients to increase the SVR rates, although the drug doses of Peg-IFN and ribavirin have been reported to affect the SVR rates in patients with an EVR. And the better efficacy of extended treatment was revealed to be limited to only those patients with earlier HCV RNA disappearance; they are good candidates for extended therapy. Further study is needed to determine whether more extended therapy; for example, 96-week treatment, would be effective for patients with later HCV RNA disappearance.

In the group with extended therapy in the present study, the Hb level at baseline was also significantly associated with SVR. We examined the relationship between Hb level at baseline and age and sex. The mean Hb levels at baseline according to age and sex were highest among male patients less than 65 years old (mean Hb, g/dl, male less than 65 years old; 15.2 ± 1.3, male more than 65 years old; 14.5 ± 0.9, female less than 65 years old; 13.5 ± 1.0, female more than 65 years old; 13.4 ± 1.0). The factors of age and sex were not selected as significant by multivariate analysis, but the Hb level at baseline did affect the SVR rate according to age and sex. In fact, among the patients with 72-week treatment in this study, the SVR rate among male patients less than 65 years old tended to be higher (84%, 21/25) than that of male patients more than 65 years old (60%, 6/10, $p = 0.19$), female patients less than 65 years old (45%, 13/29, $p < 0.01$), and female patients more than 65 years old (53%, 9/17, $p < 0.05$).

In this study, stratified analysis according to baseline factors revealed that extended therapy significantly improved the anti-viral effect, irrespective of age, sex, and Plt value. Especially, 48 weeks of standard treatment was insufficient for an anti-viral effect in aged or female patients, while extended therapy could significantly raise the SVR rate. It is of special clinical significance that extended therapy was found to be beneficial for aged patients, many of whom show an LVR. While the efficacy of extended therapy for patients with advanced liver fibrosis could not be proven in this study, it is conceivable that extended therapy could significantly raise the SVR rate in patients with a lower Plt value, which is indicative of advanced fibrosis. Further study is needed to clarify the efficacy of extended therapy for patients with advanced liver fibrosis.

The main limitation of this study is that it was not designed for randomization, and the treatment duration for patients with an LVR was decided by their physicians. Therefore, older female patients with more advanced liver fibrosis, for whom a poor treatment outcome was expected, tended to be treated for a longer period (72 weeks). However, considering the usefulness of extended therapy for patients with LVR reported in studies from the United States and Europe, there was an ethical issue against conducting an RCT in Japan which would have distributed the patients with an LVR into standard or extended therapy groups. Accordingly, we conducted a case-control study matched for age and sex, in order to compare the efficacy of 72-week treatment with that of 48-week treatment. Because it is known to be difficult to treat aged and female patients with HCV genotype 1 [25], these two factors of age and sex were chosen for minimal matching. As a result, the proportion of patients with advanced liver fibrosis (METAVIR fibrosis score 3 or 4) was not compensated for, and the selected patients in the 72-week treatment group included more patients with advanced liver fibrosis (who are difficult to treat) than the selected patients in the 48-week treatment group. Nevertheless, a higher SVR rate was obtained in the 72-week treatment group in comparison with 48-week treatment.

Recently, genetic polymorphism near the IL28B gene has been reported to be associated with the anti-viral effect of Peg-IFN plus ribavirin combination therapy [26–28]. Single-nucleotide polymorphisms (SNPs) of the IL28B gene are related to on-treatment response (rapid virologic response [RVR], EVR) and SVR [29]. However, no significant difference was observed for relapse after treatment between the major and minor types of IL28B SNPs, if HCV RNA disappeared at the same timing of the treatment [29]. Therefore, the same result as that in the present study may have been attained if the factors of IL28B SNPs had been included as evaluable factors. Further study is needed to examine the issue of the involvement of IL28B SNPs in the efficacy of 72-week Peg-IFN plus ribavirin therapy in patients with LVR.

In conclusion, our results have demonstrated that extended therapy for patients with LVR infected with HCV genotype 1 improved the SVR rate in all categories of patients, even for aged patients with an LVR. The timing of HCV RNA disappearance in patients with an LVR was a predictive factor for SVR and this suggests that response-guided therapy may be needed for later responders.

Acknowledgments Other institutions and participants in the Osaka Liver Forum are: Osaka General Medical Center, A Inoue; Higashiosaka City Central Hospital, S Iio; Itami City Hospital, Y Saji; Toyonaka Municipal Hospital, M Inada; Yao Municipal Hospital, H Fukui; Otemae Hospital, Y Doi; Osaka Medical Center for Cancer and Cardiovascular Diseases, K Katayama; Ashiya Municipal Hospital, T Kitada; National Hospital Organization Minami Wakayama Medical Center, K Fujimoto; Nishinomiya Municipal Central Hospital, H Ogawa; Saiseikai Senri Hospital, K Suzuki; Izumiotsu Municipal Hospital, S Yamagata; Osaka Kaisei Hospital, N Imaizumi; Kano General Hospital, S Kubota; Saso Hospital, M Nishiuchi; and Meiwa Hospital, Y Hayakawa. This work was supported by a Grant-in-Aid for Research on Hepatitis and BSE from the Ministry of Health, Labour and Welfare of Japan, and Scientific Research from the Ministry of Education, Science, and Culture of Japan. All authors have no financial relationships relevant to this study.

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J Gastroenterol (2011) 46:1020–1030
DOI 10.1007/s00535-011-0408-8

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

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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Received: 19 October 2010 / Accepted: 29 March 2011 / Published online: 11 May 2011
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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 cytolysis, 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 cytolysis, 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 Cytofix/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% CO_2 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 CD 56^{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

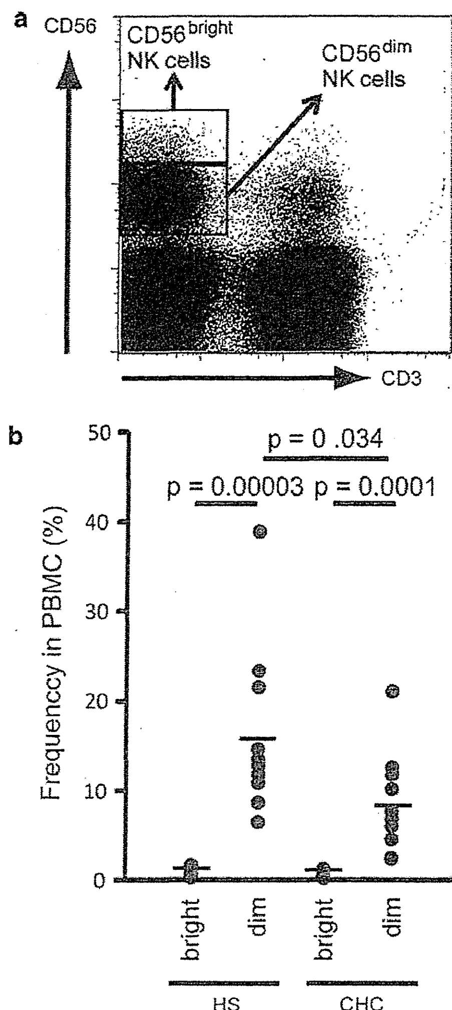

Fig. 1 Frequency of natural killer (*NK*) cell subsets in peripheral blood mononuclear cells (*PBMCs*). Frequencies of $CD56^{bright}$ NK and $CD56^{dim}$ NK cell subsets in PBMCs were evaluated by flow cytometry. PBMCs were obtained from patients with chronic hepatitis C virus (HCV) infection (*CHC*) ($n = 9$) 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 ± SD; 35.0 ± 26.8% in $CD56^{bright}$ subset and 28.7 ± 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 CD 56^{dim} NK cells phosphorylated both STAT4 and STAT1 in both subject groups (Fig. 4a). Statistical analysis, however, demonstrated that

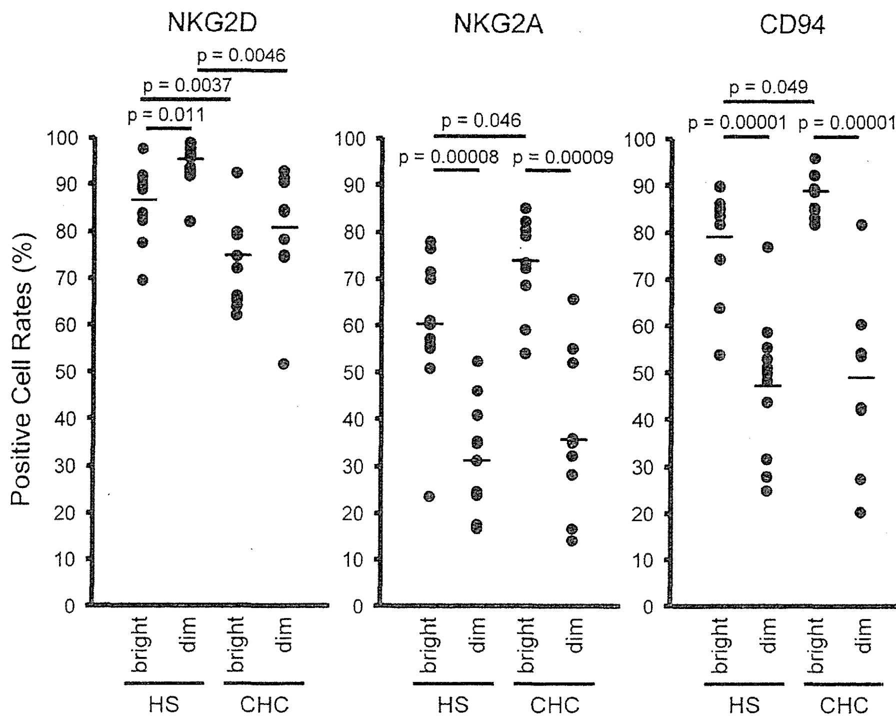

Fig. 2 NK receptor expression on NK cell subsets. The expression of NK activating or inhibitory receptors, NKG2D or NKG2A and CD94, respectively, on CD56^{bright} NK cell subset (bright) and CD56^{dim} NK cell subset (dim) was evaluated by flow cytometry with isotype control staining, electronically gating on CD56 bright CD3^{-} cells and CD56 dim CD3^{-} cells. PBMCs were derived from patients with chronic HCV infection (CHC) ($n = 9$) and healthy subjects (HS) ($n = 11$). Positive cells (positive cell rate) were determined based on isotype control staining. Comparisons of those NK receptor expression levels between bright and dim subsets in a subject group or between CHC and HS in a subset are shown as positive cell rates 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

the phosphorylation level of STAT4 occurring in response to IFN-α in the CHC patients was significantly lower than that in the HS in both CD56^{bright} NK cells and CD56^{dim} NK cells (Fig. 4b). On the other hand, the phosphorylation level of STAT1 occurring in response to IFN-α in the CHC patients was significantly greater than that in the HS in both CD56^{bright} NK cells and CD56^{dim} NK cells.

We next examined the relationship between STAT1 phosphorylation and STAT4 phosphorylation occurring in response to IFN-α in the NK cell subsets. Upon stimulation with IFN-α, the whole population of CD56^{bright} NK cells phosphorylated both STAT1 and STAT4, while some of the CD56^{dim} NK cells more strongly phosphorylated STAT1 but more weakly phosphorylated STAT4, compared with the remaining CD56^{dim} NK cells, which more weakly phosphorylated STAT1 but more strongly phosphorylated STAT4 (Fig. 5a). Moreover, the frequency of the 'high-pSTAT1 population' in response to IFN-α in CD56^{dim} NK cells in the CHC patient group was significantly greater than that in the HS group (Fig. 5a, b).

Regulation of NK receptor expression level on CD56^{bright} NK cells or CD56^{dim} NK cells occurring in response to IFN-α-based therapy in vivo

To examine whether CD56^{bright} NK cells and CD56^{dim} NK cells would respond differently to IFN-α treatment in vivo, we evaluated the frequency, the expression level of NK receptors, and the STAT1 expression level in CD56^{bright} NK cells and CD56^{dim} NK cells before and after the initiation of IFN-α-based therapy. The frequency of CD56^{bright} NK cells or CD56^{dim} NK cells did not show any significant change between before and 1 day after initiation of the therapy (data not shown). The expression levels of NKG2A/CD94 on both CD56^{bright} NK cells and CD56^{dim} NK cells were significantly decreased in response to the therapy 1 day after its initiation (Fig. 6). On the other hand, the expression level of NKG2D on CD56^{bright} NK cells or CD56^{dim} NK cells did not show any significant change between before and 1 day after initiation of the therapy. The STAT1 expression levels in both CD56^{bright} NK cells and CD56^{dim} NK cells were significantly increased in response to the therapy (data not shown).

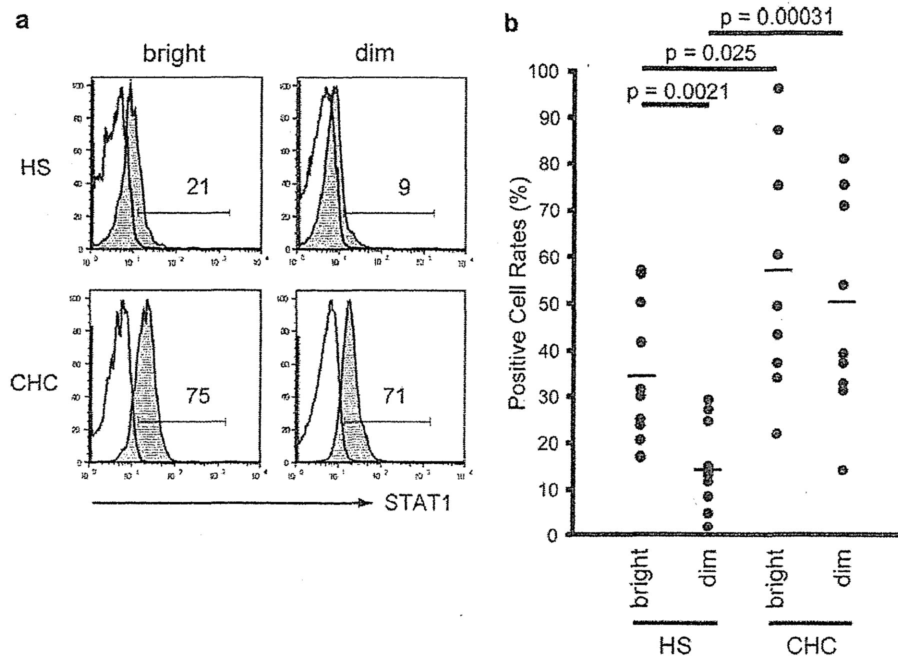

Fig. 3 Signal transducer and activator of transcription 1 (*STAT1*) expression in NK cell subsets. Intracellular STAT1 expression levels in CD56^{bright} NK cell subset (bright) and CD56^{dim} NK cell subset (dim) were evaluated by flow cytometry with isotype control staining, electronically gating on CD56 bright CD3^{-} cells and CD56 dim CD3^{-} cells. PBMCs were derived from patients with chronic HCV infection (CHC) ($n = 9$) and healthy subjects (HS) ($n = 11$). **a** Representative histograms from a patient and a healthy subject (*HS*) are shown. *Dotted lines* show staining with the isotype control. *Thick lines* with *shaded areas* show staining with the antibody. Numbers are percentages of positive cells (positive cell rate) determined based on isotype control staining. **b** Comparisons of STAT1 expression level between bright and dim subsets in a subject group or between CHC and HS in a subset are shown as positive cell rates 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

Discussion

In the present study, we found clear differences between CD56^{bright} NK cells and CD56^{dim} NK cells in their responses to cytokines, as well as the cell frequency and the surface expression level of the NK receptors. We also found some differences between these subsets in the alteration caused by chronic HCV infection. Of interest and novelty are the findings that the NK cell subsets displayed different intracellular STAT1 expression levels (Fig. 3) and responded differently to cytokine stimulation to lead to differences in the phosphorylation of STAT1/4 (Figs. 4, 5) and that some of the differences were altered in the CHC patients. Furthermore, both subsets showed alterations of IFN-α signaling in the CHC patients, compared with the HS (Fig. 4).

We have recently shown that NK cells from patients with CHC display a higher level of STAT1 expression than those from HS [24] and suggested that the up-regulation of STAT1 expression might result from a host response to HCV infection with IFN-α and/or IFN-γ production, because STAT1 itself is one of the IFN-stimulated genes (ISGs) whose expression is up-regulated by IFN-α or IFN-γ [27, 28], which has been reported to be detected in the sera of patients with CHC [29, 30]. The present study has shown that both NK cell subsets from the patients with CHC displayed a higher level of STAT1 expression than those from the HS (Fig. 3b); this might also have been induced similarly in both subsets by a host response to HCV infection. Since a host response to HCV infection would be associated with the liver inflammation and subsequent fibrosis, we examined whether the STAT1 expression level in these NK cell subsets could be correlated with the level of liver inflammation or fibrosis which had been histologically evaluated using liver biopsy samples. Although no significant correlation was observed between the STAT1 expression level in the CD56^{bright} NK cells or CD56^{dim} NK cells and the level of liver inflammation or fibrosis, there was a tendency of a higher level of inflammation or fibrosis being correlated with a higher level of STAT1 expression in NK cells, including CD56^{bright} and CD56^{dim} subsets, in our limited number of patients (T. Miyagi et al. unpublished data); further investigation should be done with a larger number of subjects. Another question that emerged was whether our findings in peripheral blood could be applied to the liver in CHC patients. Chen et al. [31]

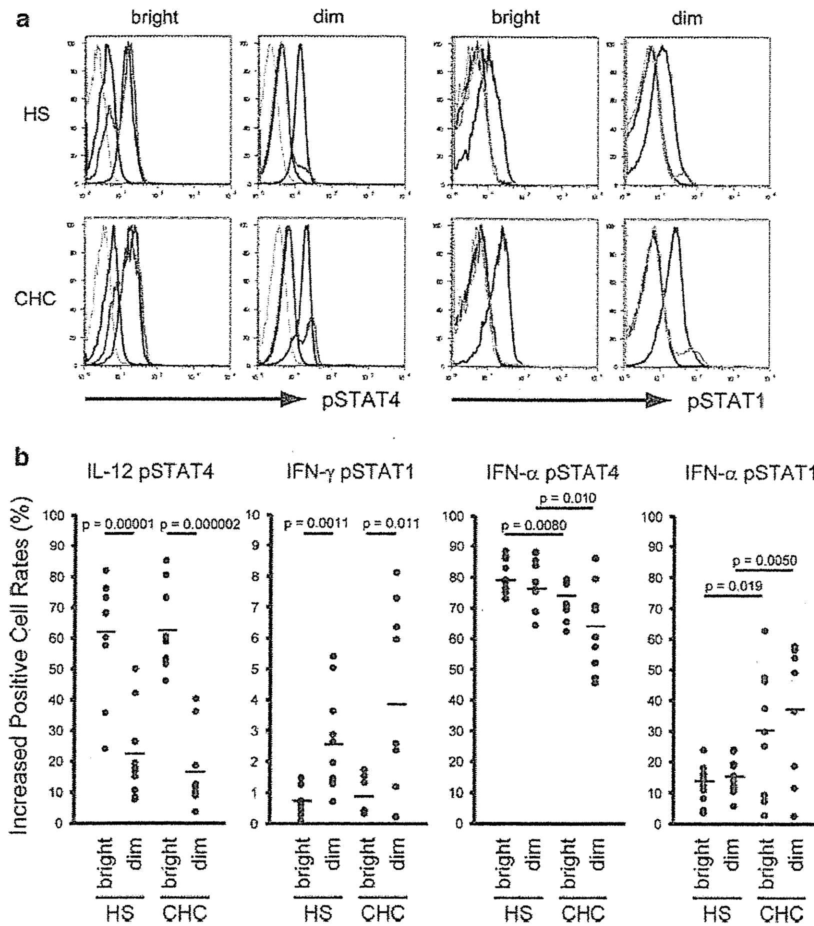

Fig. 4 Activation of STAT1/4 occurring in response to interleukin-12 (*IL-12*), interferon-γ (*IFN-γ*), or IFN-α in NK cell subsets. Phosphorylated STAT1 (*pSTAT1*) and pSTAT4 protein levels were evaluated by flow cytometry with isotype control staining. PBMCs were derived from patients with chronic HCV infection (CHC) ($n = 9$) and healthy subjects (HS) ($n = 11$). Prepared PBMCs were unstimulated or stimulated with natural IFN-α, IFN-γ, or IL-12 for 90 min in vitro, and then collected. pSTAT1 and pSTAT4 protein levels in CD56^{bright} NK (bright) and CD56^{dim} NK (dim) cell subsets were evaluated by flow cytometry, electronically gating on CD56 bright CD3^{-} cells and CD56 dim CD3^{-} cells. **a** Representative histograms of a patient and a healthy subject (*HS*) are shown. *Green lines* show staining of IFN-α-stimulated cells with isotype control. *Purple lines* show staining of unstimulated cells with the antibody. *Red*, *orange*, and *blue lines* show staining of IL-12-, IFN-γ- and IFN-α-stimulated cells, respectively, with the antibody. **b** Positive cell rates were determined based on staining with isotype controls. Increased positive cell rates were determined by subtracting the positive cell rate of unstimulated cells from those of stimulated cells. Comparisons of pSTAT1/4 level in response to IFN-α, IFN-γ, or IL-12 between bright and dim subsets in a subject group or between CHC and HS in a subset are shown as increased pSTAT1/4 positive cell rate with the statistically significant p values. Each *circle* represents individual data. *Horizontal bars* represent means. Statistical significance was analyzed using the unpaired Student's t-test

reported that the hepatic gene expression level in a subset of ISGs, including STAT1, was greater in CHC patients than in normal subjects. Sarasin-Filipowicz et al. [32] showed that the gene expression level in a subset of ISGs in CHC patients was greater in whole liver, including hepatocytes and nonparenchymal cells such as lymphocytes, than in PBMC, and suggested that chronic HCV infection had stronger local effects on the IFN system in the liver than in PBMC. Also, Tateno et al. [33] showed that the gene expression level of STAT1 in liver-infiltrating lymphocytes was about twofold greater than that in hepatocytes in CHC patients. Considering these reports, we speculate that the NK cell subsets in the liver as well as in the peripheral blood of CHC patients might display a high level of STAT1 expression. Whether our findings in peripheral blood could be applied to the liver in CHC patients requires further investigation. We also examined whether our findings with CHC patients would be observed in CHB patients. Unlike in the CHC patients, the CHB patient expression levels of STAT1 in either CD56^{bright} or CD56^{dim} subsets was not significantly higher than that in the HS, which would be consistent with the report of the