

Figure 2. Interactions between HBc and HBs. A) Co-localization of anti-HBc and anti-Calnexin staining by immunocytochemistry and PLA analysis indicate that HBc probably localizes in the ER. Overlap with B) anti-LC3B, C) anti-Rab5, and D) anti-Golgi staining suggests that HBc probably also localizes in autophagosomes, endosomes, and Golgi, respectively. E) However, no overlap was observed with anti-COX IV staining, indicating that HBc probably does not localize at mitochondria.
doi:10.1371/journal.pone.0047490.g002

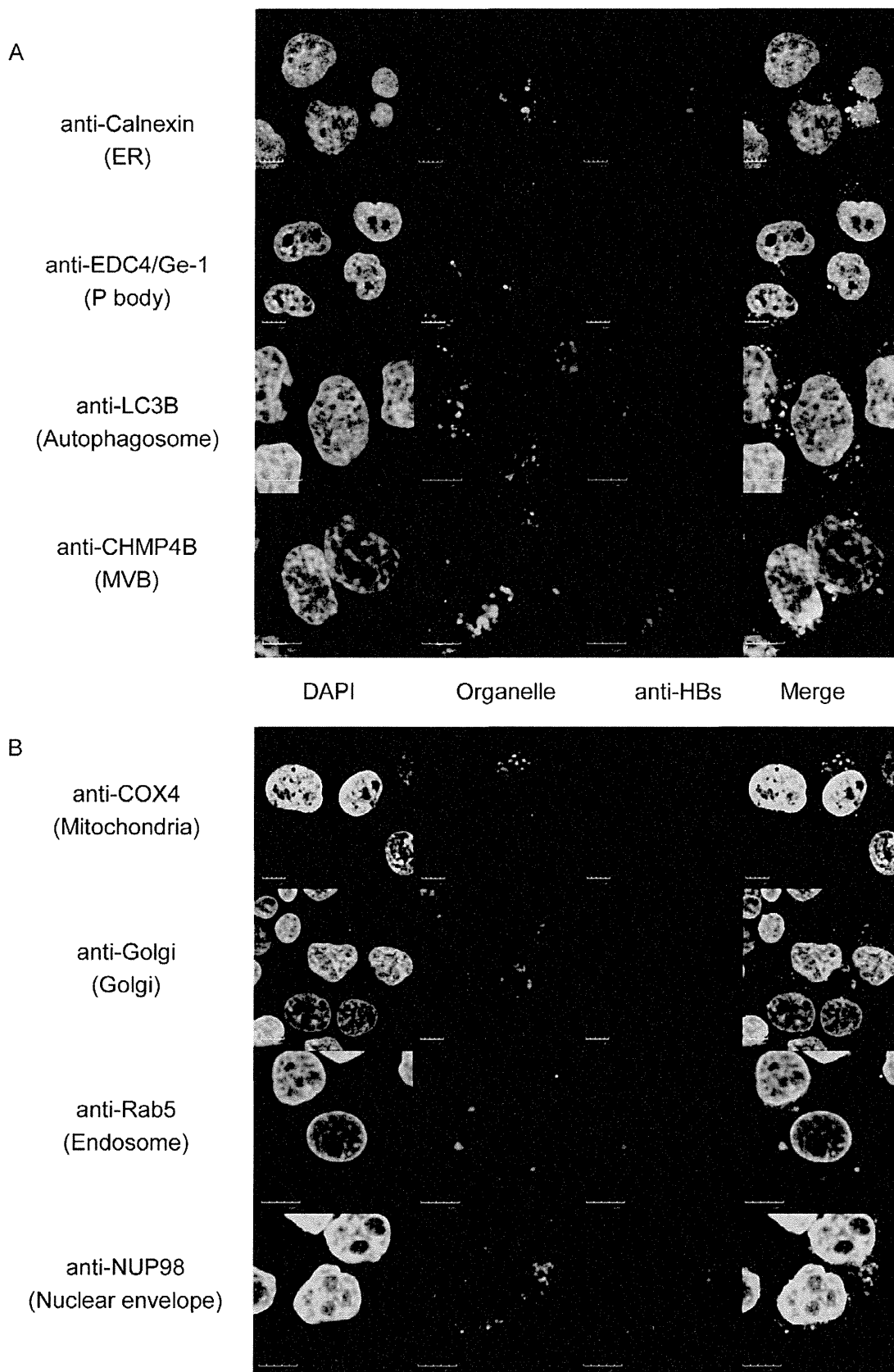


Figure 3. HBsAg localization. A) Co-localization of anti-HBs suggests that HBs localizes in the ER, processing bodies, autophagosomes, and multivesicular bodies, B) and more diffusely in mitochondria, Golgi, endosomes, and at the nuclear envelope.
doi:10.1371/journal.pone.0047490.g003

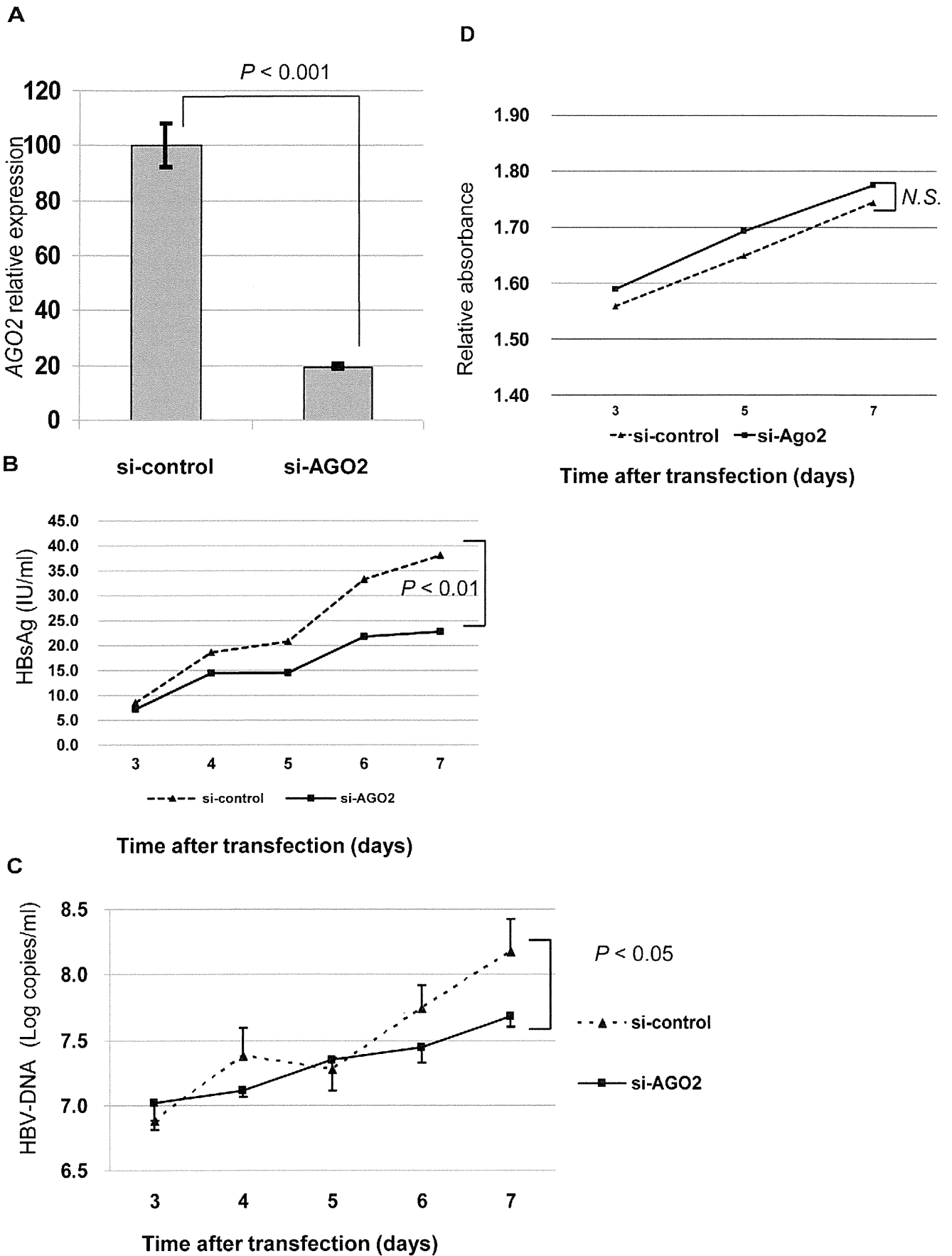


Figure 4. siRNA knock down of AGO2 expression. A) Knock down of AGO2 expression in T23 cells by specific siRNAs for AGO2 or control siRNAs, confirmed by real-time quantitative RT-PCR analysis. B) Supernatant HBs antigen, and C) HBV-DNA were measured. Both were higher in supernatant of cells transfected with si-control than in cells transfected with si-AGO2. D) There was no significant difference in cell viability between cells transfected with si-control compared to those with si-AGO2.
doi:10.1371/journal.pone.0047490.g004

controls. Mir-122, miR-22, miR-99a, and miR-125b in particular, were significantly elevated in serum of HBV patients. We also showed that AGO2, an essential component of the RNA silencing complex, co-localizes with both HBc and HBs proteins. HBc and/or HBs localize to several organelles associated with protein synthesis, processing, and degradation, including the ER, Golgi, endosomes, autophagosomes, processing bodies, and multivesicular bodies. Although we expected that depletion of AGO2 would relieve inhibition of HBV replication, we found instead that knockdown of AGO2 appears to inhibit HBV replication, implying that HBV may require AGO2 during its life cycle.

The role of AGO2 is unclear, but viruses have previously been shown to interfere with elements of the RNA-induced gene silencing pathway [17]. HCV core protein and the HIV-1 Tat protein suppress gene silencing by inhibiting Dicer, a cytoplasmic protein that processes pre-microRNA [18]. HBV down-regulates expression of Drosha, the nuclear protein involved in the first step of miRNA processing, which might globally suppress miRNA expression levels [19]. Viruses also influence expression of individual miRNAs [17].

Considering that miR-122 strongly suppresses HBV replication, it is curious that HBV is nonetheless often able to establish chronic infection in the liver [20,21,22]. In the case of HCV, miR-122/AGO2 binding stabilizes the HCV genome and prevents degradation, such that suppression of either miR-122 or AGO2 inhibits HCV replication [23,24,25]. In HBV, we also found that AGO2 knockdown suppresses replication, but Wang et al. demonstrated that anti-sense depletion of miR-122 promoted HBV replication instead of suppressing it [26]. MiR-122 suppresses HBV replication both through direct binding to HBV RNA as well as indirectly through cyclin G1-modulated p53 activity [20,27,28]. HBV might therefore be expected to down-regulate miR-122 levels to evade miR-122 binding and suppression. Wang et al. indeed found that miR-122 levels are significantly decreased in the liver of chronic HBV patient [26], whereas elevated miR-122 levels in the serum have been reported [4,29].

One explanation for the discrepancy between liver and serum miR-122 levels might be that HBV sequesters and expels AGO2-bound miR-122 inside of HBsAg particles, possibly along with other miRNAs that interfere with the viral life cycle. HBV vastly over-produces surface proteins that self-assemble into what were initially thought to be empty particles [30,31], but which may contain miRNAs stably bound to AGO2 [5]. Although HBV is a DNA virus, it relies on reverse transcription via an RNA intermediate in a way similar to retroviruses. Bouttier et al. showed that two unrelated retroviruses, HIV-1 and PFV-1, both require AGO2 interaction with viral RNA for assembly of viral particles. In these viruses, AGO2 is recruited to viral RNA and encapsidated along with it without impairing translation of viral RNA [32]. This suggests that some viruses may take advantage of another function of Argonaute, such as its role in the formation of P-bodies [33], although AGO2 possesses intrinsic exonuclease activity that must be countered. AGO2-mediated gene silencing requires recruitment of GW182 via multiple GW-rich regions [34]. While HIV-1 and PFV-1 encapsidate AGO2, they do not encapsidate GW182, which might provide a means to suppress AGO2 silencing. Some plant viruses use molecular mimicry to

inhibit RISC activity by binding to Argonaute proteins through virally encoded WG/GW motifs [35]. Although HBV proteins appear to lack WG/GW motifs, the HBV core protein may use a similar mechanism to disrupt RISC activity while preserving other AGO2 functions. One possibility involves HSP90, a chaperone involved in maintenance of the polymerase/pgRNA complex. HSP90 binds to HBV core protein dimers and is internalized in capsids, but it also binds to the N-terminus of AGO2 and may be required for miRNA loading and targeting to P-bodies [36,37]. Co-localization studies with other proteins and analysis of bound miRNAs may be necessary to elucidate the role of AGO2 in HBV replication, but we speculate that HBV proteins might suppress miRNA activity by binding to and sequestering AGO2 and their bound miRNAs.

Pathway analysis of the predicted targets of the up-regulated serum miRNAs in HBV patients showed that genes involved in phosphatase activity were significantly over-represented. Each of several miRNAs, including miR-122, miR-125b, and miR-99a, was predicted to target a different phosphorylation-associated gene. Regulation of phosphorylation appears to be important in HBV replication, as phosphorylation of the C terminal domain of the HBV core protein is essential for pgRNA packaging and HBV capsid maturation [38]. Phosphorylation also inhibits AGO2 binding of miRNA [39] and is involved in localization to P-bodies [40]. Recent studies have demonstrated that HBV enhances and exploits autophagy via the HBx and small HBs proteins to promote viral DNA replication and envelopment without increasing the rate of protein degradation [41,42]. Sir et al suggested that autophagy may affect dephosphorylation and maturation of the core protein, which protects viral DNA during replication [43]. These reports suggest that HBV exploits multiple cellular pathways in order to establish an intracellular environment conducive to replication.

Although many HBV-associated miRNAs have been reported, the functions of only a few have been examined. MiR-122, miR-125a-5p, miR-199a-3p and miRNA-210 have all been reported to bind to and directly suppress HBV RNA [8,27,44], whereas other miRNAs have been shown to promote or suppress HBV replication indirectly. MiR-1 enhances HBV core promoter activity by up-regulating FXR α , a transcription factor essential for HBV replication [45], whereas miR-141 suppresses HBsAg production in HepG2 cells by down-regulating promoter activity via PPARA [46]. The role of miR-22 and miR-99a in HBV infection is less clear, but both are involved in regulation of cell fate and are implicated in development of HCC. MiR-99a is one of the most highly expressed miRNAs in normal liver tissue and is severely down-regulated in HCC and other cancers, suggesting a role as a tumor suppressor [47]. MiR-99a alters sensitivity to TGF- β activity by suppressing phosphorylation of SMAD3 [48], whereas the HBx protein disrupts TGF- β signaling by shifting from the pSmad3C pathway to the oncogenic pSmad3L pathway [49]. MiR-22 acts as a tumor suppressor by inducing cellular senescence and is down-regulated in several cancer lines [50]. However, over-expression of miR-22 in males is associated with down-regulation of ER α expression, which compromises the protective effect of estrogen and leads to up-regulation of IL-1 α in hepatocytes under stress caused by reactive oxygen species, which is another hallmark of HBx interference [51]. Differences in

miRNA levels between hepatic and serum miRNA profiles may reveal miRNAs that play an essential role in the HBV life cycle, with potential application to miRNA-based diagnosis and therapy.

In this study we demonstrated potential interactions between AGO2 and HBc and HBs, but not HBx, in stably transfected HepG2 cells. Suppression of HBV DNA and HBsAg in the supernatant following AGO2 knockdown and the presence of HBV-associated miRNAs in the serum may indicate a dependency on AGO2 during the HBV life cycle.

Supporting Information

Figure S1 Heat map of miRNA expression. Healthy controls and patients with chronic HBV clustered separately based on serum miRNA expression. “Healthy males” and “healthy females” refer to serum mixtures of 12 uninfected males and 10 uninfected females, respectively. “HBV low” and “HBV high” refer to serum mixtures from 10 patients with low (≤ 42 IU/l) ALT levels and 10 patients with high ALT levels (>42 IU/l), respectively.
(TIF)

Figure S2 Pairwise correlations among pooled serum miRNA samples. Pooled serum samples were collected from 10 healthy males, 10 healthy females, 10 HBV patients with low ALT levels, and 10 HBV patients with high ALT levels. Pairwise correlations in miRNA expression levels among all four pooled samples were strong (>0.90 ; $P<0.001$), but correlations were strongest between the healthy male and female samples (0.98) and between the low and high ALT HBV patients (0.98), suggesting that expression of a subset of miRNAs is altered during HBV infection.
(TIF)

Figure S3 Relationship between serum miRNAs and HBsAg levels in chronic HBV patients. Serum levels of several miRNAs were significantly correlated with HBsAg levels in patients with chronic HBV. MiR-99a, miR-122, and miR-125b levels were most strongly correlated with HBsAg levels, with R^2 of 0.69, 0.56, and 0.54, respectively.
(TIF)

Figure S4 Relationship between serum miRNAs and HBV DNA levels in chronic HBV patients. Serum levels of several miRNAs were significantly correlated with HBV DNA levels in patients with chronic HBV. MiR-122, miR-99a, and miR-125b levels were most strongly correlated with HBV DNA levels, with R^2 of 0.44, 0.43, and 0.39, respectively.
(TIF)

Figure S5 Relationship between serum miRNAs and ALT levels in chronic HBV patients. Serum levels of several miRNAs were significantly but somewhat diffusely correlated with ALT levels in patients with chronic HBV. MiR-122 and miR-22 levels were correlated with ALT levels with R^2 of 0.25 and 0.21, respectively.
(TIF)

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Figure S6 Relationship between serum miRNAs and presence of HBe antigen in chronic HBV patients. Serum levels of miR-122, miR-99a, miR-720, and miR-125b were significantly elevated in patients positive for the HBe antigen.
(TIF)

Figure S7 Relationship between serum miRNAs and presence of HBe antibody in chronic HBV patients. Serum levels of miR-122, miR-99a, miR-720, and miR-125b were significantly elevated in patients negative for the HBe antibody.
(TIF)

Figure S8 Relationship between individual miRNAs in the liver and serum. Each point represents the level of a specific miRNA in non-cancerous liver tissue relative to serum in the same patient. Red points represent miRNA levels from a patient with chronic HBV, and blue and green points correspond to two different uninfected control subjects. Large red points and labels indicate the subset of miRNAs (Tables 2 and 3) that were significantly elevated in serum of chronic HBV patients. MiRNA expression levels were positively correlated ($R^2 = 0.57$; $P<2.1E-16$) between liver tissue and serum, suggesting that serum levels broadly reflect miRNA levels in the liver. There appears to be no clear discrepancy between liver and serum miRNA levels in the HBV-infected patient compared to the two uninfected patients.
(TIF)

Figure S9 Subcellular localization of HBx analyzed by immunocytochemistry. HBx localized non-specifically in the nucleus and cytoplasm, but we were unable to verify the subcellular location. Anti-Rab5 staining for endosomes is shown for illustration, but results were similar using antibodies against other compartments.
(TIF)

Table S1 Antibodies used for immunocytochemistry.
(DOC)

Table S2 Significantly up- or down-regulated miRNAs in liver samples from an HBV-infected patient compared to two non-HBV-infected patients.
(DOC)

Acknowledgments

This work was carried out at the Analysis Center of Life Science, Hiroshima University.

Author Contributions

Conceived and designed the experiments: KC CNH SA MT DM HAB HO NH. Performed the experiments: MT DM H. Abe NH MI SY H. Aikata TK YK RA KC. Analyzed the data: CNH SA MT DM HO KC. Contributed reagents/materials/analysis tools: CNH SA MT DM KC. Wrote the paper: CNH SA MT DM KC. Clinical data: KC MT DM HAB NH MI ST HAI TK YK WO. Obtained funding: KC MT DM. Critical review of the manuscript: CNH SA MT DM RA HAB HO NH MI ST HAI TK YK WO KC.

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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

Akihito Okazaki,^{1,2} Nobuhiko Hiraga,^{1,2} Michio Imamura,^{1,2} C. Nelson Hayes,^{1,2,3} Masataka Tsuge,^{1,2} Shoichi Takahashi,^{1,2} Hiroshi Aikata,^{1,2} Hiromi Abe,^{1,2,3} Daiki Miki,^{1,2,3} Hidenori Ochi,^{1,2,3} Chise Tateno,^{2,4} Katsutoshi Yoshizato,^{2,4} Hideki Ohdan,^{2,5} and Kazuaki Chayama^{1,2,3}

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 Host² and viral factors³ 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 cytolytic⁵ 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.

From the ¹Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan; ²Liver Research Project Center, Hiroshima University, Hiroshima, Japan; ³Laboratory for Digestive Diseases, Center for Genomic Medicine, RIKEN, Hiroshima, Japan; ⁴PhoenixBio Co., Ltd., Higashi-Hiroshima, Japan; and ⁵Department of Surgery, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Science, Hiroshima University, Hiroshima, Japan.

Received August 16, 2011; accepted February 4, 2012.

This study was supported, in part, by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Labor, Health, and Welfare.

development of fulminant hepatitis.^{7,8} CTLs can kill target cells using two distinct lytic pathways: the degradation 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 μ L of HBV-positive human serum.

Analysis of HBV. DNA was extracted using SMIT-EST (Genome Science Laboratories, Tokyo, Japan) and dissolved in 20 μ L of H₂O. 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'-GGTGAACAATGTTCCGGAGAC-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

Address reprint requests to: Kazuaki Chayama, M.D., Ph.D., Department of Medical and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Science, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan. E-mail: chayama@hiroshima-u.ac.jp; fax: +81-82-255-6220.

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DOI 10.1002/hep.25651

Potential conflict of interest: The authors have no conflicts to disclose.

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chimeric mice, 200 μ L of phosphate-buffered saline, containing 120 μ L 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 μ L/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 diaminobenzidine. 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 antibody²¹ and macrophages and DCs by liposome-encapsulated clodronate²² before transplantation

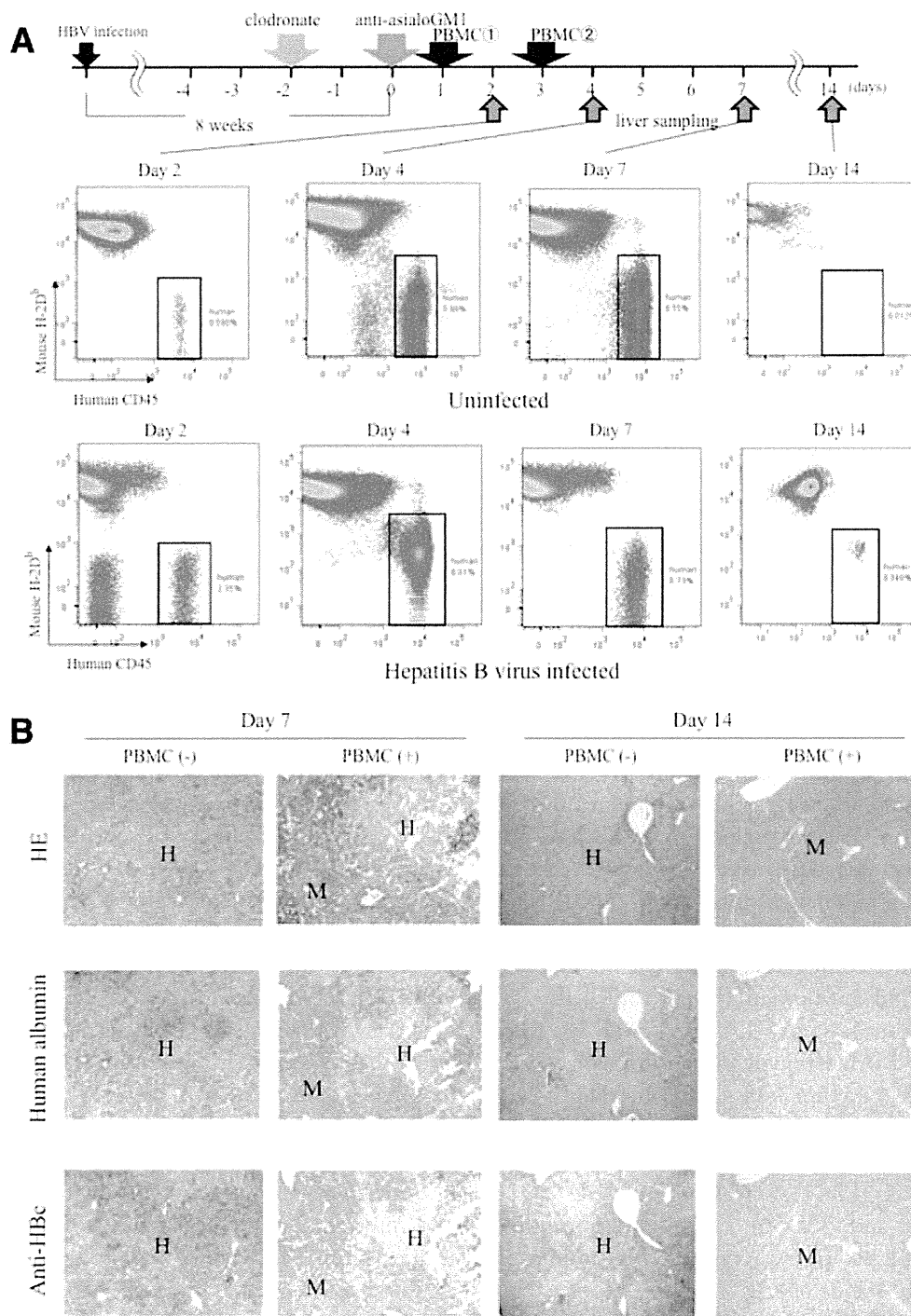


Fig. 1. Establishment of human PBMC chimerism in human hepatocyte chimeric mice. (A) Experimental protocol to establish chimerism and liver sampling is shown at the top of the figure (see Materials and Methods). Scheduling of administration of HBV-positive serum, clodronate, and anti-asialo GM1 antibody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

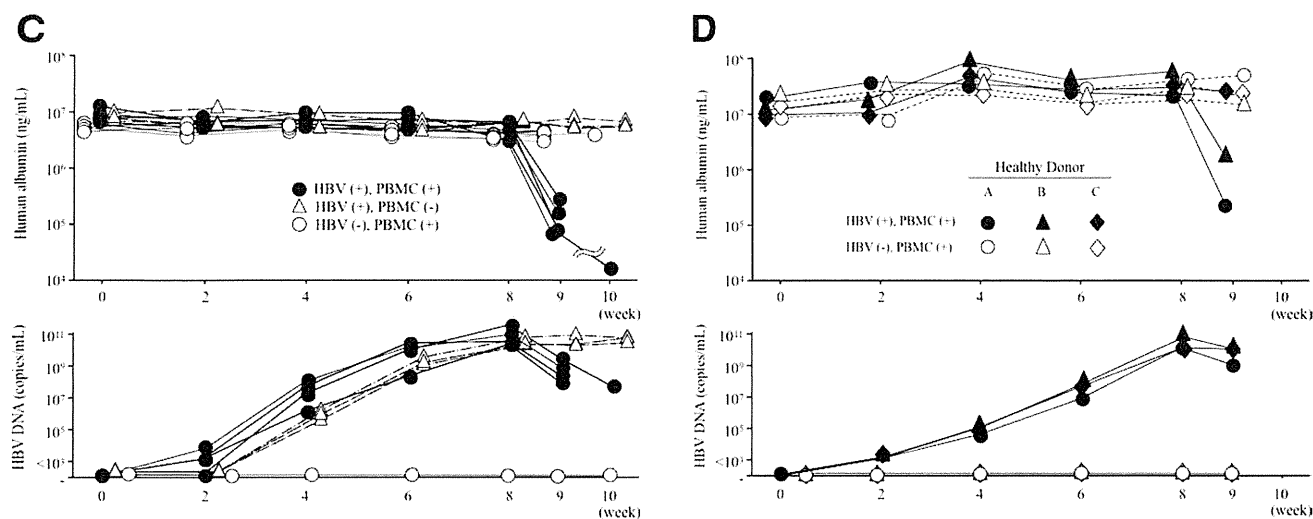


Fig. 1.

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

Day	HBV Infected				Uninfected			
	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 (by clodronate)	11	4.77	5	2.14	11	3.32	4.21	0.465
	12	1.27	39.5	2.3	12	12.9	9.06	0
DCs depleted day 7 (by clodronate)	13	2.42	24.8	2.19	13	6.31	54.1	0.131
	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.

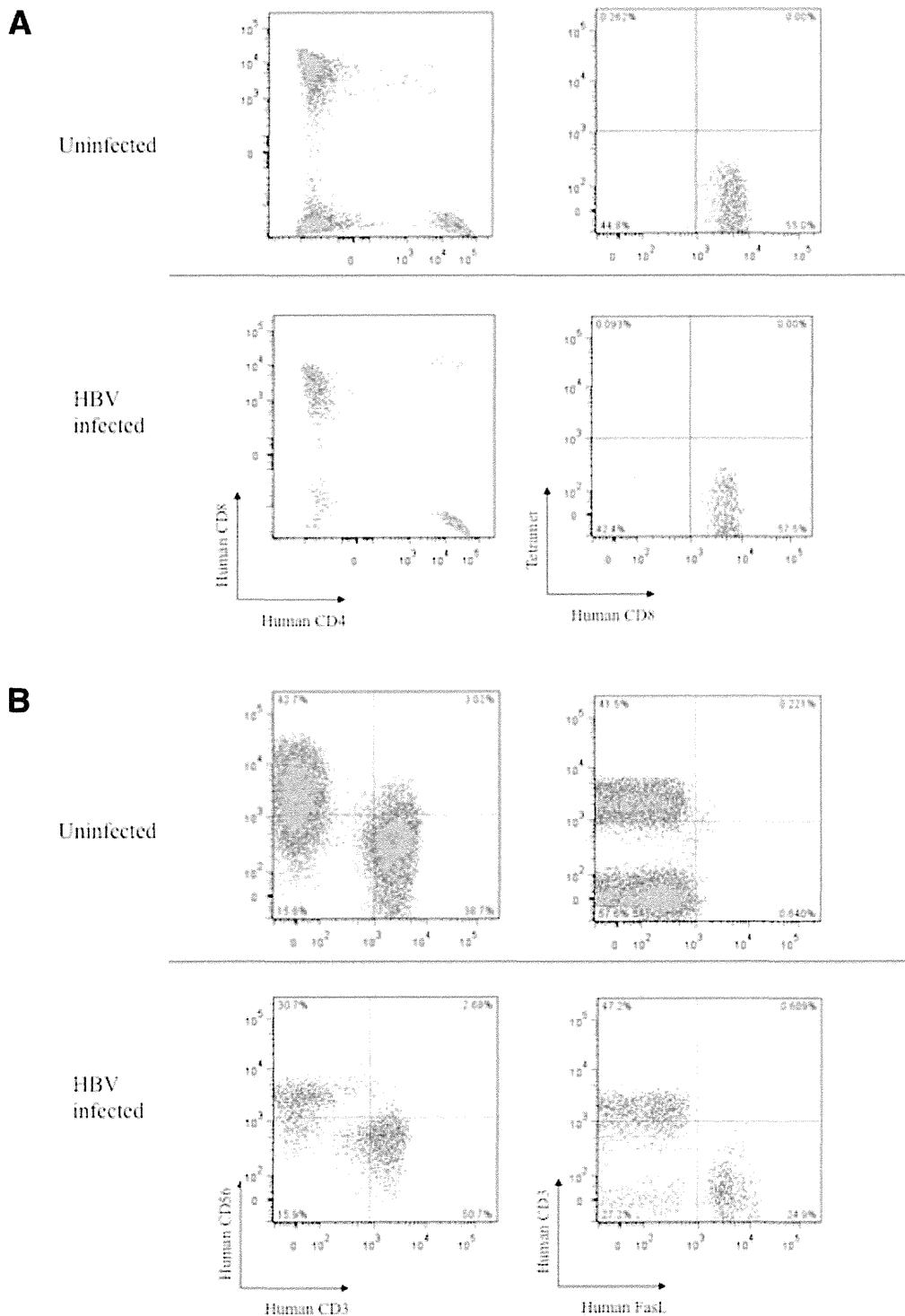


Fig. 2. Analysis of mononuclear cells isolated from day 4 chimeric mouse livers. After defining human PBMCs as mouse H-2Db-human CD45⁺ cells, we further analyzed the phenotypes of these cells. (A-C) Liver mononuclear cells of uninfected (upper panel) and HBV-infected (lower panel) mice transplanted with human PBMCs were separated with anti-human CD4 and CD8 antibody or anti-human CD8 and HLA-A2 HbCag tetramer (A), anti-human CD3 and 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).

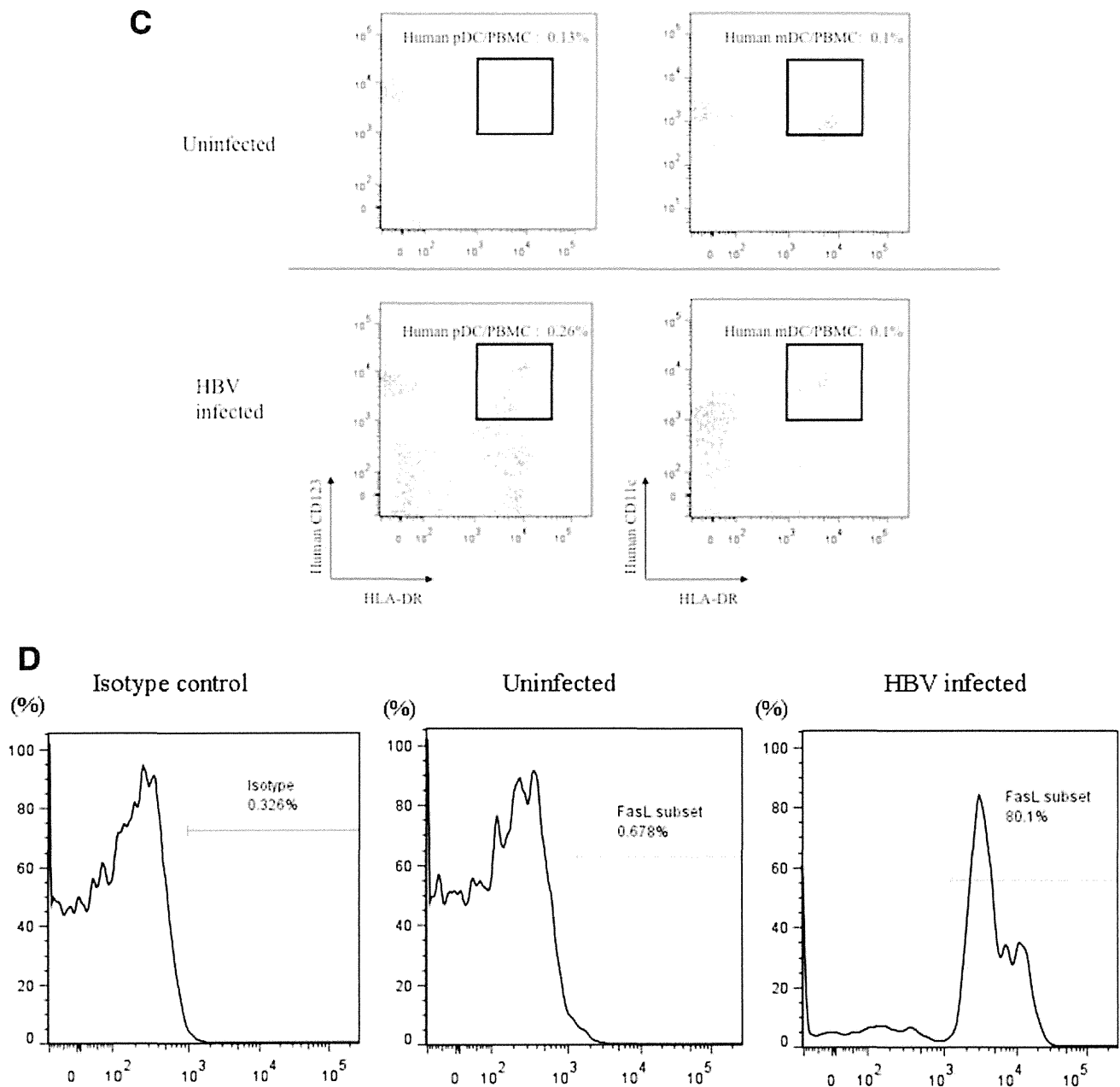


Fig. 2.

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 anti-

bodies 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

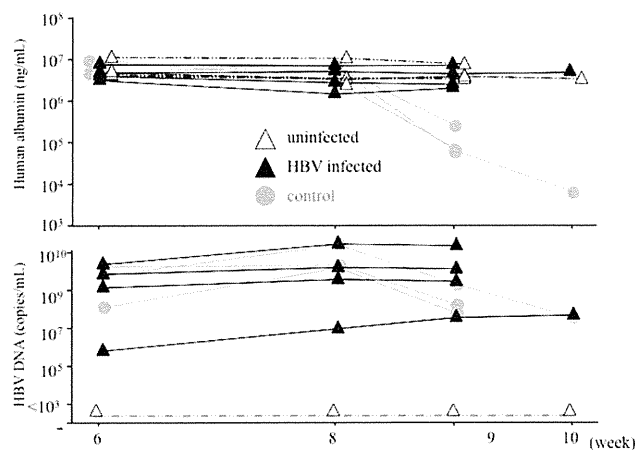


Fig. 3. Time course of mice transplanted with human PBMCs with DC depletion by clodronate 1 day before transplantation. Mice were treated with IP administration of clodronate 1 day before human PBMC transplantation. Time courses of human albumin concentration (upper panel) and HBV DNA titer (lower panel)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

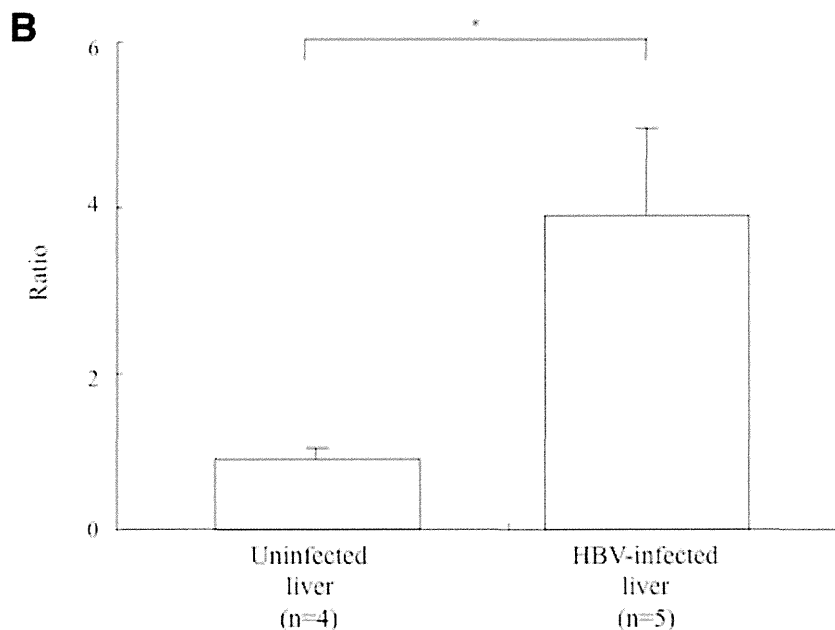
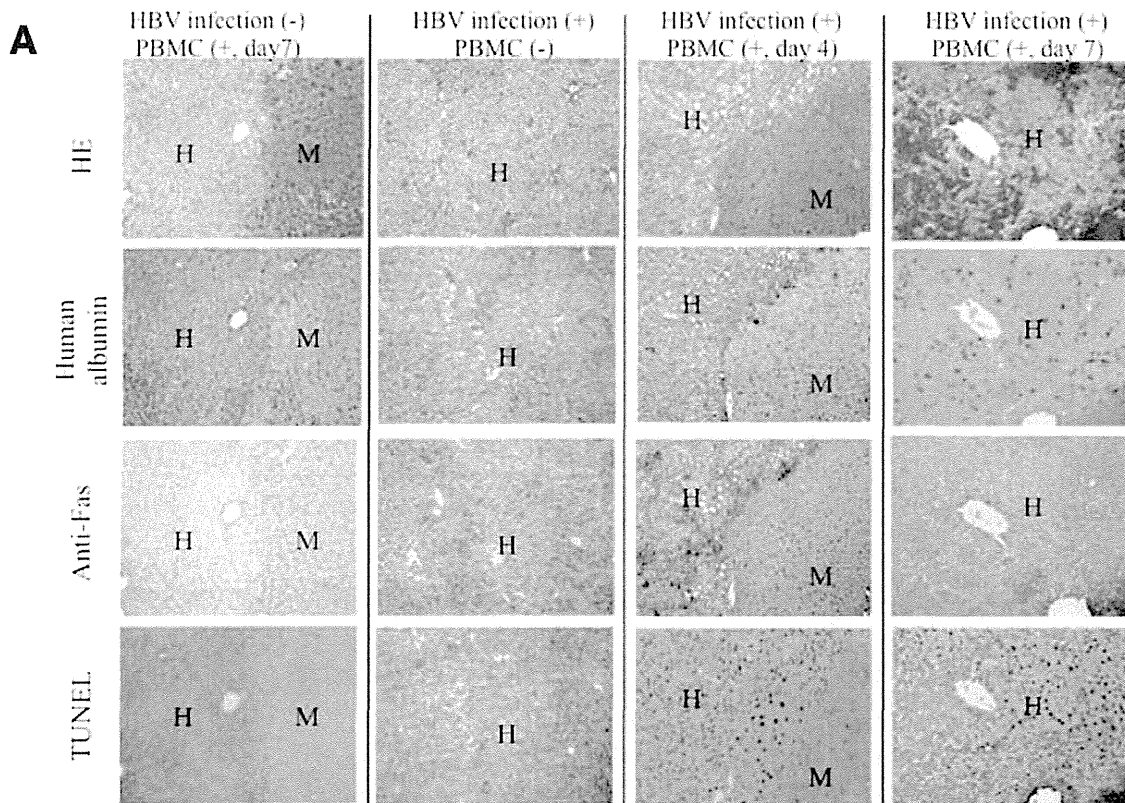


Fig. 4. Assessment of Fas expression in the liver in human hepatocyte chimeric mice. (A) Histological analysis of chimeric mice livers transplanted with human PBMCs but without HBV infection (day 7), with HBV infection but without PBMC transplantation, and with HBV infection and PBMC transplantation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

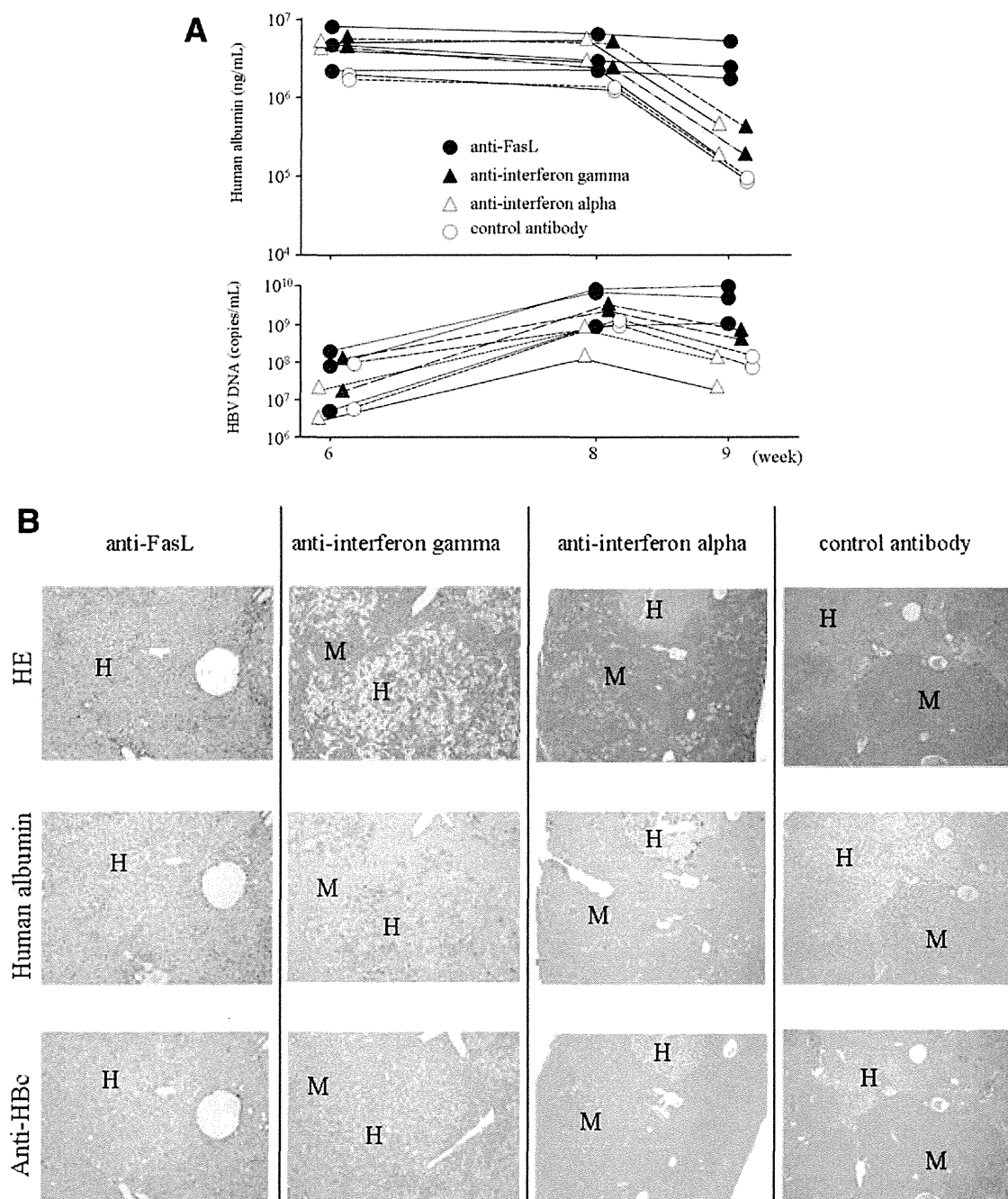


Fig. 5. Effect of anti-FasL, anti-IFN- γ and anti-IFN- α antibody administration on HSA and HBV DNA. (A) Time courses of HSA (upper panel) and HBV DNA (lower panel) before and 1 week after human PBMC transplantation are shown. Mice were pretreated with antibodies against human Fas-L, IFN- γ , and IFN-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

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.

Acknowledgment: The authors thank Rie Akiyama and Yoko Matsumoto for their expert technical assistance. This work was carried out at the Analysis Center of Life Science, Natural Science Center for Basic Research and Development, Hiroshima University.


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Research Paper

Adherence to Medication Is a More Important Contributor to Viral Breakthrough in Chronic Hepatitis B Patients Treated with Entecavir Than in Those with Lamivudine

Hidehiro Kamezaki^{1*}, Tatsuo Kanda^{1*} , Makoto Arai¹, Shuang Wu¹, Shingo Nakamoto¹, Tetsuhiro Chiba¹, Hitoshi Maruyama¹, Keiichi Fujiwara¹, Fumihiko Kanai¹, Fumio Imazeki¹, Fumio Nomura², Osamu Yokosuka¹

1. Departments of Gastroenterology and Nephrology, Chiba University, Graduate School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan;
2. Departments of Molecular Diagnosis, Chiba University, Graduate School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan.

* Hidehiro Kamezaki and Tatsuo Kanda contributed equally.

✉ Corresponding author: Tatsuo Kanda, M.D., Ph.D., Associate Professor, Department of Gastroenterology and Nephrology, Chiba University, Graduate School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan. Tel.: +81-43-226-2083, Fax: +81-43-226-2088; E-mail: kandat-cib@umin.ac.jp.

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Received: 2013.01.02; Accepted: 2013.03.13; Published: 2013.03.15

Abstract

Viral breakthrough is related to poor adherence to medication in some chronic hepatitis B patients treated with nucleos(t)ide analogues (NAs). Our study aimed to examine how adherence to medication is associated with viral breakthrough in patients treated with NAs. A total of 203 patients (135 ETV and 68 LAM) were analyzed in this retrospective analysis. Physical examination, serum liver enzyme tests, and hepatitis B virus marker tests were performed at least every 3 months. We reviewed medical records and performed medical interviews regarding to patients' adherence to medication. Adherence rates <90% were defined as poor adherence in the present study. Cumulative viral breakthrough rates were lower in the ETV-treated patients than in the LAM-treated patients ($P<0.001$). Seven ETV-treated (5.1%) and 6 LAM-treated patients (8.8%) revealed poor adherence to medication ($P=0.48$). Among ETV-treated patients, 4 (3.1%) of 128 patients without poor adherence experienced viral breakthrough and 3 (42.8%) of 7 patients with poor adherence experienced viral breakthrough ($P<0.001$). Only 3 of 38 (7.8%) LAM-treated patients with viral breakthrough had poor adherence, a lower rate than the ETV-treated patients ($P=0.039$). Nucleoside analogue resistance mutations were observed in 50.0% of ETV- and 94.1% of LAM-treated patients with viral breakthrough ($P=0.047$). Viral breakthrough associated with poor adherence could be a more important issue in the treatment with especially stronger NAs, such as ETV.

Key words: Adherence, Entecavir, Lamivudine, Hepatitis B, Viral Breakthrough.

INTRODUCTION

Two billion people have been exposed to hepatitis B virus (HBV), and 350-400 million people remain

chronically infected worldwide. In Japan, the prevalence of HBV carriers is estimated at ~1% of the pop-

ulation, but HBV is a major health issue because it causes acute hepatitis, chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) [1, 2].

Lamivudine (LAM) is a reverse-transcriptase inhibitor of HBV DNA polymerase that possesses excellent profile of safety and tolerability and causes inhibition of viral replication. LAM was the first nucleos(t)ide analogue (NA) to be approved for antiviral treatment of hepatitis B patients [3, 4]. Entecavir (ETV), a deoxyguanosine analogue, is a potent and selective inhibitor of HBV replication. The in vitro potency of ETV is 100- to 1,000-fold greater than that of LAM, and it has a selectivity index (concentration of drug required to reduce viable cell number by 50% [CC₅₀] / concentration of drug required to reduce viral replication by 50% [EC₅₀]) of approximately 8,000 [5, 6]. LAM (until 2005) and ETV (from 2006) have been used as first-line NAs for most patients with chronic hepatitis B in Japan. Most patients with chronic hepatitis B have been undergoing treatment for longer durations, and prolonged treatment is associated with increasing rates of viral breakthrough [7]. It has been reported that not all cases are associated with resistance mutations [8, 9]. We have also reported that some cases of viral breakthrough during ETV treatment were related to poor adherence to medication [10].

Adherence rates are usually lower in patients with long-term treatment regimens, such as for hypertension, than in patients with short-term regimens, such as for gastric ulcers [11]. It has been reported that 74.8% of patients with hypertension were determined to have an adherence rate $\geq 80\%$ [12], and that 55.3% of patients with chronic hepatitis B had an adherence rate $>90\%$ [8].

In the present study, we aimed to investigate whether drug adherence is related to viral breakthrough in chronic hepatitis B patients treated with LAM or ETV. We also investigated the pattern of poor adherence and suggested how adherence to medication could be improved.

MATERIALS AND METHODS

Patients

Two hundred seventy-five NA-treated naïve patients (185 ETV- and 90 LAM-treated patients), who were admitted to Chiba University Hospital between April 2000 and September 2011, were enrolled (Figure 1). Some of these patients had already been included in a previous report [10]. Between November 2011 and April 2012, doctors performed medical interviews of those patients to determine their adherence to medication. Seventy-two patients (50 ETV- and 22 LAM-treated patients) were excluded from this retrospective analysis, because their adherence to medication could not be confirmed. One hundred thirty-five patients were administered 0.5 mg of ETV daily and 68 patients were administered 100 mg of LAM daily (Table 1). In all patients, serum hepatitis B surface antigen (HBsAg) and HBV DNA were positive. All patients had negative results for hepatitis C virus or human immunodeficiency virus antibodies. Physical examinations, serum liver enzyme tests, and HBV marker tests were performed at least every 3 months. The study was carried out in accordance with the Helsinki Declaration, and was approved by the Ethics Committee of Chiba University, Graduate School of Medicine (No. 977).

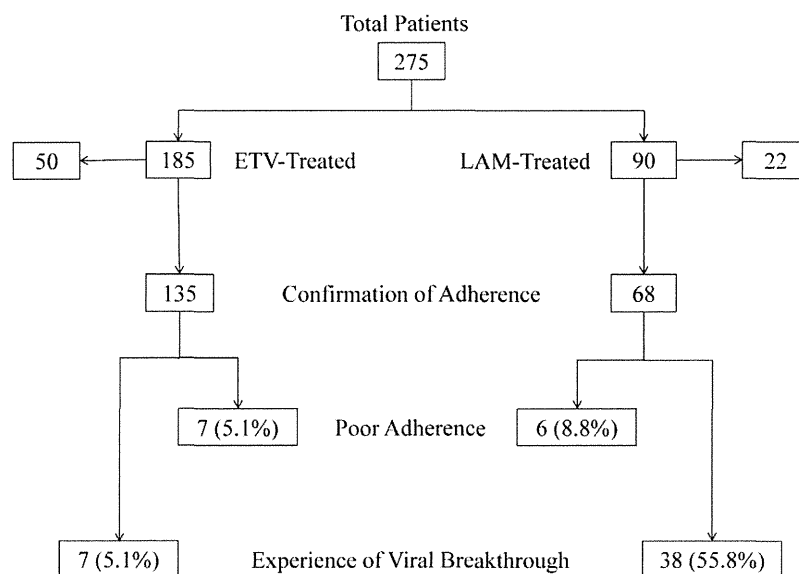


Figure 1. Patients, adherence rates, and the prevalence of viral breakthrough in this study. ETV, entecavir; LAM, lamivudine.