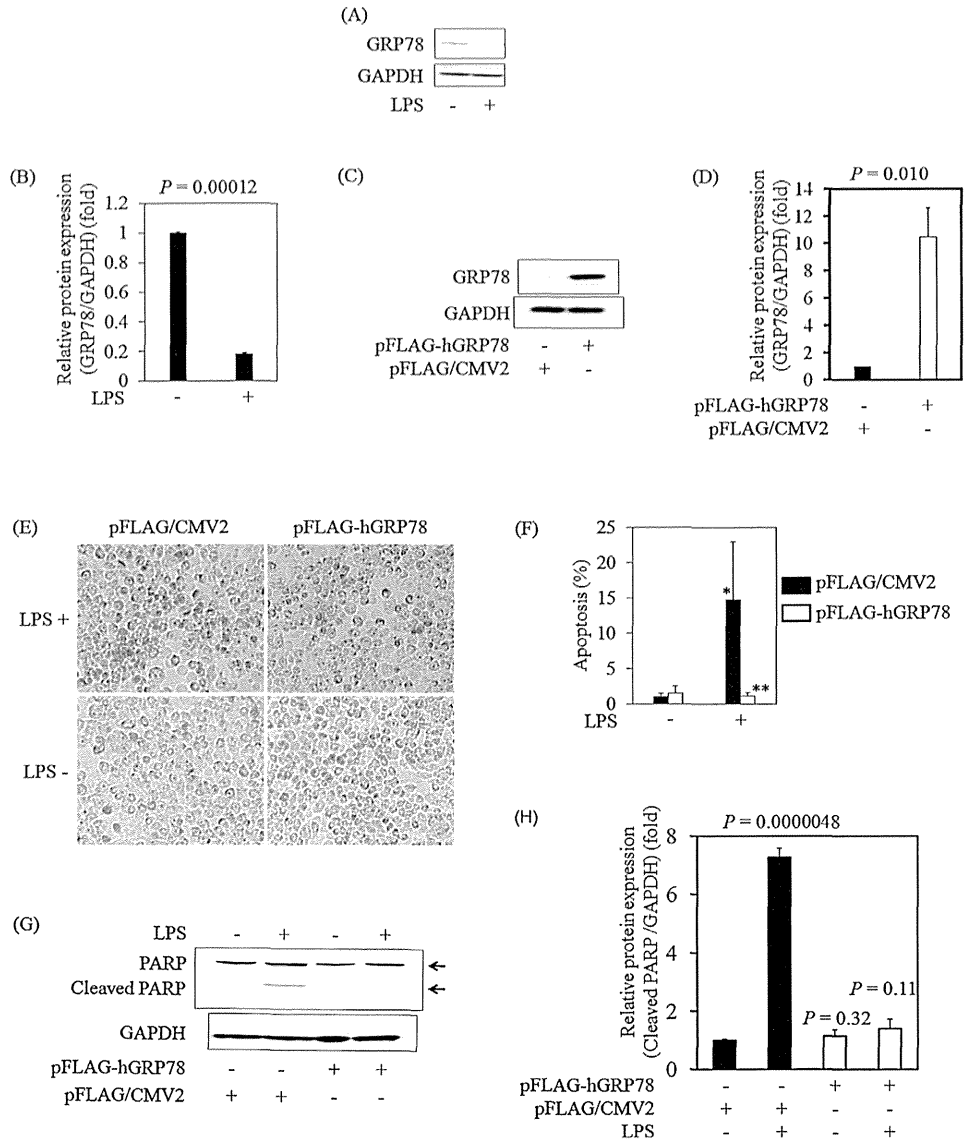

Fig. 5. Overexpression of GRP78 rescued LPS-treated HepG2 cells from cell death. (A) Western blot analysis of GRP78 expression in HepG2 treated for 24 h with or without LPS (500 ng/mL). Four micrograms of proteins was subjected to electrophoresis on 5–20% gradient polyacrylamide gels and transferred onto polyvinylidene difluoride membranes. Blots were re-probed with GAPDH-specific antibodies to assess equal protein loading. (B) GRP78/GAPDH ratios were measured using Scion Image. (C) Overexpression of human GRP78 protein separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by Western blot. Cells were transfected with 0.4 μg pFLAG-human (h) GRP78 or pFLAG/CMV2 control vectors [15] and 2 days later, cellular proteins were collected with 1× SDS lysis buffer. Four micrograms of proteins was subjected to electrophoresis on 5–20% gradient polyacrylamide gels and transferred onto polyvinylidene difluoride membranes. (D) GRP78/GAPDH ratios were measured using Scion Image. (E, F) Cell apoptosis was quantified by APOPercentage Apoptosis Assay. HepG2 cells transfected with 0.2 μg pFLAG/CMV2 control vectors (black column) or 0.2 μg pFLAG-hGRP78 (white column) were cultured for 24 h with or without LPS (500 ng/mL). Purple-red stained cells were identified as apoptotic cells using light microscopy. The number of purple-red cells/300 cells was counted as previously described [18]. *P = 0.043, compared to untreated control by Student's t-test. **P = 0.044, between HepG2 transfected with pFLAG-hGRP78 and with control vectors. (G) Overexpression of GRP78 reduced LPS-stimulated poly(ADP-ribose) polymerase (PARP) cleavage in HepG2. Western blot analyses of poly(adenosine diphosphate-ribose) polymerase (PARP) expression in pFLAG/CMV2 or pFLAG-hGRP78-transfected cells treated for 24 h with LPS. Four micrograms of proteins was subjected to electrophoresis on 7% polyacrylamide gels and transferred onto polyvinylidene difluoride membranes. Blots were reprobed with GAPDH-specific antibodies to assess equal protein loading. (H) Densitometric analysis of cleaved PARP/GAPDH showing increased cleaved PARP after transfection with pFLAG/CMV2 and LPS-treatment in HepG2 cells. Transfection of pFLAG-hGRP78 blocked LPS-induced PARP cleavage. Cleaved PARP/GAPDH ratios were measured using Scion Image. Data from three independent experiments were measured using Scion Image. Data are expressed as mean ± SD.

polyvinylidene difluoride membranes. Blots were re-probed with GAPDH-specific antibodies to assess equal protein loading. ATF6/GAPDH (F), ATF4/GAPDH (G), P-eIF2α/total eIF2α (H) and eIF2α/GAPDH (I) ratios were measured using Scion Image. Blots were re-probed with GAPDH-specific antibodies to assess equal protein loading. Results are expressed as mean ± SD of triplicate determinations from 1 experiment representative of 3 independent experiments.

pathways. It was recently reported that the commitment phase of ER stress-induced apoptosis is largely dependent on mitochondria [7]. In the present study, we did not observe the induction of CHOP by LPS in HepG2 (Fig. 3C), which is reported to cause apoptosis, although we observed LPS-induced apoptosis in HepG2. Although Zha et al. [35] reported that tunicamycin induced HepG2 apoptosis concomitantly with the upregulation of pro-apoptotic transcription factor CHOP and downregulation of Bcl-2, CHOP might not be essential in LPS-induced hepatic apoptosis. But we did not observe the enhancement of hepatic apoptosis with LPS treatment with ER stress-induced media including thapsigargin (data not shown). Further studies focusing on the relationship between CHOP and hepatic apoptosis will be needed. Recent evidence indicates that the cell homeostasis program triggered by ER stress intersects with the innate immune response [36]. Our results support the previous report that the ER stress response might be part of the conserved innate immune recognition system [37].

Previous studies [33,34] reported that HBV and HCV infections induced ER stress and oxidative stress in the liver. HBV and HCV infections induce ER stress, perhaps as a result of a weakening innate immune response including the TLR signaling pathway [17,38]. Further studies will be needed to clarify this. In the present study, we observed that UPR was reduced when LPS induced apoptosis in human hepatoma cell lines. Innate immune response seems to be closely associated with UPR. It was reported that GRP78 promotes tumor proliferation, survival, metastasis and resistance to a wide variety of therapies [9] and that GRP78 is upregulated in HCC [20]. In the present study, we also observed that the overexpression of GRP78 prevented hepatoma cells from reaching apoptosis, suggesting that GRP78 may play an important role in the resistance against therapies for HCC.

It was also reported that UPR inhibits cisplatin-induced apoptosis in HCC cells [39]. Shi et al. [40] reported that targeting autophagy enhances sorafenib lethality for HCC via ER stress-related apoptosis. The combination of ER stress-associated cell death and molecular-targeted therapy might represent a promising therapeutic strategy for the treatment of HCC. In conclusion, when LPS induced apoptosis in human hepatoma cell lines, we observed that UPR was impaired in these same cell lines. Innate immune response including TLR signaling seems to play an important role in UPR. Of interest, the interaction of inflammation and ER stress seems to control hepatoma apoptosis, presenting compelling clinical implications. Our study showed that UPR downregulation could be a collateral effect of LPS treatment, suggesting that UPR determines hepatic cell damage induced by an innate immune response including TLR signaling pathways. Still, little is known about the detailed molecular mechanisms that allow them to play different roles in hepatic cell apoptosis.

Disclosure statement

No competing financial interests exist.

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References

[1] Llovet JM, Di Bisceglie AM, Bruix J, Kramer BS, Lencioni R, Zhu AX, et al. Design and endpoints of clinical trials in hepatocellular carcinoma. J Natl Cancer Inst 2008;100:698–711.
[2] Higashi T, Hasegawa K, Kokudo N, Makuuchi M, Izumi N, Ichida T, et al. Demonstration of quality of care measurement using the Japanese liver cancer registry. Hepatol Res 2011;41:1208–15.
[3] Shiina S, Tateishi R, Arano T, Uchino K, Enooku K, Nakagawa H, et al. Radiofrequency ablation for hepatocellular carcinoma: 10-year outcome and prognostic factors. Am J Gastroenterol 2012;107:569–77.
[4] Okuda K. Hepatocellular carcinoma. J Hepatol 2000;32(1 Suppl.):225–37.
[5] Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, et al. Sorafenib in advanced hepatocellular carcinoma. N Engl J Med 2008;359:378–90.
[6] Cheng AL, Kang YK, Chen Z, Tsao CJ, Qin S, Kim JS, et al. Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomized, double-blind, placebo-controlled trial. Lancet Oncol 2009;10:25–34.
[7] Gorman AM, Healy SJ, Jager R, Samali A. Stress management at the ER: regulators of ER stress-induced apoptosis. Pharmacol Ther 2012;134:306–16.
[8] Woo CW, Cui D, Arellano J, Dorweiler B, Harding H, Fitzgerald KA, et al. Adaptive suppression of the ATF4-CHOP branch of the unfolded protein response by toll-like receptor signaling. Nat Cell Biol 2009;11:1473–80.
[9] Lee AS. GRP78 induction in cancer: therapeutic and prognostic implications. Cancer Res 2007;67:3496–9.
[10] Pfaffenbach KT, Lee AS. The critical role of GRP78 in physiologic and pathologic stress. Curr Opin Cell Biol 2011;23:150–6.
[11] Rodrigues R, Paranhos-Baccala G, Vernet G, Peyrefitte CN. Crimean-Congo hemorrhagic fever virus-induced hepatocytes induce ER-stress and apoptosis crosstalk. PLoS ONE 2012;7:e29712.
[12] Masciarelli S, Fra AM, Pengo N, Bertolotti M, Cenci S, Faqioli C, et al. CHOP-independent apoptosis and pathway-selective induction of the UPR in developing plasma cells. Mol Immunol 2010;47:1356–65.
[13] Rutkowski DT, Kaufman RJ. A trip to the ER: coping with stress. Trends Cell Biol 2004;14:20–8.
[14] Schroder M, Kaufman RJ. ER stress and the unfolded protein response. Mutat Res 2005;569:29–63.
[15] Yoo SA, You S, Yoon HJ, Kim DH, Kim HS, Lee K, et al. A novel pathogenic role of the ER chaperone GRP78/BiP in rheumatoid arthritis. J Exp Med 2012;209:871–86.
[16] Huber M, Kalis C, Keck S, Jiang Z, Georgel P, Du X, et al. R-form LPS, the master key to the activation of TLR4/MD-2-positive cells. Eur J Immunol 2006;36:701–11.
[17] Wu S, Kanda T, Imazeki F, Arai M, Yonemitsu Y, Nakamoto S, et al. Hepatitis B virus e antigen downregulates cytokine production in human hepatoma cell lines. Viral Immunol 2010;23:467–76.
[18] Tamura R, Kanda T, Imazeki F, Wu S, Nakamoto S, Tanaka T, et al. Hepatitis C virus nonstructural 5A protein inhibits lipopolysaccharide-mediated apoptosis of hepatocytes by decreasing expression of Toll-like receptor 4. J Infect Dis 2011;204:793–801.
[19] Chiou JF, Tai CJ, Huang MT, Wei PL, Wang YH, An J, et al. Glucose-regulated protein 78 is a novel contributor to acquisition of resistance to sorafenib in hepatocellular carcinoma. Ann Surg Oncol 2010;17:603–12.
[20] Shuda M, Kondoh N, Imazeki N, Tanaka K, Okada T, Mori K, et al. Activation of the ATF6, XBP1 and grp78 genes in human hepatocellular carcinoma: a possible involvement of the ER stress pathway in hepatocarcinogenesis. J Hepatol 2003;38:605–14.
[21] Choi KB, Wong F, Harlan JM, Chaudhary PM, Hood L, Karsan A. Lipopolysaccharide mediates endothelial apoptosis by a FADD-dependent pathway. J Biol Chem 1998;273:20185–8.
[22] Kuwabara T, Imajoh-Ohmi S. LPS-induced apoptosis is dependent upon mitochondrial dysfunction. Apoptosis 2004;9:467–74.
[23] Laplante P, Amireault P, Subang R, Dieude M, Levine JS, Rauch J. Interaction of β2-glycoprotein I with lipopolysaccharide leads to Toll-like receptor 4 (TLR4)-dependent activation of macrophages. J Biol Chem 2011;286:42494–503.
[24] Gilmore WJ, Hartmann G, Piquette-Miller M, Marriott J, Kirby GM. Effects of lipopolysaccharide-stimulated inflammation and pyrazole-mediated hepatocellular injury on mouse hepatic Cyp2a5 expression. Toxicology 2003;184:211–26.
[25] Endo M, Oyadomari S, Suga M, Mori M, Gotoh T. The ER stress pathway involving CHOP is activated in the lungs of LPS-treated mice. J Biochem 2005;138:501–7.
[26] Kozlov AV, Duvigneau JC, Miller I, Nurnberger S, Gesslbauer B, Kungl A, et al. Endotoxin causes functional endoplasmic reticulum failure, possibly mediated by mitochondria. Biochim Biophys Acta 2009;1792:521–30.
[27] Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K. XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. Cell 2001;107:881–91.
[28] Akazawa Y, Cazanave S, Mott JL, Elmi N, Bronk SF, Kohno S, et al. Palmitoleate attenuates palmitate-induced Bim and PUMA up-regulation and hepatocyte lipoapoptosis. J Hepatol 2010;52:586–93.
[29] Malhi H, Kaufman RJ. Endoplasmic reticulum stress in liver disease. J Hepatol 2011;54:795–809.
[30] Zhao L, Ackerman SL. Endoplasmic reticulum stress in health and disease. Curr Opin Cell Biol 2006;18:444–52.

[31] Knowlton AA. Life, death, the unfolded protein response and apoptosis. Cardiovasc Res 2007;73:1–2.
[32] Ma KL, Ruan XZ, Powis SH, Chen Y, Moorhead JF, Varghese Z. Inflammatory stress exacerbates lipid accumulation in hepatic cells and fatty livers of apolipoprotein E knockout mice. Hepatology 2008;48:770–81.
[33] Banerjee A, Ray RB, Ray R. Oncogenic potential of hepatitis C virus proteins. Viruses 2010;2:2108–33.
[34] Na B, Huang Z, Wang Q, Qi Z, Tian Y, Lu CC, et al. Transgenic expression of entire hepatitis B virus in mice induces hepatocarcinogenesis independent of chronic liver injury. PLoS ONE 2011;6:e26240.
[35] Zha L, Fan L, Sun G, Wang H, Ma T, Zhong F, et al. Melatonin sensitizes human hepatoma cells to endoplasmic reticulum stress-induced apoptosis. J Pineal Res 2012;52:322–31.
[36] Glimcher LH, Martinon F, Modlin RL. Editorial overview. Curr Opin Immunol 2011;23:1–2.
[37] Modlin RL, Glimcher LH. Regulation of innate immunity by signaling pathways emerging from the endoplasmic reticulum. Curr Opin Immunol 2011;23:35–40.
[38] Wu S, Kanda T, Imazeki F, Nakamoto S, Tanaka T, Arai M, et al. Hepatitis B virus e antigen physically associates with receptor-interacting serine/threonine protein kinase 2 and regulates IL-6 gene expression. J Infect Dis 2012;206:415–20.
[39] Chen R, Dai RY, Duan CY, Liu YP, Chen SK, Yan DM, et al. Unfolded protein response suppresses cisplatin-induced apoptosis via autophagy regulation in human hepatocellular carcinoma cells. Folia Biol (Praha) 2011;57:87–95.
[40] Shi YH, Ding ZB, Zhou J, Hui B, Shi GM, Ke AW, et al. Targeting autophagy enhances sorafenib lethality for hepatocellular carcinoma via ER stress-related apoptosis. Autophagy 2010;7:1159–72.

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Research Paper

Efficacy of Lamivudine or Entecavir against Virological Rebound after Achieving HBV DNA Negativity in Chronic Hepatitis B Patients

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Abstract

Nucleos(t)ide analogues (NAs) lead to viral suppression and undetectable hepatitis B virus (HBV) DNA in some individuals infected with HBV, but the rate of virological rebound has been unknown in such patients. We examined the prevalence of virological rebound of HBV DNA among NA-treated patients with undetectable HBV DNA. We retrospectively analyzed 303 consecutive patients [158 entecavir (ETV)- and 145 lamivudine (LAM)-treated] who achieved HBV DNA negativity, defined as HBV DNA < 3.7 log IU/mL for at least 3 months. They were followed up and their features, including their rates of viral breakthrough, were determined. Viral rebound after HBV DNA negativity was not observed in the ETV-group. Viral rebound after HBV DNA negativity occurred in 38.7% of 62 HBe antigen-positive patients in the LAM-group. On multivariate analysis, age was an independent factor for viral breakthrough among these patients (P = 0.035). Viral rebound after HBV DNA negativity occurred in 29.1% of 79 HBe antigen-negative patients in the LAM-group. Differently from LAM, ETV could inhibit HBV replication once HBV DNA negativity was achieved. In contrast, LAM could not inhibit HBV replication even if HBV negativity was achieved in the early phase. Attention should be paid to these features in clinical practice.

Key words: Entecavir, HBeAg, HBV DNA, Lamivudine, Virological rebound.

INTRODUCTION

Hepatitis B virus (HBV) infection remains a major health problem and one of the risk factors for the development of hepatocellular carcinoma (HCC) worldwide [1,2]. Chronic HBV infection has been linked epidemiologically to the development of HCC for more than 30 years [3]. To date, the mechanism of HBV-related hepatocarcinogenesis is not clear. Although effective vaccine exists for preventing HBV

infection [4], acute liver failure due to HBV or acute exacerbation of chronic hepatitis B is also a life-threatening disease [5,6].

Positivity for hepatitis B e antigen (HBeAg), which in serum indicates active viral replication in hepatocytes, is associated with an increased risk of HCC [7]. Chronic HBV carriers with high-titer viremia are also at increased risk for HCC [8]. The risk for cirrhosis and that for HCC increase significantly with increasing HBV DNA levels [9, 10]. Thus, it cannot be overstated that HBV DNA should be directly suppressed to prevent the development of HCC.

There are several nucleos(t)ide analogues (NAs) for the treatment of chronic hepatitis B [11]. Currently, the Japanese national health insurance system approves lamivudine (LAM) and entecavir (ETV) as first-line therapy for treatment-naïve patients with chronic hepatitis B, although some patients are treated with standard interferon-alfa or peginterferon-alfa-2a [6,12]. In general, LAM, the first oral NA available for the treatment of chronic hepatitis B, is associated with high rates of drug-resistance, with ~76% after 8 years of treatment [13,14]. ETV is found to be superior to LAM from the point of view that ETV is stronger than LAM and that resistance to ETV is rare, about 1.2% after 5 years of ETV treatment [14,15].

The aim of this study was to determine the efficacy and the rates of virological rebound after achieving HBV DNA negativity in the use of ETV or LAM in clinical practice. Our study showed that ETV could inhibit HBV replication if HBV DNA negativity had been achieved, but LAM was unable to inhibit HBV replication even if HBV negativity was achieved in the early phase.

MATERIALS AND METHODS

Patients and Study Design

This was a retrospective analysis comparing the rates of virological rebound in patients treated with ETV versus those in patients treated with LAM. A total of 303 patients were examined from Chiba University Hospital, Chiba, Japan, and 4 affiliated hospitals between the period of January 2000 and December 2011. NAs-naïve chronic hepatitis B patients daily receiving 0.5 mg of ETV (ETV group, N=158) or receiving 100 mg of LAM (LAM group, N=145) with undetectable HBV DNA (< 3.7 log IU/mL) for three months were enrolled. Some of the included patients had been previously reported [12, 16]. All patients had serum hepatitis B surface antigen (HBsAg) detectable for at least 6 months, regardless of their HBeAg status. They were negative for hepatitis C virus and human immunodeficiency virus antibodies. This study was approved by the Ethics Committee of Chiba University, Graduate School of Medicine (No. 977).

Definition of Virological Rebound of HBV

We defined virological rebound as $\geq$ 3.7 log IU/mL for at least 3 months after achieving undetectable HBV DNA.

Monitoring of HBV DNA, Serum Liver Function Tests and Hematological Tests

The primary outcome of this study was the virological rebound. Patients were followed up at least every 3 months to examine physical status and to monitor liver biochemistry and virology. All clinical laboratory tests including hematological data, biochemical data, and HBV serologies were performed at the Central Laboratory of Chiba University Hospital. HBsAg, HBeAg and anti-HBe antibody were determined by ELISA (Abbott, Chicago, IL, USA) or CLEIA (Fujirebio, Tokyo, Japan) [17]. HBV genotype was determined from patients' sera by ELISA (Institute of Immunology, Tokyo, Japan) as reported by Usuda et al [18]. HBV DNA was measured by transcription-mediated amplification (TMA) assay, COBAS Amplicor HBV Monitor assay, or COBAS TaqMan (Roche Diagnostics, Branchburg, NJ, USA). The clinical efficacy of NAs was assessed as the proportion of patients achieving HBV DNA negativity, defined as an HBV DNA level of < 3.7 log IU/mL.

Statistical analysis

Data were expressed as mean $\pm$ standard deviation (SD). Differences were evaluated by Student's *t*-test, chi-square test, or Fisher's exact test. $P < 0.05$ was considered statistically significant. Variables with $P < 0.05$ at univariate analysis were retained for multivariate logistic-regression analysis. For all tests, two-sided P-values were calculated and the results were considered statistically significant at $P < 0.05$. Statistical analysis was performed using the Excel-statistics program for Windows, version 7 (SSRI, Tokyo, Japan).

RESULTS

A total 303 patients were recruited into either the ETV group (n = 158) or the LAM group (n = 145), with a follow-up period of 33.7 $\pm$ 11.3 months (28.6 $\pm$ 11.3 months or 39.3 $\pm$ 31.4 months, respectively). Baseline demographic and laboratory data are summarized in Table 1. There were no differences in age, gender, HBV DNA, alanine aminotransferase (ALT) levels, ultrasound findings/presence of cirrhosis, and periods from the initial administration of ETV or LAM to

undetectable HBV DNA, between the ETV and LAM groups, although the proportion of HBeAg-positive patients in the ETV group (55%) tended to be higher than that in the LAM group (44%).

Virological Rebound

The patient flow and outcome are summarized in Figure 1. We excluded 9 patients, whose HBeAg status at baseline was unknown, from this analysis. When comparing the baseline characteristics of patients according to HBeAg status, HBeAg-positive patients were younger, had higher ALT levels and HBV DNA levels, and less cirrhotic findings by ultrasound than HBeAg-negative patients (Table 2). The period from the initial administration of ETV or LAM to the determination of undetectable HBV DNA in the HBeAg-negative group tended to be shorter than that in the HBeAg-positive group (Table 2).

In the ETV group, none of the patients had virological rebound during the follow-up periods. In the LAM group, 24 and 23 patients of 62 HBeAg-positive and 79 HBeAg-negative patients at baseline, respectively, developed evidence of virological rebound. In the 24 HBeAg-positive patients at baseline with virological rebound, 9, 8, 3, 1, 2, and 1 had virological rebound at ≤ 1, $1 \sim \leq 2$, $2 \sim \leq 3$, $3 \sim \leq 4$, $4 \sim \leq 5$, and details unknown, respectively. In the 23 HBeAg-negative patients at baseline with virological rebound, 10, 8, 3, 0, 1, and 1 had virological rebound at ≤ 1, $1 \sim \leq 2$, $2 \sim \leq 3$, $3 \sim \leq 4$, $4 \sim \leq 5$ and details unknown, respectively. Baseline characteristics of patients treated with ETV or LAM according to HBeAg status are shown in Table 3. In the ETV group, the period from the initial administration of ETV to the determination of undetectable HBV DNA in the HBeAg-negative group was the same as that in the HBeAg-positive group (Table 3). In the LAM group, the period from the initial administration of LAM to undetectable HBV DNA in the HBeAg-negative group was shorter than that in the HBeAg-positive group (Table 3). In the HBeAg-positive patients, the period from the initial administration to undetectable HBV DNA in the ETV group was shorter than that in the LAM group (Table 3).

Predictors of Virological Rebound in Patients treated with LAM

To clarify the predictors of virological rebound in patients treated with LAM, we compared the pretreatment factors between patients with and without virological rebound according to HBeAg status (Table 4A & 4B). Univariative analysis showed that age, HBV DNA, ALT levels and the period from the initial administration of LAM to the determination of undetectable HBV DNA in HBeAg-positive patients contributed to the occurrence of virological rebound (Table 4A). Factors significantly associated with virological rebound in HBeAg-positive patients treated with LAM by univariate analysis were also analyzed by multivariate logistic regression analysis. Virological rebound was attained independently of age in HBeAg-positive patients treated with LAM (Table 4C). In HBeAg-negative patients, no significant factors contributing to virological rebound could be found (Table 4B).

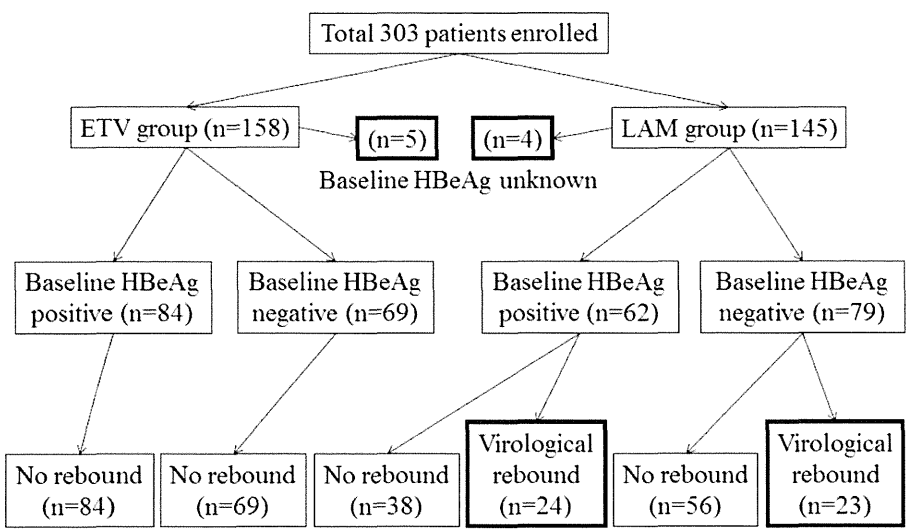

Figure 1. Study design and patient flow for both groups.

Table 1. Baseline characteristics of patients treated with entecavir (ETV) or lamivudine (LAM).

	Total	ETV group	LAM group	*P*-values
Number	303	158	145	
Age (years)	51 ± 12	51 ± 12	50 ± 12	*N.S.*
Gender (male)	205	101	104	*N.S.*
HBeAg (+)	146	84	62	*0.079*
HBV DNA (log IU/mL)	6.5 ± 1.5	6.6 ± 1.7	6.4 ± 1.3	*N.S.*
ALT (IU/L)	203 ± 280	187 ± 290	220 ± 266	*N.S.*
US: Cirrhosis (+)	113	56	57	*N.S.*
Periods to undetectable HBV DNA (months)	10.0 ± 18.2	8.5 ± 11.9	11.8 ± 23.3	*N.S.*

Data are expressed as mean ± SD. ETV group, patients receiving 0.5 mg of ETV daily; LAM group, patients receiving 100 mg of LAM daily; *P*-values, *P*-values between ETV and LAM groups; HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase; US, ultrasound findings; *N.S.*, no statistically significant difference.

Table 2. Baseline characteristics of patients according to HBeAg status.

HBeAg	Positive group	Negative group	*P*-values
Number	146	148	
Age (years)	46 ± 12	55 ± 11	*< 0.001*
Gender (male)	101	97	*N.S.*
HBV DNA (log IU/mL)	7.2 ± 1.1	5.8 ± 1.4	*< 0.001*
ALT (IU/L)	257 ± 332	156 ± 211	*0.002*
US: Cirrhosis (+)	41	70	*< 0.001*
Periods to undetectable HBV DNA (months)	11.0 ± 18.1	7.4 ± 14.4	*0.063*

Data are expressed as mean ± SD. *P*-values, *P*-values between HBeAg-positive and HBeAg-negative groups; HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase; US, ultrasound findings; *N.S.*, no statistically significant difference.

Table 3. Baseline characteristics of patients treated with entecavir (ETV) or lamivudine (LAM) according to HBeAg status.

	ETV group		LAM group	
HBeAg	Positive	Negative	Positive	Negative
Number	84	69	62	79
Age (years)	48 ± 12	56 ± 11*	44 ± 11 ##	54 ± 11**
Gender (male)	53	45	48	52**
HBV DNA (log IU/mL)	7.5 ± 1.1	5.7 ± 1.5*	6.9 ± 1.1$	5.9 ± 1.3**
ALT (IU/L)	219 ± 325	159 ± 246	309 ± 334	154 ± 174**
US: Cirrhosis (+)	25	29	16	41
Periods to undetectable HBV DNA (months)	8.3 ± 10.5	7.3 ± 11.0	15.0 ± 24.7$$	7.5 ± 16.9#

Data are expressed as mean ± SD. HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase; US, ultrasound findings; *$P < 0.001$, compared to HBeAg-positive of ETV group; **$P < 0.001$ and #$P = 0.034$, compared to HBeAg-positive of LAM group; ##$P = 0.041$, $P = 0.001$ and $$P = 0.027$, compared to HBeAg-positive of ETV group.

Table 4A. Predictors of virological rebound in patients treated with lamivudine (LAM). (A) Comparison of HBeAg-positive patients with or without virological rebound by univariate analysis.

Virological rebound	No	Yes	*P-values*
Number	38	23	
Age (years)	42 ± 11	49 ± 11	*0.019*
Gender (male)	30	17	*N.S.*
HBV DNA (log IU/mL)	6.9 ± 1.2	6.8 ± 0.9	*N.S.*
ALT (IU/L)	379 ± 377	196 ± 205	*0.037*
US: Cirrhosis (+)	7	9	*N.S.*
Periods to undetectable HBV DNA (months)	20.6 ± 29.1	4.1 ± 3.1	0.009

Data are expressed as mean ± SD. *P*-values, *P*-values between patients with or without virological rebound groups; HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase; US, ultrasound findings; *N.S.*, no statistically significant difference.

Table 4B. (B) Comparison of HBeAg-negative patients with or without virological rebound by univariate analysis.

Virological rebound	No	Yes	*P-values*
Number	56	22	
Age (years)	54 ± 11	54 ± 10	*N.S.*
Gender (male)	40	12	*N.S.*
HBV DNA (log IU/mL)	5.9 ± 1.4	5.9 ± 1.0	*N.S.*
ALT (IU/L)	163 ± 179	137 ± 163	*N.S.*
US: Cirrhosis (+)	30	11	*N.S.*
Periods to undetectable HBV DNA (months)	7.3 ± 14.8	3.1 ± 2.1	*N.S.*

Data are expressed as mean ± SD. *P*-values, *P*-values between patients with or without virological rebound groups; HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase; US, ultrasound findings; *N.S.*, no statistically significant difference.

Table 4C. (C) Factor associated with virological rebound among HBeAg-positive patients treated with LAM by multivariate analysis.

Factor	Category	Odds ratio	95% CI	*P-value*
Age ≤ 44.5 (years)	(+/-)	0.222	0.0547-0.9023	0.0354

DISCUSSION

To date, there is not much data regarding virological rebound after achieving HBV DNA negativity in the use of ETV or LAM. A recent report supported the merit of the change from LAM to ETV [14]. This study concluded that prior optimal viral suppression with ETV did not confer any significant advantage for patients who switched to LAM.

The present study revealed that ETV could suppress HBV replication after achieving HBV DNA negativity, although additional longer follow-up studies will be needed. On the other hand, LAM could not suppress HBV replication even after achieving HBV DNA negativity (Figure 1), although most cases with virological rebound were observed within 2 years of the start of LAM medication. We could not check the emergence of YMDD motif mutations [19] in all of the cases because the present study was performed as part of regular clinical practice. Of 2 of the HBeAg-positive patients at baseline with virological rebound, one showed YVDD motif (50%). In 4 of the HBeAg-negative patients at baseline with virological rebound, one YVDD motif (25%) and three YIDD motifs (75%) were seen. Virological rebound may not mean the emergence of NA-resistance mutations [12].

We do not know the reason why virological rebound was attained independently of age in HBeAg-positive patients treated with LAM. HBeAg to anti-HBe antibody seroconversions were found in 20 and 11 patients with and without virological rebound, that is, the HBeAg to anti-HBe antibody seroconversion rates were similar in the two groups (data not shown), although the number of study patients seemed small in the present study. Further studies might be needed. In any event, it might be important to consider the LAM-to-ETV switch in HBeAg-positive patients treated with LAM, although some of our patients in the LAM group remained HBV-negative throughout the observation period.

In the present study, 95.3% (122 of 128), 82.3% (14 of 17) and 89.2% (25 of 28) had an adherence rate >90% [16] in ETV-treated, LAM-treated with virological rebound and LAM-treated patients without virological rebound, respectively. These results supported our previous study that viral breakthrough associated with poor adherence could be a more important issue in the treatment with especially stronger NAs, such as ETV [12,16], although we cannot ensure durable HBV negativity after NAs are discontinued. We and others reported that HBeAg could impair both innate and adaptive immune responses to promote chronic HBV infection [16,20,21]. Of interest, the virological rebound with the use of LAM seemed unrelated to the HBeAg status, suggesting that it was dependent on resistant mutation.

Recently, other effective antiviral therapies such as peginterferon [22,23] and tenofovir [24,25] were reported to be useful for the control of HBV infection. These drugs might also be candidates for treating virological rebound. Fung et al. [14] reported that prior optimal viral suppression with ETV did not confer any significant advantage for patients who switched to LAM. Our results also supported the previous studies that ETV was much more efficient than LAM [26-29]. In conclusion, ETV could inhibit HBV replication if HBV DNA negativity had been achieved. In contrast, LAM could not inhibit HBV replication even if HBV negativity was achieved in the early phase. Attention should be paid to these features in clinical

practice.

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CONFLICT OF INTEREST

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ABBREVIATIONS

ALT: alanine aminotransferase; ETV: Entecavir; HBeAg: Hepatitis B e antigen; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; LAM: lamivudine; NA: nucleos(t)ide analogue.

REFERENCES

1. Dandri M, Locarnini S. New insight in the pathobiology of hepatitis B virus infection. Gut. 2012; 61: i6-i17.
2. Di Bisceglie AM. Hepatitis B and hepatocellular carcinoma. Hepatology. 2009; 49 (5 Suppl): S56-S60.
3. Beasley RP, Hwang LY, Lin CC, et al. Hepatocellular carcinoma and hepatitis B virus. A prospective study of 22707 men in Taiwan. Lancet. 1981; 2: 1129-1133.
4. Lavanchy D. Viral hepatitis: global goals for vaccination. J Clin Virol. 2012; 55:296-302.
5. Imamura T, Yokosuka O, Kurihara T, et al. Distribution of hepatitis B virus genotypes and mutations in the core promoter and precore regions in acute form of liver disease in patients from Chiba, Japan. Gut. 2003; 52: 1630-1637.
6. Kanda T, Shinozaki M, Kamezaki H, et al. Efficacy of lamivudine or entecavir on acute exacerbation of chronic hepatitis B. Int J Med Sci. 2012; 9: 27-32.
7. Yang HI, Lu SN, Liaw YF, et al. Hepatitis B e antigen and the risk of hepatocellular carcinoma. N Engl J Med. 2002; 347: 168-174.
8. Harris RA, Chen G, Lin WY, et al. Spontaneous clearance of high-titer serum HBV DNA and risk of hepatocellular carcinoma in a Chinese population. Cancer Causes Control. 2003; 14: 995-1000.
9. Chen CJ, Yang HI, Su J, et al. Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. JAMA. 2006; 295: 65-73.
10. Iloeje UH, Yang HI, Su J, et al. Predicting cirrhosis risk based on the level of circulating hepatitis B viral load. Gastroenterology. 2006; 130: 678-686.
11. Liaw YF, Kao JH, Piratvisuth T, et al. Asian-Pacific consensus statement on the management of chronic hepatitis B: a 2012 update. Hepatol Int. 2012; 6: 531-561.
12. Kamezaki H, Kanda T, Wu S, et al. Emergence of entecavir-resistant mutations in nucleos(t)ide-naïve Japanese patients infected with hepatitis B virus: virological breakthrough is also dependent on adherence to medication. Scand J Gastroenterol. 2011; 46: 1111-1117.
13. Yuen MF, Seto WK, Chow DH, et al. Long-term lamivudine therapy reduces the risk of long-term complications of chronic hepatitis B infection even in patients without advanced disease. Antivir Ther. 2007; 12: 1295-1303.
14. Fung J, Lai CL, Yuen J, et al. Randomized trial of lamivudine versus entecavir in Entecavir-treated patients with undetectable hepatitis B virus DNA: outcome at 2 years. Hepatology. 2011; 53: 1148-1153.
15. 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: 1503-1514.
16. Kamezaki H, Kanda T, Makoto A, et al. Adherence to medication is a more important contributor to viral breakthrough in chronic hepatitis B patients treated with entecavir than in those with lamivudine. Int J Med Sci. 2013; 10: 567-574.
17. Wu S, Kanda T, Imazeki F, et al. Hepatitis B virus e antigen downregulates cytokine production in human hepatoma cell lines. Viral Immunol. 2010; 23: 467-476.
18. Usuda S, Okamoto H, Iwanari H, et al. Serological detection of hepatitis B virus genotypes by ELISA with monoclonal antibodies to type-specific epitopes in the preS2-region product. J Virol Methods. 1999; 80: 97-112.
19. Seta T, Yokosuka O, Imazeki F, et al. Emergence of YMDD motief mutations of hepatitis B virus during lamivudine treatment of immunocompetent type B hepatitis patients. J Med Virol. 2000; 60: 8-16.
20. Chen M, Sallberg M, Hughes J, et al. Immune tolerance split between hepatitis B virus precore and core proteins. J Virol. 2005; 79: 3016-3027.
21. Wu S, Kanda T, Imazeki F, et al. Hepatitis B virus e antigen physically associates with receptor-interacting serine/threonine protein kinase 2 and requires IL-6 gene expression. J Infect Dis. 2012; 206: 415-420.
22. Marcellin P, Lau GK, Bonino F, et al. Peginterferon alfa-2a alone, lamivudine alone, and the two in combination in patients with HBeAg-negative chronic hepatitis B. N Engl J Med. 2004; 351: 1206-1217.
23. Lau GK, Piratvisuth T, Luo KX, et al. Peginterferon Alfa-2a, lamivudine, and the combination for HBeAg-positive chronic hepatitis B. N Engl J Med. 2005; 352: 2682-2695.
24. Schildgen O, Sirma H, Funk A, et al. Variant of hepatitis B virus with primary resistance to adefovir. N Engl J Med. 2006; 354: 1807-1812.
25. Marcellin P, Heathcote EJ, Buti M, et al. Tenofovir disoproxil fumarate versus adefovir dipivoxil for chronic hepatitis B. N Engl J Med. 2008; 359: 2442-2455.
26. Chang TT, Gish RG, de Man R, et al. A comparison of Entecavir and lamivudine for HBeAg-positive chronic hepatitis B. N Engl J Med. 2006; 354: 1001-1010.
27. Lai CC, Shouval D, Lok AS, et al. Entecavir versus lamivudine for patients with HBeAg-negative chronic hepatitis B. N Engl J Med. 2006; 354: 1011-1020.
28. Veenstra DL, Sullivan SD, Clarke L, et al. Cost effectiveness of Entecavir versus lamivudine with adefovir salvage in HBe-positive chronic hepatitis B. Pharmacoeconomics. 2007; 25: 963-977.
29. Lacey L, Chien RN, Chuang WL, et al. Economic evaluation of chronic hepatitis B treatments in Taiwan. J Gastroenterol Hepatol. 2008; 23: 571-579.

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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

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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% [CC_{50}] / concentration of drug required to reduce viral replication by 50% [EC_{50}]) 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 ≥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).

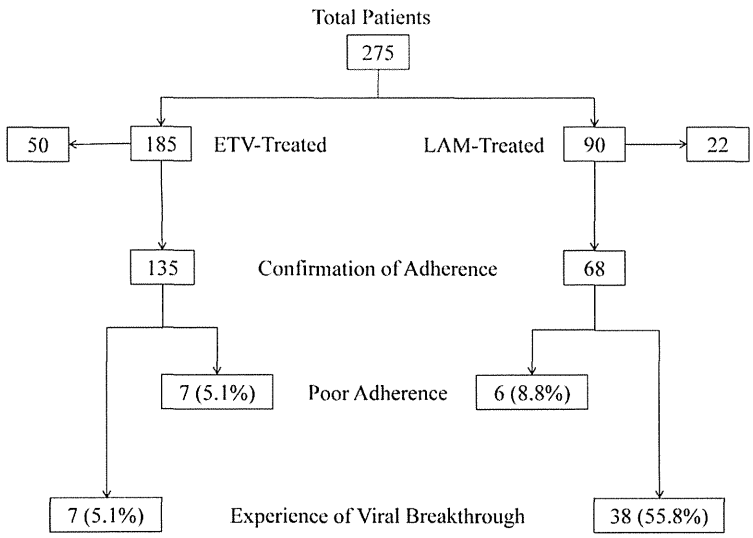


Figure 1. Patients, adherence rates, and the prevalence of viral breakthrough in this study. ETV, entecavir; LAM, lamivudine.

Table 1. Baseline characteristics of patients.

	ETV	LAM	P-values
Number of cases	135	68	
Age (years)	51.7 ± 11.7	45.5 ± 12.1	<0.001
Gender (male/female)	83/52	49/19	0.135
HBeAg (+/-)	64/71	45/23	0.011
Genotype (A/B/C/unknown)	0/11/78/46	1/6/57/4	0.427
HBV DNA (log IU/mL) (≤5.0/> 5.0/unknown)	27/108/0	3/55/10	0.009
ALT (IU/L)	161 ± 195	353 ± 394	<0.001
Platelets ($\times 10^4/mm^3$)	16.3 ± 5.9	16.9 ± 7.0	0.556
APRI	2.49 ± 4.19	6.52 ± 6.98	<0.001
Follow-up period (months)	26.9 ± 21.6	49.0 ± 39.7	<0.001

ETV, entecavir; LAM, lamivudine; HBeAg, hepatitis B e antigen; N.D., not determined; HBV DNA, hepatitis B virus deoxyribonucleic acid; ALT, alanine aminotransferase; APRI, aspartate aminotransferase platelet ratio index. Continuous variables are expressed as mean ± standard deviation.

Blood examinations

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels, and platelet counts were reviewed in the present study. We also calculated the aspartate aminotransferase platelet ratio index [APRI: AST (IU/L)/ 35/platelet count ($10^3/\mu L$) x 100], which is significantly correlated with the staging of liver fibrosis, with a higher correlation coefficient than platelet count or AST level alone [13].

Detection of HBV markers

HBsAg, hepatitis B e antigen (HBeAg) and anti-HBe antibody were determined by ELISA (Abbott, Chicago, IL, USA) or CLEIA (Fujirebio, Tokyo, Japan)[14]. HBV genotype was determined by ELISA (Institute of Immunology, Tokyo, Japan) [15]. HBV DNA was measured by Roche Amplicor PCR assay (detection limits: 2.6 log IU/mL; Roche Diagnostics, Tokyo, Japan).

Follow-up period

The follow-up period ended when the NA was switched to another NA or another NA was added, or it was discontinued for various reasons.

Definition of adherence to medication

To obtain information regarding adherence to medication, we reviewed medical records. We also interviewed patients about their adherence to medication. We expressed the rate of adherence to medication as a percentage calculated by the number of days of taking a pill divided by the follow-up period (days). Adherence rates <90% were defined as poor adherence in the present study.

Definition of viral breakthrough

Viral breakthrough was defined as an increase of ≥ 1 log IU/mL in serum HBV DNA level from nadir.

Sequence analysis of HBV DNA

The YMDD motif was analyzed by PCR-ELMA in sera of patients who had experienced viral breakthrough, as reported by Kobayashi et al [16]. HBV polymerase/reverse transcriptase (RT) substitutions were also analyzed in sera of ETV-treated patients who had experienced viral breakthrough. Briefly, HBV DNA was extracted from 100 μL of sera using SepaGene (Sanko Junyaku, Tokyo, Japan). Nested PCR was performed using LA Taq polymerase (Takara Bio, Otsu, Shiga, Japan) under the following conditions: 5-min denaturation at 94°C, 35 cycles with denaturation at 94°C for 40 s, annealing at 58°C for 1 min, and extension at 68°C for 1.5 min [2]. An 862 base-pair fragment (nt 242-1103) containing the polymerase RT domain was amplified on the PCR Thermal Cycler Dice Model TP600 (Takara Bio). The primers for the first PCR were 5′-CAG AGT CTA GAC TCG TGG-3′ (sense, nt 242-258) and 5′-GGC GAG AAA GTG AAAGCC-3′ (antisense, nt 1103-1086). The PCR product was sequenced using the primers: 5′-TGG CTC AGT TTA CTAGTG CC -3′ (nt 668-687) and 5′-GGC ACT AGT AAA CTGAGC CA-3′ (nt 687-668), and these primers were also used for the second PCR. To prepare the sequence template, PCR products were treated with ExoSAP-ITR (Affymetrix, Inc., Santa Clara, CA, USA), and then sequenced using the BigDye(R) Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Tokyo, Japan). Sequences were performed with Applied Biosystems 3730xl (Life Technologies) [17].

Statistical analysis

Statistical analyses were performed using SAS 9.3 Software (SAS Institute, Cary, NC, USA). Continuous variables were expressed as mean ± standard deviation and were compared by Student's t-test or

Welch's t-test. Categorical variables were compared by chi-square test or Fisher's exact probability test. The Kaplan-Meier method was used to calculate viral breakthrough rates. Baseline was taken as the date when the first dose of LAM or ETV was taken. Statistical significance was considered at a *P-value* < *0.05*.

RESULTS

Baseline characteristics of patients

Baseline characteristics of patients are shown in Table 1. In ETV-treated patients, the age was higher, the prevalence of HBeAg-negative patients was higher, HBV DNA was lower, ALT levels were lower, and APRI was lower (ie., liver fibrosis was milder) than in LAM-treated patients. HBV genotype C was dominant in both groups. The follow-up period in ETV-treated patients was shorter than that in LAM-treated patients, based on the fact that ETV was a newer drug and many ETV-treated patients had started treatment more recently.

Adherence to medication, and viral breakthrough between ETV- and LAM-treated patients

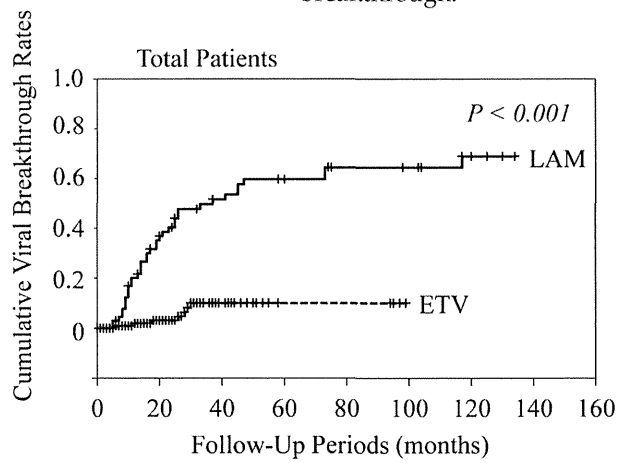
Most patients presented good adherence to medication in the present study. Seven ETV-treated (5.1%) and 6 LAM-treated patients (8.8%) had poor adherence (Figure 1). The number of patients with poor adherence was not significantly different between the ETV- and LAM-treated groups (*P=0.48*). The characteristics of the 13 patients with poor adherence are shown in Table 2. Cumulative viral breakthrough rates were lower in the ETV-treated patients than in the LAM-treated patients (*P<0.001*) (Figure 2).

Viral breakthrough in HBeAg-positive and -negative patients

Among the LAM-treated patients, cumulative viral breakthrough rates in HBeAg-positive patients at baseline (n=45; 25.0% at 1 year, 55.1% at 3 years, and 67.0% at 5 years) were similar to those in HBeAg-negative patients at baseline (n=23; 9.5% at 1 year, 38.2% at 3 years, and 44.4% at 5 years; *P=0.16*). Among the ETV-treated patients, cumulative viral breakthrough rates in HBeAg-positive patients at baseline (n=64; 2.2% at 1 year, 18.1% at 3 years, and 18.1% at 5 years) were also similar to those in HBeAg-negative patients at baseline (n=71; 1.6% at 1 year, 1.6% at 3 years, and 1.6% at 5 years; *P=0.050*).

Among the LAM-treated patients who were HBeAg-positive at baseline, cumulative viral breakthrough rates in patients who converted to HBeAg-seronegative were lower than those in patients who maintained HBeAg seropositivity (*P<0.001*) (Figure 3). All LAM-treated patients who did not become HBeAg-seronegative experienced viral breakthrough. Among the ETV-treated patients who were positive for HBeAg at baseline, conversion to HBeAg seronegativity did not affect the rate of viral breakthrough (*data not shown*).

There were no differences in HBV viral loads at study entry between HBeAg-positive patients with and without viral breakthrough. There were also no differences in HBV viral loads between HBeAg-negative patients with and without viral breakthrough.



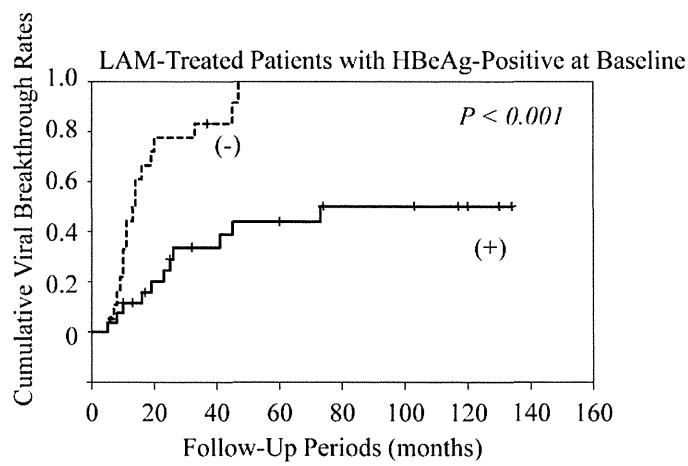
	Drug	Baseline	12	24	36	48	60
Number of Patients	LAM	68	52	34	27	20	19
	ETV	135	95	69	34	19	5

Figure 2. Cumulative viral breakthrough rates. ETV, entecavir; LAM, lamivudine.

Table 2. Patients with poor adherence to medication.

Case	Drug	Adherence rate (%)	Age (years)	Gender	Genotype	HBeAg	HBV DNA (log IU/mL)	ALT (IU/L)	APRI	HBeAg-seronegative	HBV DNA negativity	VT	Duration of treatment before VT (months)	Resistance mutations	Treatment after VT	Clinical outcome
1	ETV	50	55	F	B	-	3.8	16	0.33	N.A.	+	+	6	-	ETV	good
2	ETV	75	49	M	C	+	7.3	107	1.60	+	+	+	28	+	LAM+ADV	good
3	ETV	85	38	M	C	+	6.9	59	2.80	-	+	+	29	N.D.	ETV	good
4	ETV	80	39	M	C	+	5.8	51	0.63	+	+	-	N.A.	N.A.	ETV	good
5	ETV	85	37	F	C	+	6.9	160	2.25	+	+	-	N.A.	N.A.	ETV	good
6	ETV	85	66	M	N.D.	+	7.7	68	0.95	-	-	-	N.A.	N.A.	ETV	good
7	ETV	85	38	M	C	+	6.5	478	7.94	-	+	-	N.A.	N.A.	ETV	good
8	LAM	50	47	F	C	+	6.5	455	2.54	+	+	+	45	-	LAM	good
9	LAM	80	36	M	C	+	7.0	110	4.25	+	+	+	41	+	LAM+ADV	good
10	LAM	85	23	M	C	+	>7.6	161	3.53	-	+	+	11	-	cessation	flare
11	LAM	85	32	M	C	+	>7.6	343	1.30	+	+	-	N.A.	N.A.	LAM	good
12	LAM	85	54	F	C	-	4.1	196	2.68	N.A.	+	-	N.A.	N.A.	LAM	good
13	LAM	85	36	M	C	+	6.7	1576	15.78	+	+	-	N.A.	N.A.	LAM	good

Cases 2 and 3 had already been included in a previous report.[10] HBeAg, hepatitis B e antigen; HBV DNA, hepatitis B virus deoxyribonucleic acid, ALT, alanine aminotransferase; APRI, aspartate aminotransferase platelet ratio index; VT, viral breakthrough; ETV, entecavir; LAM, lamivudine; ADV, adefovir; F, female; M, male; N.D., not determined; N.A., not available; HBeAg-seronegative, conversion to HBeAg-seronegative after administration of a nucleoside analogue; HBV DNA negativity, achieving HBV DNA negativity after administration of a nucleoside analogue; flare, fluctuating ALT after treatment after VT.



	HBeAg-Seronegative	Baseline	12	24	36	48	60
Number of Patients	(-)	19	10	4	3	0	0
	(+)	26	22	17	13	11	11

Figure 3. Cumulative viral breakthrough rates in lamivudine (LAM)-treated patients with HBe antigen (HBeAg)-positive at baseline. (-), maintaining HBeAg seropositivity; (+), conversion to HBeAg-seronegative.

Viral breakthrough in patients who achieved, and did not achieve HBV DNA negativity

Among the LAM-treated patients, cumulative viral breakthrough rates in patients who did not achieve HBV DNA negativity were higher than in those who achieved HBV DNA negativity (*P<0.001*) (Figure 4). All patients who did not achieve HBV DNA negativity experienced viral breakthrough. In

contrast, among the ETV-treated patients, cumulative viral breakthrough rates in patients who did not achieve HBV DNA negativity were similar to the rates in those who achieved HBV DNA negativity (*data not shown*).

Correlation between adherence to medication and viral breakthrough

We also compared viral breakthrough rates according to adherence to medication. Among 62 LAM-treated patients who did not have poor adherence, 35 patients (56.4%) experienced viral breakthrough (Figure 5). Among 6 LAM-treated patients with poor adherence, 3 patients (50.0%) experienced viral breakthrough. In LAM treatment, poor adherence did not contribute to viral breakthrough (*P=0.89*). However, among 128 ETV-treated patients who did not have poor adherence, 4 patients (3.1%) experienced viral breakthrough. Among 7 ETV-treated patients with poor adherence, 3 patients (42.8%) experienced viral breakthrough. In the treatment with ETV, poor adherence contributed to viral breakthrough (*P<0.001*).

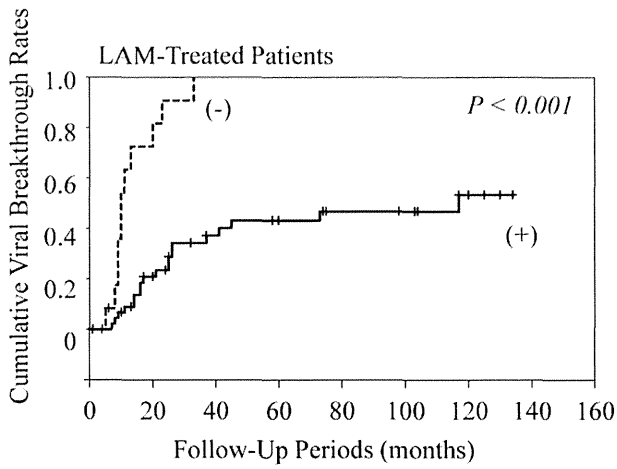
Resistance mutations

Resistance mutations were analyzed in some patients who experienced viral breakthrough. They were analyzed in 34 LAM-treated patients and 4 ETV-treated patients (Table 3). Thirty-two LAM-resistant patients had 10 YVDD, 17 YIDD, and 5 YV/IDD motifs, and 2 ETV-resistant patients had two YVDD motifs. Resistance mutations were not observed in 2 LAM-treated patients (5.8%) and 2 ETV-treated patients (50.0%) (*P=0.047*).

Table 3. Patients with viral breakthrough.

	ETV		LAM	
Adherence rate	≥90%	<90%	≥90%	<90%
Resistance mutation (+)	1	1	31	1
L180M	1	1	N.D.	N.D.
T184A	1	0	N.D.	N.D.
S202G	0	1	N.D.	N.D.
M204V	1	1	9	1
M204I	0	0	17	0
M204V/I	0	0	5	0
M250V	0	0	N.D.	N.D.
Resistance mutation (-)	1	1	0	2

ETV, entecavir; LAM, lamivudine; N.D., not determined. Numbers of amino acid positions were according to Refs. 2 and 10.



	HBV DNA Negativity	Baseline	12	24	36	48	60
Number of Patients	(-)	12	4	1	0	0	0
	(+)	47	39	30	23	19	18

Figure 4. Cumulative viral breakthrough rates in lamivudine (LAM)-treated patients who achieved HBV DNA negativity and those who did not. (-), maintaining HBV DNA positivity; (+), achieving HBV DNA negativity. HBV DNA negativity was unknown in 9 patients because of lack of data.

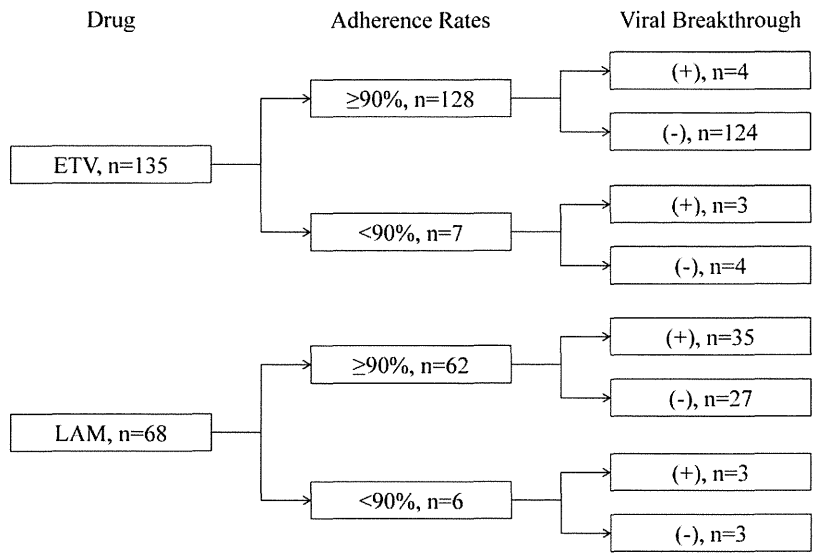


Figure 5. Association between adherence to medication and viral breakthrough.

DISCUSSION

The current study found that ETV-treated patients were not likely to acquire any resistance mutations and experience an ALT flare. Therefore, patients with poor liver residual function, such as liver cirrhosis, were likely to be administered ETV rather than LAM. Unexpectedly, HBsAg loss was observed in 3 of 28 LAM-treated patients without viral breakthrough (10.7%) and in 3 of 118 ETV-treated patients without viral breakthrough (2.5%). Long-term treatment with these drugs might result in HBsAg loss, although several reports have stated that one-year treatment with peg-interferon led to more HBsAg loss than these drugs [18-25].

In the current study, adherence to medication of most patients was excellent. The reasons for this might be as follows: (1) Our setting was a University Hospital, and this may have strengthened their will to succeeded with the treatment; (2) some patients with poor adherence might have been excluded because they did not see a doctor during the interview period; and (3) the rate of adherence to medication was based on patient self-assessment. A previous report showed that adherence might be underestimated by the Medication Event Monitoring System, a system that automatically records whenever a drug bottle is opened, and might be overestimated by pill counting and at interviews [26]. We classified the adherence rate as good at 90% or more, and as poor at less than 90%. However, we could not prove any significant influence of this classification on viral breakthrough as well as resistance mutation.

In the 13 patients with poor adherence (Table 2), we examined the reasons for their failure to take the pills. All 13 patients displayed some carelessness about taking pills. Two ETV-treated patients did not see a doctor and could not take pills continuously for a certain period of time, which particularly appeared to affect their viral breakthrough.

In LAM-treated patients, conversion of HBeAg to seronegative and achieving HBV DNA negativity was one of the important factors for successful treatment (Figures 3 & 4). In contrast, among ETV-treated patients, maintaining HBeAg seropositivity or HBV DNA positivity was not associated with viral breakthrough in the present study. Because of the stronger effect of ETV, it has been reported that long-term ETV treatment leads to a viral response in the vast majority of patients with detectable HBV DNA after 48 weeks [27]. Moreover, in the current study, poor adherence to medication was a major factor of viral breakthrough in the ETV-treated patients, but not in the LAM-treated patients. Ha et al. [9] also reported that medication non-adherence is likely to be a more important contributor to treatment failure than antiviral resistance, especially with new anti-HBV agents such as ETV and tenofovir. In LAM-treated or ETV-treated patients, viral breakthrough without resistance mutations might occur to some degree because of poor adherence to medication. In the present study, in LAM-treated patients, emergence of viral breakthrough with resistance mutations was common. Therefore, viral breakthrough due to poor adherence to LAM might not be important, compared with ETV-treated patients. However, in ETV-treated pa-

tients, viral breakthrough with resistance mutations was rare, and therefore, viral breakthrough due to poor adherence to ETV might be important.

In conclusion, viral breakthrough associated with poor adherence could be an important issue in the treatment with strong nucleoside analogues, such as ETV.

ABBREVIATIONS

ALT: alanine aminotransferase
ETV: entecavir
HBeAg: hepatitis B e antigen
HBsAg: hepatitis B surface antigen
HBV: hepatitis B virus
HCC: hepatocellular carcinoma
NA: nucleos(t)ide analogue
LAM: lamivudine

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Contributors

HK, TK, FI, and OY designed the study. HK, TK, MA, TC, HM, KF, FK, FI, FN and OY saw patients and conducted the interview. HK, TK, WS, and SN analyzed the data. HK and TK drafted the paper and all authors approved the paper.

COMPETING INTERESTS

Dr. Tatsuo Kanda reports receiving lecture fees from Chugai Pharmaceutical, MSD, and Ajinomoto, and Prof. Osamu Yokosuka reports receiving grant support from Chugai Pharmaceutical, Bayer, MSD, Daiichi-Sankyo, Mitsubishi Tanabe Pharma, and Bristol-Myers Squibb.

References

1. Lok AS, McMahon BJ. Chronic hepatitis B. Hepatology. 2007; 45: 507-539.
2. Wu S, Fukai K, Imazeki F, et al. Initial virological response and viral mutation with adefovir dipivoxil added to ongoing Lamivudine therapy in Lamivudine-resistant chronic hepatitis B. Dig Dis Sci. 2011; 56: 1207-1214.
3. Dienstag JL, Perrillo RP, Schiff ER, et al. A preliminary trial of lamivudine for chronic hepatitis B infection. N Engl J Med. 1995; 333: 1657-1661.
4. Lai CL, Chien RN, Leung NW, et al. A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. N Engl J Med. 1998;339:61-8.
5. Innaimo SF, Seifer M, Bisacchi GS, et al. Identification of BMS-200475 as a potent and selective inhibitor of hepatitis B virus. Antimicrob Agents Chemother. 1997; 41: 1444-1448.
6. Ono SK, Kato N, Shiratori Y, et al. The polymerase L528M mutation cooperates with nucleotide binding-site mutations, increasing hepatitis B virus replication and drug resistance. J Clin Invest. 2001; 107: 449-455.
7. Hashimoto Y, Suzuki F, Hirakawa M, et al. Clinical and virological effects of long-term (over 5 years) lamivudine therapy. J Med Virol. 2010; 824: 684-691.
8. Chotiyaputta W, Peterson C, Ditah FA, et al. Persistence and adherence to nucleos(t)ide analogue treatment for chronic hepatitis B. J Hepatol. 2011; 54: 12-8.
9. Ha NB, Ha NB, Garcia RT, et al. Medication nonadherence with long-term management of patients with hepatitis B e antigen-negative chronic hepatitis B. Dig Dis Sci. 2011; 56: 2423-31.
10. Kamezaki H, Kanda T, Wu S, et al. Emergence of entecavir-resistant mutations in nucleos(t)ide-naive Japanese patients infected with hepatitis B virus: virological breakthrough is also dependent on adherence to medication. Scand J Gastroenterol. 2011; 46: 1111-1117.
11. Haynes RB, McDonald HP, Garg AX. Helping patients follow prescribed treatment: clinical applications. JAMA. 2002; 288: 2880-2883.
12. Bramley TJ, Gerbino PP, Nightengale BS, et al. Relationship of blood pressure control to adherence with antihypertensive monotherapy in 13 managed care organizations. J Manag Care Pharm. 2006; 12: 239-245.
13. Ishibashi H, Maruyama H, Takahashi M, et al. Assessment of hepatic fibrosis by analysis of the dynamic behaviour of microbubbles during contrast ultrasonography. Liver Int 2010; 30: 1355-1363.
14. Wu S, Kanda T, Imazeki F, et al. Hepatitis B virus e antigen downregulates cytokine production in human hepatoma cell lines. Viral Immunol. 2010; 23: 467-476.
15. Usuda S, Okamoto H, Iwanari H, et al. Serological detection of hepatitis B virus genotypes by ELISA with monoclonal antibodies to type-specific epitopes in the preS2-region product. J Virol Methods. 1999; 80: 97-112.
16. Kobayashi S, Shimada K, Suzuki H, et al. Development of a new method for detecting a mutation in the gene encoding hepatitis B virus reverse transcriptase active site (YMDD motif). Hepatol Res. 2000; 17: 31-42.
17. Kanda T, Jeong SH, Imazeki F, et al. Analysis of 5' nontranslated region of hepatitis A viral RNA genotype I from South Korea: comparison with disease severities. PLoS One. 2010; 5: e15139.
18. Lau GK, Piratvisuth T, Luo KX, et al. Peginterferon alfa-2a, lamivudine, and the combination for HBeAg-positive chronic hepatitis B. N Engl J Med. 2005; 352: 2682-2695.
19. Chan HL, Leung NW, Hui AY, et al. A randomized, controlled trial of combination therapy for chronic hepatitis B: comparing pegylated interferon-α2b and lamivudine with lamivudine alone. Ann Intern Med. 2005; 142: 240-250.
20. Liaw YF, Jia JD, Chan HL, et al. Shorter durations and lower doses of peginterferon alfa-2a are associated with inferior hepatitis B e antigen seroconversion rates in hepatitis B virus genotypes B or C. Hepatology. 2011; 54: 1591-1599.
21. Buster EH, Flink HJ, et al. Sustained HBeAg and HBsAg loss after long-term follow-up of HBeAg-positive patients treated with peginterferon α-2b. Gastroenterology. 2008; 135: 459-467.
22. Wong VW, Wong GL, Yan KK, et al. Durability of peginterferon alfa-2b treatment at 5 years in patients with hepatitis B e antigen-positive chronic hepatitis B. Hepatology. 2010; 51: 1945-1953.
23. Marcellin P, Lau GK, Bonino F, et al. Peginterferon alfa-2a alone, lamivudine alone, and the two in combination in patients with HBeAg-negative chronic hepatitis B. N Engl J Med. 2004; 351: 1206-1217.
24. Papadopoulous VP, Chrysagis DN, Protopapas AN, et al. Peginterferon alfa-2b as monotherapy or in combination with lamibudine in patients with HBeAg-negative chronic hepatitis B: a randomised study. Med Sci Monit. 2009; 15: CR56-CR61.
25. Marcellin P, Bonino F, Lau GK, et al. Sustained response of hepatitis B e antigen-negative patients 3 years after treatment with peginterferon alpha-2a. Gastroenterology. 2009; 136: 2169-2179.
26. Liu H, Golin CE, Miller LG, et al. A comparison study of multiple measures of adherence to HIV protease inhibitors. Ann Intern Med. 2001; 134: 968-977.
27. Zoutendijk R, Reijnders JG, Brown A, et al. Entecavir treatment for chronic hepatitis B: adaptation is not needed for the majority of naïve patients with a partial virological response. Hepatology. 2011; 54: 443-451.

Hepatitis B Virus e Antigen Physically Associates With Receptor-Interacting Serine/Threonine Protein Kinase 2 and Regulates *IL-6* Gene Expression

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We previously reported that hepatitis B virus (HBV) e antigen (HBeAg) inhibits production of interleukin 6 by suppressing NF-κB activation. NF-κB is known to be activated through receptor-interacting serine/threonine protein kinase 2 (RIPK2), and we examined the mechanisms of interleukin 6 regulation by HBeAg. HBeAg inhibits RIPK2 expression and interacts with RIPK2, which may represent 2 mechanisms through which HBeAg blocks nucleotide-binding oligomerization domain-containing protein 1 ligand–induced NF-κB activation in HepG2 cells. Our findings identified novel molecular mechanisms whereby HBeAg modulates intracellular signaling pathways by targeting RIPK2, supporting the concept that HBeAg could impair both innate and adaptive immune responses to promote chronic HBV infection.

Hepatitis B virus (HBV) nucleoprotein exists in 2 forms [1, 2]. Nucleocapsid, designated HBV core antigen (HBcAg), is an intracellular, 21-kDa protein that self-assembles into particles that encapsidate viral genome and polymerase and is essential for function and maturation of virion. HBV also secretes a nonparticle second form of the nucleoprotein, designated precore or HBV e antigen (HBeAg) [1, 2]. Precore and core proteins are translated from 2 RNA species that have different 5′ initiation sites. Precore messenger RNA (mRNA) encodes a hydrophobic signal sequence that directs precore protein to the endoplasmic reticulum, where it undergoes N- and C-terminal cleavage within the secretory pathway and is secreted as an 18-kDa monomeric protein [3–5].

Nucleotide-binding oligomerization domain–containing protein 1 (NOD1) and NOD2 are cytosolic pattern-recognition receptors involved in the sensing of bacterial peptidoglycan subcomponents [6]. NOD1 and NOD2 stimulation activates NF-κB through receptor-interacting serine/threonine protein kinase 2 (RIPK2; also known as RIP2, RICK, or CARDIAK), a caspase-recruitment domain-containing kinase. RIPK2 is also involved in Toll-like receptor (TLR)–signaling pathway and plays an important role in the production of inflammatory cytokines through NF-κB activation [6, 7].

We previously reported that HBeAg inhibits the production of interleukin 6 (IL-6) through suppression of NF-κB activation [4]. In the present study, we investigated the molecular mechanism of HBeAg functions for the requirement of RIPK2 in NF-κB transcriptional regulation.

METHODS

Cell Culture and Plasmids

HepG2, Huh7, HT1080, COS7, and HEK293T cells were used in the present study. Stable cell lines were obtained as previously described [4]. Briefly, HepG2, Huh7, and HT1080 were transfected with pCXN2-HBeAg(+) or pCXN2-HBeAg(−) in Effectene (Qiagen). After G418 screening, HBeAg-positive and -negative HepG2/Huh7/HT1080 cell lines were collected for further analysis [4]. The plasmid pCXN2-HBeAg(+), which can produce both HBeAg and core peptides, and the plasmid pCXN2-HBeAg(−), which can produce only core peptides, were obtained as described previously [4]. pNF-κB–luc, which expresses luciferase upon promoter activation by NF-κB, was purchased from Stratagene [4]. pGFP–human RIPK2 (kindly provided by Prof John C. Reed, Sanford-Burnham Institute for Medical Research) can express GFP-human $RIP2^{WT}$ [8].

HepG2 cells were transfected with plasmid control–small hairpin RNA (shRNA) or with RIPK2-shRNA (Santa Cruz). After puromycin screening, individual colonies were picked up and examined for expression of endogenous RIPK2, and clones HepG2-shC and HepG2-shRIPK2-3 were selected for subsequent studies.

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Luciferase Assays and Treatment of Cells With NOD Ligands

Around 1.0×10^5 HepG2 and Huh7 cells were plated in 6-well plates (Iwaki Glass, Tokyo, Japan) for 24 hours and transfected with 0.4 μg of pNF-κB–luc. For luciferase assay of NF-κB activation, cells were treated for 4 hours with or without NOD1 ligand (C12-iEDAP, 2.5 μg/mL) and NOD2 ligand (muramyl dipeptide [MDP], 10 μg/mL) (InvivoGen) at 44 hours after transfection [9]. After 48 hours, cells were lysed with reporter lysis buffer (Promega), and luciferase activity was determined as described previously [4].

RNA Extraction, Complementary DNA (cDNA) Synthesis, Real-Time Polymerase Chain Reaction (PCR) Analysis, and PCR Array

Total RNA was isolated by RNeasy Mini Kit (Qiagen). A total of 5 μg of RNA was reverse transcribed using the First Strand cDNA Synthesis Kit (Qiagen) [4]. Quantitative amplification of cDNA was monitored with SYBR Green by real-time PCR in a 7300 Real-Time PCR system (Applied Biosystems). Gene expression profiling of 84 TLR-related genes was performed using RT^2 profiler PCR arrays (Qiagen) in accordance with the manufacturer's instructions [4].

Gene expression was normalized to 2 internal controls (GAPDH and/or β-actin) to determine the fold-change in gene expression between the test sample (HBeAg-positive HepG2/Huh7/HT1080) and the control sample (HBeAg-negative HepG2/Huh7/HT1080) by the 2^{-ddCT} (comparative cycle threshold) method [4]. Three sets of real-time PCR arrays were performed. Some results of HepG2 cells were previously reported [4].

Coimmunoprecipitation

Cells were cotransfected with 2.5 μg pCXN2-HBeAg(+) or 2.5 μg pCXN2-HBeAg(−), as well as with 2.5 μg pGFP–human RIPK2, and cell lysates were prepared after 48 hours, using lysis buffer containing a cocktail of protease inhibitors. Cell lysates were incubated with anti-GFP rabbit polyclonal antibody (Santa Cruz) or anti-HBe mouse monoclonal antibody (Institute of Immunology, Tokyo, Japan) for 3 hours at 4°C, followed by overnight incubation with protein G–Sepharose beads (Santa Cruz). Immunoprecipitates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electroblotted onto a nitrocellulose membrane. Immunoblotting was performed by incubating the membrane for 1 hour with anti-HBe antibody. Proteins were detected by enhanced chemiluminescence (GE Healthcare), using an image analyzer (LAS-4000, Fuji Film). The membrane was reprobed with a monoclonal antibody to GFP or RIPK2 (Cell Signaling).

Transfection of pGFP–Human RIPK2 and Confocal Microscopy

Formaldehyde (3.7%)–fixed cells were incubated with anti-HBe antibody and stained with fluorochrome-conjugated secondary antibody (Alexa Fluor 555 conjugate, Cell Signaling). Cells were mounted for confocal microscopy (ECLIPSE TE 2000-U, Nikon). Whenever necessary, images were merged digitally to monitor colocalization. Cotransfection of 0.1 μg pCXN2-HBeAg(+) or 0.1 μg pCXN2-HBeAg(−) with 0.3 μg pGFP–human RIPK2 into the cells was performed. After 48 hours, intracellular localization of RIPK2 was visualized by confocal microscopy.

Enzyme-Linked Immunosorbent Assay (ELISA) for IL-6

Cell culture fluid was analyzed for IL-6 by ELISA (KOMA-BIOTECH, Seoul, Korea), in accordance with the manufacturer's protocol [4].

Small Interfering RNA (siRNA) Transfection and Wound-Healing Assay

Control siRNA (siC) and siRNA specific for RIPK2 (siRIPK2) were purchased from Thermo Fisher Scientific. Cells were transfected with siRNA by electroporation. After 48 hours, cells were treated with 10 ng/mL tumor necrosis factor α (TNF-α) (Wako Pure Chemical, Osaka, Japan), while the wound-healing (ie, scratch) assay was performed using a p-200 pipette tip to induce RIPK2 [10]. Up to 12 hours after scratching, the cells were observed by microscopy. Cell migration was measured using Scion Images (SAS). Migration by siC-transfected cells was set at 1.

Statistical Analysis

Results are expressed as mean values ± SD. The Student *t* test was used to determine statistical significance.

RESULTS

HBeAg Downregulates RIPK2 Expression

To explore the effect of HBeAg on TLR-related gene expression, we generated HepG2, Huh7, and HT1080 cell lines that stably expressed HBV core region with or without precore region. HT1080, a primate fibrosarcoma cell line, is useful for the study of interferon signaling. HBeAg and HBV core–related antigen (HBcrAg) levels of these cell lines demonstrated that expression of HBV core region without HBV precore region did not allow HBeAg secretion by cells (data are cited elsewhere [4] or not shown). First, we performed real-time RT-PCR analysis of these cell lines, using focused gene arrays (Figure 1*A*). We observed that, in 3 cell lines, 5 genes (*RIPK2, TLR9, TNF, CD180,* and *IL1A*) were downregulated ≥1.3-fold in HBeAg-positive cells than in HBeAg-negative cells. We chose to focus our investigation on RIPK2 because HBeAg inhibits the production of IL-6 through the suppression of NF-κB activation [4], and NF-κB is known to be activated through RIPK2 [4]. RIPK2 expression was >100-, 1.41-, and 1.45-fold lower in HBeAg-positive HepG2, Huh7, and HT1080 cells, respectively, compared with their HBeAg-negative counterparts

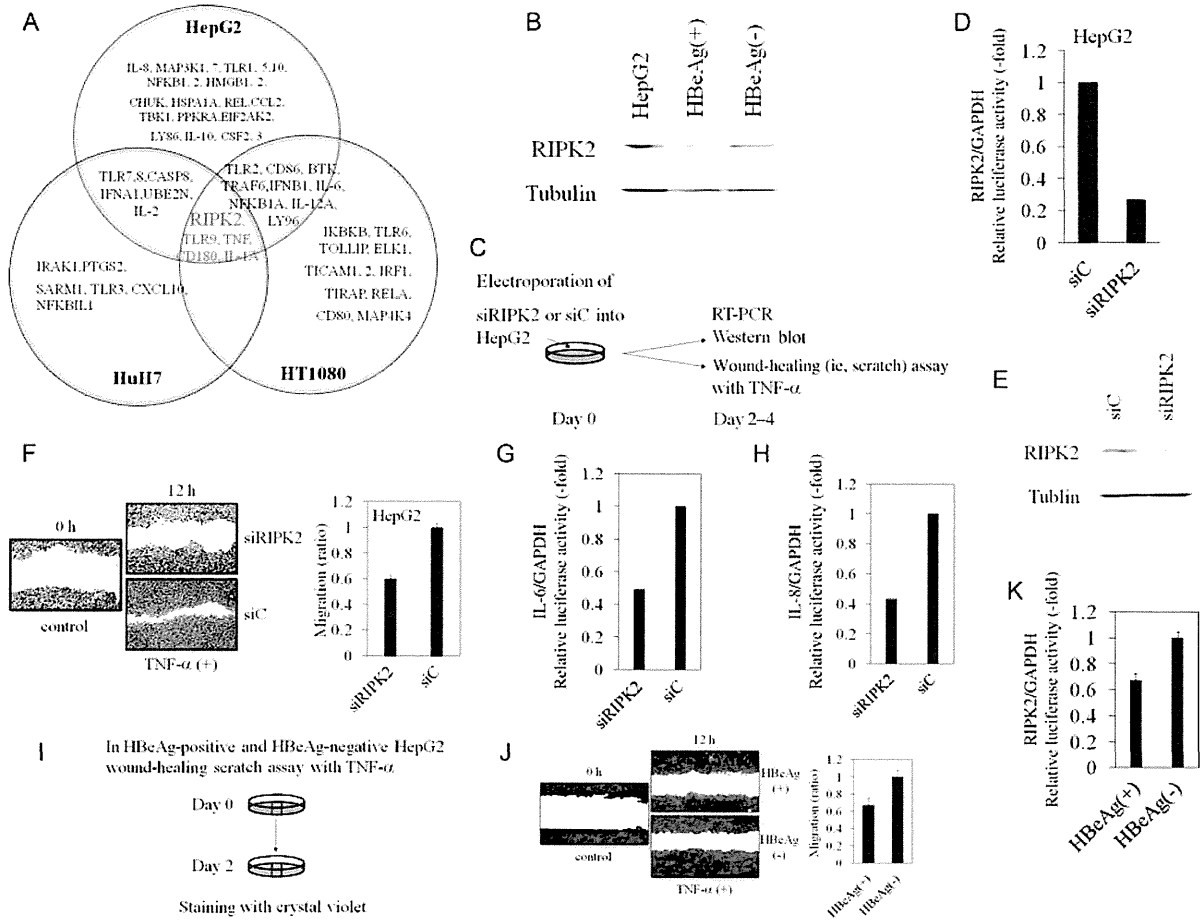

Figure 1. Receptor-interacting serine/threonine protein kinase 2 (RIPK2) expression is downregulated by hepatitis B virus e antigen (HBeAg), and knockdown of RIPK2 and HBeAg impairs hepatic wound repair. *A*, Venn diagram representing Toll-like receptor (TLR)–related genes downregulated ≥1.3-fold in HBeAg-positive HepG2/Huh7/HT1080 cells, compared with HBeAg-negative cells. Cellular RNA was extracted and analyzed with focused array, quantifying 84 genes. Gene expression levels were normalized to actin and GAPDH expression levels. *B*, HBeAg downregulates RIPK2 expression in HepG2 cells. Western blot analysis of RIPK2 and tubulin expression in HepG2, HBeAg(+) HepG2, and HBeAg(−) HepG2. *C*, Experimental protocol of electroporation of control (siC) and RIPK2 (siRIPK2) small interfering RNA (siRNA) into HepG2 cells. *D* and *E*, Real-time polymerase chain reaction (PCR; *D*) and Western blot (*E*) analyses of RIPK2 expression in siC- or siRIPK2-expressing HepG2 cells. RIPK2 messenger RNA (mRNA) levels were normalized to GAPDH levels. *F–H*, siC- and siRIPK2-transfected HepG2 cells were scratch wounded and incubated with 10 ng/mL tumor necrosis factor α (TNF-α), and cell migration was analyzed after 12 hours and quantified using Scion Image (*F*). Interleukin 6 (IL-6; *G*) and interleukin 8 (IL-8; *H*) mRNA expression are quantified by real-time reverse transcription–PCR (RT-PCR) and expressed relative to GAPDH mRNA expression. *I*, Protocol of wound-healing (ie, scratch) assay in HBeAg(+) and HBeAg(−) HepG2 cells. TNF-α was used at 10 ng/mL. *J*, Cell migration was analyzed using Scion Image. *K*, RIPK2 mRNA expression was quantified by real-time RT-PCR and expressed relative to GAPDH mRNA expression. Primers specific for RIPK2 were 5′-AGACACTACTGACATCCAAG-3′ (sense) and 5′-CACAAGTATTTCCGGGTAAG-3′ (antisense), and primers for other genes were as described previously [4]. Data are mean values ± SD of 3 independent experiments.

(Figure 1*A*). Western blot analyses confirmed lower levels of RIPK2 in HBeAg-positive HepG2 than in HBe-negative HepG2 or parental HepG2 (Figure 1*B*). The fact that RIPK2 is one of the targets for the ubiquitin proteasome system and uses a ubiquitin-dependent mechanism to achieve NF-κB activation [6] might be a reason for the differences between RIPK2 mRNA and protein expression status. We also observed lower levels of RIPK2 mRNA expression (0.18-fold) in HepG2.2.15 cells, which secrete complete HBV virion and HBeAg, compared with expression in HepG2 cells (data not shown).

Knockdown of RIPK2 and HBeAg Impairs Hepatic Cell Migration

It has recently been reported that RIPK2 expression is induced by TNF-α plus scratch wounding in keratinocytes [10]. Therefore, we next examined whether RIPK2 affected hepatic