development of fulminant hepatitis.[7,8] CTLs can kill target cells using two distinct lytic pathways: the degranulation pathway, in which perforin is used to puncture the membranes of infected cells, and the Fas-based pathway, in which the interaction between Fas ligand (FasL) expressed on cytolytic lymphocytes and Fas on target cells triggers apoptosis and target cell death.[9] However, the role of innate immune cells, especially natural killer (NK) cells, in fulminant hepatitis remains obscure. NK cells have recently been reported to contribute to the pathogenesis of human hepatitis and animal models of liver injury.[10,11] Replication of HBV is host cell dependent, and the study of cellular immune response in hepatitis B has long been hampered by the lack of a small animal model that supports the replication of HBV and elimination of infected cells by immune response. Before the advent of human hepatocyte chimeric mice,[12,13] only chimpanzees had been used as a model for HBV infection and inflammation, although fulminant hepatitis B (FHB) had never been reported, and severe liver inflammation is rare in chimpanzees.[14] We previously established an HBV-infection animal model using chimeric mice, in which the livers were extensively repopulated with human hepatocytes.[15-17] In this study, we attempted to establish an animal model of HBV-infected human hepatocytes with human immunity by transplanting human peripheral mononuclear cells (PBMCs) to HBV-infected human hepatocyte chimeric mice.

Materials and Methods

Generation of Human Hepatocyte Chimeric Mice. Generation of the urokinase-type plasminogen activator $(uPA)^{+/+}$/severe combined immunodeficiency $(SCID)^{+/+}$ mice and transplantation of human hepatocytes with human leukocyte antigen (HLA)-A0201 were performed as described previously.[15,16] All mice were transplanted with frozen human hepatocytes obtained from the same donor. Infection, extraction of serum samples, and euthanasia were performed under ether anesthesia. Concentration of human albumin, which is correlated with the repopulation index,[15] was measured in mice as described previously.[16] All animal protocols described in this study were performed in accord with the *Guide for the Care and Use of Laboratory Animals* and the local committee for animal experiments, and the experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of the Graduate School of Biomedical Sciences at Hiroshima University (Hiroshima, Japan).

Human Serum Samples. Human serum samples, containing high titers of genotype C HBV DNA (5.3×10^6 copies/mL), were obtained from patients with chronic hepatitis who provided written informed consent. Individual serum samples were divided into aliquots and stored in liquid nitrogen. Six weeks after hepatocyte transplantation, chimeric mice were injected intravenously with 50 μL of HBV-positive human serum.

Analysis of HBV. DNA was extracted using SMITEST (Genome Science Laboratories, Tokyo, Japan) and dissolved in 20 μL of H_2O. HBV DNA was measured by real-time polymerase chain reaction (PCR) using a light cycler (Roche, Mannheim, Germany). Primers used for amplification were 5′-TTTGGGCATGGACATTGAC-3′ and 5′-GGTGA ACAATGTTCCGGAGAC-3′. Amplification conditions included initial denaturation at 95°C for 10 minutes, followed by 45 cycles of denaturation at 95°C for 15 seconds, annealing at 58°C for 5 seconds, and extension at 72°C for 6 seconds. The lower detection limit of this assay was 300 copies.

Preparation of Human Blood Mononuclear Cells and Transplantation of Human PBMCs Into Human Hepatocyte Chimeric Mice. PBMCs were isolated from healthy blood donors with HLA-A0201 and successfully vaccinated with recombinant yeast-derived hepatitis B surface antigen (HBsAg) vaccine (Bimmugen; Chemo-Sero Therapeutic Institute, Kumamoto, Japan) using Ficoll-Hypaque density gradient centrifugation. Neither monocytes nor macrophages were observed in the isolated PBMCs (Supporting Fig. 1). PBMCs isolated from 3 healthy, unvaccinated blood donors were also transplanted. Eight weeks after HBV inoculation, human PBMCs were transplanted into human hepatocyte chimeric mice. To deplete mouse NK cells and prevent the elimination of human PBMCs from human hepatocyte

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chimeric mice, 200 μL of phosphate-buffered saline, containing 120 μL of anti–ganglio-N-tetraosylceramide (asialo GM1) antibody (Wako, Osaka, Japan), were administered intraperitoneally (IP) 1 day before (day 0; Fig. 1) the initial IP transplantation (day 1) of human PBMC. Then, 10 μL/g of liposome-encapsulated clodronate (Sigma-Aldrich, St. Louis, MO) were also administered 4 days before PBMC transplantation (day −2) to deplete mouse macrophages and DC cells. The second PBMC administration (4×10^7 cells/mouse) was performed 2 days after the initial administration (day 3).

To assess the effect of the depletion of human DC, NK, or CD8-positive CTL cells from administered PBMCs on hepatitis formation, the BD IMag separation system (BD Biosciences, Franklin Lakes, NJ) was used. Alternatively, mice were treated with an IP administration of clodronate, as described above, 1 day before PBMC transplantation.

To analyze the effect of inhibition of the Fas/FasL system, IFN-γ, IFN-α, antihuman FasL monoclonal antibody (mAb) (1.5 mg/mouse; R&D Systems, Minneapolis, MN), antihuman IFN-γ mAb (1.5 mg/mouse; R&D Systems), and antihuman IFN-α mAb (1.5 mg/mouse; PBL Biomedical Laboratories, Piscataway, NJ) were injected 1 day before transplantation of human PBMCs.

Flow Cytometry. Reconstructed human PBMC proliferation in mice was determined by flow cytometry with the following mAbs used for PBMC surface staining: allophycocyanin (APC)-H7 antihuman CD3 (clone SK7); APC-conjugated anti-CD4 (clone SK); BD Horizon V450 antihuman CD8 (clone RPA-T8); APC-conjugated antihuman CD11c (clone B-ly6); HU HRZN V500 MAB-conjugated antihuman CD45 (clone H130); Alexa Fluor 488–conjugated antihuman CD56 (clone B159); PerCP-Cy5.5 antihuman CD123 (clone 7G3); fluorescein isothiocyanate–conjugated Lineage cocktail 1 (Lin-1) (anti-CD3, CD14, CD16, CD19, CD20, and CD56); APC-H7 antihuman HLA-DR (clone L243); phycoerythrin (PE)-conjugated antihuman FasL (clone NOK-1); and biotin-conjugated antimouse H-2D^b (clone KH95). The biotinylated mAbs were visualized using PE-Cy7-streptavidin. Each of the above mAbs were purchased from BD Biosciences. PE-conjugated HBV core-derived immunodominant CTL epitope (HBcAg93)[18] (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan). Dead cells identified by light scatter and propidium iodide staining were excluded from the analysis. Flow cytometry was performed using a FACSAria II flow cytometer (BD Biosciences), and results were analyzed with FlowJo software (Tree Star, Inc., Ashland, OR).

DCs can be classified into two main subsets: plasmacytoid DCs (pDCs) and myeloid DCs (mDCs).[19,20] pDCs were defined as CD45$^+$Lin-1$^-$HLA-DR$^+$CD123$^+$ cells, whereas mDCs were defined as CD45$^+$ Lin-1$^-$HLA-DR$^+$CD11c$^+$ cells.

Histochemical Analysis of Mouse Liver and Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling Assay. Histochemical analysis and immunohistochemical staining using an antibody against human serum albumin (HSA; Bethyl Laboratories, Inc., Montgomery, TX), an antibody against hepatitis B core antigen (HBcAg) (Dako Diagnostika, Hamburg, Germany) and antibody against Fas (BD Biosciences, Tokyo, Japan) were performed as described previously.[16] Immunoreactive materials were visualized using a streptavidin-biotin staining kit (Histofine SAB-PO kit; Nichirei, Tokyo, Japan) and diainobenzidine. For the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay in sliced tissues, we used an *in situ* cell death detection kit (POD; Roche Diagnostics Japan, Tokyo, Japan).

Dissection of Mouse Livers and Isolation of RNA and Measurement of Messenger RNAs of Fas by Reverse-Transcription PCR. Mice were sacrificed by anesthesia with diethyl ether, and livers were excised, dissected into small sections, and then snap-frozen in liquid nitrogen. Total RNA was extracted from cell lines using the RNeasy Mini Kit (Qiagen, Valencia, CA). One microgram of each RNA sample was reverse transcribed with ReverseTra Ace (Toyobo Co., Tokyo, Japan) and Random Primer (Takara Bio Inc., Kyoto, Japan). We analyzed the messenger RNA (mRNA) levels of Fas by reverse-transcription PCR, as previously reported, using Fas forward primer 5′-GGGCATCTGGACCCTCCTA-3′ and Fas reverse primer 5′- GGCATTAACACTTTTGGACGATAA-3′.

Statistical Analysis. mRNA expression levels of Fas and interferon-stimulated genes (ISGs) were compared using Mann-Whitney's U test and unpaired *t* tests. A *P* value less than 0.05 was considered statistically significant.

Results

Establishment of an Animal Model of Fulminant Hepatitis Using HBV-Infected Human Hepatocyte Chimeric Mice and Human PBMC Transplantation. Administration of 2×10^7 PBMCs twice after suppression of mice NK cells by anti–asialo GM1 antibody[21] and macrophages and DCs by liposome-encapsulated clodronate[22] before transplantation

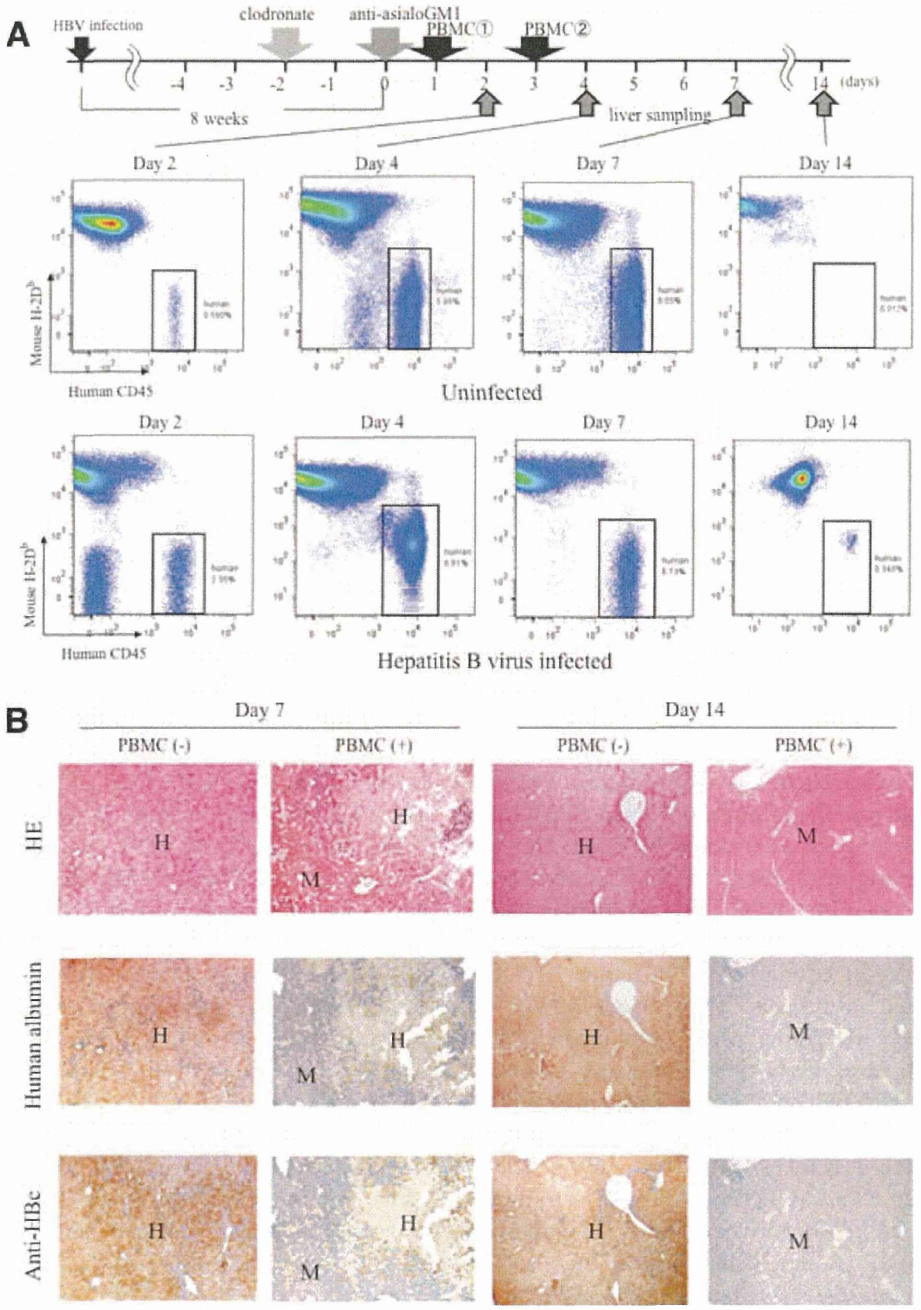

Fig. 1. Establishment of human PBMC chimerism in human hepatocyte chimeric mice. (A) Experimental protocol to establish chimerism and liver sampling is shown at the top of the figure (see Materials and Methods). Scheduling of administration of HBV-positive serum, clodronate, and anti-asialo GM1 antibody and liver sampling by scarification are shown by arrows. Liver mononuclear cells isolated from uninfected (upper panel) and HBV-infected (lower panel) human hepatocyte chimeric mice transplanted with human PBMCs were separated with antibodies for human CD45 and mouse H-2D^b and were analyzed by flow cytometry. Percentage of human mononuclear cells is shown in each panel. Representative figures of two experiments with similar results are shown. (B) Histological analysis of livers of HBV-infected mice. Liver samples obtained from mice with or without human PBMCs at weeks 9 (day 7) and 10 (day 14) were stained with hematoxylin and eosin staining (HE), anti-human albumin antibody, or anti-hepatitis B core antibody. Regions are shown as human (H) and mouse (M) hepatocytes, respectively (original magnification, 40×). (C) Time course of human albumin concentration (upper panel) and HBV DNA titer (lower panel) in mouse serum. Time course of 4 HBV-infected mice transplanted with human PBMCs, 3 HBV-infected mice without human PBMC transplantation, and 4 uninfected mice transplanted with human PBMC are shown. (D) Time course of human albumin concentration (upper panel) and HBV DNA titer (lower panel) in mice. Mice with or without HBV-infection were transplanted with PBMCs obtained from 3 healthy donors who were not vaccinated against hepatitis B.

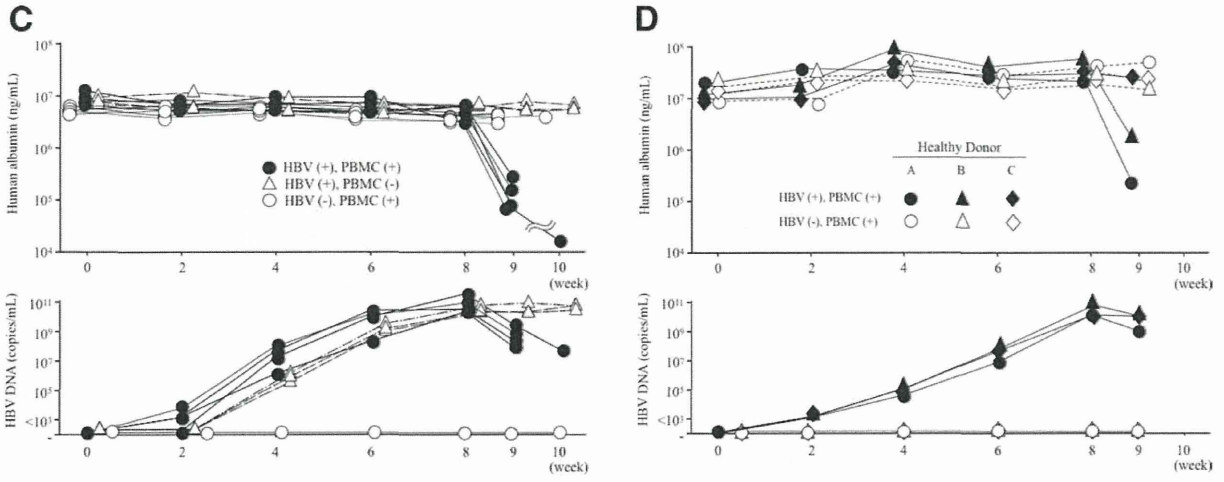

Fig. 1.

enabled us to establish a human PBMC chimerism in uPA-SCID mice. We observed an up to 7% human mononuclear cell chimerism among the liver-resident mononuclear cells of uninfected and HBV-infected mice 2-14 days after the initial injection of PBMC (Fig. 1A; Table 1). Chimerism was most prominent 4 days after initial PBMC administration and almost undetectable by day 14 (Fig. 1A). Histological examination of chimeric mice livers showed extensive human liver cell death, comparable to the massive liver cell death observed in fulminant hepatitis, only in HBV-infected and PBMC-treated mice liver (Fig. 1B). Human hepatocytes were almost completely eliminated and replaced by human albumin-negative mouse hepatocytes at days 7 and 14. Consistent with these histological changes, we observed a rapid decline of HSA levels and HBV DNA only in HBV-infected and PBMC-treated mice (Fig. 1C). The decline of mice HSA levels and HBV DNA was also observed in 2 of 3 HBV-infected mice transplanted with PBMCs isolated from healthy blood donors without HBsAg vaccination (Fig. 1D and Supporting Fig. 2).

Analysis of Liver-Infiltrating Human Lymphocytes Necessary to Establish Massive Hepatocyte Degeneration. We then analyzed liver-infiltrating cells with flow cytometry. Unexpectedly, we did not detect CD8-positive and tetramer-positive CTLs, as reported previously (Fig. 2A). Instead, we observed substantial numbers of CD3-negative and CD56-positive NK cells (Fig. 2B) and small numbers of pDCs and mDCs (Fig. 2C). The majority of NK cells of HBV-infected mice were FasL positive (Fig. 2D). In contrast, such FasL-positive NK cells were not detected in uninfected

Table 1. Analysis of Liver-Infiltrating Cells by Flow Cytometry

	HBV Infected				Uninfected			
Day	No.	Chimerism (%)	Human NK (%)	Fas (+) NK (%)	No.	Chimerism (%)	Human NK (%)	FasL (+) NK (%)
2	1	1.77	2.51	0	1	0.59	12.8	0
	2	2.35	3.02	0.143	2	0.774	58.8	1.1
4	3	6.81	30.7	80.1	3	5.95	42.7	0.678
	4	1.08	68.7	94.7	4	7.11	4.98	0.027
	5	6.60	23.2	58.7	5	5.02	23.1	0.314
7	6	6.73	13.2	0.383	6	6.55	42.1	0.103
	7	5.70	12.5	2.01	7	1.24	13.6	0.025
	8	1.46	3.83	0	8	2.04	1.49	4.03
14	9	0.34	ND	ND	9	0.012	ND	ND
	10	NA*	NA	NA	10	0.013	ND	ND
DCs depleted day 4	11	4.77	5	2.14	11	3.32	4.21	0.465
(by clodronate)	12	1.27	39.5	2.3	12	12.9	9.06	0
DCs depleted day 7	13	2.42	24.8	2.19	13	6.31	54.1	0.131
(by clodronate)	14	1.41	10.6	0.103	14	4.69	1.68	0.12

Abbreviations: NA, not analyzed; ND, not detectable.
*Mouse died just before liver analysis.

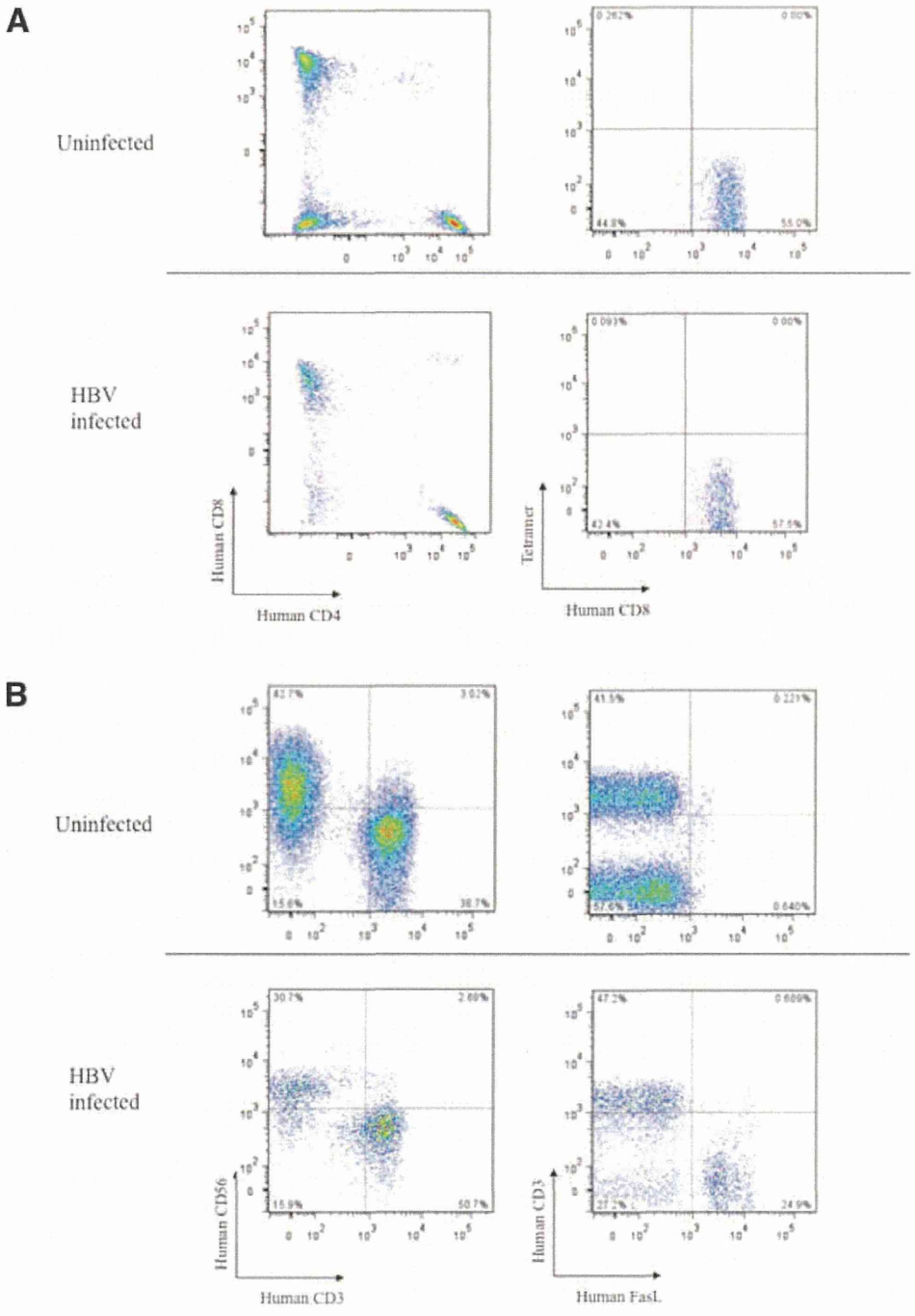

Fig. 2. Analysis of mononuclear cells isolated from day 4 chimeric mouse livers. After defining human PBMCs as mouse H-2Db-human CD45^{+} cells, we further analyzed the phenotypes of these cells. (A-C) Liver mononuclear cells of uninfected (upper panel) and HBV-infected (lower panel) mice transplanted with human PBMCs were separated with anti-human CD4 and CD8 antibody or anti-human CD8 and HLA-A2 HBcAg tetramer (A), anti-human CD3and CD56 or human CD3 and FasL (B), and anti-human HLA-DR and CD123 and HLA-DR and CD11c (C). (D) Frequency of FasL-positive cells in NK cells were analyzed in uninfected and HBV-infected mice. All figures are representative of two experiments with similar results.

mice livers (Table 1; Fig. 2D), suggesting that these NK cells were activated in HBV-infected mice. These activated NK cells and DCs were detectable in mice livers only 4 days after the initial PBMC injection, but were undetectable after 2 and 7 days (Supporting Figs. 3 and 4, respectively).

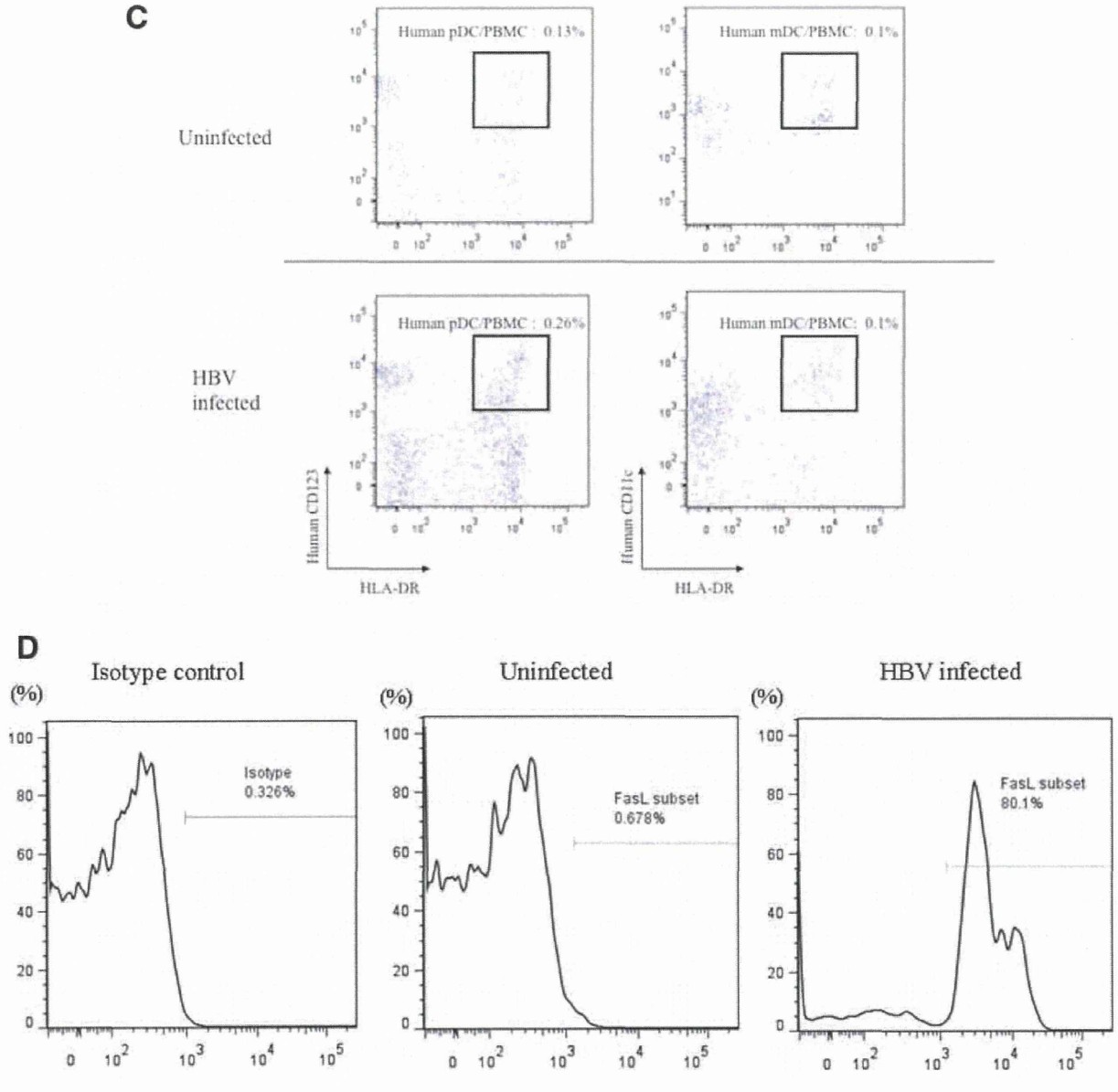

Fig. 2.

Effect of DC Depletion on Establishment of Massive Hepatocyte Degeneration. To confirm the necessity of both DCs and NK cells to complete hepatocyte destruction, we depleted DCs or NK cells with negative selection using antibody-coated magnetic beads before the administration of PBMC. Depletion of either DCs or NK cells completely abolished the decline of human albumin as well as HBV DNA (Supporting Fig. 5A). However, analysis of liver-infiltrating cells revealed that chimerism with human PBMC was poorly established in these animals, probably the result of the loss or damage of human cells by bound antibodies during separation and/or subsequent incubation in mice (Supporting Fig. 5B; Supporting Table 1).

To overcome possible confounding resulting from poor chimerism resulting in poor human hepatocyte degeneration in mice, we attempted to remove DCs from transplanted human PBMCs by alternate means. We attempted to deplete human DCs by administering clodronate 1 day before PBMC transplantation, because we thought that clodronate remaining in the mouse body would impair transplanted human DCs. As expected, we observed an almost complete elimination of DCs by this procedure without impairing

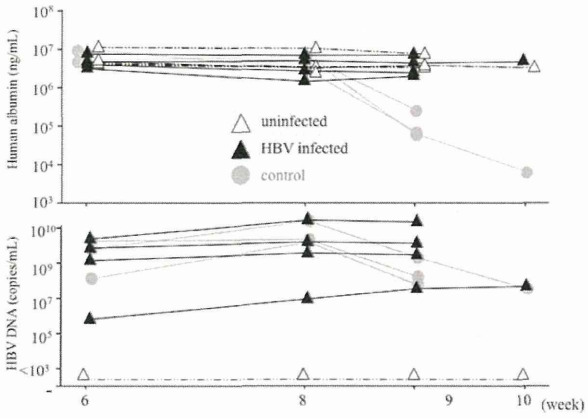

Fig. 3. Time course of mice transplanted with human PBMCs with DC depletion by clodronate 1 day before transplantation. Mice were treated with IP administration of clodronate 1 day before human PBMC transplantation. Time courses of human albumin concentration (upper panel) and HBV DNA titer (lower panel) in mouse serum are shown. Open and closed triangles correspond to 3 uninfected and 4 HBV-infected mice, respectively. Time courses of 3 mice infected with HBV and transplanted with human PBMC 3 days before transplantation (see Fig. 1C) are shown for comparison (shaded closed circle).

PBMC chimerism (Supporting Figs. 6A and 7A; Supporting Table 1). Activation of NK cells was not observed in this setting (Supporting Figs. 6B and 7B; Supporting Table 1). Depletion of DCs completely abolished the decline of both human albumin and HBV DNA (Fig. 3). Histological examination showed that hepatocyte degeneration was absent, and that there were no TUNEL-staining–positive cells (data not shown). Clodronate lyposomes may also nonspecifically deplete macrophages and monocytes in addition to DCs, but no monocytes or macrophages were observed when transplanted PBMCs were analyzed using Ficoll-Hypaque density gradient centrifugation, indicating that the clodronate administration was specifically associated with DC depletion in this study.

Analysis of Fas/FasL System in Massive HBV-Infected Hepatocyte Degeneration Model. We then assessed the importance of the Fas/FasL system and the occurrence of apoptosis in NK-cell–mediated human hepatocyte degeneration. Only HBV-infected human hepatocytes positive for HSA were positive for Fas antibody staining (Fig. 4A). TUNEL staining was also positive only in mice infected with HBV and inoculated with PBMCs (days 4 and 7). Measurement of mRNA levels in infected and uninfected livers showed that expression levels of Fas mRNA increased significantly upon HBV infection (Fig. 4B). To confirm that apoptosis of human hepatocytes was mediated by the Fas/FasL pathway and to determine whether IFN-α or IFN-γ played a role in the establishment of liver cell degeneration, we administered a blocking mAb against FasL, IFN-α, and IFN-γ 1 day before PBMC transplantation. Treatment of mice with antibody against FasL before PBMC completely abolished the decline of human albumin and HBV DNA (Fig. 5A). This abolishment of human albumin decline in mouse serum suggests that the Fas/FasL pathway almost exclusively eliminated infected hepatocytes in this model, which also suggests that Fas-mediated apoptosis could play an important role in FHB. Antibodies against IFN-α and IFN-γ inhibited IFN-induced ISG expression in mice livers (Supporting Fig. 8); however, these antibodies did not disturb the decline of HSA levels (Fig. 5A) and histological inflammation (Fig. 5B). Contact-dependent and -independent activation of NK cells by DCs has been reported previously.[23-25] Although IFN-α and IFN-γ play a role in their activation,[23,25,26] our results indicate that the effects of IFN-α are almost negligible in our experiments (Fig. 5A), suggesting that direct contact among these cells, or cytokines other than IFN-α and IFN-γ, are necessary to activate NK cells in this setting. NK cells have also been reported to exert antiviral effects by secreting IFN-γ. However, our results suggest that this mechanism does not work well in our model (Fig. 5A).

Discussion

In this study, we established a small animal model in which massive hepatocyte degeneration similar to FHB in humans is observed. Our initial attempts to detect human PBMCs in blood or any organ in transplanted mice failed even after injecting 2×10^7 cells, which is sufficient to establish human PBMC chimerism in SCID mice.[27] We assumed that failure to develop chimerism was the result of the activity of NK cells and macrophages because the activity of these cells in uPA-SCID mice is higher than in SCID mice.[28,29] Therefore, we attempted to eliminate these effects by administering clodronate and anti–asialo GM1 antibody, which are known to effectively eliminate these cells.[30,31] This assumption appears to be valid, because we were able to establish human PBMC chimerism and massive hepatocyte degeneration by suppressing these cells (Fig. 1).

HBV-specific CTLs have been reported to play an important role in eliminating the virus.[32-34] Accordingly, we attempted to detect HBV-specific CTLs in mice with massive hepatocyte degeneration. Unexpectedly, we failed to detect HBV-specific CTLs (Fig. 2A and Supporting Fig. 9) and instead found that infiltrating cells in the liver were CD3-negative NK cells

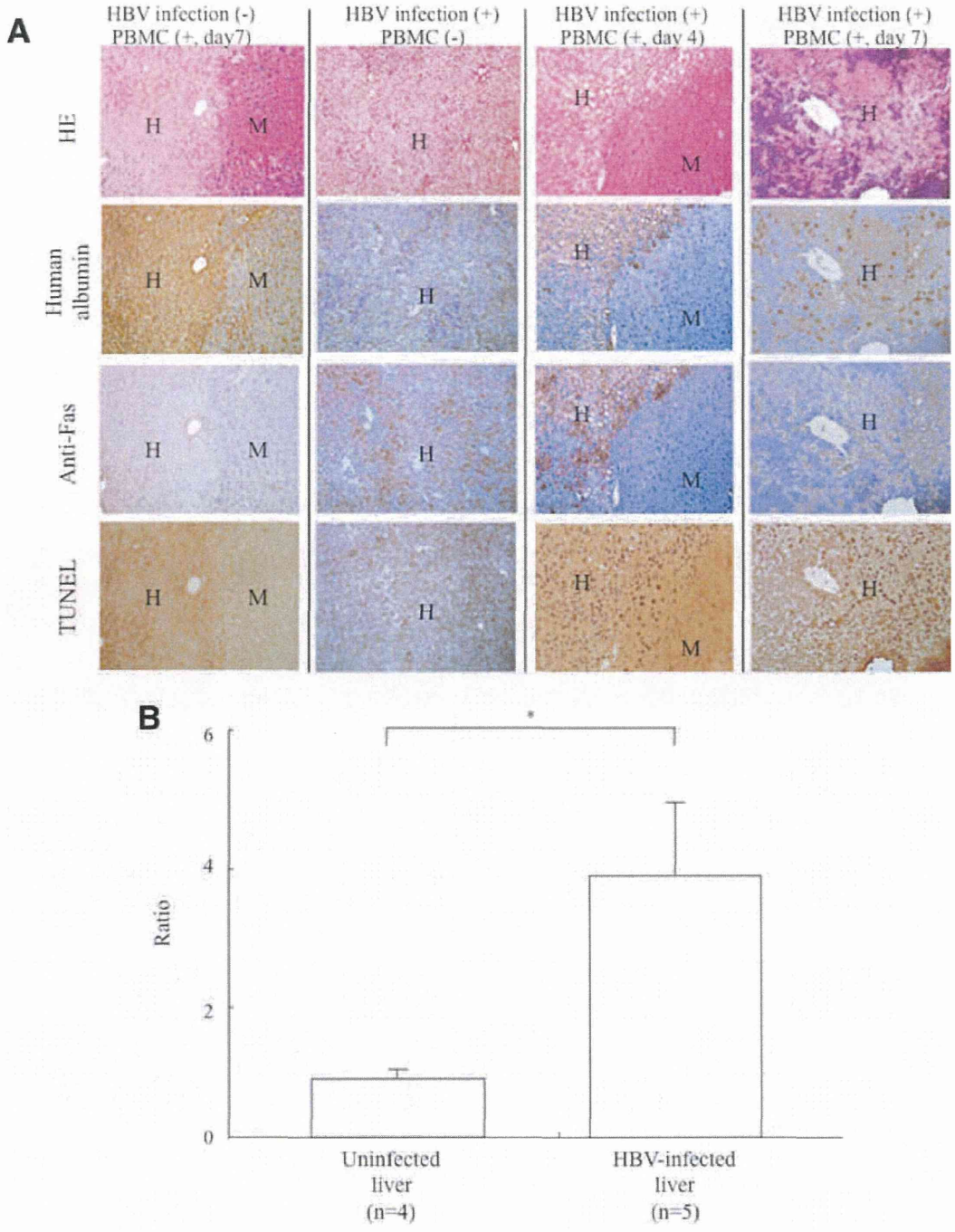

Fig. 4. Assessment of Fas expression in the liver in human hepatocyte chimeric mice. (A) Histological analysis of chimeric mice livers transplanted with human PBMCs but without HBV infection (day 7), with HBV infection but without PBMC transplantation, and with HBV infection and PBMC transplantation at days 4 and 7. Liver samples were stained with hematoxylin and eosin staining (HE), anti-human albumin antibody, anti-human Fas antibody, and TUNEL staining. Regions are shown as human (H) and mouse (M) hepatocytes, respectively (original magnification, 100×). Note that Fas antigen was expressed only in HBV-infected human hepatocytes, and TUNEL staining is only positive for HBV-infected and human PBMC-transplanted mice livers. Mouse hepatocytes were negative for all three stains. (B) Expression of Fas mRNA levels in uninfected and HBV-infected human hepatocytes. Data are represented as mean ± standard deviation. $*P < 0.001$.

(Fig. 2B,D and Supporting Fig. 10). The reason for the absence of CTLs in our experiment is unknown, but this suggests that massive hepatocyte degeneration resembling fulminant hepatitis can be caused by NK cells as a main player, and recent reports demonstrating that NK cells contribute to severe acute and

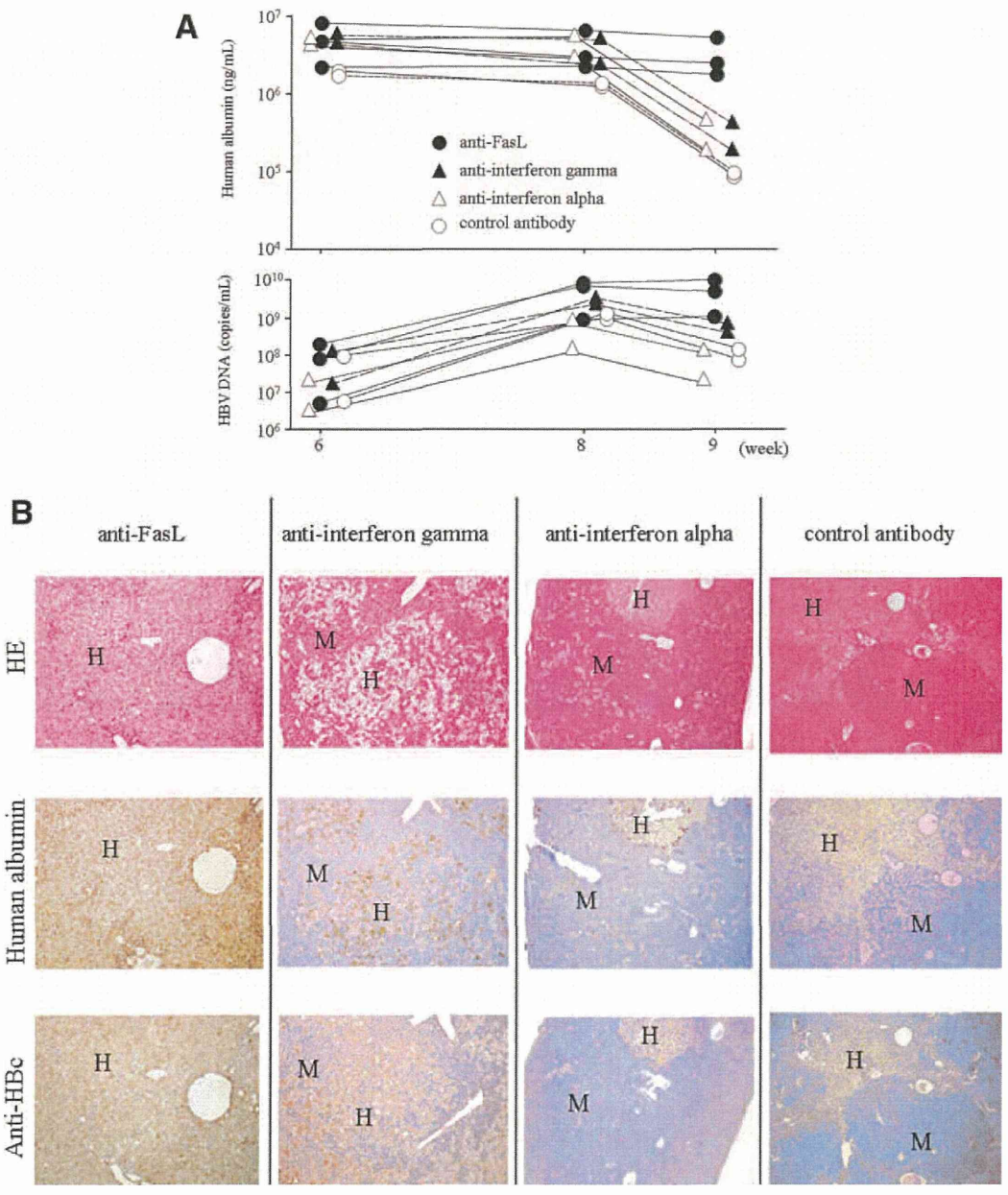

Fig. 5. Effect of anti-FasL, anti-IFN-γ and anti-IFN-α antibody administration on HSA and HBV DNA. (A) Time courses of HSA (upper panel) and HBV DNA (lower panel) before and 1 week after human PBMC transplantation are shown. Mice were pretreated with antibodies against human Fas-L, IFN-γ, and IFN-α before PBMC transplantation, as described in Materials and Methods. Isotype antibody was used as a control. (B) Histological analysis of livers of HBV-infected mice injected with anti-human FasL mAb, IFN-γ, IFN-α, and control antibody. Liver samples obtained from mice with human PBMCs at weeks 9 (day 7) were stained with hematoxylin and eosin staining (HE), antihuman albumin antibody, or antihepatitis B core antibody. Regions are shown as human (H) and mouse (M) hepatocytes, respectively (original magnification, 40×).

chronic hepatitis B (CHB) support this assertion.[11,35] We attempted to collect CTLs from HBV-infected patients and to establish hepatitis in chimeric mice. However, we rarely detected tetramer-positive CTLs in blood samples from chronically infected patients and were therefore unable to establish hepatitis using CD8-positive T cells. Consequently, a limitation of this study is that differential roles of NK cells and CTLs in massive liver cell death could not be examined.

Although it is not clear in this study how profoundly DC and NK cell activity plays a role in patients with FHB, our results suggest that the immune system can trigger severe hepatocyte

degeneration. The importance of the activation of NK cells by DCs was evident, because depletion of DCs almost completely abolished the massive hepatocyte degeneration in this model (Supporting Fig. 10; Table 1). The interaction between NK cells and DCs is not well characterized, although it has been established that antigen-presenting accessory cells provide both indirect (i.e., soluble) and direct (i.e., contact-dependent) signals to T cells. Experiments in which NK cells are separated from pathogens and antigen-presenting cells by semipermeable membranes are cultured with supernatants from pathogen-activated DCs or in which cytokines are neutralized with blocking antibodies. These reports indicate that both soluble and contact-dependent signals may contribute to the activation of NK cells.[23,25,26]

The importance of the Fas/FasL system in hepatocyte damage in acute and chronic HBV infection has been reported previously.[37,38] However, the extent to which this system plays a role in human hepatitis B, especially fulminant hepatitis, is unknown. As shown in this study (Fig. 5A), inhibition of the Fas/FasL system by anti-Fas antibody dramatically reduced the effect of human PBMC transplantation. This showed the possibility that the Fas/FasL system plays an important role in the degeneration of infected hepatocytes in FHB. Further studies should be conducted to evaluate what immunological responses play important roles in human hepatitis B.

The importance of NK-cell activity suggests that the suppression of DCs and NK-cell activity or the Fas/FasL system might have therapeutic implications for FHB.[11,35] If DCs and NK-cell activity or Fas/FasL activity could be controlled in the early stages of severe acute or fulminant hepatitis, we might be able to control hepatitis activity and prevent subsequent liver failure. Of course, it would be necessary to monitor the development of chronic hepatitis after such treatment because DCs and NK cells contribute to early host defenses and shape subsequent adaptive immune response through complex cross-talk regulating the early phase of the immune response.[19,24,39,40]

We analyzed liver damage using HBV genotype C–infected mice in this study. However, HBV genotype C is associated with more severe histological liver damage than genotype B,[41] and future studies should compare immunological differences between genotypes B and C.

In summary, we established an animal model of FHB using highly repopulated human hepatocyte chimeric mice and transplanted human PBMCs. Modifications of this model will facilitate further research into acute and CHB using human immune cells, including HBV-directed CTL clones, suppressor and regulatory T cells, as well as immunological experiments to study interactions between DCs and NK cells. Such models may be useful to develop and evaluate new therapeutic strategies against HBV infection.

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Case Report

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Achievement of Sustained Viral Response after Switching Treatment from Pegylated Interferon α-2b to α-2a and Ribavirin in Patients with Recurrence of Hepatitis C Virus Genotype 1 Infection after Liver Transplantation: A Case Report

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Key Words
PEG-IFN α-2a · PEG-IFN α-2b · *IL28B* · Hepatitis C virus · Liver transplantation

Abstract
We report a case in which sustained viral response was achieved after switching treatment from pegylated interferon (PEG-IFN) α-2b to α-2a and ribavirin (RBV) in patients with recurrence of hepatitis C virus (HCV) infection after living donor liver transplantation. The patient was a 62-year-old man with liver cirrhosis due to HCV genotype 1b infection. The patient had 8 amino acid (aa) substitutions in the interferon sensitivity-determining region, and had substitutions for mutant and wild-type at aa70 and aa91, respectively, in the core region. The patient had minor genotype (GG) *IL28B* single nucleotide polymorphisms (rs8099917). He had initially received interferon α-2b and RBV for 2 years, and later developed hepatocellular carcinoma (HCC). After surgical resection of HCC, he subsequently received PEG-IFN α-2b and RBV for 1.5 years, without undetectable viremia during the treatment course. Due to recurrence of HCC, the patient received a living donor liver transplantation. Later on, hepatitis C relapsed. For the management of relapse, he received another course of PEG-IFN α-2b and RBV. However, breakthrough viremia occurred. PEG-IFN was thus switched from α-2b to α-2a and RBV for another 17 months. The patient eventually achieved a sustained viral response.

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Introduction

Currently, pegylated interferon (PEG-IFN) α and ribavirin (RBV) are used as standard therapy for the treatment of patients with hepatitis C virus (HCV) infection; successful outcomes with PEG-IFN and RBV have been achieved in approximately 60% of the treated cases [1]. However, about 50% of the patients treated with this therapy have been reported to show an increase in the viral load and/or serum alanine aminotransferase (ALT) level during therapy [2, 3]. The event of increase in the viral load is called 'breakthrough viremia'. No treatment regimens have been established for patients who develop breakthrough viremia during treatment with or relapse following PEG-IFN and RBV. In some reports, PEG-IFN α-2a and RBV has been reported to result in a higher sustained viral response (SVR) than that achieved by PEG-IFN α-2b and RBV [4]. Moreover, PEG-IFN α-2a and RBV was reported to be effective for treatment of some HCV patients who experienced relapse after PEG-IFN α-2b and RBV [5]. Although there are reports about interferon (IFN) therapy in patients with HCV genotype 1 infection after liver transplantation (LT) [6–8], there are no reported cases where SVR was achieved by switching treatment with PEG-IFN from α-2b to α-2a and RBV in patients with HCV genotype 1 infection after LT.

We report a case in whom SVR was achieved after switching treatment from PEG-IFN α-2b to α-2a and RBV in a patient with recurrence of HCV genotype 1 infection after LT.

Case Report

The patient was a 62-year-old man with liver cirrhosis due to HCV genotype 1b infection. The patient's height was 168 cm, weight 70.4 kg, and body mass index 24.9.

HCV RNA was 1,200 kIU/ml. The patient had undergone IFN therapy with conventional IFN α-2b (6 MU) plus RBV (800 mg) for 24 months since 2002. He was administered IFN α-2b (6 MU) for thrombopenia, but the therapy was stopped since he showed no response to the therapy.

The patient developed hepatocellular carcinoma (HCC) and was treated by hepatic resection and had stage F3 fibrosis in September 2005. After that, IFN therapy was started with PEG-IFN α-2b (60 μg) and RBV (200 mg) in December 2005. At that time, HCV RNA was 2,400 kIU/ml. However, RBV was stopped since the patient developed itching. However, HCV RNA never reached undetectable levels. After that, HCC recurred. Therefore, the patient underwent splenectomy, and hepatectomy for HCC recurrence in August 2006. At the time, the patient showed stage F3 fibrosis. After that, IFN therapy was restarted.

Table 1. Laboratory data at the start of IFN therapy after LT

CBC	
WBC/μl	3,160
RBC/μl	4.50×10^6
Hb, g/dl	13.0
Ht, %	37.4
Plt/μl	257×10^3
Blood coagulation test	
PT, %	118
Blood chemistry	
T-bil, mg/dl	2.4
AST, IU/l	89
ALT, IU/l	45
LDH, IU/l	269
ALP, IU/l	497
γGTP, IU/l	377
TP, g/dl	6.9
Alb, g/dl	3.1
TC, mg/dl	129
TTT, U	5
ZTT, U	12
BUN, mg/dl	13
Cr, mg/dl	1.17
CRP, mg/dl	<0.2
FBS, mg/dl	267
HbA_{1c}, %	6.6
NH_3, μg/ml	47
Tumor marker	
AFP, ng/ml	27.1
HCV virus marker	
HCV RNA, kIU/ml	27,000
MELD score	6
Child-Pugh	A
aa substitution in ISDR	eight
aa70 in the core region	mutant
aa91 in the core region	wild
IL28B, genotype	GG

AFP = α-Fetoprotein; Alb = albumin; ALP = alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate aminotransferase; Cr = creatinine; CRP = C-reactive protein; FBS = fasting blood sugar level; Hb = hemoglobin; LDH = lactate dehydrogenase; Plt = platelets; PT = prothrombin time; RBC = red blood cells; T-bil = total bilirubin; TC = total cholesterol; TTT = thymol turbidity test; WBC = white blood cells; ZTT = zinc sulfate turbidity test; aa substitution in ISDR = amino acid substitutions in the IFN sensitivity-determining region.

Tumor stage was stage III [9]. Treatment with curative intent was not possible owing to the presence of multiple HCC lesions. Although the MELD score was 6 and Child-Pugh score A, his sister wished to be the donor for LT; LT was performed with informed consent in June 2007. At the time, the patient showed stage F4 fibrosis.

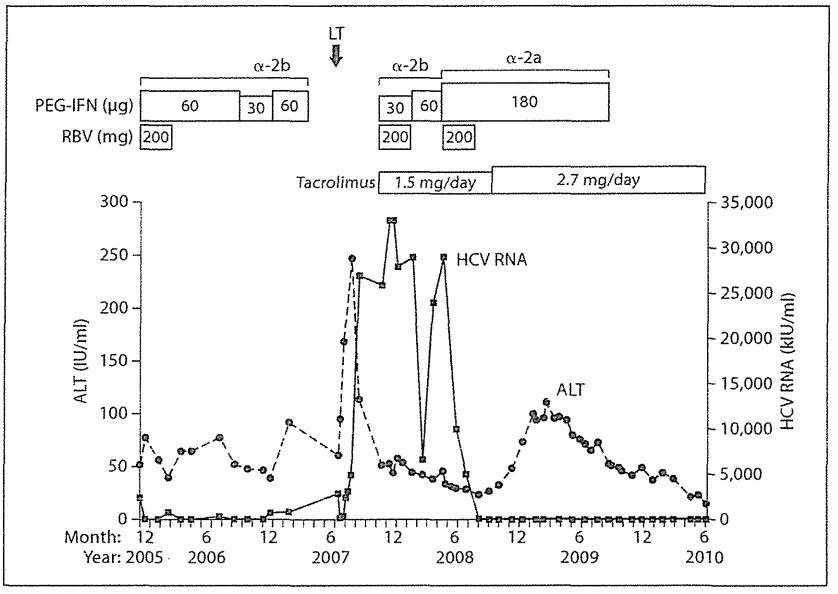

Fig. 1. Course of IFN therapy.

IFN therapy was restarted at 4 months after living donor LT in November 2007. Laboratory test data at the start of IFN therapy after LT are shown in table 1. His platelet count was 257 × 10^3/μl and ALT level 45 IU/l. Eight amino acid (aa) substitutions were detected in the IFN sensitivity-determining region (ISDR), and substitutions for mutant and wild-type were detected at aa70 and aa91, respectively, in the core region. The patient had minor genotype (GG) *IL28B* single nucleotide polymorphisms (SNPs) (rs8099917).

He was treated with PEG-IFN α-2b (60 μg) and RBV (200 mg). However, RBV administration was stopped since the patient developed itching. Although HCV RNA had decreased from 29,000 to 6,700 kIU/ml, it re-increased from 6,700 to 24,000 kIU/ml. Therefore, PEG-IFN α-2b and RBV was switched to PEG-IFN α-2a and RBV in April 2008. As a result, he was treated with PEG-IFN α-2b for 5 months.

In September 2008, 5 months after PEG-IFN α-2a and RBV, the serum HCV RNA titer became undetectable. PEG-IFN α-2a and RBV was continued until September 2009 for 12 months after the serum HCV RNA titer became undetectable, according to our protocol [7]. PEG-IFN α-2a was administered for a total of 17 months. Immunosuppressive therapy, tacrolimus 1.5 mg/day, was used at the start of IFN therapy in April 2008. Because ALT was elevated in October 2008, the dose of tacrolimus was raised up to 2.7 mg/day. As a result, ALT became normal. Finally, SVR was achieved (fig. 1).

Discussion

Recent studies have shown that various hosts and viral factors are significant predictors of the efficacy of IFN treatment. With regard to the viral factors, the number of aa substitutions in the ISDR correlated with the SVR rate in patients with HCV genotype 1b infection who underwent IFN therapy [10, 11]. Akuta et al. [12–16] reported that the substitutions at aa70 and/or aa91 in the HCV core region are independent and significant predictors of virological responses such as SVR and non-viral response to combination therapy. Our patient had 8 aa substitutions in the ISDR and substitutions for mutant and wild-type at aa70 and aa91, respectively, in the core region.

Recently, Fukuhara et al. [17] reported that mutations of the HCV core and ISDR of HCV genome were associated with the SVR rates in 50 patients. On the other hand, we reported that mutations of the HCV core and ISDR of HCV genome were not associated with the SVR rates in our previous study [7]. It was already known that IFN monotherapy for 24 weeks is enough to eradicate HCV RNA in the case of acute hepatitis C [18–20]. There was no report that HCV core mutant and substitution of aa of the ISDR region affect the SVR rate in the cases of acute hepatitis C. Since recurrence of hepatitis C in LT is thought to be another acute hepatitis C, we concluded that the mutations of the HCV core and ISDR of HCV genome do not affect the SVR rate.

Furthermore, the effect of PEG-IFN and RBV in patients with HCV genotype 1b infection is associated with several SNPs at the *IL28B* locus [21–24]. This patient had minor genotype (GG) *IL28B* SNPs (rs8099917). Recently, Fukuhara et al. [25] reported that the SVR rate

is 10–20% on minor genotype (GG) *IL28B* SNPs (rs8099917) in HCV recipients. Furthermore, Lange et al. [26] reported that the donor's *IL28B* rather than the recipient's *IL28B* affects the SVR rate. In this case the donor was his sister. Although the donor's SNP was not checked, there was a very small possibility that the sister's SNP was a major genotype (TT), since the recipient's parents had the necessary G allele in one allele of two alleles respectively.

Breakthrough viremia can be attributed to a variety of reasons. One possible cause is the development of antibodies against PEG-IFN α-2b. Vallbracht et al. [27] first reported the development of neutralizing immunoglobulin-G antibodies against natural human fibroblast IFN in a patient treated with the said IFN in 1981. Furthermore, several studies have reported neutralizing anti-IFN antibodies due to administration of IFN [28–35]. Achievement of a complete SVR in patients with HCV infection by switching the previously administered IFN with another has been reported in several studies [2, 5, 36]. Therefore, we think this patient achieved SVR by switching treatment with PEG-IFN from α-2b to α-2a. The other possible cause for the occurrence of breakthrough viremia is the generation of HCV escape mutants during IFN therapy [37]. In addition, downregulation of specific IFN cell receptors due to IFN therapy may also be a cause of breakthrough viremia [38]. Another possible cause is that the dosage of RBV was suboptimal. RBV was stopped due to itching. A further reason might be that the initial dose of PEG-IFN α-2b was insufficient. The dose of PEG-IFN α-2b was intentionally administrated at 30 μg because of the patient's general fatigue, and then the PEG-IFN dose was elevated to 60 μg. The PEG-IFN dose was going to be increased, however breakthrough viremia occurred. Therefore, we switched PEG-IFN from α-2b to α-2a and RBV.

In summary, we have reported a male patient in whom SVR was achieved by switching treatment with PEG-IFN from α-2b to α-2a and RBV for recurrence of HCV genotype 1 infection after LT. Switching an originally administered IFN with another type may be effective for the treatment of patients with HCV infection after LT.

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ORIGINAL ARTICLE

Reliability and validity of a new living liver donor quality of life scale

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Abstract
Purpose To develop a living liver donor (LLD) quality of life (QOL) scale and test its reliability and validity.
Methods We sent a draft questionnaire comprising 38 questions to 965 LLDs from five hospitals. To evaluate test–retest reliability, the questionnaire was re-sent 2 weeks later to some of the donors from one hospital.
Results Of the 447 (54.5 %) donors who responded, 15 were excluded. Factor analysis of 26 items extracted 7 subscales; namely, damage from the operation, scarring, satisfaction, burden, after-effects, digestive symptoms, and lack of understanding of donor health. We analyzed construct validity on the basis of factor analysis and observed significant correlations among the seven subscales. Criterion-related validity was confirmed by significant correlation with the 36-item Short-Form Health Survey scores. None of the subscales showed unreasonable values. We evaluated the subscale reliability for internal consistency ($\alpha = 0.670$–0.868, except for "digestive symptoms", $\alpha = 0.431$) and test–retest reliability ($r = 0.749$–0.918). The factor "digestive symptoms" needs careful consideration because of low internal consistency.
Conclusion The findings of this study confirmed the reliability and validity of the LLD QOL scale, which can be used for quantitatively evaluating the QOL of LLDs.

Keywords Living liver donor · Quality of life · Liver transplantation · Psychosomatic scale

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Introduction

Living donor liver transplantation (LDLT) began in Japan in 1989. By the end of 2009, 5653 transplantations had been performed, with more than 400 transplantations since 2001[1]. The outcomes of LDLT recipients have been studied well, but the physical and psychosocial effects of LDLT on donors also need evaluation, and quality of life (QOL) is a suitable index for this. Many QOL evaluations of living liver donors (LLDs) in different countries have been conducted using the 36-item Short Form Health Survey (SF-36) questionnaire, and the results indicate that the QOL of LLDs is equal to or better than that of similar individuals in the general population [2–8]. Longitudinal study results also show that although QOL is temporarily compromised postoperatively, it recovers to close to the preoperative levels after approximately 6 months [9] and remains relatively favorable over the long term [10, 11].

In contrast, the results of a survey of LLDs by the Japanese Liver Transplantation Society showed that in addition to symptoms such as tightness of the wound, only about 50 % felt that they had "recovered completely" 3 years after surgery [12]. Furthermore, according to a recent publication, biliary complications are the most common postoperative complication, with an incidence ranging from 0.4 to 11.1 % [13]. The SF-36 evaluates the subjective symptoms that greatly influence health or lifestyle, which might account for the variation in the results, with donors seeming to have better QOL even though they do have some symptoms. The results of this study suggest that LLDs are aware of the effects of the surgery until a number of years afterward, but this is not revealed by the score of the SF-36. Although these symptoms could be considered minor, it is important that their impact is understood more precisely, to evaluate the outcomes of hepatectomy for donors and to establish what medical assistance should be provided to them.

The scales normally used for QOL evaluation can be classified as either generic instruments, such as the SF-36, which are not restricted to patients with particular diseases, or disease-specific instruments, which examine the effects and symptoms of a disease and its treatment [14]. An advantage of generic instruments is that they can be used to compare subjects with members of the general population. On the other hand, disease-specific instruments have the advantage of being able to sensitively evaluate issues specific to patients with those diseases. At present, there is no specific instrument for measuring the QOL of LLDs. Problems with generic instruments have also been outlined in a review of the QOL of LLDs, which reinforces the necessity for a scale that measures the donor-specific aspects [15]. A scale created specifically for LLDs would allow us a more detailed understanding of the QOL of LLDs.

As a preliminary step in the development of a scale designed specifically for LLDs, we investigated and reported on the conceptual framework of the QOL of LLDs [16]. This resulted in the hypothesis that LLD QOL consists of seven elements: "scars", "digestive symptoms", "loss of organ", "postoperative damage", "lack of understanding of the donors' health", "burden of expense", and "satisfaction of decision making". However, this framework is nothing more than a qualitative descriptive analysis of the results of an interview survey, and it has not been statistically verified. Thus, we conducted a large-scale survey to verify whether the QOL elements hypothesized in our prior research could be supported statistically to develop a QOL scale for LLDs, in which questions are selected carefully, assuming that the scale would be put into practice, and to verify the scale's reliability and validity.

Methods

Subjects

The subjects of this study were 965 LLDs who underwent hepatectomy at least 1 month prior to inclusion, at one of the following five medical facilities: Osaka University Hospital, Kyushu University Hospital, Hokkaido University Hospital, Hiroshima University Hospital, or Tohoku University Hospital. We excluded ten people who had previously refused to participate in research or who were overseas residents.

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Questionnaire content

LLD-QOL scale drafts

Based on the results of semi-structured interviews of 20 LLDs and the conceptual framework provided by our previous research [16], we created original question items and constructed a draft QOL scale. Responses were assessed at five levels. Points (1–5) were assigned, with a higher numerical value indicating better QOL. We asked five LLDs to respond to this draft scale as a pretest and then asked them for their opinions. This led us to confirm the apparent validity of the scale, based on which we corrected and selected the 38 questions posed in the second draft of the LLD-QOL scale.

Japanese version SF-36v2™

The Japanese version SF-36v2™ was used to confirm the concurrent validity of the temporary LLD-QOL scale. The SF-36 is a generic instrument that measures QOL, and its reliability and validity have been verified. In Japan, SF-36v2™, which has been completely standardized, is used as the standard version [17]. It comprises eight subscales: "physical functioning", "role limitations because of physical problems", "body pain", "general health perceptions", "vitality", "social functioning", "role limitations because of emotional problems", and "mental health". Each subscale is assigned a score of 0–100 with higher scores indicating better QOL.

Attributes and background

We included questions that asked the subjects about their attributes, such as gender and age; and elements of their background relevant to their transplantation surgery, such as the year of liver donation surgery, the relationship of the donor to the recipient, the age of the recipient at the time of surgery, and the recipient's condition.

Method of data collection

Anonymous questionnaires which included the 38 questions were posted to all the subjects. To investigate test–retest reliability, the subjects from one facility, Osaka University Hospital, were requested to answer the same questionnaire again after an interval of 2 weeks. The second questionnaire included only the "Draft LLD-QOL Scale". Data were collected between February and June 2011.

Ethical considerations

To keep personal information confidential, questionnaires were posted to the subjects from the facility at which the transplantation surgery was performed. Participation was voluntary and subjects were not required to enter data that would reveal their identity. They were requested to return the questionnaires by post to a researcher not affiliated with the facility where they underwent the surgery. A brief description of the research was sent with the questionnaire, outlining the protection of privacy and explicitly stating that participation was voluntary. Response to the questionnaire was regarded as an indication of the subject's consent. This study was conducted with the approval of the clinical research ethics committee of Osaka University Hospital and the ethics committees of each facility involved in the study.

Analytical procedures

Missing values, averages of scores, and standard deviations for the 38 items on the temporary LLD-QOL scale were calculated, and inappropriate items were excluded. We also calculated the Spearman rank correlation coefficient between each pair of questions to identify unnecessary items. Next, we performed an exploratory factor analysis to carefully select questions for the LLD-QOL scale and to investigate the validity of the constructive concept. After calculating the subscale scores on the basis of the acquired factor structure, we calculated the Pearson product–moment correlation coefficient with the SF-36 subscales to compare two scales and investigate the criterion-related validity. For some subscales, we predicted that a difference because of the number of postoperative years, and to analyze these subscales, the subjects were divided into groups according to the year of their surgery. We then examined whether there was a significant difference. To investigate reliability, we calculated Cronbach's α-coefficient to assess the internal consistency of the subscales. We also calculated the Pearson product–moment correlation coefficient of subscale scores in the first and second questionnaires and used this as an index of the scale's test–retest reliability.

Statistical processing

SPSS19.0 was used for data analysis.

Results

Participant backgrounds

The domicile or address of 135 of a possible 955 subjects was unknown, so initial questionnaires were distributed to 820 subjects, and we received 447 responses, representing a response rate of 54.5 %. Second questionnaires were

distributed to 142 of 162 subjects, and we received 90 responses, representing a response rate of 63.4 %. Of these 90 subjects, 76 responded to both questionnaires, allowing us to compare the responses of the same subject. After the exclusion of 15 subjects whose questionnaires had many missing values, questionnaires from 432 subjects were included in the final analysis.

The 432 subjects in the final analysis comprised 216 men (50.0 %) and 210 women (48.6 %), and 6 who did not specify their gender (1.4 %). The mean age and standard deviation was 44.1 ± 12.4 years (19–75 years), and the median year of surgery was 2006 (range 1992–2011). The relationship of the recipient to the donor was their child for 122 (28.3 %), a parent for 151 (35.0 %), a spouse for 82 (19.0 %), a sibling for 52 (13.0 %), and other relationships for 18 (4.2 %), while 3 donors did not specify their relationship to the recipient (0.7 %). At the time of surgery, 96 (22.2 %) recipients were younger than 18 years of age, 332 (76.9 %) were 18 years or older, and 4 (0.9 %) donors did not specify the age of the recipient. Eighty-six donors (19.9 %) reported death of the recipient.

Exclusion of inappropriate questions

Investigation of missing values and response distribution

We excluded five questions for which the proportion of missing values exceeded 2 %, making them either too difficult to answer or inappropriate. One question was excluded for its ceiling effect. If the sum of the standard deviation and mean for a particular value exceeded the maximum score value of 5 considerably, we regarded it as having a ceiling effect. The number of questions was thereby reduced to 32.

Correlation coefficients between questions

We calculated the Spearman rank correlation coefficient between each pair of questions to identify duplicated question content. Three questions with a correlation coefficient of approximately 0.70 were examined for duplicated content and were considered to increase the redundancy of the scale. We removed two of these questions and retained one, leaving 30 questions.

Verification of validity

Verification of construct validity

We performed exploratory factor analysis to assess factor validity, by principal factor analysis and promax rotation of all 30 questions, and examined the results using eigenvalues and a scree plot as reference. We deleted values with a factor loading of less than 0.3 or those with low commonality after the extraction of factors, repeated the factor analysis, and excluded a total of four questions. We ultimately extracted seven factors and 26 questions and determined that they exhibited sufficient values, because their cumulative contribution ratio was 64.2 % and each factor had a factor loading of 0.3 or above (Table 1).

Five questions related to the first factor, "damage from the operation", signifying loss of appetite and modification of lifestyle to avoid pain and burden. Four questions related to the second factor, "scars", representing the problems and effects caused by the wound. Four questions related to the third factor, "satisfaction", concerning the person's sense of acceptance and satisfaction as a donor. Four questions related to the fourth factor, "burden", signifying somewhat negative feelings such as the inconvenience and burden (including financial) of being a donor. Four questions related to the fifth factor "after-effects", suggesting postoperative physical changes and changes in the subjects' overall feeling of well-being. Three questions related to the sixth factor, "digestive symptoms", and asked about heartburn, diarrhea, and constipation. Two questions related to the seventh factor, "lack of understanding of donor health", asking about the donors' perception of others around them.

The mean values of the items comprising each subscale were later analyzed as subscale scores. When we calculated the Pearson product–moment correlation coefficient between subscale scores, we found that "damage from the operation" was significantly correlated with "scars", "burden", "after-effects", and "digestive symptoms". There was a significant correlation between "satisfaction" and "lack of understanding of donor health", and there were no apparent inconsistencies in the concepts behind the names of the factors (Table 2). These results enabled us to confirm factor validity.

We also divided the subjects into groups to find out if there were any differences in the subscales predicted to exhibit changes related to the number of postoperative years. Using the median year of surgery as a reference, we divided the subjects into a long-term postoperative group (prior to 2006; $n = 184$) and a short-term postoperative group (2006 or later; $n = 244$) and conducted the Mann–Whitney test. We found significant differences in the factors "damage from operation" ($p < 0.01$) and "scars" ($p < 0.05$) with higher subscale scores for the long-term postoperative group than for the short-term postoperative group. We considered these results to confirm construct validity.

Verification of criterion-related validity (concurrent validity)

Calculation of the Spearman rank correlation coefficient for the LLD-QOL and SF-36 subscale scores revealed a