Table 1 Baseline characteristics of hepatitis B virus (HBV) e antigen (HBeAg)-negative patients

	Total	Normal ALT	Elevated ALT	*P*
Number	244	158	86	
Age(years) : (mean ± SD)	44.1 ± 12.5	44.1 ± 13.1	44.0 ± 11.4	NS*
<30	35 (14.3%)	24 (15.2%)	11 (12.8%)	
30–39	52 (21.3%)	32 (20.3%)	20 (23.2%)	
40–49	66 (27.0%)	44 (27.8%)	22 (25.6%)	
50–	91 (37.3%)	58 (36.7%)	33 (38.4%)	
Sex				<0.001**
Male	141 (57.8%)	76 (48.1%)	66 (75.9%)	
Female	103 (42.2%)	82 (51.9%)	21 (24.1%)	
Alanine aminotransferase (ALT) (IU/L) (mean ± SD)	58.9 ± 108.1	20.9 ± 8.7	127.9 ± 160	<0.001*
<20	84			
21–30	47			
31–40	27			
42–84	47			
85–	39			
Platelet count ($\times 10^4/mm^3$) (mean ± SD)	205.5 ± 69.6	211.4 ± 60	193.3 ± 81.8	NS*
HBV-DNA (log copies/mL) (mean ± SD)	4.3 ± 1.5	3.8 ± 1.1	5.1 ± 1.7	<0.001*
<4.0	116 (47.5%)	91 (57.6%)	25 (29.1%)	
4.0–4.9	54 (22.1%)	38 (24.1%)	16 (18.6%)	
5.0–5.9	27 (11.1%)	18 (11.4%)	9 (10.5%)	
6.0–6.9	26 (10.7%)	5 (3.2%)	21 (24.4%)	
7.0–	16 (6.6%)	3 (1.9%)	13 (15.1%)	
Genotype				NS**
A	3 (1.2%)	2 (1.3%)	1 (1.2%)	
B	30 (12.3%)	16 (10.1%)	14 (16.3%)	
C	87 (35.7%)	49 (31%)	38 (44.2%)	
Not detected	124 (50.8%)	91 (57.6%)	33 (38.4%)	
Liver Histology ($n = 44$)				
Fibrosis 4/3/2/1	7/8/9/20	0/1/4/13	7/7/5/7	NS**
Activity 3/2/1	7/16/21	1/4/13	6/12/8	NS**
Use of anti-Diabetes drug	20 (8.2%)	3 (1.9%)	6 (7.0%)	NS**
Body mass index (kg/m^2) (mean ± SD)	23.3 ± 3.3	23.1 ± 3.2	24.0 ± 3.5	NS**
Smoker/ ever smoker/ non-smoker	32/15/89	16/5/56	16/10/33	NS**
Daily alcohol consumption	46 (27.1%)	24 (23.1%)	22 (33.3%)	NS**
Follow-up (months) (mean ± SD)	103.6 ± 74.8	109.5 ± 76.1	101.8 ± 74.6	NS*

*Unpaired *t*-test and **χ^2 test. NS, not significant difference.

interval from a visit with a normal ALT to a visit with an elevated ALT was used for Kaplan–Meier and Cox regression analysis. Kaplan–Meier curves were constructed for ALT and HBV-DNA levels (Fig. 2).

Risk factors for future use of antiviral drugs for HBV in HBeAg-negative patients

Seventeen (7.0%) patients used an antiviral drug (lamivudine in 8 and entecavir in 9). We investigated the risk factors for future use of antiviral drugs for HBV. The time interval from baseline to the use of an antiviral drug for HBV was used for Cox regression analysis. HBV-DNA levels, use of antidiabetic drugs, and daily alcohol consumption were predictive of future antiviral drug use for HBV, according to the results of multivariate Cox hazard regression analysis. Hazard ratios for HBV-DNA levels, antidiabetic drug use, and daily alcohol consumption were 1.519 (1.130–2.042, 95% confidence interval [CI]), 3.769 (1.203–11.81), and 3.011 (1.086–8.348), respectively. We repeated the univariate analysis, changing the threshold for HBV DNA from 3.5 to 7.0 log copies/mL in 0.1 log increments. We determined the threshold when the probability value was lowest; the HBV-DNA threshold level was 5.7 log copies/mL. Kaplan–Meier curves were constructed for HBV-DNA levels, antidiabetic drug use, and daily alcohol consumption (Fig. 3).

Risk factors for hepatocellular carcinoma in HBeAg-negative patients

In 10 patients (4.1%), HCC was detected. We investigated the risk factors for HCC in HBeAg-negative patients. The time interval from baseline to occurrence of HCC was used for Cox regression analysis. According to the results of multivariate Cox regression analysis, PLT was predictive of the development of HCC. The hazard ratio for PLT was 0.807 (0.724–0.899, 95% CI). We performed univariate analyses, changing the PLT threshold from 8.0 to $30.0 \times 10^4/mm^3$ in $1.0 \times 10^4/mm^3$ increments. We determined the threshold when the value of probability was smallest; the

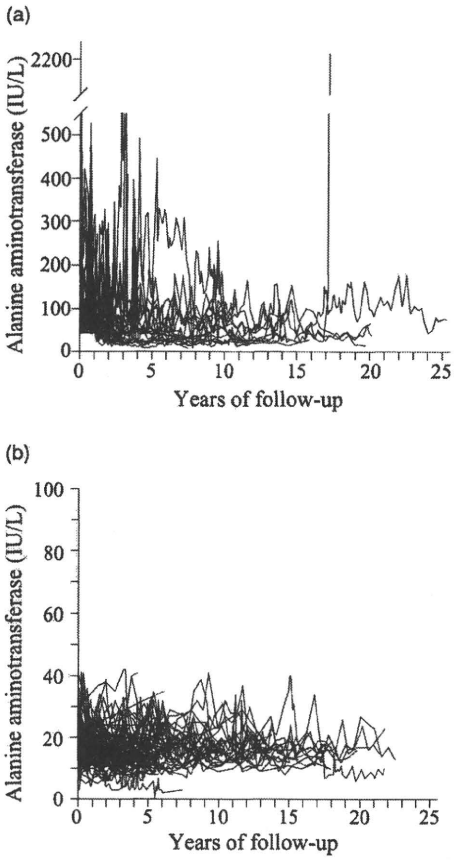

Figure 1 Level of alanine aminotransferase (ALT) in (a) patients with normal ALT at baseline and intermittently elevated ALT during follow-up ($n = 73$) and (b) patients with normal ALT at baseline and during follow-up ($n = 85$).

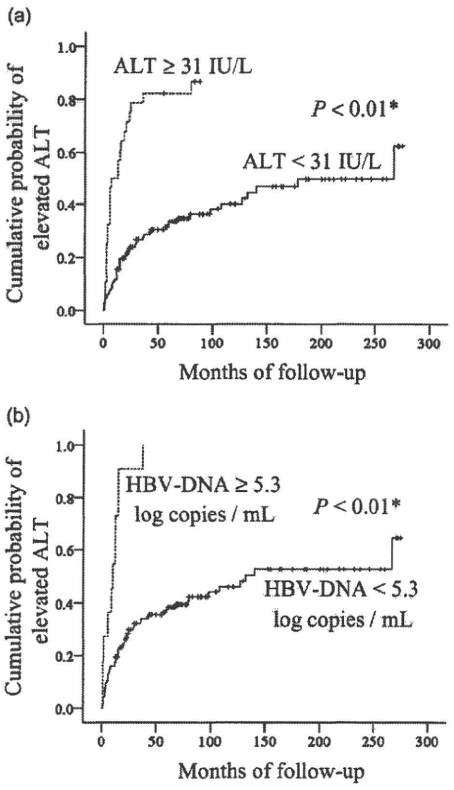

Figure 2 Cumulative occurrence of abnormal alanine aminotransferase (ALT) in HBeAg-negative patients with normal ALT based on (a) ALT and (b) HBV-DNA levels. We determined the threshold for ALT and HBV-DNA levels when the probability value was lowest in the univariate analysis. Kaplan–Meier curves show the time to ALT elevation. Solid lines indicated the control group. *A significant difference was determined by log-rank test.

Table 2 Univariate and multivariate analysis of factors associated with alanine aminotransferase (ALT) elevation in hepatitis B virus (HBV) e antigen (HBeAg)-negative patients with normal ALT levels

	Univariate analysis				Multivariate analysis			
	Standard error	Wald statistic	*P*-value	Hazard ratio (95% confidence interval)	Standard error	Wald statistic	*P*-value	Hazard ratio (95% confidence interval)
Sex (Male)	0.263	0.203	0.652	1.126 (0.673–1.885)				
Age (years)	0.011	5.704	0.017	1.027 (1.005–1.049)	0.252	0.068	0.794	1.015 (0.572–1.534)
HBV-DNA	0.109	17.773	<0.001	1.587 (1.280–1.966)	0.111	10.602	0.001	1.437 (1.155–1.788)
Genotype								
B	0.459	0.22	0.639	0.806 (0.328–1.982)				
C	0.435	0.055	0.815	1.107 (0.472–2.600)				
Alanine aminotransferase	0.014	42.440	<0.001	1.097 (1.067–1.128)	0.015	29.496	<0.001	1.086 (1.054–1.119)
Platelet count	0.019	5.928	0.015	0.955 (0.920–0.991)	0.021	0.754	0.385	0.982 (0.942–1.023)
Use of anti-diabetes drug	0.427	0.470	0.493	1.340 (0.581–3.091)				
Body mass index (kg/m^2)	0.042	0.033	0.855	0.992 (0.913–1.078)				
Smoker and ever smoker	0.374	0.111	0.739	1.133 (0.544–2.359)				
Daily alcohol consumption	0.333	0.512	0.474	1.269 (0.661–2.435)				

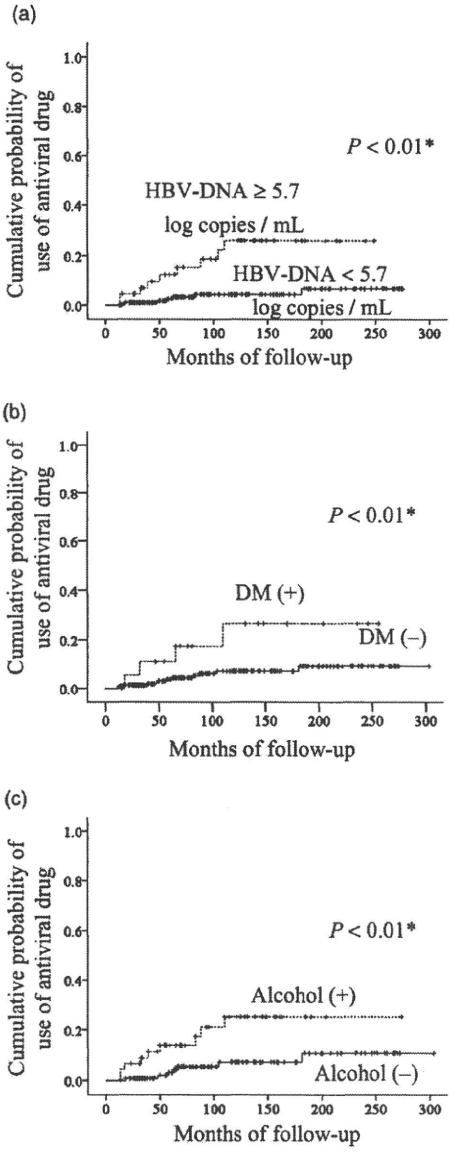

Figure 3 Cumulative occurrence of antiviral drug use for hepatitis B virus (HBV) in HBeAg-negative patients based on (a) HBV-DNA levels (b) use of antidiabetic drug, and (c) daily alcohol consumption. We determined the threshold for HBV-DNA levels when the probability value was lowest in the univariate analysis. Kaplan–Meier curves show the time to use of antiviral drugs for HBV. Solid lines indicated the control group. *A significant difference was determined by log-rank test.

PLT threshold was $10.0 \times 10^4/mm^3$. Kaplan–Meier curves were constructed for PLT (Fig. 4).

Stratification analyses of risk factors for clinical outcomes in HBeAg-negative patients by age, sex, and HBV genotype

The stratification analyses by age, sex, and HBV-genotype were performed to evaluate the risk factors for future ALT elevation in patients with normal ALT levels, future use of antiviral drugs for HBV, and HCC in HBeAg-negative patients (Table 3). The age threshold was 45 years, which was the average age of all the patients. We did not perform stratification analysis for patients infected with HBV genotype B because the number of such cases was very small.

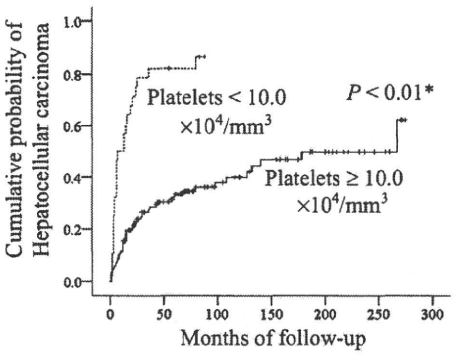

Figure 4 Cumulative occurrence of hepatocellular carcinoma (HCC) based on the platelet counts. We determined the threshold for HBV-DNA levels when the probability value was lowest in the univariate analysis. Kaplan–Meier curves show the time to HCC. Solid lines indicated the control group. *A significant difference was observed by log-rank test.

Discussion

Most patients who have undergone HBeAg seroconversion have normal serum ALT levels, which is indicative of a good clinical outcome.[10] Therefore, various therapies for early seroconversion have been used.[5] Recently, HBeAg-negative viral mutants have been shown to be responsible for continuous HBV-DNA replication.[7] That is, there exists the possibility that liver disease will get worse after HBeAg seroconversion. In fact, previous reports revealed that HBeAg status is not a predictive factor for HCC,[11,12] and fulminant hepatitis can occur by the infection of HBV with HBeAg-negative.[13] HBeAg-negative patients should be monitored closely, even though most of these patients show normal ALT levels and no progressive liver disease.[14] Therefore, predictive factors for active liver disease in HBeAg-negative patients need to be identified in order to facilitate optimal disease management. This study provides data regarding the prediction of future active liver disease, i.e. ALT elevation, unavoidable use of antiviral drugs, and occurrence of HCC.

Many previous reports have attempted to define a threshold HBV-DNA level that corresponds to the presence of active liver disease.[15] A National Institute of Health workshop demonstrated that an HBV-DNA level of 10^5 copies/mL could be used to distinguish active HBV infection from inactive HBV infection.[16] Other studies also suggested that the threshold HBV-DNA level lies somewhere between 10^4 and 10^6 copies/mL.[8] In this study, in order to clarify the natural course of HBeAg-negative patients with normal ALT levels, we used a HBV-DNA threshold of $10^{5.3}$ copies/mL. By log rank analysis, the ALT levels in patients with $>10^{5.3}$ copies/mL HBV-DNA level were significantly higher than in patients with HBV-DNA below this level. In HCV patients, ALT is

Table 3 Stratification analysis multivariate analysis of factors associated with alanine aminotransferase (ALT) elevation in hepatitis B virus (HBV) e antigen (HBeAg)-negative patients with normal ALT levels, future use of antiviral drugs for HBV, and occurrence of hepatocellular carcinoma

		Age (years)						Sex						Genotype			
		≥45 years		<45 years			Male			Female			C				
		$n = 126$		$n = 118$			$n = 141$			$n = 103$			$n = 87$				
	Factors	Hazard ratio (95% CI)	*P*-value	Factors	Hazard ratio (95% CI)	*P*-value	Factors	Hazard ratio (95% CI)	*P*-value	Factors	Hazard ratio (95% CI)	*P*-value	Factors	Hazard ratio (95% CI)	*P*-value	Factors	Hazard ratio (95% CI)	*P*-value
Future ALT elevation in the patients with normal ALT level	HBV-DNA	1.535 (1.146–2.057)	0.004	ALT	1.106 (1.059–1.156)	<0.001	ALT	1.194 (1.075–1.325)	<0.001	ALT	1.060 (1.015–1.108)	0.008	ALT	1.149 (1.075–1.228)	<0.001			
	ALT	1.077 (1.035–1.122)	<0.001							HBV-DNA	1.739 (1.213–2.492)	0.003	HBV-DNA	1.902 (1.223–2.956)	0.004			
Future use of anti-viral drugs for HBV	Alcohol	4.744 (1.362–16.52)	0.014	HBV-DNA	2.238 (1.107–4.526)	0.025	HBV-DNA	1.486 (1.053–2.098)	0.024	DM†	86.14 (1.842–4027.3)	0.023	Alcohol	5.617 (1.431–22.05)	0.013			
										BMI†	0.408 (0.187–0.890)	0.024						
Occurrence of hepatocellular carcinoma	PLT	0.772 (0.659–0.905)	0.001	(–)†			PLT	0.832 (0.731–0.948)	0.006	PLT†	0.775 (0.635–0.947)	0.013	PLT	0.833 (0.732–0.948)	0.006			

†Three patients were used in subgroup for HBV antiviral drugs and HCC occurrence.
Alcohol, Daily alcohol consumption; BMI, Body mass index; CI, confidence interval; DM, use of antidiabetic medication; PLT, platelet count.

a poor surrogate marker for inflammation and fibrosis.[17] Therefore, even if the patient's ALT level was within normal limits, they should still be monitored closely, and HCV eradication therapy is recommended under certain circumstances. Similarly, even if the ALT levels are within normal limits in HBV-infected patients who are HBeAg-negative, the higher their ALT levels were, the more frequently their ALT levels would be high in the future, which might cause progressive liver disease.[18]

Some of the patients with progressive liver disease caused by HBV infection were treated with the antiviral drugs lamivudine and entecavir. The use of lamivudine or entecavir might result in mutant HBV resistance to antiviral drugs[19,20] and the associated costs are not trivial. The baseline levels of HBeAg, ALT, and HBV-DNA, and the presence of either chronic hepatitis or cirrhosis have been established as determinants for eligibility for antiviral treatment.[21] According to treatment guidelines in the Unites States (National Guideline Clearinghouse, http://www.guideline.gov), patients with HBeAg-negative chronic hepatitis B should be considered for antiviral treatment based on their HBV-DNA and ALT levels (serum HBV-DNA >20 000 IU/mL and elevated ALT >2 times normal). In this study, only four out of 17 patients treated with an antiviral drug showed normal ALT levels at baseline, and all four patients showed elevated ALT levels in 8–57 months later. Therefore, this study revealed that patients with high HBV-DNA levels tended to have high ALT levels at baseline or in the future; as a result, such patients have a tendency for future treatment with and antiviral drug.

Hepatocellular carcinoma occurrence was noted in only 10 cases (4.1%). The only predictive factor for HCC occurrence was PLT, which meant that patients with advanced liver disease tended to develop HCC later, because the decrease in PLT corresponded to the extent of liver fibrosis. Four patients (1.6%) died of liver-related diseases and one (0.4%) died of cancer in another organ. The number of deaths was too small to determine the predictive factors for death of HBeAg-negative HBV carriers. Further analysis is needed to properly address this factor.

Stratification analyses of risk factors for clinical outcomes by age, sex, and HBV-genotype were performed. Because the numbers of female patients with future use of antiviral drugs for HBV ($n = 3$), HCC occurrence ($n = 3$), or who were under 45 years old with HCC occurrence ($n = 3$) were very small, it was not possible to properly evaluate these subgroups. The risk factors among subgroups for future ALT elevation in patients with normal ALT levels, and for HCC were almost equal to those of the entire patient population. However, daily alcohol consumption, not HBV-DNA level, was predictive of future use of antiviral drugs for HBV in patients ≥45 years old or in patients infected with HBV genotype C. In these subgroups, alcohol consumption was an important factor for predicting the clinical course of HBV carriers; i.e. advising patients to abstain from drinking might reduce the need for antiviral drugs in the future.

Coffee or caffeine consumption is reported to be strongly related to ALT levels and HCC occurrence.[22–24] In our study, we did not survey caffeine consumption; therefore, further analysis is needed to determine the importance of coffee or caffeine consumption as a predictive factor of the clinical course in HBeAg-negative HBV carriers.

In conclusion, we established that low HBV-DNA levels and ALT levels at baseline were good predictors for future ALT eleva-

tion in HBeAg-negative HBV carriers with normal ALT levels. In addition, this study provides data on the prediction of unavoidable antiviral drug use and HCC occurrence.

References

1 Fattovich G, Bortolotti F, Donato F. Natural history of chronic hepatitis B: special emphasis on disease progression and prognostic factors. *J. Hepatol.* 2008; **48**: 335–52.
2 Di Marco V, Lo Iacono O, Camma C *et al.* The long-term course of chronic hepatitis B. *Hepatology* 1999; **30**: 257–64.
3 Fattovich G, Olivari N, Pasino M *et al.* Long-term outcome of chronic hepatitis B in Caucasian patients: mortality after 25 years. *Gut* 2008; **57**: 84–90.
4 Hoofnagle JH, Dusheiko GM, Seeff LB *et al.* Seroconversion from hepatitis B e antigen to antibody in chronic type B hepatitis. *Ann. Intern. Med.* 1981; **94**: 744–8.
5 Korenman J, Baker B, Waggoner J *et al.* Long-term remission of chronic hepatitis B after alpha-interferon therapy. *Ann. Intern. Med.* 1991; **114**: 629–34.
6 Sumi H, Yokosuka O, Seki N *et al.* Influence of hepatitis B virus genotypes on the progression of chronic type B liver disease. *Hepatology* 2003; **37**: 19–26.
7 Lin CL, Liao LY, Liu CJ *et al.* Hepatitis B viral factors in HBeAg-negative carriers with persistently normal serum alanine aminotransferase levels. *Hepatology* 2007; **45**: 1193–8.
8 Feld JJ, Ayers M, El-Ashry D *et al.* Hepatitis B virus DNA prediction rules for hepatitis B e antigen-negative chronic hepatitis B. *Hepatology* 2007; **46**: 1057–70.
9 Orito E, Ichida T, Sakugawa H *et al.* Geographic distribution of hepatitis B virus (HBV) genotype in patients with chronic HBV infection in Japan. *Hepatology* 2001; **34**: 590–4.
10 Lai CL, Yuen MF. The natural history of chronic hepatitis B. *J. Viral. Hepat.* 2007; **14** (Suppl. 1): 6–10.
11 Murata K, Sugimoto K, Shiraki K *et al.* Relative predictive factors for hepatocellular carcinoma after HBeAg seroconversion in HBV infection. *World J. Gastroenterol.* 2005; **11**: 6848–52.
12 Pokorski RJ, Ohlmer U. Long-term morbidity and mortality in Chinese insurance applicants infected with the hepatitis B virus. *J. Insur. Med.* 2001; **33**: 143–64.
13 Fujiwara K, Yokosuka O, Ehata T *et al.* The two different states of hepatitis B virus DNA in asymptomatic carriers: HBe-antigen-positive versus anti-HBe-positive asymptomatic carriers. *Dig. Dis. Sci.* 1998; **43**: 368–76.
14 Kumar M, Sarin SK, Hissar S *et al.* Virologic and histologic features of chronic hepatitis B virus-infected asymptomatic patients with persistently normal ALT. *Gastroenterology* 2008; **134**: 1376–84.
15 Mels GC, Bellati G, Leandro G *et al.* Fluctuations in viremia, aminotransferases and IgM antibody to hepatitis B core antigen in chronic hepatitis B patients with disease exacerbations. *Liver* 1994; **14**: 175–81.
16 Hoofnagle JH, Doo E, Liang TJ *et al.* Management of hepatitis B: summary of a clinical research workshop. *Hepatology* 2007; **45**: 1056–75.
17 Sanai FM, Benmousa A, Al-Hussaini H *et al.* Is serum alanine transaminase level a reliable marker of histological disease in chronic hepatitis C infection? *Liver Int.* 2008; **28**: 1011–18.
18 Lai M, Hyatt BJ, Nasser I *et al.* The clinical significance of persistently normal ALT in chronic hepatitis B infection. *J. Hepatol.* 2007; **47**: 760–7.
19 Villet S, Ollivet A, Pichoud C *et al.* Stepwise process for the development of entecavir resistance in a chronic hepatitis B virus infected patient. *J. Hepatol.* 2007; **46**: 531–8.
20 Sa-nguanmoo P, Tangkijvanich P, Payungporn S *et al.* Dynamics of HBV DNA levels, HBV mutations and biochemical parameters during antiviral therapy in a patient with HBeAg-negative chronic hepatitis B. *Asian Pac. J. Allergy Immunol.* 2007; **25**: 183–8.
21 Buster EH, van Erpecum KJ, Schalm SW *et al.* Treatment of chronic hepatitis B virus infection—Dutch national guidelines. *Neth. J. Med.* 2008; **66**: 292–306.
22 Ruhl CE, Everhart JE. Coffee and caffeine consumption reduce the risk of elevated serum alanine aminotransferase activity in the United States. *Gastroenterology* 2005; **128**: 24–32.
23 Ruhl CE, Everhart JE. Coffee and tea consumption are associated with a lower incidence of chronic liver disease in the United States. *Gastroenterology* 2005; **129**: 1928–36.
24 Larsson SC, Wolk A. Coffee consumption and risk of liver cancer: a metaanalysis. *Gastroenterology* 2007; **132**: 1740–5.

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ORIGINAL ARTICLE

Initial Virological Response and Viral Mutation with Adefovir Dipivoxil Added to Ongoing Lamivudine Therapy in Lamivudine-Resistant Chronic Hepatitis B

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Abstract
Background Although adefovir dipivoxil (ADV) has been used for antiviral treatment of lamivudine (LAM)-resistant chronic hepatitis B (CHB) patients, the long-term efficacy of this treatment is not well understood. Initial virological response (IVR) has been reported to be an important factor in relation to the development of ADV-resistance.
Aims We therefore examined the factors associated with IVR and ADV mutation in these patients.
Methods Forty-nine LAM-resistant CHB patients with ADV add-on LAM therapy, 47% of whom were hepatitis B e-antigen (HBeAg)-positive with median treatment duration of 23 months, were enrolled in this study. Patients were classified into IVR and non-IVR groups on the basis of viral suppression status. Mutational analysis of the HBV polymerase/reverse transcriptase (rt) domain was performed by PCR-direct sequencing.
Results Serum HBV DNA was undetectable (<2.6 $\log_{10}$ copies/mL) in 67, 82, and 84% of patients at 24, 48, and 96 weeks, respectively, after ADV add-on LAM therapy. IVR was achieved in 82% of patients, and ALT normalized at week 24 in 90% of IVR and 78% of non-IVR patients. The lower pretreatment HBV DNA level and virus-containing mutations other than double mutation of rtL180M + rtM204V were significantly associated with IVR ($P = 0.002$ and $P = 0.014$, respectively). ADV-resistant mutations in the RT motif, reported previously, were not detected.
Conclusion IVR is useful for predicting the antiviral efficacy of ADV and LAM combination therapy in LAM-resistant CHB.

Keywords Chronic hepatitis B · Adefovir dipivoxil · Lamivudine · Initial virological response · Mutation

Abbreviations

ADV	Adefovir dipivoxil
ALT	Alanine aminotransferase
CHB	Chronic hepatitis B
HBV	Hepatitis B virus
IVR	Initial virological response
LAM	Lamivudine
rt	Reverse transcriptase

Introduction

Because of the frequent development of life-threatening sequelae, for example liver cirrhosis and hepatocellular carcinoma (HCC), chronic hepatitis B (CHB) infection is a major public health problem worldwide, affecting over 350 million people [1], especially in Asia and Africa [2–4]. The levels of circulating hepatitis B virus (HBV) DNA reflect the status of HBV replication in the liver and are thought to be related to future incidence of cirrhosis, HCC [2, 5–8], and HCC-related mortality [9]. Therefore, complete and sustained suppression of viral replication is the most important objective of treatment of chronic HBV infection. Long-term administration of nucleos(t)ide analogues may

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prevent these complications. Lamivudine (LAM) has been used as first-choice therapy for CHB patients, regardless of HBeAg status, because of its potency, safety profile, and relatively low cost [10]. However, the efficacy of long-term therapy with LAM is compromised by viral resistance; the annualized incidence rate of LAM-resistant mutations was 22% [11] and reached 71% in year 4 [12].

Adefovir dipivoxil, an oral pro-drug of adefovir (ADV), is a synthetic adenine nucleotide analogue that has been shown to be effective in suppression of HBV DNA, HBeAg seroconversion, alanine aminotransferase (ALT) normalization, and histological improvement, regardless of HBeAg status [13–15]. The drug has been shown to have antiviral activity against not only wild-type HBV [13, 14] but also LAM-resistant HBV mutants both in vitro and in vivo [16, 17]. In contrast with LAM therapy, the benefit of ADV therapy is the delayed and infrequent selection of drug-resistant viruses [14, 18–20]. The cumulative incidence of an ADV-resistant mutation emerging in nucleos(t)ide treatment-naïve CHB patients at 48, 96, 144, 192, and 240 weeks was 0, 0.8–3, 11, 18%, and up to 29%, respectively [13, 21–25].

The antiviral activity of ADV has been reported to be lower in LAM-resistant CHB patients than in treatment-naïve patients [26–28]. However, the factors associated with antiviral efficacy of ADV are still not well understood.

We have previously studied the association between lamivudine sensitivity and amino acid substitutions in the reverse transcriptase (RT) region of HBV polymerase and found that sequence analysis of the RT domain is useful for predicting sensitivity to LAM therapy [29].

In this study we assessed the long-term efficacy of ADV add-on therapy for CHB patients with LAM-resistance, analyzed the relationship between amino acid substitution in the RT domain and sensitivity to ADV add-on LAM therapy for LAM-resistant CHB patients, and determined the risk factors associated with the initial virological response (IVR).

Materials and Methods

Patients

CHB patients ($n = 49$) who received 10 mg daily of ADV as add-on therapy to ongoing LAM (100 mg daily) after the emergence of LAM resistance were enrolled at Chiba University Hospital between 2004 and 2009. All patients were negative for hepatitis C, hepatitis D, and human immunodeficiency virus antibodies. Sera obtained from patients at the commencement of ADV add-on LAM therapy were stored at −20°C until analysis. This study was approved by the Ethics Committee of Chiba University Hospital.

Serological Examination

HBsAg, HBeAg, and anti-HBe antibody were determined by enzyme-linked immunosorbent assay (ELISA; Abbott Laboratory, Chicago, IL, USA). HBV genotype was determined from patients' sera by ELISA (HBV Genotype EIA; Tokushu-Meneki Laboratory, Tokyo, Japan) based on the method described by Usuda et al. [30]. Serum HBV DNA levels were monitored every four weeks using the Roche Amplicor Monitor test (Roche Diagnostics, Tokyo, Japan), which has a lower detection limit of 2.6 log copies/mL.

Viral Genome Sequencing

Pretreatment sera were obtained from 31 patients and nucleotide sequences could be analyzed in 22 patients. Sequence analysis for detection of HBV-DNA mutations in serum samples in the non-IVR group was performed after 24, 48, and 96 weeks of treatment. To amplify the region encompassing the polymerase reverse transcriptase (RT) domain, DNA extracted from 200 μL serum was used as a template and long-range PCR and nested PCR were performed in a 50-μL reaction using LA Taq polymerase (TaKaRa Bio, Kyoto, Japan) under the following conditions: 5-min activation at 94°C, 35 cycles or 30 cycles with denaturation at 94°C for 40 s, annealing at 58°C for 1 min, and extension at 68°C for 90 s and 1 min in the first and second round, respectively. The last cycle was followed by a final extension at 72°C for 7 min. An 862 base-pair fragment (nt 242-1103) containing the polymerase RT domain was amplified. The primers for the first round of PCR were 5′-CCT CAG GCT CAG GGC ATA-3′ (sense, nt 3082-3099) and 5′-GAC GGG ACG TAG ACA AAG G-3′ (antisense, nt 1436-1418). The primers for the second round of PCR were 5′-CAG AGT CTA GAC TCG TGG-3′ (sense, nt 242-258) and 5′-GGC GAG AAA GTG AAA GCC-3′ (antisense, nt 1103-1086). The PCR product was sequenced using the primers: 5′-TGG CTC AGT TTA CTA GTG CC -3′ (nt 668-687), 5′-GGC ACT AGT AAA CTG AGC CA-3′ (nt 687-668), and the primers for the second round of PCR. The amino acid sequence of each protein was deduced from the nucleotide sequence. The HBV genotype was also confirmed on the basis of the viral sequence data obtained.

Definition of Initial Virological Response and Undetectable HBV DNA

An initial virological response (IVR) was defined as HBV DNA < 4 $\log_{10}$ copies/mL after treatment for 24 weeks [26]. HBV DNA < 2.6 $\log_{10}$ copies/mL was regarded as "serum HBV DNA undetectable".

Statistical Analysis

Categorical variables between groups were compared by use of Fisher's exact test. The Mann–Whitney U test was used for assessing the association between baseline factors and the occurrence of IVR. Results were considered statistically significant at $P < 0.05$.

Results

Clinical and Biochemical Data of the Patients

A total of 49 patients were included in this analysis. Thirty-six (71%) were men, the median age when ADV was added to LAM treatment was 55 years (range: 35–71 years), and 24 patients (47%) were HBeAg-positive. Pretreatment ALT levels ranged from 14 to 1495 IU/L (median: 129 IU/L), and the median pretreatment HBV DNA level was 6.9 $\log_{10}$ copies/mL (range: 2.8–8.8 $\log_{10}$ copies/mL). The median duration of treatment with LAM was 25.5 months (range: 3–78 months). The median duration of combination treatment with ADV and LAM was 29 months (range: 8–63 months) (Table 1).The median duration of treatment with LAM was 26 months (range: 3–78 months) and 23 months (range: 12–50 months) in the IVR and non-IVR groups, respectively (P = N.S.).

Frequency of Undetectable HBV DNA Levels

In all patients, sequential monitoring revealed that 24, 48, and 96 weeks after addition of ADV to ongoing LAM therapy serum HBV DNA levels were undetectable (<2.6 $\log_{10}$ copies/mL) in 67, 82, and 84%, respectively, (Fig. 1a). Among the 24 HBeAg-positive patients, HBeAg seroconversion was observed to be 5 and 16% after 48 and 96 weeks, respectively.

Table 1 Clinical and biochemical data of patients infected with hepatitis B virus

Number of patients	49
Median age, years (range)	55 (35–71)
Male sex, number (%) of patients	36 (71%)
HBeAg positive, number (%) of patients	24 (47%)
Median pretreatment ALT level, IU/L (range)	129 (14–1495)
Median pretreatment HBV DNA level, $\log_{10}$ copies/mL (range)	6.9 (2.8–8.8)
Median duration of LAM therapy, months (range)	26 (3–78)
Median duration of ADV therapy, months (range)	29 (8–63)

ALT, alanine aminotransferase; HBV, hepatitis B virus; ADV, adefovir dipivoxil

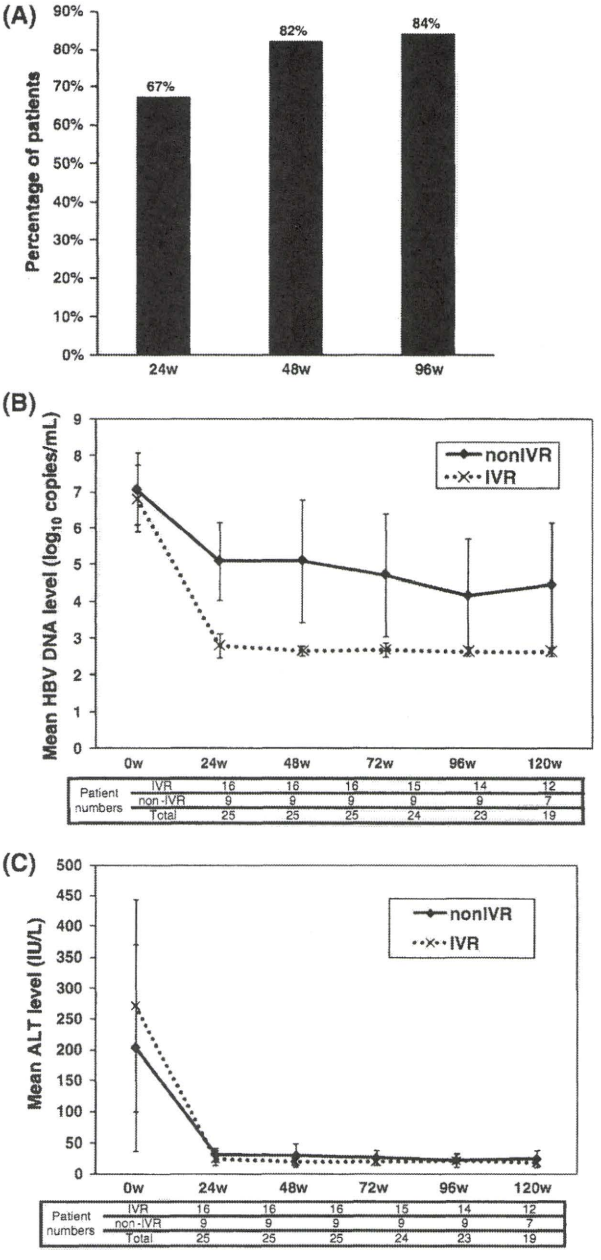

Fig. 1 **a** Percentages of patients with reduction in HBV DNA by <2.6 $\log_{10}$ copies/mL 24, 48, and 96 weeks after ADV add-on LAM. **b, c** Sequential HBV DNA and ALT levels according to initial virological response (IVR). Patients who achieved IVR are represented by the *dashed line*; those who did not are represented by the *solid line*. Data represent mean ± SD. An HBV DNA level below 2.6 $\log_{10}$ copies/mL was regarded as being approximately equal to 2.6 $\log_{10}$ copies/mL. The numbers of IVR and non-IVR patients, and total patient numbers, at each time point are also shown in the table

Comparison of Characteristics Between the IVR and Non-IVR Groups

According to the IVR definition, patients were classified into two groups, an IVR group and a non-IVR group, for further analyses (Table 2). IVR was achieved in 82% of the 49 patients. As expected, patients who achieved IVR had a more marked drop in HBV DNA levels during the first 24 weeks and this reduction lasted throughout the follow-up period (Fig. 1b). The rates of ALT normalization at week 24 were 90 and 78% in the IVR and non-IVR groups, respectively (Fig. 1c).

Comparison of patient characteristics in the IVR and non-IVR groups showed that the HBV DNA level at baseline was lower in the IVR group than in the non-IVR group ($P = 0.002$). The non-IVR group had a high percentage of HBeAg-positive patients at baseline compared with the IVR group (78% vs. 42%, $P = 0.054$). More women than men achieved IVR (Table 2, $P = 0.036$). There were no significant differences between the baseline characteristics age, body mass index, or baseline serum ALT levels of the two groups. Genotype was determined in 27 patients. There were 25 with genotype C and 2 with genotype A; genotype C was detected in 16/18 of the IVR group and in 9/9 of the non-IVR group (P = N.S.).

Amino Acid Sequences of RT Motif Domains Between the IVR and Non-IVR Groups

The polymerase RT domains were sequenced to investigate the relationship with sensitivity to ADV add-on LAM therapy. We compared the deduced amino acid sequences of A, B, C, D, and E domains of the RT motif between the two groups (13 and 9 patients in the IVR and non-IVR groups, respectively), but found neither ADV-resistant mutations nor any significant differences, except for the substitutions at rtL180 and rtM204. Double mutation (rtL180M + M204V/I) or single mutation (rtM204I, rtM204V) was observed among the patients studied (Fig. 2). However, the HBV-containing double mutant (rtL180M + M204V) was observed more frequently in the non-IVR group than in the IVR group (78% vs. 28%, $P = 0.014$).

Characteristics of Patients with Lasting High HBV DNA Levels

Most of the patients achieved undetectable levels of HBV DNA after 48 weeks of ADV add-on LAM therapy, but six patients in the non-IVR group were found to have sustained high HBV DNA levels (>5 $\log_{10}$ copies/mL) beyond 48 weeks of ADV add-on LAM treatment. Two of these patients (n-IVR 5 and n-IVR 9) developed virological breakthrough (VBT, elevation of >1 $\log_{10}$ copies/mL from nadir). VBT occurred at weeks 68 and 48 in patients n-IVR 5 and n-IVR 9, respectively. To investigate the additional amino acid substitution in the RT domain during treatment of these patients we analyzed the amino acid sequences at several time points during combination therapy. Four cases were found to be infected with HBV carrying the rtL180M + M204V double mutation at the commencement of ADV add-on LAM therapy (Table 3). Sequential analysis of RT mutations of the four patients is shown in Fig. 3. Because no additional amino acid substitutions, including ADV-resistant mutations, were detected in any samples tested, an alternate mechanism is likely to be responsible for the insufficient response of these patients to therapy.

Discussions

In LAM therapy for patients with chronic HBV infection, emergence of a LAM-resistant YMDD mutant virus is a serious problem, because it inevitably restricts the antiviral efficacy of LAM. For this reason, LAM has been replaced

Table 2 Comparison of patient characteristics between IVR and non-IVR groups at enrollment

	IVR group ($n = 40$)	Non-IVR group ($n = 9$)	P value
Median age, years (range)	54 (35–71)	50 (38–67)	0.588[b]
Male/female	26/14	9/0	0.036[a]
Median body mass index, kg/m^2 (range)	22.0 (17.5–27.9)	22.8 (19.4–25.2)	0.795[b]
HBeAg positive rates	42%	78%	0.054[a]
Median ALT level, IU/L (range)	117 (14–1495)	199 (35–710)	0.439[b]
Median HBV DNA level, $\log_{10}$ copies/mL (range)	6.8 (2.8–8.7)	8.0 (7.1–8.8)	0.002[b]
rtL180M + M204V	28%	78%	0.014[a]

ALT, alanine aminotransferase

[a] Fisher's exact test

[b] Mann–Whitney U test

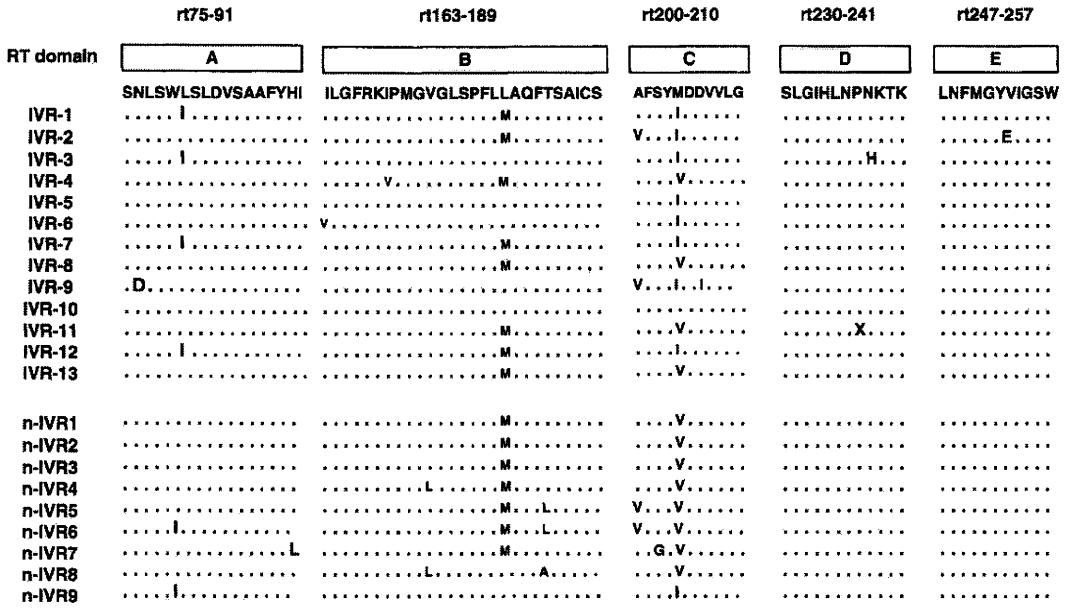

Fig. 2 Amino acid sequences of A, B, C, D, E domains of the RT motif are shown for the initial virological response (IVR) group and the non-IVR group. Double mutation of rtL180M + rtM204V is predominant in the non-IVR group compared with the IVR group (78% vs. 28%, $P = 0.014$)

Table 3 Pretreatment characteristics of patients with sustained elevation of HBV DNA levels after 48 weeks of ADV treatment

	n-IVR 1	n-IVR 3	n-IVR 5	n-IVR 7	n-IVR 8	n-IVR 9
Age (years)	61	41	44	42	51	64
Gender	Male	Male	Male	Male	Male	Male
HBeAg	Positive	Positive	Positive	Negative	Positive	Positive
HBV-DNA level ($\log_{10}$ copies/mL)	8	7.5	7.3	7.1	8	8
ALT level (IU/L)	358	389	35	416	85	56
Mutation in the RT region	rt180M + rtM204V	rt180M + rtM204V	rt180M + rtM204V	rt180M + rtM204V	rtM204V	rtM204I
Virological breakthrough	Negative	Negative	Positive	Negative	Negative	Positive

RT, reverse transcriptase

by newly developed nucleos(t)ide analogues, for example ADV and entecavir (ETV), for treatment of chronic hepatitis B. ETV has been reported to be more effective at reducing HBV DNA, and induces the drug-resistant mutant virus less frequently than LAM in nucleos(t)ide-naïve patients [24, 31].

The IVR, which was recently defined as HBV DNA < 4 $\log_{10}$ copies/mL after 24 weeks on treatment [26], was reported to be associated with the antiviral efficacy of ADV and the emergence of an ADV-resistant mutation in LAM-resistant CHB [32–34]. Several previous studies have suggested that lower pretreatment HBV DNA levels, higher pretreatment ALT, HBeAg negativity, and the presence of liver cirrhosis were associated with the virological response. In agreement with a previous report [33], this study showed that patients without IVR exhibited higher baseline HBV DNA levels than patients with IVR (8.0% vs. 6.8%, $P = 0.002$). Other studies have identified HBV virological rebounds during LAM or ADV treatment in the absence of mutation associated with drug resistance [22, 35]. The possibility of patient dosing adherence may be one of the factors leading to non-IVR.

The analyses of the amino acid sequence of the RT motif at the commencement of ADV add-on therapy revealed that it was difficult to achieve optimum viral suppression in patients who were infected with the virus carrying the rtL180M + M204V double mutation compared with other mutational patterns, for example the rtL180M + rtM204I double mutation, or rtM204V and rtM204I single mutations. Because the number of samples

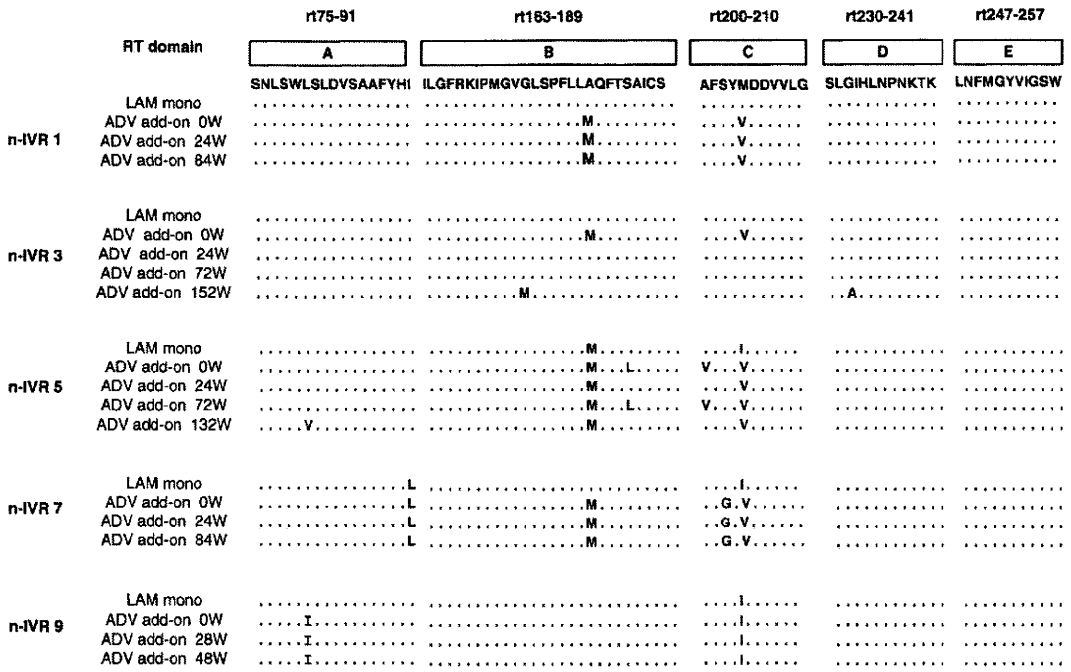

Fig. 3 Amino acid sequences of five representative patients in the non-IVR group are shown. Emergence of the rtL180M + M204V double mutation was observed in four of five patients from the commencement of ADV add-on LAM combination therapy

detected containing these mutations was small, and the alleged association was negative, further study will be needed to confirm this result.

Suzuki et al. [36] reported that the rtM204I mutant was associated with an earlier virological response as compared with the rtM204V mutant, and virological suppression of the mutation rtL180M was linked to that of rtM204I or rtM204V [36]. Furthermore, Suzuki et al. [36] showed that when viral loads of both mutants (rtM204V and rtM204I) were similar at the commencement of ADV therapy in patients with mixed-type virus, rtM204V predominated over rtM204I at 52 weeks. In our study, six patients in the non-IVR group had sustained elevation of HBV DNA levels (>5 $\log_{10}$ copies/mL), yet endured ADV add-on LAM co-administration for more than 48 weeks, and four of the six patients had mutant virus carrying the rtL180M + M204V double mutation (Table 3).

Cha et al. [37] assessed the patterns of LAM-resistant mutations and the effect of such mutations on virological response to ADV monotherapy in LAM-resistant CHB. They established the mutational patterns, for example rtM204V ± rtL180M ± rtV173L, rtM204I ± rtL180M, rtM204I ± rtL80I, compared the IVR status with these mutations, and found that the antiviral effect of ADV did not differ significantly among these patterns. Lada et al. [38] studied the susceptibility of LAM-resistant HBV to ADV in vitro. They reported that in samples with triple LAM resistance-associated amino acid changes rtV173L + L180M + M204V, HBV DNA reduction at week 48 was lower than for samples which had only the rtL180M + M204V mutations. In our study, rtV173L was observed only in the non-IVR group but the incidence did not differ significantly between groups. Our results are partially discordant with these previous studies, and differences between the studies, for example the additional mutations and use of ADV monotherapy, may be a possible explanation for the different outcomes.

In a randomized controlled study of ADV therapy in 42 patients who had genotypic LAM resistance with virological and clinical breakthrough, Rapti et al. [39] found that ADV resistance was not detected in the 28 patients undergoing ADV add-on LAM combination therapy but was detected in three patients (21%) upon viral/biochemical breakthrough after switching to ADV monotherapy. In our study, most of the patients treated with ADV add-on LAM therapy exhibited sustained viral suppression, except for two patients who had emergent virological breakthroughs. The sequencing analyses, however, demonstrated no ADV-resistant mutations (rtN236T, rtA181V/T, and rtI233V), suggesting the other mechanisms, for example viral mutation in the remaining part of the sequences or host factors, may be responsible for the reduced efficacy of the combination therapy in these two patients.

In conclusion, ADV add-on LAM therapy for LAM-resistant CHB patients was effective in suppressing viral replication and normalizing ALT levels. However, in cases

with high pre-treatment HBV DNA levels and the rtL180M + rtM204V double mutation, the antiviral effect of ADV is likely to be weak. Careful monitoring for the emergence of ADV-resistant mutation during prolonged treatment is critical.

References

1. Lavanchy D. Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. *J Viral Hepatol.* 2004;11:97–107.
2. Merican I, Guan R, Amarapuka D, et al. Chronic hepatitis B virus infection in Asian countries. *J Gastroenterol Hepatol.* 2000;15: 1356–1361.
3. O'Sullivan BG, Gidding HF, Law M, Kaldor JM, Gilbert GL, Dore GJ. Estimates of chronic hepatitis B virus infection in Australia, 2000. *Aust N Z J Public Health.* 2004;28:212–216.
4. Burnett RJ, Francois G, Kew MC, et al. Hepatitis B virus and human immunodeficiency virus co-infection in sub-Saharan Africa: a call for further investigation. *Liver Int.* 2005;25: 201–213.
5. 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.
6. Iloeje UH, Yang HI, Su J, Jen CL, You SL, Chen CJ. Predicting cirrhosis risk based on the level of circulating hepatitis B viral load. *Gastroenterology.* 2006;130:678–686.
7. Liaw YF. Hepatitis B virus replication and liver disease progression: the impact of antiviral therapy. *Antiviral Ther.* 2006;11:669–679.
8. WEt Delaney, Borroto-Esoda K. Therapy of chronic hepatitis B: trends and developments. *Curr Opin Pharmacol.* 2008;8: 532–540.
9. Iloeje UH, Yang HI, Jen CL, et al. Risk and predictors of mortality associated with chronic hepatitis B infection. *Clin Gastroenterol Hepatol.* 2007;5:921–931.
10. Lok AS, McMahon BJ. Chronic hepatitis B: update of recommendations. *Hepatology.* 2004;39:857–861.
11. Zoulim F, Poynard T, Degos F, et al. A prospective study of the evolution of lamivudine resistance mutations in patients with chronic hepatitis B treated with lamivudine. *J Viral Hepatol.* 2006;13:278–288.
12. Lok AS, Lai CL, Leung N, et al. Long-term safety of lamivudine treatment in patients with chronic hepatitis B. *Gastroenterology.* 2003;125:1714–1722.
13. Hadziyannis SJ, Tassopoulos NC, Heathcote EJ, et al. Adefovir dipivoxil for the treatment of hepatitis B e antigen-negative chronic hepatitis B. *N Engl J Med.* 2003;348:800–807.
14. Marcellin P, Chang TT, Lim SG, et al. Adefovir dipivoxil for the treatment of hepatitis B e antigen-positive chronic hepatitis B. *N Engl J Med.* 2003;348:808–816.
15. Marcellin P, Chang TT, Lim SG, et al. Long-term efficacy and safety of adefovir dipivoxil for the treatment of hepatitis B e antigen-positive chronic hepatitis B. *Hepatology.* 2008;48: 750–758.
16. Xiong X, Flores C, Yang H, Toole JJ, Gibbs CS. Mutations in hepatitis B DNA polymerase associated with resistance to lamivudine do not confer resistance to adefovir in vitro. *Hepatology.* 1998;28:1669–1673.
17. Peters MG, Hann Hw H, Martin P, et al. Adefovir dipivoxil alone or in combination with lamivudine in patients with lamivudine-resistant chronic hepatitis B. *Gastroenterology.* 2004;126: 91–101.
18. Westland C, Delaney Wt, Yang H, et al. Hepatitis B virus genotypes and virologic response in 694 patients in phase III studies of adefovir dipivoxil1. *Gastroenterology.* 2003;125: 107–116.
19. Perrillo R, Hann HW, Mutimer D, et al. Adefovir dipivoxil added to ongoing lamivudine in chronic hepatitis B with YMDD mutant hepatitis B virus. *Gastroenterology.* 2004;126:81–90.
20. Westland CE, Yang H, Delaney WEt, et al. Activity of adefovir dipivoxil against all patterns of lamivudine-resistant hepatitis B viruses in patients. *J Viral Hepatol.* 2005;12:67–73.
21. Yang H, Westland CE, Delaney WEt, et al. Resistance surveillance in chronic hepatitis B patients treated with adefovir dipivoxil for up to 60 weeks. *Hepatology.* 2002;36:464–473.
22. Westland CE, Yang H, Delaney WEt, et al. Week 48 resistance surveillance in two phase 3 clinical studies of adefovir dipivoxil for chronic hepatitis B. *Hepatology.* 2003;38:96–103.
23. Angus P, Vaughan R, Xiong S, et al. Resistance to adefovir dipivoxil therapy associated with the selection of a novel mutation in the HBV polymerase. *Gastroenterology.* 2003;125: 292–297.
24. Hadziyannis SJ, Tassopoulos NC, Heathcote EJ, et al. Long-term therapy with adefovir dipivoxil for HBeAg-negative chronic hepatitis B for up to 5 years. *Gastroenterology.* 2006;131:1743–1751.
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. Fung SK, Chae HB, Fontana RJ, et al. Virologic response and resistance to adefovir in patients with chronic hepatitis B. *J Hepatol.* 2006;44:283–290.
27. Lee YS, Suh DJ, Lim YS, et al. Increased risk of adefovir resistance in patients with lamivudine-resistant chronic hepatitis B after 48 weeks of adefovir dipivoxil monotherapy. *Hepatology.* 2006;43:1385–1391.
28. Yeon JE, Yoo W, Hong SP, et al. Resistance to adefovir dipivoxil in lamivudine resistant chronic hepatitis B patients treated with adefovir dipivoxil. *Gut.* 2006;55:1488–1495.
29. Fukai K, Zhang KY, Imazeki F, Kurihara T, Mikata R, Yokosuka O. Association between lamivudine sensitivity and the number of substitutions in the reverse transcriptase region of the hepatitis B virus polymerase. *J Viral Hepatol.* 2007;14:661–666.
30. 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.
31. Colonno RJ, Rose R, Baldick CJ, et al. Entecavir resistance is rare in nucleoside naive patients with hepatitis B. *Hepatology.* 2006;44:1656–1665.
32. Chen CH, Wang JH, Lee CM, et al. Virological response and incidence of adefovir resistance in lamivudine-resistant patients treated with adefovir dipivoxil. *Antiviral Ther.* 2006;11:771–778.
33. Kim IH, Kim SH, Kim HC, et al. Effect of initial virologic response to adefovir on the development of resistance to adefovir in lamivudine-resistant chronic hepatitis B. *Korean J Hepatol.* 2007;13:349–362.
34. Gallego A, Sheldon J, Garcia-Samaniego J, et al. Evaluation of initial virological response to adefovir and development of adefovir-resistant mutations in patients with chronic hepatitis B. *J Viral Hepatol.* 2008;15:392–398.
35. Pillay D, Cane PA, Ratcliffe D, Atkins M, Cooper D. Evolution of lamivudine-resistant hepatitis B virus and HIV-1 in co-infected individuals: an analysis of the CAESAR study. CAESAR coordinating committee. *AIDS.* 2000;14:1111–1116.

36. Suzuki F, Kumada H, Nakamura H. Changes in viral loads of lamivudine-resistant mutants and evolution of HBV sequences during adefovir dipivoxil therapy. *J Med Virol.* 2006;78:1025–1034.
37. Cha CK, Kwon HC, Cheong JY, et al. Association of lamivudine-resistant mutational patterns with the antiviral effect of adefovir in patients with chronic hepatitis B. *J Med Virol.* 2009;81:417–424.
38. Lada O, Benhamou Y, Cahour A, Katlama C, Poynard T, Thibault V. In vitro susceptibility of lamivudine-resistant hepatitis B virus to adefovir and tenofovir. *Antiviral Ther.* 2004;9:353–363.
39. Rapti I, Dimou E, Mitsoula P, Hadziyannis SJ. Adding-on versus switching-to adefovir therapy in lamivudine-resistant HBeAg-negative chronic hepatitis B. *Hepatology.* 2007;45:307–313.

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Hepatitis B Virus e Antigen Downregulates Cytokine Production in Human Hepatoma Cell Lines

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Abstract

Disease activities of hepatitis B are affected by the status of hepatitis B e antigen (HBeAg). The function of the hepatitis B virus (HBV) precore or HBeAg is unknown. We assumed that HBeAg blocks aberrant immune responses, although HBeAg is not required for viral assembly, infection, or replication. We examined the interaction of HBeAg and the immune system, including cytokine production. The inflammatory cytokine TNF, IL-6, IL-8, IL-12A, IFN-α1, and IFN-β mRNA were downregulated in HBeAg-positive HepG2, which stably expresses HBeAg, compared to HBeAg-negative HepG2 cells. The results of real-time RT-PCR-based cytokine-related gene arrays showed the downregulation of cytokine and IFN production. We also observed inhibition of the activation of NF-κB- and IFN-β-promoter in HBeAg-positive HepG2, as well as inhibition of IFN and IL-6 production in HBeAg-positive HepG2 cell culture fluids. HBeAg might modify disease progression by inhibiting inflammatory cytokine and IFN gene expression, while simultaneously suppressing NF-κB-signaling- and IFN-β-promoter activation.

Introduction

More than 2 billion people have been exposed to hepatitis B virus (HBV), and 350 million remain chronically infected worldwide. HBV is a noncytopathic DNA virus with a partially double-stranded 3.2-kb genome. HBV causes acute and chronic hepatitis, cirrhosis, and hepatocellular carcinoma (2,6,21,25,37). Viral clearance and its pathogenesis during acute HBV infection require the induction of a vigorous $CD8^+$ T-cell response, and the induction of hepatic immunopathology, including cytokine responses.

The HBV genome consists of four open reading frames coding for the surface, core, polymerase, and X proteins. Viral DNA, upon entry into cells during productive infection, undergoes a repair process and forms covalently closed circular DNA. Transcription of this DNA produces longer (precore) and shorter (pregenomic) 3.5-kb RNAs. The pregenomic RNA is packaged into nucleocapsides along with the viral polymerase, and serves as the template for viral genome replication. Precore and pregenomic RNAs encode core, polymerase (by pregenomic RNA), and hepatitis B e antigen (HBeAg) (by precore RNA) (47).

Disease severity of hepatitis B is affected by the status of HBeAg. The presence of HBeAg in serum is also known to be a marker of a high degree of viral infectivity. Although there are diverse opinions, fulminant hepatitis may occur in persons who are negative for HBeAg in highly endemic areas (29). Infants born to HBeAg-positive mothers tend to be HBsAg-positive more than those born to HBeAg-negative mothers (44). HBeAg-positive asymptomatic carriers (ASCs) have higher viral load, but most do not display any liver dysfunction (10). These clinical cases can be assumed to have immune tolerance for HBeAg.

The core gene of 183 codons (at least for genotypes B and C) is preceded by an in-frame pre-ATG codon that extends the protein by 29 hydrophobic amino acids (Fig. 1A). Proteins like this are translated from a 3.5-kb precore RNA and converted to HBeAg by two proteolytic cleavage events in the secretory pathway (12,26,38). First, the N-terminal 19 residues encoded by the precore region serve as the signal peptide for translocation of the precore/core protein into the endoplasmic reticulum lumen, where the peptide is clipped away by a signal peptidase. Next, 30 residues are removed from the C terminus in a post-endoplasmic reticulum compartment to generate mature HBeAg of ~17 kDa (12). A single point mutation has been reported to produce a stop codon in the precore region of HBV DNA and prevent the formation of the precore protein required to make HBeAg (7). HBeAg is thought to involve immune tolerance via an unknown mechanism, although it is not required for viral

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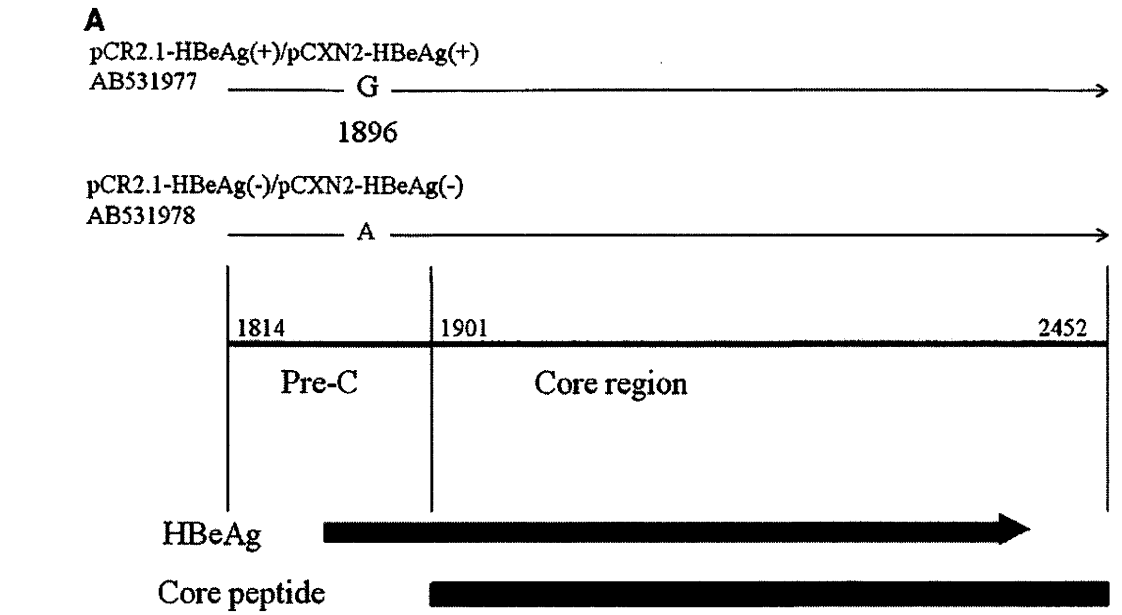

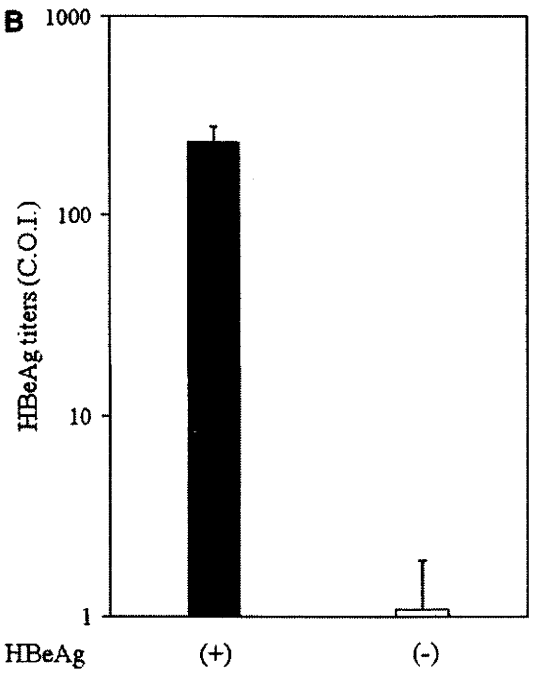

FIG. 1. (A) Schematic diagram of the precore and core peptides of HBV genotype C and cDNAs used in this study. Nucleotide A makes a stop codon at 1896, resulting in the absence of HBeAg production. Plasmid pCXN2-HBeAg(+) can produce both HBeAg and core, whereas pCXN2-HBeAg(−) can produce only core peptide. HBeAg and core peptides are indicated by the bold arrow and bold line, respectively. (B) Titers of HBeAg in cell culture fluid from HBeAg-positive and HBeAg-negative HepG2 cells were measured by the CLEIA method. The cut-off index (C.O.I.) is shown. The titer of HepG2 control cells is ~0.4 C.O.I.

assembly, infection, or replication (3,42). Visvanathan *et al.* (43) reported that the expression of TLR2 on hepatocytes, Kupffer cells, and peripheral monocytes, was significantly reduced in HBeAg-positive chronic hepatitis B patients. Although the precise function of HBV precore or HBeAg is unknown, it is possible that HBeAg suppresses the TLR pathways, thereby allowing HBV to establish persistent infection in the host (43).

Toll-like receptors (TLRs) play important roles in the innate immune response and are thought to have therapeutic potential for infectious diseases and cancers (18). Some of them are expressed on many different cells, including hepatocytes (32,36). Preiss *et al.* (32) demonstrated mRNA transcription for most TLRs, with the exception of TLR8. TLR5 mRNA was not detectable in HepG2 cells. Hepatocytes may themselves play an active role in innate immune responses to viruses such as HBV (32). Once these pattern recognition receptors (PRRs) have identified the pathogen-associated molecular patterns (PAMPs), the effector cells function and respond immediately. Ligand recognition by TLRs leads to the recruitment of various TIR domain-containing adaptors, such as myeloid differentiation primary

response gene (88) (MyD88), toll-interleukin 1 receptor domain containing adaptor protein (TIRAP), TIR domain-containing adapter inducing interferon-β (TRIF), and TRIF-related adapter molecule (TRAM), which in turn triggers the cascade of the signaling pathway, and ultimately the activation of transcription factors such as nuclear factor-κB (NF-κB) and interferon regulatory factors (IRFs), leading to the expression of various cytokines (e.g., tumor necrosis factor [TNF], interleukin-6 [IL-6], IL-8, interferon-α1 [IFN-α1], and IFN-β). Hepatic cytokines also play an important role in the progression of hepatitis B-associated liver diseases. A number of viruses have been shown to encode proteins that have the potential to inhibit antiviral activity of the innate and adaptive immune responses. Inflammatory cytokines contributing to viral clearance in HBV infection may have therapeutic value (20). In the present study, we assumed that HBeAg blocks aberrant immune responses, and we examined the role of HBeAg protein in cytokine production to test the interaction between HBeAg and the immune system in human hepatocytes. Our results demonstrated that cytokine production is inhibited by HBeAg, and that it also enhances IFN-sensitive hepatitis C virus (HCV) replication.

Materials and Methods

Plasmids

pNF-κB-luc, which expresses luciferase upon promoter activation by NF-κB, was purchased from Stratagene (La Jolla, CA). This vector has five repeats of the binding site for NF-κB (TGGGGACTTTCCGC). pIFN-β-luc, which expresses luciferase under the control of an IFN-β-dependent promoter, was kindly provided by Dr. N. Kato (Institute of Medical Science, University of Tokyo, Japan). To construct plasmids including HBV precore and core regions, HBV DNA was used from the serum of a genotype C HBeAg-positive asymptomatic carrier (ASC) patient as previously described (10). The DNA sequence information from this study will appear at GenBank (accession numbers AB531977 and AB531978). To make pCR2.1-HBeAg(+), the PCR product was cloned into pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA). Using the Quickchange II site-directed mutagenesis kit (Stratagene), precore stop codon mutant G1896A was induced into pCR2.1-HBeAg(+) to pCR2.1-HBeAg(−) according to the manufacturer's instructions. To obtain the mammalian cell expression vectors, we performed subcloning using the EcoRI site of pCXN2 (kindly provided by Prof. J. Miyazaki, Osaka University, Osaka, Japan), a mammalian expression vector with a β-actin-based CAG promoter and SV40 origin (28). The constructs pCXN2-HBeAg(+) and pCXN2-HBeAg(−) were generated by this method (Fig. 1A). All sequences of these plasmids were confirmed using Big Dye Terminator on a 3730 DNA sequencer (Applied Biosystems, Foster City, CA).

Cell culture

Human hepatoma cells, HepG2 and Huh7 cells, were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO_2. Approximately 1×10^5 HepG2 cells were placed on 35-mm tissue culture dishes (Iwaki Glass, Tokyo, Japan) 24 h prior to transfection (13). The cells were transfected with pCXN2-HBeAg(+) or pCXN2-HBeAg(−) in Effectene transfection reagent (Qiagen, Hilden, Germany). After 48 h, G418 was added at 1000 μg/mL for the selection of stable cell lines, and HBeAg-positive and HBeAg-negative HepG2 cells were designated. After 3 wk, to avoid monoclonal selection, all cells were collected for further analysis.

RNA extraction, cDNA synthesis, and real-time PCR

The cells were seeded into 6-well plates, and total cellular RNA was extracted 48 h later using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The RNA samples were then stored at −80°C until use. RNA quality was examined using the A_{280}/A_{260} ratio (Pharmacia Biotech, Bedford, MA). cDNA synthesis was performed using a random hexamer. For RNA quantitation, real-time PCR was conducted using SyBr Green I (ABI PRISM 7300; Applied Biosystems). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization, and data were analyzed by the comparative threshold cycle (C_T) method (16). The primers used are shown in Table 1.

Real-time PCR arrays

Gene expression profiling for TLR target genes was performed using RT^2 profiler PCR arrays (SuperArray, Frederick, MD) according to the manufacturer's instructions. In brief, 1 μg RNA was reverse-transcribed with the RT^2 profiler PCR array first-strand synthesis assay (SuperArray), followed by

TABLE 1. PRIMERS USED FOR QUANTITATIVE REAL-TIME PCR

Gene name	*Sequences (forward/reverse)*
GAPDH	5′-ACCCACTCCTCCACCTTTG-3′/ 5′-CTCTTGTGCTCTTGCTGGG-3′
TLR7	5′-GGAGGTATTCCCACGAACACC-3′/ 5′-GACCCCAGTGGAATAGGTACAC-3′
TNF	5′-CCAGACCAAGGTCAACCTC-3′/ 5′-CCAGATAGATGGGCTCATACC-3′
IL-6	5′-AAAAGTCCTGATCCAGTTC-3′/ 5′-GAGATGAGTTGTCATGTCC-3′
IL-8	5′-ACATACTCCAAACCTTTCCAC-3′/ 5′-CCAGACAGAGCTCTCTTCC-3′
IL-12A	5′-CCCTTGCACTTCTGAAGAG-3′/ 5′-AGGCAACTCTCATTCTTGG-3′
IFN-α1	5′-GGGATGAGGACCTCCTAGAC-3′/ 5′-GGAGTCCGCATTCATCAGG-3′
IFN-β	5′-GATTCATCTAGCACTGGCTGG-3′/ 5′-CTTCAGGTAATGCAGAATCC-3′
LY96 (MD-2)	5′-ATTTGCCGAGGATCTGATG-3′/ 5′-GGTGTAGGATGACAAACTCC-3′
RIPK2	5′-AGACACTACTGACATCCAAG-3′/ 5′-CACAAGTATTTCCGGGTAAG-3′
NF-κB1	5′-GAAGAAAATGGTGGAGTCTG-3′/ 5′-GGTTCACTAGTTTCCAAGTC-3′
MAP3K1	5′-CCACTGCATGTCAATTTGGG-3′/ 5′-CGTGGCTGTAGAAATCATGAG-3′
HCV	5′-TCTGCGGAACCGGTGAGTA-3′/ 5′-TCAGGCAGTACCACAAGGC-3′

real-time PCR with RT^2 real-time PCR master mix SyBr green (SuperArray). Gene expression was normalized to two internal controls (GAPDH and β-actin), to determine the fold change in gene expression between the test sample (HBeAg-positive HepG2) and the control sample (HBeAg-negative HepG2) by the 2^{-ddCT} (comparative cycle threshold) method (17). Data were analyzed with RT^2 Profiler™ PCR Array Data Analysis software (http://www.superarray.com/pcrarraydataanalysis.php). Genes with more than twofold change were also confirmed by real-time RT-PCR in at least triplicate. For this we used GAPDH for normalization.

Transfection and reporter assay

Approximately 1×10^5 cells were placed on 6-well plates (Iwaki Glass) 24 h prior to transfection. Cells were transfected with 0.4 μg of plasmid pIFN-β-luc or pNF-κB-luc in Effectene (Qiagen). For luciferase assay of NF-κB activation, cells were treated for 4 h with 0.5 or 5 ng/mL TNF-α, 10 or 50 μg/mL TLR4 ligand:lipopolysaccharide (LPS), or none at 44 h post-transfection (22,31,34,35,40). For IFN-β promoter assay, 50 μg/mL TLR3 ligand:poly(I-C), or none was added to cell culture fluid at 32 h post-transfection (16). At 48 h post-transfection, the cells were lysed with reporter lysis buffer (Promega, Madison, WI), and luciferase activity was determined by luminometer (Luminescencer-JNR II AB-2300; ATTO Bio Instruments, Tokyo, Japan) as previously described (16). Relative luciferase activity was measured at 48 h post-transfection and compared with that of an untreated control. Relative luciferase activity of HBeAg-negative cells was set as 1.

Chemiluminescent enzyme immunoassay

The supernatants of these cell lines were used for measuring the levels of HBeAg by the chemiluminescent enzyme immunoassay (CLEIA) system (Fujirebio Inc., Tokyo, Japan).

ELISA

Cell culture fluid was analyzed for IL-6 by enzyme-linked immunosorbent assay (ELISA; KOMA Biotech Inc., Seoul, Korea) following the manufacturer's protocol. Briefly, cell culture fluid samples were incubated in plates at 4°C overnight, followed by incubation with biotinylated monoclonal antibodies. Avidin-conjugated peroxidase was added to the plates, and enzyme activity was detected with an ELISA plate reader.

MTS assay

MTS assays were performed with the CellTiter 96 AQ One Solution Cell Proliferation Assay (Promega) (15). Twenty microliters/well of the MTS reagent was added to 100 μL of media containing cells in each well of 96-well plates, and left for 4 h at 37°C in a humidified 5% CO_2 atmosphere. For analysis, absorbance at 490 nm was measured using a Bio-Rad iMark microplate reader (Bio-Rad, Hercules, CA).

Antiviral assay using HCV subgenomic replicon

Huh7 cells harboring HCV genotype 1b subgenomic replicon, termed C13-3 cells, were used for antiviral bioassay (14). Intracellular HCV subgenomic RNA was measured by real-time RT-PCR. C13-3 cells were incubated in cell culture supernatant from HBeAg-positive, HBeAg-negative HepG2, or control HepG2 cells for 24–48 h. Post-incubation, RNA was extracted and stored at −80°C until analysis.

Statistical analysis

Results were expressed as mean ± SD. Student's *t*-test was used to determine statistical significance.

Results

Detection of stable expression of HBeAg by CLEIA

First, we examined the HBeAg production in cell culture fluid in HepG2 stably expressing HBV precore and core regions. HBeAg was detected in cell culture supernatants of HBV precore and core region-expressing cells (HBeAg-positive HepG2, 241 ± 47.9 C.O.I.) by CLEIA (cut-off index [C.O.I.]). On the other hand, expression of the core region without precore did not produce HBeAg in cell culture fluid (HBeAg-negative HepG2, 1.1 ± 0.84 C.O.I.) (Fig. 1B). Next, we performed an MTS assay to examine whether HBeAg affected cell proliferation or cell viability in our system. Cell proliferation/viability of HBeAg-positive cells (100 ± 0.87% at 24 h [n = 4]; 98.5 ± 0.7% at 48 h [n = 4]) was not statistically different from that of HBeAg-negative HepG2 (100 ± 0.4% at 24 h [n = 4]; 100 ± 1.21% at 48 h [n = 4]).

HepG2 cells respond to TLR3 ligand, TLR4 ligand, and tumor necrosis factor

Next we examined whether human hepatoma cell lines HepG2 and Huh7 respond to TLR3 ligand, TLR4 ligand, and tumor necrosis factor (TNF). Here we examined the NF-κB- and IFN-signaling pathways in HepG2 and Huh7 cells. To examine whether HepG2 possesses a functional TLR4 pathway, we initially characterized LPS-induced activation of NF-κB in HepG2 and Huh7 by luciferase reporter assay.

TABLE 2. NUCLEAR FACTOR (NF)-κB ACTIVATION FOLLOWING EXPOSURE TO LIPOPOLYSACCHARIDE (LPS), AND FOLLOWING EXPOSURE TO TUMOR NECROSIS FACTOR (TNF)-α, AND INTERFERON (IFN)-β-PROMOTER ACTIVATION FOLLOWING EXPOSURE TO POLY(I-C) BY LUCIFERASE ASSAYS

Ligand	*HepG2 (fold)*	*Huh7 (fold)*
NF-κB activation		
LPS (10 μg/mL)	23.3 ± 3.11**	1.82 ± 0.17*
LPS (50 μg/mL)	56.0 ± 13.6*	3.01 ± 0.69*
TNF-α (0.5 ng/mL)	9.47 ± 1.37**	1.45 ± 0.27
TNF-α (5 ng/mL)	14.4 ± 0.82***	8.59 ± 1.18**
IFN-β-promoter activation		
Poly (I-C) (50 μg/mL)	1.69 ± 0.14*	0.93 ± 0.10

Cells were transfected with 0.4 μg of plasmid pIFN-β-luc or pNF-κB-luc in Effectene (Qiagen). For the luciferase assay of NF-κB activation, cells were treated for 4 h with 0.5 or 5 ng/mL TNF-α, 10 or 50 μg/mL LPS, or none, at 44 h post-transfection (22,31,34,35,40). For the IFN-β promoter assay, 50 μg/mL poly(I-C) or none was added to cell culture fluid at 32 h post-transfection (16). Relative luciferase activity was measured at 48 h post-transfection and compared with that of an untreated control. Results are expressed as mean ± SD.

*$p < 0.01$, **$p < 0.001$, and ***$p < 0.0001$ in HepG2 or Huh7 induced by each ligand compared with untreated controls.

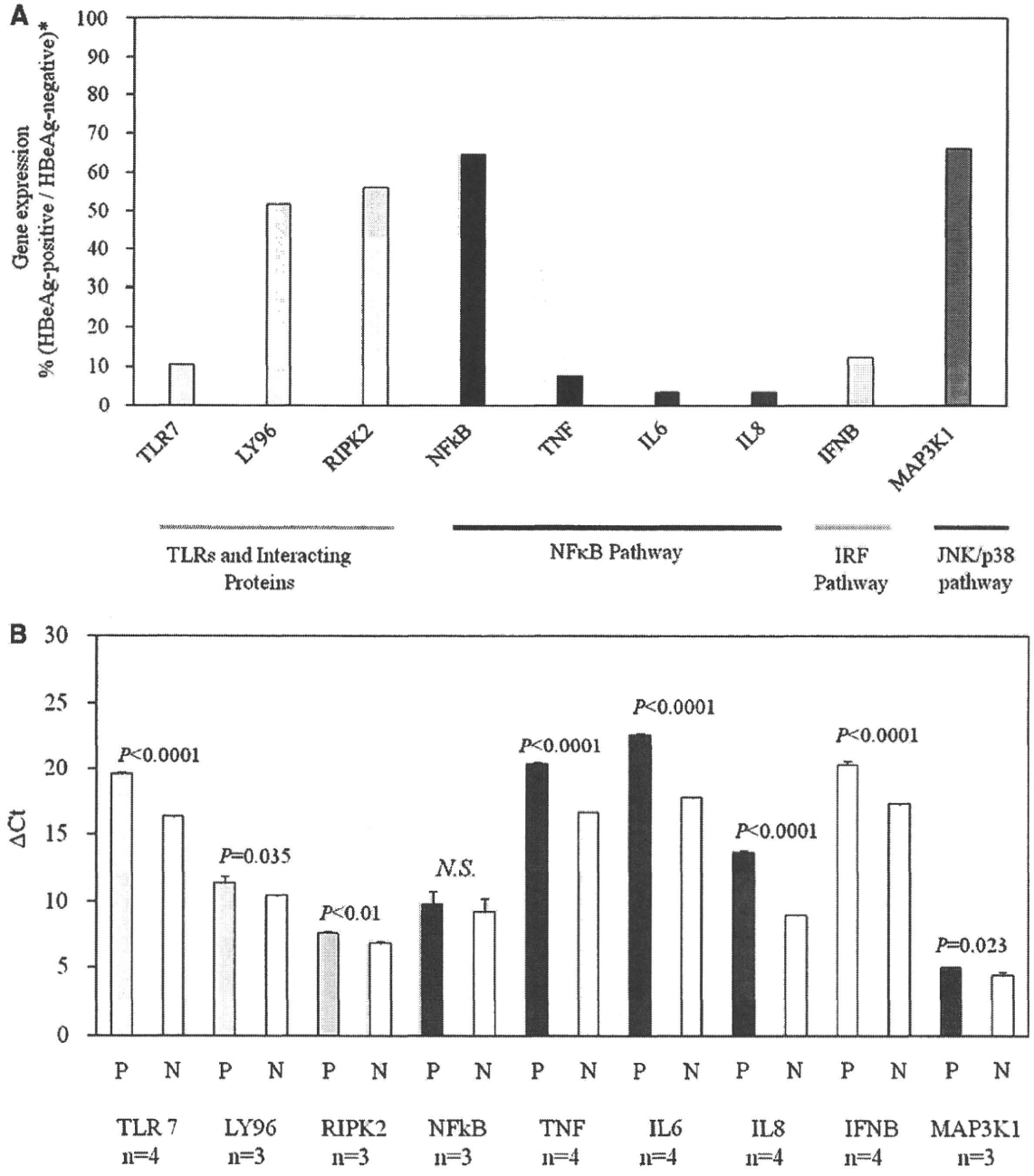

FIG. 2. Effects of HBeAg on toll-like receptor (TLR) signaling-related gene expression (comparison of genes expressed in HBeAg-positive HepG2 with those in HBeAg-negative HepG2). (**A**) TLR target gene expression examined by real-time RT-PCR in at least triplicate. GAPDH was used for normalization. These genes were screened by real-time PCR arrays, as described in the materials and methods section *(value of HBeAg-positive cells/value of HBeAg-negative cells)×100. (**B**) Statistical analysis of TLR signaling-related gene expression in HBeAg-positive and HBeAg-negative HepG2 cellular RNA by real-time RT-PCR by ΔCt. Results are expressed as mean ± SD (N.S., not statistically significant by Student's *t*-test; P, HBeAg-positive HepG2; N, HBeAg-negative HepG2; TLR7, toll-like receptor 7; LY96 [MD-2], lymphocyte antigen 96; RIPK2, receptor-interacting serine-threonine kinase 2; NFκB1, nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 [p105]; IL-6, interleukin-6 [interferon-β2]; IL-8, interleukin-8; IFN-β, interferon-β1; MAP3K1, mitogen-activated protein kinase kinase kinase 1).

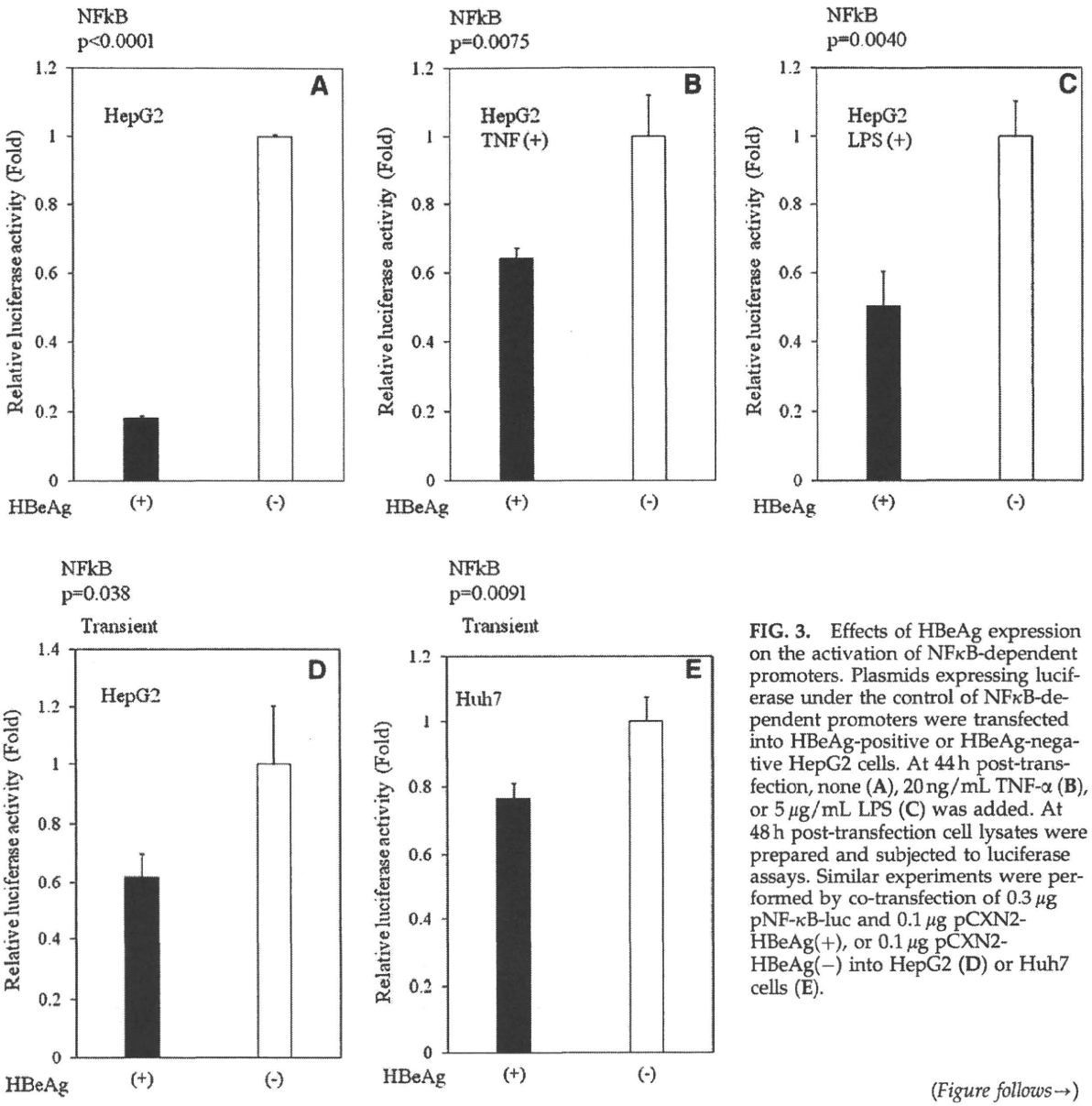

FIG. 3. Effects of HBeAg expression on the activation of NFκB-dependent promoters. Plasmids expressing luciferase under the control of NFκB-dependent promoters were transfected into HBeAg-positive or HBeAg-negative HepG2 cells. At 44 h post-transfection, none (**A**), 20 ng/mL TNF-α (**B**), or 5 μg/mL LPS (**C**) was added. At 48 h post-transfection cell lysates were prepared and subjected to luciferase assays. Similar experiments were performed by co-transfection of 0.3 μg pNF-κB-luc and 0.1 μg pCXN2-HBeAg(+), or 0.1 μg pCXN2-HBeAg(−) into HepG2 (**D**) or Huh7 cells (**E**).

(*Figure follows→*)

TLR4 plays an important role in the activation of NF-κB following exposure to extracellular LPS. When LPS was added to the cell culture medium of HepG2 and Huh7, approximately 23∼56-fold and 1.8∼3.0-fold activation, respectively, of NF-κB activity were observed (Table 2). Similarly, when TNF-α, another NF-κB activator, was added to the cell culture medium of HepG2 and Huh7, respectively, approximately 9∼14-fold and 1.4∼8.6-fold activation, respectively, of NF-κB activity were observed (Table 2). However, to examine for a functional TLR3 pathway by luciferase reporter assay, when poly(I-C) was added to the cell culture medium of HepG2 and Huh7, respectively, approximately 1.69-fold and 0.93-fold activation, respectively, of IFN-β-promoter activity were observed (Table 2), supporting the view that Huh7 cells are defective in the TLR3 and RIG-I pathway (16,39). Our results suggested that HepG2 possesses functional TLR3 and TLR4 pathways to some extent, but Huh7 does not possess a functional TLR3 pathway.

Downregulation of IFN and cytokine gene expression by HBeAg

Since HBeAg is associated with immune tolerance (3,42), we wanted to determine whether this might be related to HBeAg suppressing the host innate response, including the production of cytokines. To confirm the downregulation of IFN and cytokine genes, we performed real-time RT-PCR

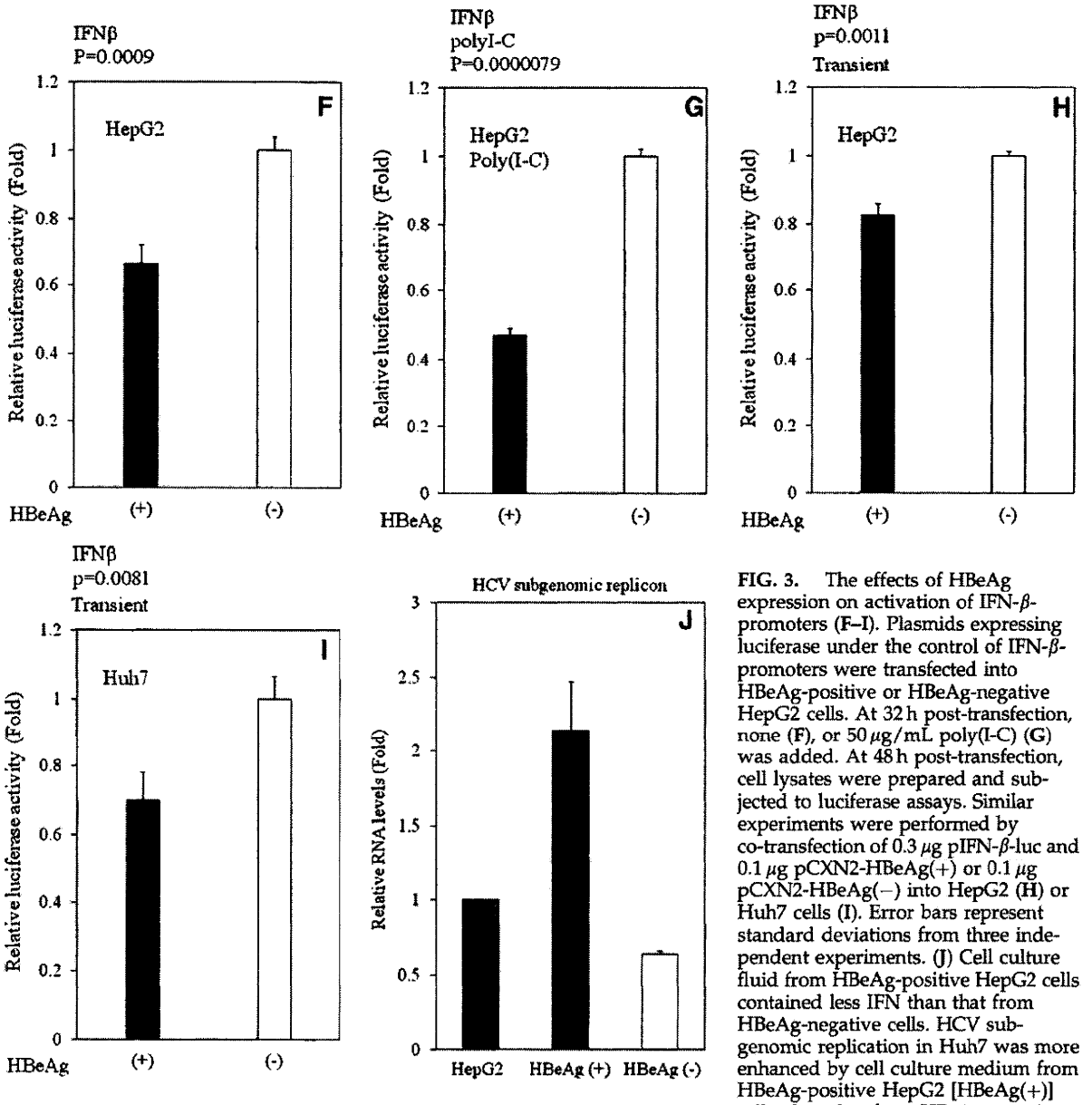

FIG. 3. The effects of HBeAg expression on activation of IFN-β-promoters (F–I). Plasmids expressing luciferase under the control of IFN-β-promoters were transfected into HBeAg-positive or HBeAg-negative HepG2 cells. At 32 h post-transfection, none (F), or 50 μg/mL poly(I-C) (G) was added. At 48 h post-transfection, cell lysates were prepared and subjected to luciferase assays. Similar experiments were performed by co-transfection of 0.3 μg pIFN-β-luc and 0.1 μg pCXN2-HBeAg(+) or 0.1 μg pCXN2-HBeAg(−) into HepG2 (H) or Huh7 cells (I). Error bars represent standard deviations from three independent experiments. (J) Cell culture fluid from HBeAg-positive HepG2 cells contained less IFN than that from HBeAg-negative cells. HCV subgenomic replication in Huh7 was more enhanced by cell culture medium from HBeAg-positive HepG2 [HBeAg(+)] cells, than that from HBeAg-negative HepG2 [HBeAg(−)] cells, and that from control HepG2 cells. Total cellular RNA was extracted at 24 h after adding cell culture medium. Intracellular gene expression levels of HCV and GAPDH were measured by real-time RT-PCR. The ratios of HCV/GAPDH are presented as *n*-fold relative to that in control HepG2 cells. The results are presented as means of data from three independent experiments.

assays. We compared six IFN and cytokine (IFN-α1, IFN-β, IL-6, IL-8, IL-12A, and TNF) gene expressions in HBeAg-positive HepG2 cells with those in HBeAg-negative HepG2 cells. The mRNAs of IFN-α1 and IL-12A were inhibited (6.7% and 11.6%, respectively, of those in HBeAg-negative HepG2), and ΔCt of HBeAg-positive HepG2/ΔCt of HBeAg-negative HepG2 in IFN-α1 mRNA and those in IL-12A mRNA were $14.36 \pm 0.11/10.47 \pm 0.02$ ($p < 0.001$, $n = 3$), and $17.74 \pm 0.11/14.65 \pm 0.17$ ($p < 0.001$, $n = 3$), respectively. As shown in Fig. 2, more inhibition of IFN-β, IL-6, IL-8, and TNF mRNA in HBeAg-positive HepG2 were also observed, compared with HBeAg-negative HepG2.

Effects of TLR-dependent target gene expression by HBeAg

To explore the upstream mechanism of IFN and cytokine production, we performed RT^2 profiler array assays to analyze important TLR-activated genes (84 target genes were included in the RT^2 profiler array), that could be modulated by