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Severe Necroinflammatory Reaction Caused by Natural Killer Cell-Mediated Fas/Fas Ligand Interaction and Dendritic Cells in Human Hepatocyte Chimeric Mouse

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The necroinflammatory reaction plays a central role in hepatitis B virus (HBV) elimination. Cluster of differentiation (CD)8-positive cytotoxic T lymphocytes (CTLs) are thought to be a main player in the elimination of infected cells, and a recent report suggests that natural killer (NK) cells also play an important role. Here, we demonstrate the elimination of HBV-infected hepatocytes by NK cells and dendritic cells (DCs) using urokinase-type plasminogen activator/severe combined immunodeficiency mice, in which the livers were highly repopulated with human hepatocytes. After establishing HBV infection, we injected human peripheral blood mononuclear cells (PBMCs) into the mice and analyzed liver pathology and infiltrating human immune cells with flow cytometry. Severe hepatocyte degeneration was observed only in HBV-infected mice transplanted with human PBMCs. We provide the first direct evidence that massive liver cell death can be caused by Fas/Fas ligand (FasL) interaction provided by NK cells activated by DCs. Treatment of mice with anti-Fas antibody completely prevented severe hepatocyte degeneration. Furthermore, severe hepatocyte death can be prevented by depletion of DCs, whereas depletion of CD8-positive CTLs did not disturb the development of massive liver cell apoptosis. ***Conclusion:*** **Our findings provide the first direct evidence that DC-activated NK cells induce massive HBV-infected hepatocyte degeneration through the Fas/FasL system and may indicate new therapeutic implications for acute severe/fulminant hepatitis B.** (HEPATOLOGY 2012;56:555-566)

Between 4% and 32% of fulminant hepatitis cases, characterized by acute massive hepatocyte degeneration and subsequent development of hepatic encephalopathy and liver failure, are caused by acute hepatitis B virus (HBV) infection.[1] Host[2] and viral factors[3] may influence the development of fulminant hepatitis, but these factors have not been fully elucidated.

Innate and adaptive immunity both play a role in the elimination of viral infections. In the innate immune response, cytoplasmic and membrane-bound receptors recognize viruses and induce interferon (IFN)-β production, which, in turn, up-regulates IFN-α and induces an antiviral state in surrounding cells.[4] In the adaptive immune response, viruses are recognized by dendritic cells (DCs), which activate cluster of differentiation (CD)8-positive T cells to reduce viral replication through cytolytic[5] and noncytolytic mechanisms.[6] The role of immune cells, especially HBV-specific cytotoxic T lymphocytes (CTLs), is crucial in the

Abbreviations: APC, allophycocyanin; asialo GM1, ganglio-N-tetraosylceramide; CD, cluster of differentiation; CHB, chronic hepatitis B; CTLs, cytotoxic T lymphocytes; DC, dendritic cell; FasL, Fas ligand; FHB, fulminant hepatitis B; HBcAg, hepatitis B core antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HLA, human leukocyte antigen; HSA, human serum albumin; IFN, interferon; IP, intraperitoneally; ISG, interferon-stimulated gene; mAb, monoclonal antibody; mDC, myeloid DC; mRNA, messenger RNA; NK, natural killer; PBMCs, peripheral blood mononuclear cells; PCR, polymerase chain reaction; pDC, plasmacytoid DC; SCID, severe combined immunodeficiency; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; uPA, urokinase-type plasminogen activator.

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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-$1^-HLA\text{-}DR^+CD123^+$ cells, whereas mDCs were defined as $CD45^+$ Lin-$1^-HLA\text{-}DR^+CD11c^+$ 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

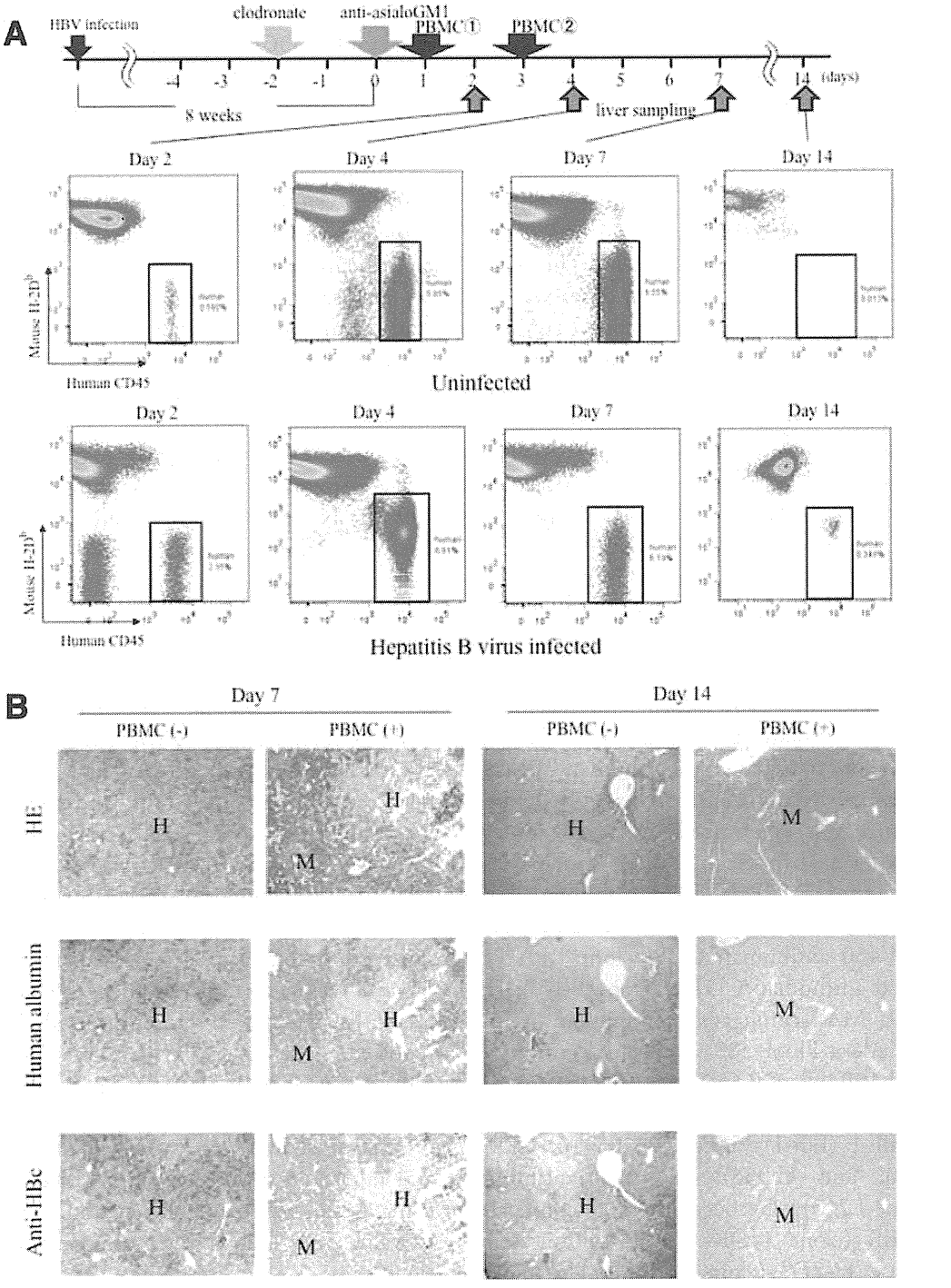

Fig. 1. Establishment of human PBMC chimerism in human hepatocyte chimeric mice. (A) Experimental protocol to establish chimerism and liver sampling is shown at the top of the figure (see Materials and Methods). Scheduling of administration of HBV-positive serum, clodronate, and anti-asialo GM1 antibody 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.

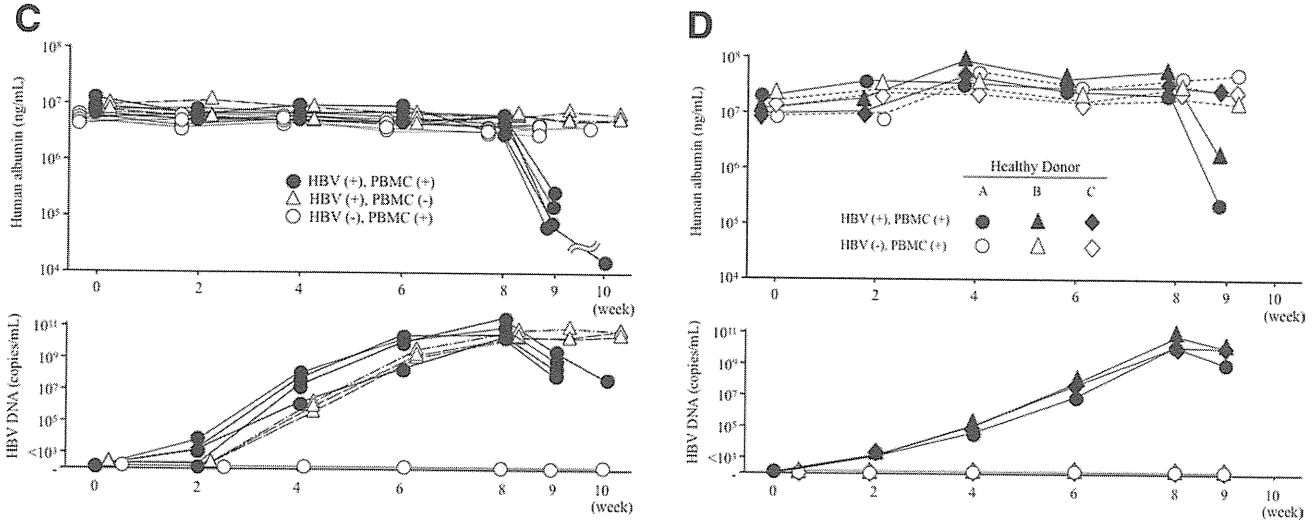

Fig. 1.

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.

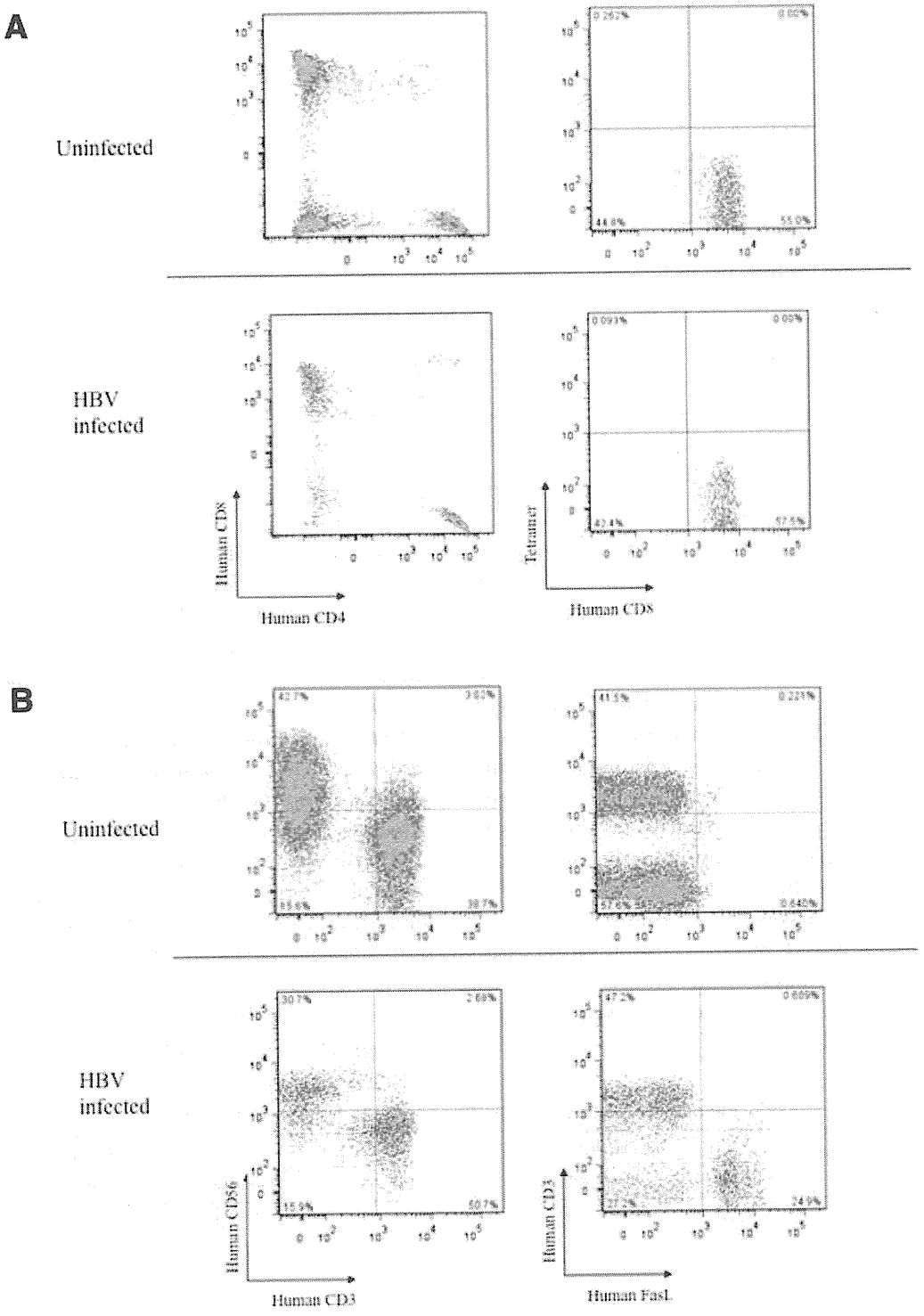

Fig. 2. Analysis of mononuclear cells isolated from day 4 chimeric mouse livers. After defining human PBMCs as mouse H-2Db-human CD45^{+} cells, we further analyzed the phenotypes of these cells. (A-C) Liver mononuclear cells of uninfected (upper panel) and HBV-infected (lower panel) mice transplanted 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).

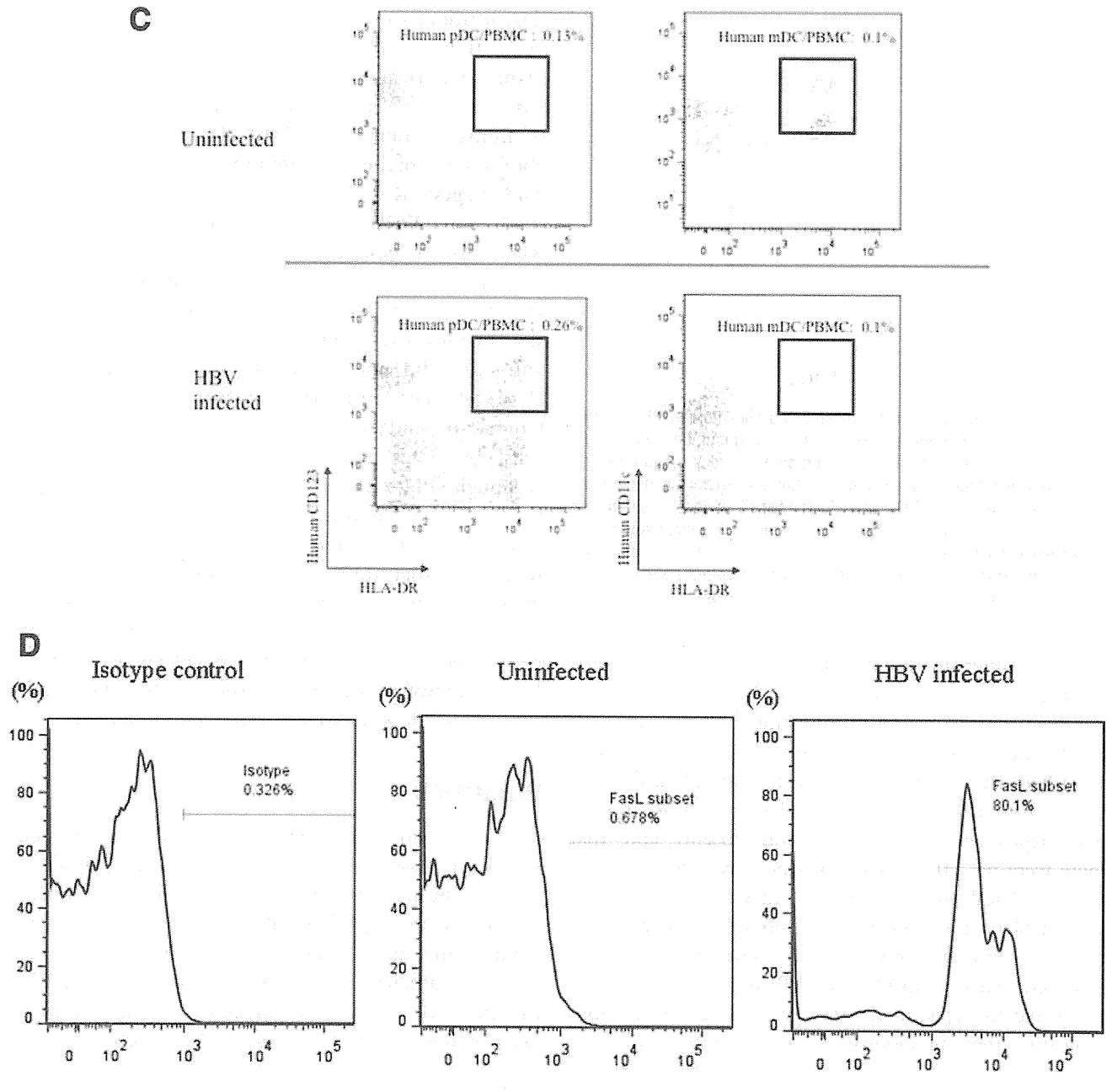

Fig. 2.

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

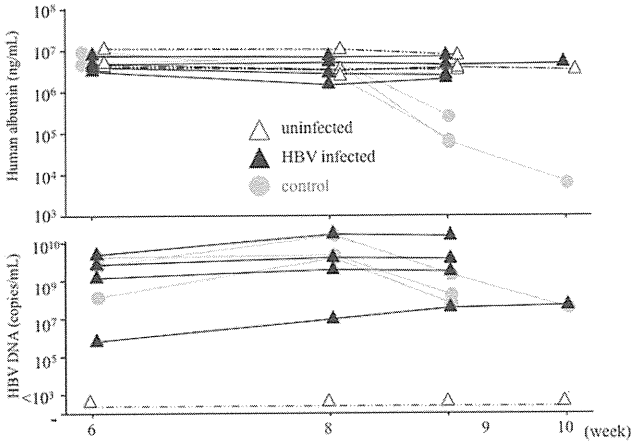

Fig. 3. Time course of mice transplanted with human PBMCs with DC depletion by clodronate 1 day before transplantation. Mice were treated with IP administration of clodronate 1 day before human PBMC transplantation. Time courses of human albumin concentration (upper panel) and HBV DNA titer (lower panel) 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

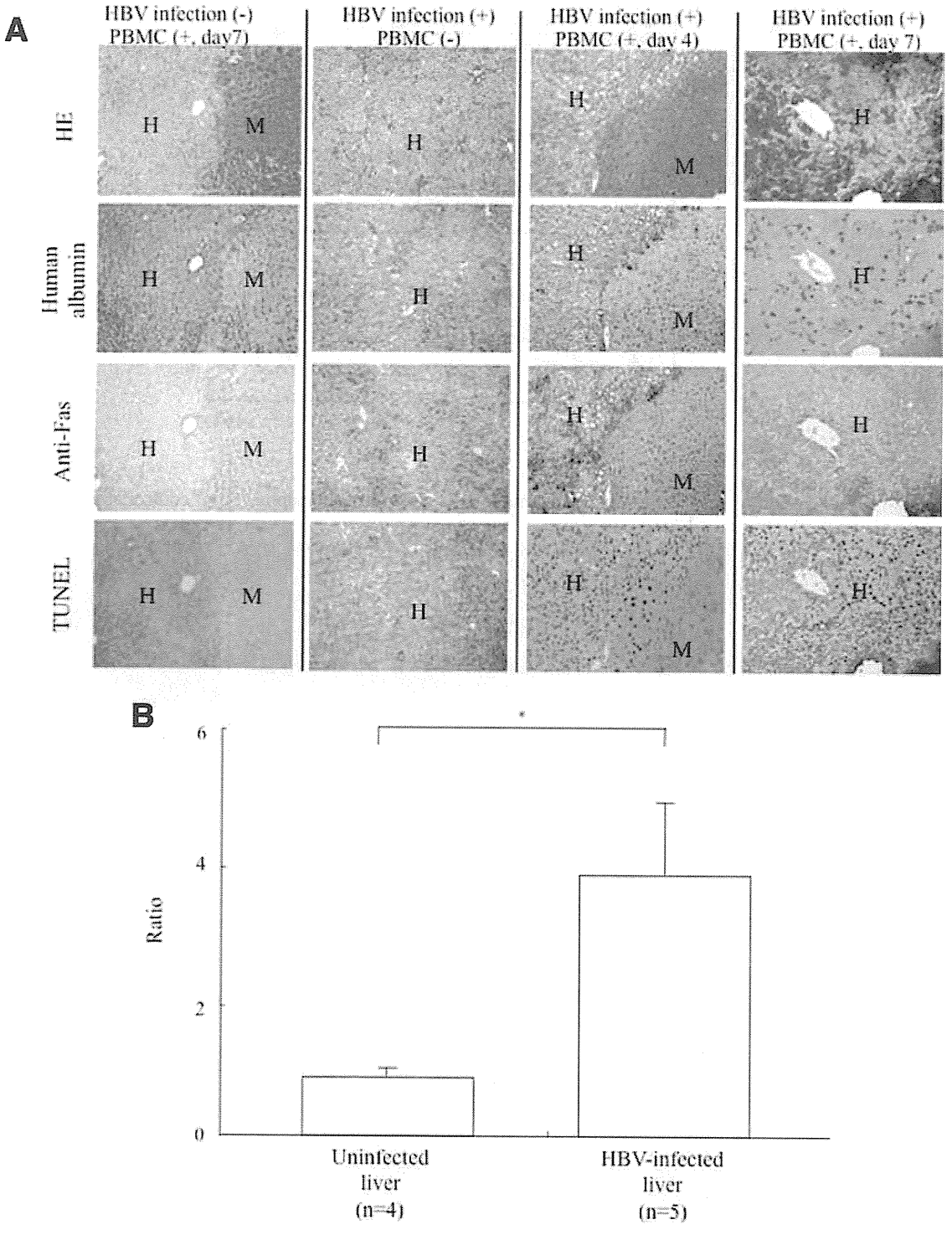

Fig. 4. Assessment of Fas expression in the liver in human hepatocyte chimeric mice. (A) Histological analysis of chimeric mice livers transplanted with human PBMCs but without HBV infection (day 7), with HBV infection but without PBMC transplantation, and with HBV infection and PBMC transplantation 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

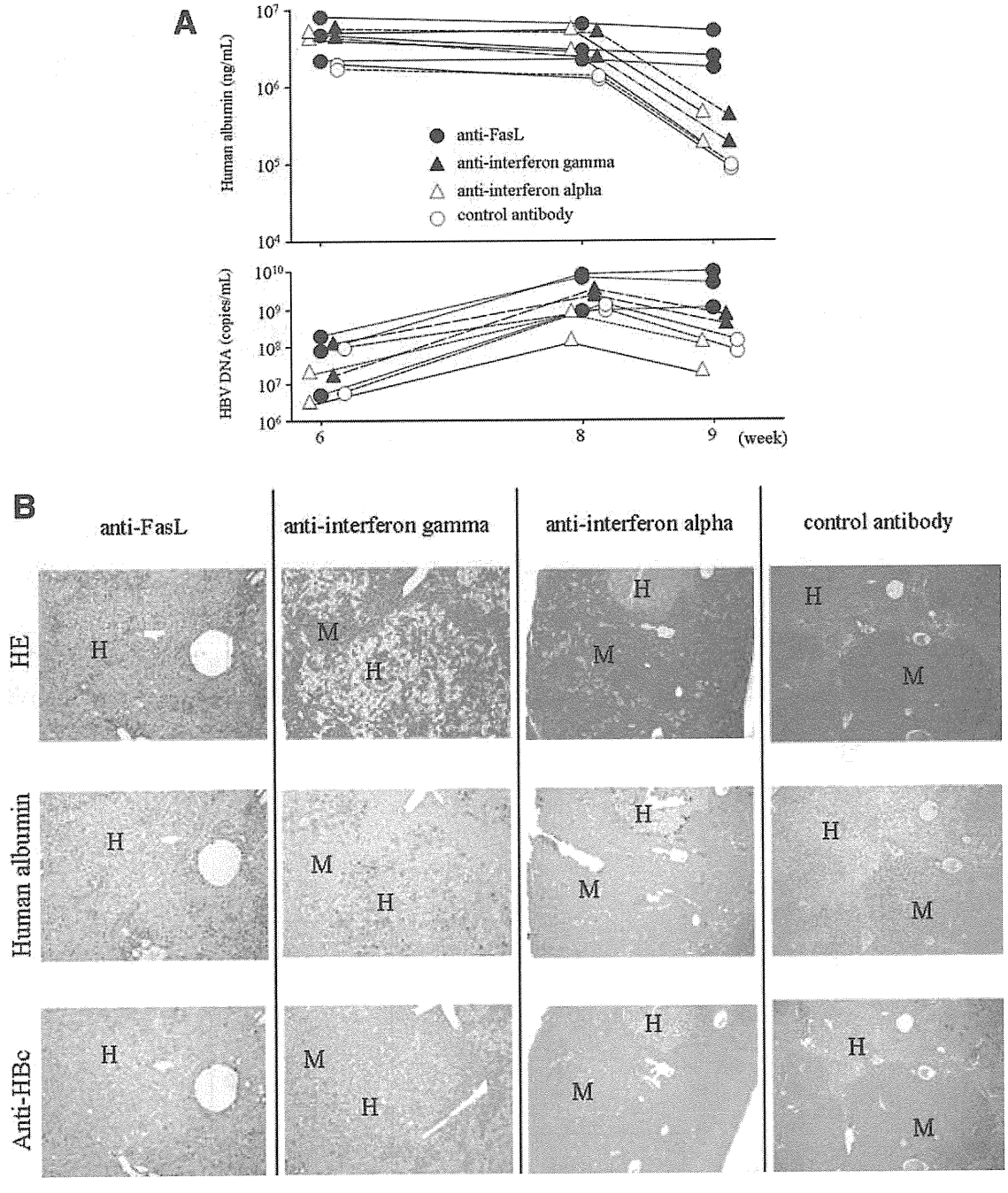

Fig. 5. Effect of anti-FasL, anti-IFN-γ and anti-IFN-α antibody administration on HSA and HBV DNA. (A) Time courses of HSA (upper panel) and HBV DNA (lower panel) before and 1 week after human PBMC transplantation are shown. Mice were pretreated with antibodies against human Fas-L, IFN-γ, and IFN-α before 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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References

1. Bernal W, Auzinger G, Dhawan A, Wendon J. Acute liver failure. Lancet 2010;376:190-201.
2. Leifeld L, Cheng S, Ramakers J, Dumoulin FL, Trautwein C, Sauerbruch T, Spengler U. Imbalanced intrahepatic expression of interleukin 12, interferon gamma, and interleukin 10 in fulminant hepatitis B. HEPATOLOGY 2002;36:1001-1008.
3. Ozasa A, Tanaka Y, Orito E, Sugiyama M, Kang JH, Hige S, et al. Influence of genotypes and precore mutations on fulminant or chronic outcome of acute hepatitis B virus infection. HEPATOLOGY 2006;44: 326-334.
4. Kawai T, Akira S. Toll-like receptor and RIG-I-like receptor signaling. Ann N Y Acad Sci 2008;1143:1-20.
5. Maini MK, Boni C, Lee CK, Larrubia JR, Reignat S, Ogg GS, et al. The role of virus-specific CD8(+) cells in liver damage and viral control during persistent hepatitis B virus infection. J Exp Med 2000;191: 1269-1280.
6. Guidotti LG, Ando K, Hobbs MV, Ishikawa T, Runkel L, Schreiber RD, Chisari FV. Cytotoxic T lymphocytes inhibit hepatitis B virus gene expression by a noncytolytic mechanism in transgenic mice. Proc Natl Acad Sci U S A 1994;91:3764-3768.
7. Ando K, Moriyama T, Guidotti LG, Wirth S, Schreiber RD, Schlicht HJ, et al. Mechanisms of class I restricted immunopathology. A transgenic mouse model of fulminant hepatitis. J Exp Med 1993;178: 1541-1554.
8. Cote PJ, Toshkov I, Bellezza C, Ascenzi M, Roneker C, Ann Graham L, et al. Temporal pathogenesis of experimental neonatal woodchuck hepatitis virus infection: increased initial viral load and decreased severity of acute hepatitis during the development of chronic viral infection. HEPATOLOGY 2000;32:807-817.
9. Chisari FV. Rous-Whipple Award Lecture. Viruses, immunity, and cancer: lessons from hepatitis B. Am J Pathol 2000;156:1117-1132.
10. Dunn C, Brunetto M, Reynolds G, Christophides T, Kennedy PT, Lampertico P, et al. Cytokines induced during chronic hepatitis B virus infection promote a pathway for NK cell-mediated liver damage. J Exp Med 2007;204:667-680.
11. Zhang Z, Zhang S, Zou Z, Shi J, Zhao J, Fan R, et al. Hypercytolytic activity of hepatic natural killer cells correlates with liver injury in chronic hepatitis B patients. HEPATOLOGY 2011;53:73-85.
12. Dandri M, Burda MR, Torok E, Pollok JM, Iwanska A, Sommer G, et al. Repopulation of mouse liver with human hepatocytes and *in vivo* infection with hepatitis B virus. HEPATOLOGY 2001;33:981-988.
13. Petersen J, Burda MR, Dandri M, Rogler CE. Transplantation of human hepatocytes in immunodeficient UPA mice: a model for the study of hepatitis B virus. Methods Mol Med 2004;96:253-260.
14. Ogata N, Miller RH, Ishak KG, Purcell RH. The complete nucleotide sequence of a pre-core mutant of hepatitis B virus implicated in fulminant hepatitis and its biological characterization in chimpanzees. Virology 1993;194:263-276.

15. Tateno C, Yoshizane Y, Saito N, Kataoka M, Utoh R, Yamasaki C, et al. Near completely humanized liver in mice shows human-type metabolic responses to drugs. Am J Pathol 2004;165:901-912.
16. Tsuge M, Hiraga N, Takaishi H, Noguchi C, Oga H, Imamura M, et al. Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis B virus. HEPATOLOGY 2005;42:1046-1054.
17. Tsuge M, Hiraga N, Akiyama R, Tanaka S, Matsushita M, Mitsui F, et al. HBx protein is indispensable for development of viraemia in human hepatocyte chimeric mice. J Gen Virol 2010;91:1854-1864.
18. Kuhober A, Pudollek HP, Reifenberg K, Chisari FV, Schlicht HJ, Reimann J, Schirmbeck R. DNA immunization induces antibody and cytotoxic T cell responses to hepatitis B core antigen in H-2b mice. J Immunol 1996;156:3687-3695.
19. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998;392:245-252.
20. Shortman K, Liu YJ. Mouse and human dendritic cell subtypes. Nat Rev Immunol 2002;2:151-161.
21. Sandhu J, Shpitz B, Gallinger S, Hozumi N. Human primary immune response in SCID mice engrafted with human peripheral blood lymphocytes. J Immunol 1994;152:3806-3813.
22. Gonzalez SF, Lukacs-Kornek V, Kuligowski MP, Pitcher LA, Degn SE, Kim YA, et al. Capture of influenza by medullary dendritic cells via SIGN-R1 is essential for humoral immunity in draining lymph nodes. Nat Immunol 2010;11:427-434.
23. Fernandez NC, Lozier A, Flament C, Ricciardi-Castagnoli P, Bellet D, Suter M, et al. Dendritic cells directly trigger NK cell functions: cross-talk relevant in innate anti-tumor immune responses *in vivo*. Nat Med 1999;5:405-411.
24. Ferlazzo G, Tsang ML, Moretta L, Melioli G, Steinman RM, Munz C. Human dendritic cells activate resting natural killer (NK) cells and are recognized via the NKp30 receptor by activated NK cells. J Exp Med 2002;195:343-351.
25. Andoniou CE, van Dommelen SL, Voigt V, Andrews DM, Brizard G, Asselin-Paturel C, et al. Interaction between conventional dendritic cells and natural killer cells is integral to the activation of effective antiviral immunity. Nat Immunol 2005;6:1011-1019.
26. Walzer T, Dalod M, Robbins SH, Zitvogel L, Vivier E. Natural-killer cells and dendritic cells: "l'union fait la force". Blood 2005;106:2252-2258.
27. Mosier DE, Gulizia RJ, Baird SM, Wilson DB, Spector DH, Spector SA. Human immunodeficiency virus infection of human-PBL-SCID mice. Science 1991;251:791-794.
28. Kawahara T, Douglas DN, Lewis J, Lund G, Addison W, Tyrrell DL, et al. Critical role of natural killer cells in the rejection of human hepatocytes after xenotransplantation into immunodeficient mice. Transpl Int 2010;23:934-943.
29. Morosan S, Hez-Deroubaix S, Lunel F, Renia L, Giannini C, Van Rooijen N, et al. Liver-stage development of *Plasmodium falciparum*, in a humanized mouse model. J Infect Dis 2006;193:996-1004.
30. Dorshkind K, Pollack SB, Bosma MJ, Phillips RA. Natural killer (NK) cells are present in mice with severe combined immunodeficiency (SCID). J Immunol 1985;134:3798-3801.
31. Ito M, Hiramatsu H, Kobayashi K, Suzue K, Kawahata M, Hioki K, et al. NOD/SCID/gamma(c)(null) mouse: an excellent recipient mouse model for engraftment of human cells. Blood 2002;100:3175-3182.
32. Thimme R, Wieland S, Steiger C, Ghrayeb J, Reimann KA, Purcell RH, Chisari FV. CD8(+) T cells mediate viral clearance and disease pathogenesis during acute hepatitis B virus infection. J Virol 2003;77:68-76.
33. Rehermann B, Fowler P, Sidney J, Person J, Redeker A, Brown M, et al. The cytotoxic T lymphocyte response to multiple hepatitis B virus polymerase epitopes during and after acute viral hepatitis. J Exp Med 1995;181:1047-1058.
34. Webster GJ, Reignat S, Maini MK, Whalley SA, Ogg GS, King A, et al. Incubation phase of acute hepatitis B in man: dynamic of cellular immune mechanisms. HEPATOLOGY 2000;32:1117-1124.
35. Zou Y, Chen T, Han M, Wang H, Yan W, Song G, et al. Increased killing of liver NK cells by Fas/Fas ligand and NKG2D/NKG2D ligand contributes to hepatocyte necrosis in virus-induced liver failure. J Immunol 2010;184:466-475.
36. Newman KC, Riley EM. Whatever turns you on: accessory-cell-dependent activation of NK cells by pathogens. Nat Rev Immunol 2007;7:279-291.
37. Galle PR, Hofmann WJ, Walczak H, Schaller H, Otto G, Stremmel W, et al. Involvement of the CD95 (APO-1/Fas) receptor and ligand in liver damage. J Exp Med 1995;182:1223-1230.
38. Rivero M, Crespo J, Fábrega E, Casafont F, Mayorga M, Gomez-Fleitas M, Pons-Romero F. Apoptosis mediated by the Fas system in the fulminant hepatitis by hepatitis B virus. J Viral Hepat 2002;9:107-113.
39. Moretta A. Natural killer cells and dendritic cells: rendezvous in abused tissues. Nat Rev Immunol 2002;2:957-964.
40. Asselin-Paturel C, Trinchieri G. Production of type I interferons: plasmacytoid dendritic cells and beyond. J Exp Med 2005;202:461-465.
41. Orito E, Ichida T, Sakugawa H, Sata M, Horiike N, Hino K, et al. Geographic distribution of hepatitis B virus (HBV) genotype in patients with chronic HBV infection in Japan. HEPATOLOGY 2001;34:590-594.

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Review Article

Treatment of chronic hepatitis B with nucleos(t)ide analogues

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Recently antiviral therapies for chronic hepatitis B using nucleos(t)ide analogues have become standard treatment modalities on the basis of several independent guidelines, starting with those of the American Association for the Study of Liver Diseases (AASLD) and other such organizations and bodies, including the European Association for the Study of the Liver (EASL), the Asian Pacific Association for the Study of the Liver (APASL), and the Japanese Ministry of Health, Labour and Welfare (MHLW)'s research team. The philosophies underlying such treatment strategies are considered basically equivalent. MHLW's guidelines define subjects for medical intervention to be cases measuring alanine aminotransferase (ALT) ≥31 IU/L, with serological hepatitis B virus (HBV) DNA level ≥5 log copies/mL for hepatitis B e antigen (HBeAg)-positive cases, and serological HBV DNA level ≥4 log copies/mL for HBeAg-negative cases. These Japanese guidelines advocate entecavir as the first-line treatment option for nucleos(t)ide-naïve patients, and combination treatment of lamivudine and adefovir as the basis of treatment for patients with lamivudine- and/or entecavir-resistant viruses. Of particular note for patients undergoing lamivudine treatment with persistent HBV DNA level < 2.1 log copies/mL is the recommendation of a switch to entecavir. Early detection of drug-resistant virus is desirable after initiation of nucleos(t)ide analogue treatment, but such a procedure is not uniformly available at all medical institutions. Nevertheless, timely estimation of potential early-stage drug-resistant virus development is crucial for getting a head start on treatment. HBV core-related antigen (HBcrAg) level or HBV DNA level are considered useful markers for the appearance of such drug-resistant viruses.

Key words: drug-resistant virus, HBV core-related antigen, HBV DNA, HBV genotype, interferon, nucleos(t)ide analogue

INTRODUCTION

THE AIM OF antiviral therapy for chronic hepatitis B is considered to be seroconversion from hepatitis B e antigen (HBeAg) positive to hepatitis B e antibody (anti-HBe) positive, sustained hepatitis B virus (HBV) DNA-negative status by polymerase chain reaction (PCR) assay, and normalization of alanine aminotransferase (ALT) level. Further, the most desirable endpoints of such treatment are elimination of hepatitis B surface antigen (HBsAg) and appearance of the hepatitis B surface antibody (anti-HBs). However, the number of chronic hepatitis B patients attaining such a "complete response" among the Japanese, a population with most carriers infected through the mother/child pathway, is relatively small.

As for recent treatments of chronic hepatitis B, in addition to the conventional interferon (IFN) therapy, which targets seroconversion, nucleos(t)ide analogues such as lamivudine, adefovir, and entecavir have become widely used due to the drugs' strong antiviral properties based on inhibition of HBV reverse transcriptase. In the United States, such new drugs as telbivudine and tenofovir, among others, have received regulatory approval.[1] With lamivudine, the first such drug available for use in Japan, lamivudine-resistant viral strains occurred at high frequency (24% after one year, 53% after 3 years).[2] For that reason, adefovir was introduced as the first-line drug treatment choice for lamivudine-resistant virus. In Japan, combination

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treatment with adefovir and lamivudine became possible in 2004, with adefovir monotherapy approved for use in 2008. However, because incidence of adefovir-resistant virus with this monotherapy was relatively high, the standard treatment at present is the combination of adefovir and lamivudine. On the other hand, the American Association for the Study of Liver Diseases (AASLD) guidelines recommend combination treatment against lamivudine-resistant virus with the addition of adefovir or tenofovir, or by the replacement of lamivudine with truvada (comprised of a combination pill with emtricitabine [200 mg] and tenofovir [300 mg]).

Entecavir, which was introduced to Japan in 2006, has the lowest rate of drug-resistant virus development (less than 2% after five years) among the nucleos(t)ide analogues currently available for use in nucleos(t)ide-naïve cases. Entecavir has therefore become the first-line drug treatment in such instances.[3,4] The AASLD and EASL guidelines also recommend tenofovir (no appearance of resistant virus after about 3 years), as well as entecavir, as first-line drug choices for treatment.

This manuscript focuses its explanation on the recent treatment guidelines for chronic hepatitis B formulated by the Japanese Ministry of Health, Labour and Welfare (MHLW)'s Study Group for the Standardization of Treatment of Viral Hepatitis including Cirrhosis, the body responsible for the successful standardization of use of such antiviral drugs, with the 2009 AASLD guidelines used as reference for comparison and complementary purposes. Moreover, the paper will touch on differences in incidence of drug-resistant virus by HBV genotype, and effective markers for estimation of the potential for appearance of such viruses.

CURRENT NUCLEOS(T)IDE ANALOGUE TREATMENT BASED ON GUIDELINES

IN THE RECENT MHLW guidelines, patients considered for antiviral therapy of chronic hepatitis B comprise those measuring ALT ≥31 IU/L, with HBV DNA level ≥5 log copies/mL in cases testing positive for HBeAg, and HBV DNA level ≥4 log copies/mL in cases testing negative for HBeAg, with treatment recommended for liver cirrhosis cases measuring HBV DNA level ≥3 log copies/mL.[5,6]

For younger patients (less than 35 years of age), the guidelines' fundamental treatment philosophy is application of IFN monotherapy or sequential therapy comprising IFN and nucleos(t)ide analogues, aiming at drug-free lives for patients and, ultimately, attainment of undetectable HBsAg level. According to the 2011 version of the said guidelines, such sequential treatment for cases testing HBeAg negative because of nucleos(t)ide analogue treatment or for cases testing HBeAg negative from the outset should be administered using nucleos(t)ide analogues for a sufficient duration of time. For cases in which one year or more has passed since undetectable HBV DNA level was attained, with hepatitis B virus core-related antigen (HBcrAg) measuring 4.0 log U/mL or lower, two treatment methods are available: (i) combination treatment with IFN and nucleos(t)ide analogues continued for a period of one month, following which IFN is administered for 5 months, and (ii) after conclusion of nucleos(t)ide analogue treatment, IFN is administered for a period of 6 months and then discontinued. However, for advanced cases with platelet counts of less than 15 ($\times 10^4/\mu L$) and liver fibrosis stage of at least F2, the use of nucleos(t)ide analogues should be considered from the start, according to the guidelines, regardless of patient age. On the other hand, for middle-aged and elderly patients (35 years and older), the basis of treatment is long-term administration of such nucleos(t)ide analogues with the aim of consistently undetectable HBV DNA level, normalized ALT level, and the ultimate goal of undetectable HBsAg. However, even for chronic hepatitis B cases with HBV genotypes A and B in patients 35 years and older, IFN treatment remains effective (Table 1), and the guidelines thus call for, to the extent possible, consideration of IFN as the primary treatment option in such cases.[7]

According to the AASLD guidelines, cases considered for antiviral therapy against chronic hepatitis B comprise those with ALT level > 2 × the upper limit of normal (ULN), regardless of HBeAg status, and HBV DNA level > 20 000 IU/mL (or, $>10^5$ copies/mL), and for compensated cirrhosis, cases with HBV DNA level > 2000 IU/mL (or, $>10^4$ copies/mL).[1] Based on data from cohort research including first-time blood donors and healthy volunteers, these reports recommend as the upper limit of standards for ALT level to be 30 IU/L for males and 19 IU/L for females.[8–10]

These guidelines consider treatment if HBeAg seroconversion for HBeAg-positive cases meeting the above conditions is not detected over the natural course of disease, on the basis of observation for a period of from 3–6 months. However, for cases measuring ALT ≤2 × ULN, the disease course is followed up, with treatment considered from the time when ALT level increases. In addition, for those aged 40 years or older with persistent ALT level of 1–2 × ULN, liver biopsy is conducted as the occasion demands, with treatment

Table 1 Effects of antiviral therapy by hepatitis B virus (HBV) genotype[18–25]

	HBV genotype		
	A	B	C
IFN therapy			
Loss of HBeAg	High	High	Low
Nucleos(t)ide analogue treatment			
Lamivudine			
HBeAg seroconversion	Very high	Not significant between genotypes B and C	
Incidence of drug-resistant virus		Low	High
Adefovir			
HBeAg seroconversion		Not significant among HBV genotypes	
Incidence of drug-resistant virus		Not significant between genotypes B and C	

HBeAg, hepatitis B e antigen; IFN, interferon.

considered in cases exhibiting findings of moderate/severe inflammation or significant fibrosis. For HBeAg-negative cases with ALT level of 1–2 × ULN and persistent HBV DNA level of 2000–20 000 IU/mL, treatment is considered in cases with liver biopsy findings of moderate/severe inflammation or significant fibrosis. As initial therapy for nucleos(t)ide-naïve patients, IFNα/Peg IFN-α, entecavir, and tenofovir are recommended. Moreover, because adefovir's antiviral effects decline after administration for a period of at least one year and incidence of adefovir-resistant virus is high, the drug is considered by the guidelines to be a second-line treatment for chronic hepatitis B.

PREDICTIVE FACTORS ON THE EFFECTS OF NUCLEOS(T)IDE ANALOGUE TREATMENT

NUCLEOS(T)IDE ANALOGUES HAVE superior antiviral effects. However, the treatment has been found to be associated with several concerns: the drugs are difficult to discontinue once use is initiated, drug-resistant virus can develop after long-term use, and HBV tends to repopulate, resulting in exacerbation of hepatitis (breakthrough hepatitis). Therefore, using a highly sensitive detection assay to detect drug-resistant virus in the early stages of development,[11,12] although such testing is not available at all medical institutions, and predicting the emergence of drug-resistant virus immediately following the start of nucleos(t)ide analogue treatment would enable timely initiation of treatment against drug-resistant virus appearance and thereby minimize hepatitis exacerbation.

Change in HBV DNA level due to such nucleos(t)ide analogue treatments as lamivudine or entecavir[13–15] was reported to be associated not only with responsiveness to the treatment itself but also with incidence of drug-resistant virus development. Reports have shown that in the 24th week after initiation of lamivudine treatment, chronic hepatitis B cases with sustained HBV DNA level have higher drug-resistant virus incidence, compared with those cases no longer with detectable HBV DNA level (Fig. 1). Additionally, it is known that drug-resistant viruses tend to appear readily in cases where HBV DNA level is high prior to start of nucleos(t)ide analogue treatment and in HBeAg-positive cases.[13,16,17]

Reports in recent years have shown that HBV genotype differences cause variation not only in virological characteristics but also in chronic hepatitis B pathologies and treatment effectiveness. With respect to HBV genotype and pathology, the HBeAg-positive rate for genotype B is lower than that for genotype C, whereas genotype B has a higher rate of HBeAg seroconversion. Genotype C, however, tends toward early-stage development of cirrhosis and hepatocellular carcinoma as well as a higher rate of progression of liver fibrosis compared with genotype B, leading to poor prognosis for this genotype.[18–21] Regarding HBV genotype and treatment results (Table 1), genotype B has a higher rate of HBeAg loss compared with that of genotype C, with good treatment results.[22,23] Nevertheless, seroconversion rates of nucleos(t)ide analogue treatments such as lamivudine and adefovir do not differ significantly among the HBV genotypes. Moreover, one report indicated that while incidence of lamivudine-resistant virus is higher for genotype C than for genotype B, incidence of adefovir-resistant virus does not differ significantly between the two genotypes.[24] Genotype A, however, which is known to cause acute infection in adults that progresses to a carrier state in about 10% of cases, exhibits the highest incidence of lamivudine-resistant virus.[25]

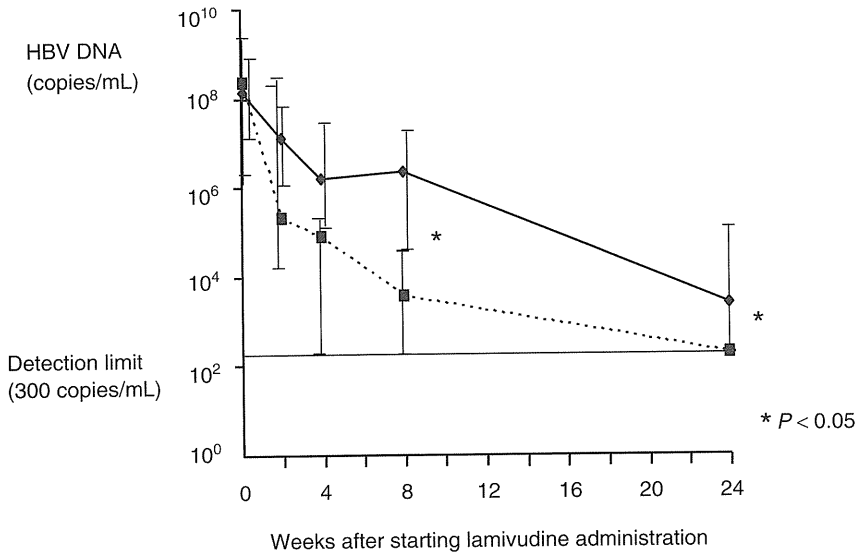

Figure 1 Changes of hepatitis B virus (HBV) DNA in lamivudine-resistant virus-positive and -negative cases (modified from Ohishi *et al.*[13]). —•—, Lamivudine-resistant virus + (12 cases); ···▪···, Lamivudine-resistant virus − (12 cases).

HBcrAg was recently added as a new serological HBV marker,[26] and represents a general term used to cover three different types of antigen proteins (hepatitis B core antigen (HBcAg), HBeAg, and p22crAg) translated by the mRNA of transcriptional products of covalently closed circular DNA (cccDNA), which is generated by the HBV replication process in hepatocytes. HBcrAg level reflects intrahepatic cccDNA level, and in the natural course of disease, serological HBcrAg level and HBV DNA level are linearly correlated, reflecting HBV proliferative status in hepatocytes.[27] However, during administration of nucleos(t)ide analogues, divergence between HBV DNA and HBcrAg levels becomes apparent.[14] Specifically, HBV DNA level quickly decreases, and in most cases, becomes undetectable, while HBcrAg level decreases slowly, but remains present and persistent. The reason for such a difference is that, during nucleos(t)ide analogue administration, reverse transcription from pregenome RNA to DNA in the HBV replication process is inhibited, leading to a marked decrease in virus particles (HBV DNA) secreted into the bloodstream. Since HBcrAg, however, is translated directly from mRNA, which is transcribed from cccDNA, it is hypothesized that HBcrAg continues to be secreted from hepatocytes into the bloodstream, without being directly inhibited by the nucleos(t)ide analogues.

HBcrAg level, a marker indicative of intrahepatic cccDNA level, is effective for monitoring of viral load during nucleos(t)ide analogue treatment, much as is HBV DNA level, with reports pointing to its association with drug-resistant virus appearance and hepatitis recurrence following conclusion of treatment.[14,28] Tanaka *et al.* investigated a cohort of cases divided into HBcrAg > 4.7 logU/mL and < 4.7 log U/mL groups at 6 months after initiation of lamivudine treatment. The researchers concluded in their report that the group measuring HBcrAg < 4.7 logU/mL had significantly lower cumulative incidence of lamivudine-resistant virus.[14]

TREATMENT FOR RESISTANCE TO NUCLEOS(T)IDE ANALOGUES

AT PRESENT, THE incidence of drug-resistant virus development in nucleos(t)ide-naïve patients is, among approved medications, highest with lamivudine and lowest with entecavir and tenofovir.[29] For treatment of drug-resistant HBV, approved nucleos(t)ide analogues are more limited in number in Japan than in the USA, and therefore, recommended treatment methods in the guidelines of AASLD and MHLW differ slightly.

In Japan, the foundation for treatment of lamivudine- and/or entecavir-resistant virus is combination treatment of lamivudine and adefovir. Compared with lamivudine-resistant virus cases, one report indicated that incidence of adefovir-resistant virus resulting from adefovir monotherapy is 21% after one year, but with combination treatment of both lamivudine and adefovir, the rate drops dramatically to 1.6% after 2 years.[30] This is assumed to be because each of the lamivudine- and adefovir-resistant viruses is sensitive to the other of the two drugs. Therefore, with such combination treatment, development of these drug-resistant viruses can

be held at bay. Another report showed that when drug-resistant virus appears after such combination treatment, tenofovir monotherapy is effective.[31] In Japan, however, tenofovir's use is not yet approved.

On the other hand, the AASLD guidelines consider the foundation for treatment of lamivudine-resistant virus to be a combination of lamivudine with adefovir or tenofovir, or truvada as a replacement for lamivudine after discontinuation of that drug's use. Moreover, the guidelines add that the basis for treatment of entecavir-resistant virus is a switch to tenofovir or truvada, from entecavir, or simply the addition of one of the drugs. That adefovir and tenofovir have antiviral activity against entecavir-resistant HBV was confirmed by *in vitro* experiments,[32] although clinical data are as yet limited.

TREATMENT DESIGN FOR DECREASE IN APPEARANCE OF LAMIVUDINE-RESISTANT VIRUS

IN JAPAN, IN conjunction with the introduction of entecavir in 2006, the 2007 version of MHLW's guidelines recommended a switch from lamivudine, with its high incidence of drug-resistant virus, to entecavir, for which the incidence of drug-resistant virus is low.[5,6] Dividing of the administration period into 3 years or more and less than 3 years, the guidelines initially called for a proactive switch to entecavir in cases for which the duration of lamivudine administration was less than 3 years and accompanied by persistent HBV DNA level <2.6 log copies/mL (afterward, 2.1 log copies/mL). Even in cases for which the administration period is 3 years or more accompanied by normal ALT level and persistent HBV DNA level <2.6 log copies/mL, the potential is high for diagnosis of lamivudine-resistant virus. The guidelines therefore now call for combination therapy by addition of adefovir when viral breakthrough is observed, alongside continued administration of lamivudine. Nevertheless, one paper reporting on a randomized control study of cases treated with lamivudine for a period of at least 3 years accompanied by HBV DNA level <2.6 log copies/mL, based on comparison of drug-resistant virus incidence between cases whose treatment was switched to entecavir and cases for whom lamivudine treatment was continued, found that the group treated with continued lamivudine exhibited drug-resistant virus development, while the group treated with entecavir experienced no such appearance of drug-resistant virus.[33]

With such a situation as background, beginning with the 2010 version, the MHLW guidelines recommend[34] that, for cases with persistent HBV DNA level <2.1 log copies/mL, regardless of duration of lamivudine administration, a switch to entecavir be made as a general rule (Table 2).

Table 2 2010–2011 guidelines of the Japanese Ministry of Health, Labour and Welfare's research team[34]: Lamivudine-to-entecavir switching treatment in lamivudine-pretreated patients

HBV DNA level	Regimen of therapy
<2.1 log copies/mL, sustained	As a general rule, switch to entecavir (0.5 mg/day)
≥2.1 log copies/mL	
VBT –	Switch to entecavir (0.5 mg/day) is feasible.
VBT +	Add adefovir (10 mg/day)

HBV, hepatitis B virus; VBT, viral breakthrough.

CONCLUSION

THE BASIC POLICIES between Japan and the USA for treatment of chronic hepatitis B with nucleos(t)ide analogues do not differ substantially, despite the fact that such drugs approved for use in Japan are fewer in number than those approved in the USA. For nucleos(t)ide-naïve patients, for example, both policies recommend use of entecavir or tenofovir, drugs with the lowest appearance of drug-resistant virus, in addition to combination therapy to which is added adefovir or tenofovir as treatment for lamivudine-resistant virus. Moreover, Japanese standards recommend that a switch be made to entecavir for cases being treated with lamivudine measuring HBV DNA level <2.1 log copies/mL, aiming at inhibition of drug-resistant virus development, on the basis of reports of actual therapeutic effects from such treatment. In the future, verification of efficacy of this switching treatment based on a larger number of cases will be necessary, but the treatment method does appear at present to be effective.[35]

In addition, as a measure to counter drug resistance in cases treated with nucleos(t)ide analogues, regular strict monitoring of HBV DNA and ALT levels is crucial. Consideration of differences in efficacy of lamivudine treatment based on HBV genotype, as well as use of HBV DNA level or HBcrAg level 6 months after initiation of treatment as markers associated with drug-resistant virus appearance, are thought to have potential in

helping support the minimization of hepatitis exacerbation through early initiation of drug-resistant virus treatment.

REFERENCES

1 Lok AS, McMahon BJ. Chronic hepatitis B: update 2009. *Hepatology* 2009; **50**: 1–36.
2 Lai CL, Dienstag J, Schiff E *et al.* Prevalence and clinical correlates of YMDD variants during lamivudine therapy for patients with chronic hepatitis B. *Clin Infect Dis* 2003; **36**: 687–96.
3 Tenney DJ, Rose RE, Baldick CJ *et al.* Long-term monitoring shows hepatitis B virus resistance to entecavir in nucleoside-naïve patients is rare through 5 years of therapy. *Hepatology* 2009; **49**: 1503–14.
4 Yokosuka O, Takaguchi K, Fujioka S *et al.* Long-term use of entecavir in nucleoside-naïve Japanese patients with chronic hepatitis B infection. *J Hepatol* 2010; **52**: 791–9.
5 Ohishi W, Chayama K. Current treatment for chronic hepatitis B in Japan. *Clin J Gastroenterol* 2009; **2**: 325–30.
6 Kumada H, Okanoue T, Onji M *et al.* Guidelines for the treatment of chronic hepatitis and cirrhosis due to hepatitis B virus infection for the fiscal year 2008 in Japan. *Hepatol Res* 2010; **40**: 1–7.
7 Yokosuka O, Kurosaki M, Imazeki F *et al.* Management of hepatitis B: consensus of the Japan Society of Hepatology 2009. *Hepatol Res* 2011; **41**: 1–21.
8 Keeffe EB, Dieterich DT, Han SH *et al.* A treatment algorithm for the management of chronic hepatitis B virus infection in the United States: 2008 update. *Clin Gastroenterol Hepatol* 2008; **6**: 1315–41.
9 Prati D, Taioli E, Zanella A *et al.* Updated definitions of healthy ranges for serum alanine aminotransferase levels. *Ann Intern Med* 2002; **137**: 1–10.
10 Kariv R, Leshno M, Beth-Or A *et al.* Re-evaluation of serum alanine aminotransferase upper normal limit and its modulating factors in a large-scale population study. *Liver Int* 2006; **26**: 445–50.
11 Chayama K, Suzuki Y, Kobayashi M *et al.* Emergence and takeover of YMDD motif mutant hepatitis B virus during long-term lamivudine therapy and re-takeover by wild type after cessation of therapy. *Hepatology* 1998; **27**: 1711–6.
12 Ohishi W, Shirakawa H, Kawakami Y *et al.* Identification of rare polymerase variants of hepatitis B virus using a two-stage PCR with peptide nucleic acid clamping. *J Med Virol* 2004; **72**: 558–65.
13 Ohishi W, Chayama K, Kumada H. Viral kinetics in patients with chronic hepatitis B during lamivudine therapy by real-time PCR. *Acta Hepatol Jpn* 2002; **43**: 214. (In Japanese.)
14 Tanaka E, Matsumoto A, Suzuki F *et al.* Measurement of hepatitis B virus core-related antigen is valuable for identifying patients who are at low risk of lamivudine resistance. *Liver Int* 2006; **26**: 90–6.
15 Colonno RJ, Rose R, Baldick CJ *et al.* Entecavir resistance is rare in nucleoside naïve patients with hepatitis B. *Hepatology* 2006; **44**: 1656–65.
16 Nafa S, Ahmed S, Tavan D *et al.* Early detection of viral resistance by determination of hepatitis B virus polymerase mutations in patients treated by lamivudine for chronic hepatitis B. *Hepatology* 2000; **32**: 1078–88.
17 Lau DT, Khokhar MF, Doo E *et al.* Long-term therapy of chronic hepatitis B with lamivudine. *Hepatology* 2000; **32**: 828–34.
18 Orito E, Ichida T, Sakugawa H *et al.* Geographic distribution of hepatitis B virus (HBV) genotype in patients with chronic HBV infection in Japan. *Hepatology* 2001; **34**: 590–94.
19 Sumi H, Yokosuka O, Seki N *et al.* Influence of hepatitis B virus genotypes on the progression of chronic type B liver disease. *Hepatology* 2003; **37**: 19–26.
20 Sakugawa H, Nakasone H, Nakayoshi T *et al.* Preponderance of hepatitis B virus genotype B contributes to a better prognosis of chronic HBV infection in Okinawa, Japan. *J Med Virol* 2002; **67**: 484–9.
21 Orito E, Mizokami M. Hepatitis B virus genotypes and hepatocellular carcinoma in Japan. *Intervirology* 2003; **46**: 408–12.
22 Kao JH, Wu NH, Chen PJ, Lai MY, Chen DS. Hepatitis B genotypes and the response to interferon therapy. *J Hepatol* 2000; **33**: 998–1002.
23 Wai CT, Chu CJ, Hussain M *et al.* HBV genotype B is associated with better response to interferon therapy in HBeAg(+) chronic hepatitis than genotype C. *Hepatology* 2002; **36**: 1425–30.
24 Li X, Wang L, Zhong Y *et al.* Hepatitis B virus (HBV) subgenotypes C2 and B2 differ in lamivudine- and adefovir-resistance-associated mutational patterns in HBV-infected Chinese patients. *J Clin Microbiol* 2010; **48**: 4363–9.
25 Tanaka Y, Hasegawa I, Kato T *et al.* A case-control study for differences among hepatitis B virus infections of genotypes A (subtypes Aa and Ae) and D. *Hepatology* 2004; **40**: 747–55.
26 Kimura T, Rokuhara A, Sakamoto Y *et al.* Sensitive enzyme immunoassay for hepatitis B virus core-related antigens and their correlation to virus load. *J Clin Microbiol* 2002; **40**: 439–45.
27 Rokuhara A, Tanaka E, Matsumoto A *et al.* Clinical evaluation of a new enzyme immunoassay for hepatitis B virus core-related antigen; a marker distinct from viral DNA for monitoring lamivudine treatment. *J Viral Hepat* 2003; **10**: 324–30.
28 Matsumoto A, Tanaka E, Minami M *et al.* Low serum level of hepatitis B core-related antigen indicates unlikely reactivation of hepatitis after cessation of lamivudine therapy. *Hepatol Res* 2007; **37**: 661–6.
29 Lok AS. Drug therapy: tenofovir. *Hepatology* 2010; **52**: 743–7.

30 Yatsuji H, Suzuki F, Sezaki H *et al.* Low risk of adefovir resistance in lamivudine-resistant chronic hepatitis B patients treated with adefovir plus lamivudine combination therapy: two-year follow-up. *J Hepatol* 2008; **48**: 923–31.
31 van Bömmel F, de Man RA, Wedemeyer H *et al.* Long-term efficacy of tenofovir monotherapy for hepatitis B virus-monoinfected patients after failure of nucleoside/nucleotide analogues. *Hepatology* 2010; **51**: 73–80.
32 Yatsuji H, Hiraga N, Mori N *et al.* Successful treatment of an entecavir-resistant hepatitis B virus variant. *J Med Virol* 2007; **79**: 1811–7.
33 Matsuura K, Tanaka Y, Kusakabe A *et al.* Recommendation of lamivudine-to-entecavir switching treatment in chronic hepatitis B responders: randomized controlled trial. *Hepatol Res* 2011; **41**: 505–11.
34 Kumada H, Scientific Research Grant of Ministry of Health, Labour and Welfare. Research of hepatitis overcome urgent strategy. 2010. Research report of the standardization of viral hepatitis treatment including cirrhosis. (In Japanese.)
35 Ide T, Sata M, Chayama K *et al.* Evaluation of long-term entecavir treatment in stable chronic hepatitis B patients switched from lamivudine therapy. *Hepatol Int* 2010; **4**: 594–600.

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Review Article

Hepatocellular carcinoma: Towards personalized medicine

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Over the past several years, the success of genome-wide association studies (GWAS) and pharmacogenomics has gradually begun to enable personalized medicine in some fields. In the field of liver diseases, host genetic factors are now very useful in clinical practice for predicting treatment outcome and adverse reactions for pegylated interferon plus ribavirin combination therapy against chronic hepatitis C virus (HCV) infection. Recently, three virus-related hepatocellular carcinoma (HCC) GWAS were reported from Asia. One study examined hepatitis B virus-related HCC in China, where hepatitis B is very prevalent, and the other two examined HCV-related HCC in Japan. We identified a common variant in the *DEPDC5* locus associated with HCV-related HCC, and another group identified an association involving the *MICA* locus. In this review, we compare the results of these GWAS and earlier candidate gene studies. Further research is needed to determine the role of these single nucleotide polymorphisms on HCC risk, but identification of these markers could make it possible to assess the magnitude of the risk of cancer based on each patient's genetic background. Consideration of the genetic background of the patients will likely play a role in personalized medicine for HCC, and understanding the mechanism underlying the association could suggest novel promising therapeutic targets in the future. (*Cancer Sci* 2012; 103: 846–850)

Over the last several years, the success of GWAS and the International HapMap Project, a large-scale database of SNPs, has identified genetic risk factors for more than 150 diseases, as well as genetic differences in drug response.[1–4] The success of these studies as well as pharmacogenomics has gradually begun to enable personalized medicine in some fields.[5–8] The goal of personalized medicine is to optimize the medical care and outcomes for each patient based on clinical, genetic, and environmental information.[9] In the field of liver diseases, host genetic factors are now very useful in clinical practice for predicting treatment outcome and adverse reactions of PEG-IFN-α plus ribavirin combination therapy against chronic HCV infection,[10–17] which causes chronic hepatitis and HCC.

Epidemiology and Risk Factors of HCC

Hepatocellular carcinoma is the third leading cancer-related cause of death and the seventh most common form of cancer worldwide.[18] There are 750 000 new cases of HCC and nearly 700 000 deaths each year, making it a lethal form of cancer.[18] A variety of risk factors for HCC have been reported, including hepatitis viruses, vinyl chloride, tobacco, aflatoxin B1, alcohol consumption, non-alcoholic fatty liver disease, diabetes mellitus, obesity, diet, coffee, oral contraceptives, and hemochromatosis.[19] Incidence of HCC varies around the world, largely reflecting the distribution of HBV and HCV. As HBV infection is highly prevalent in many Asian countries and in Africa, HBV is the most common etiology of HCC in these regions, whereas in many developed countries, including Japan, HCV infection is the most common risk factor for HCC.[18–21] Chronic hepatitis caused by HCV often leads to fibrosis and cirrhosis (stage F4 fibrosis), which markedly increase the risk of developing HCC.[22] However, the incidence and progression of HCC varies by region, and only a fraction of HCV-infected patients develop HCC. To date, many studies have examined patients with HCV and identified several predictive factors for HCC, including liver fibrosis, age, male gender, alcohol consumption, diabetes mellitus, obesity, ethnicity, and co-infection with HBV.[18,23–25] In contrast to chronic HBV carriers, the influence of viral load and viral genotype on HCC is still controversial in chronic HCV carriers.[26] In addition to these factors, multiple host genetic factors are thought to contribute to HCV-related HCC development. Single nucleotide polymorphisms are the most common form of genomic variation, involving change at a single nucleotide in either coding or non-coding DNA. The contribution of SNPs in the development of HCC has been investigated by various means. For decades, numerous studies have been undertaken using a candidate gene approach, in which candidate genes are selected prior to analysis on the basis of known functions thought to be relevant to disease risk, for example, inflammatory genes and oncogenes, and the corresponding genomic region is intensively screened for disease-associated SNPs. For example, the association between HCV-related HCC and SNPs in the region of the *IL-1beta, MDM2*, and *UGT1A7* genes have been reported from Japan and other countries.[27–32] It has been reported that these gene polymorphisms are also associated with HBV-related HCC.[33–36] In addition, the influence of *HFE* and *MnSOD* gene polymorphisms on HCV-related HCC has been reported from many countries, although not from East Asian countries.[37–39] Gene polymorphisms associated with activity of hepatitis and liver fibrosis progression, which contribute to the development of HCC, have also been reported in HCV patients.[40,41] In spite of this effort, most studies had insufficient sample sizes, and the associations with HCV-related HCC were not robust. Therefore, better predictive genetic markers are still needed.

Genome-Wide Association Studies of HCV Treatment Response

Recently, methods for searching SNPs associated with diseases or drug responses have been changing dramatically. In contrast

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