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

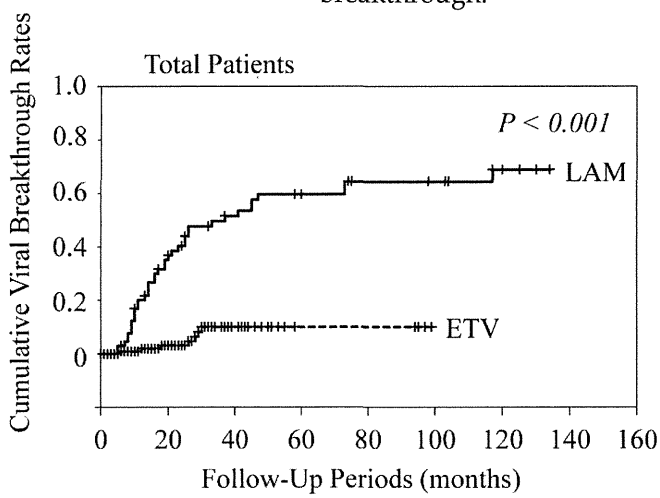
Most patients presented good adherence to medication in the present study. Seven ETV-treated (5.1%) and 6 LAM-treated patients (8.8%) had poor adherence (Figure 1). The number of patients with poor adherence was not significantly different between the ETV- and LAM-treated groups (*P=0.48*). The characteristics 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.



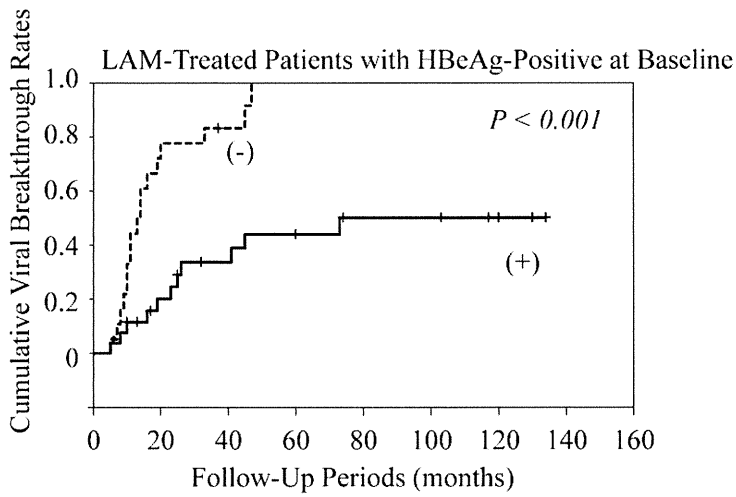
	Drug	Baseline	12	24	36	48	60
Number of Patients	LAM	68	52	34	27	20	19
	ETV	135	95	69	34	19	5

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*).

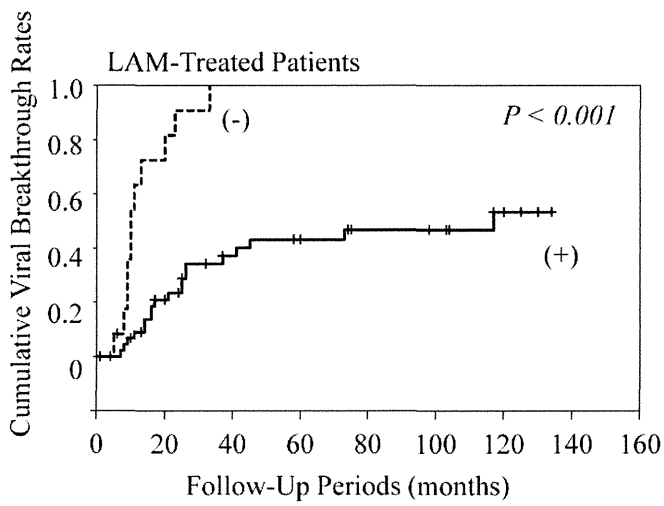
Resistance mutations

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.

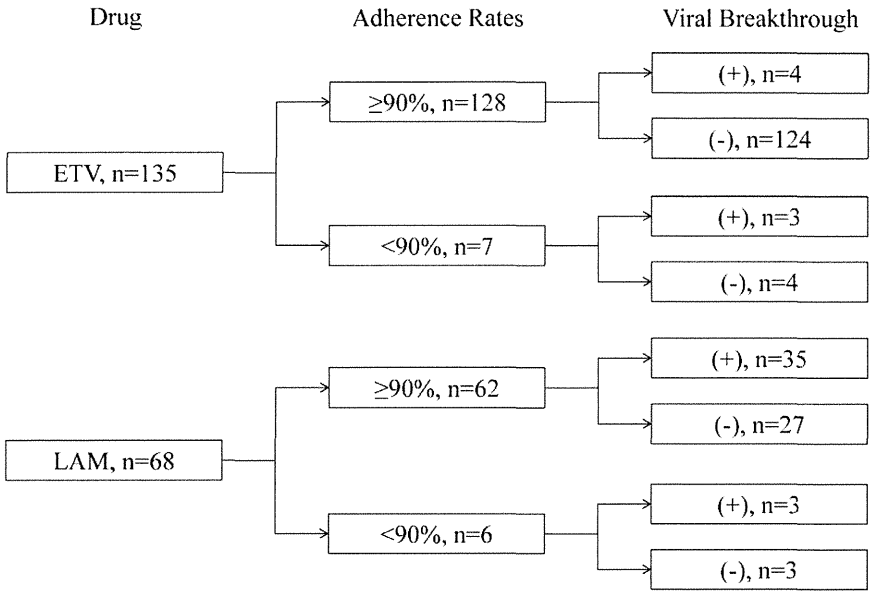


Figure 5. Association between adherence to medication and viral breakthrough.

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.

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Original Article

Hepatitis A, B, C and E virus markers in Chinese residing in Tokyo, Japan

Jun Yan,[1,2] Tatsuo Kanda,[1] Shuang Wu,[1] Fumio Imazeki[1] and Osamu Yokosuka[1]

[1]*Department of Medicine and Clinical Oncology, Graduate School of Medicine, Chiba University, Chiba, and* [2]*Kyowa Clinic, Tokyo, Japan*

Aim: Recently, the number of foreigners living in Japan has been increasing, with the majority originating from China. It is important for us to know the prevalence of hepatitis virus markers among them, as proper medical practices and vaccinations should be prepared when seeing them and their offspring.

Methods: We examined the relationship between the prevalence of hepatitis virus markers: hepatitis B surface antigen (HBsAg), anti-HBs, anti-hepatitis C virus (HCV), anti-hepatitis A virus (HAV) and anti-hepatitis E virus immunoglobulin (Ig)G, and background such as age, birthplace and length of stay in Japan, of 568 Chinese residing in Tokyo, and also of 55 indigenous Japanese.

Results: The prevalence of HBV and HAV markers in Chinese staying in Tokyo is higher than in indigenous Japanese (HBsAg, 10% vs 1.8%; anti-HBs, 45% vs 9.0%; anti-HAV, 90% vs 14%). There were no differences in anti-HCV and anti-HEV IgG between the two groups.

Conclusion: Indigenous Japanese subjects have less immunity against HAV and HBV. The HBV carrier rate is higher in Chinese subjects, and attention should be paid to this issue in clinical practice. It might be important to control hepatitis viruses in Chinese subjects when doctors see them in Japan.

Key words: Chinese, HAV, HBV, HCV, HEV, Tokyo

INTRODUCTION

HEPATITIS A, B, C and E virus (HAV, HBV, HCV and HEV, respectively) cause acute hepatitis, and occasionally fulminant hepatitis, and HBV and HCV also lead to chronic hepatitis, cirrhosis and hepatocellular carcinoma in Japan as well as throughout the world.[1–6] In general, the prevalence of hepatitis viruses follows a wide range of diverse patterns, being dependent on different areas and countries.[7–10]

In Japan, hepatitis B surface antigen (HBsAg) and antibody to HCV (anti-HCV), respectively, were detected in 0.63% and 0.49% in sera from first-time blood donors aged 16–64 years.[5] It was also reported that only fewer than 50% of people have immunity against HAV, estimated from anti-HAV prevalence.[11] Of qualified blood donors, 3.4% were regarded as positive for anti-HEV immunoglobulin (Ig)G.[12] On the other hand, as an example, in China, the prevalence of HBsAg, anti-HBs, anti-HCV, anti-HAV and anti-HEV IgG was reported to be 5.84%, 41.3%, 0.58%, 72.8% and 17.66%, respectively, although this prevalence pattern is well known to differ among different areas in China.[13]

By the end of 2009, 2 186 121 foreigners were living in Japan, and the largest proportion, 31.6%, was born in China, Taiwan and Hong Kong.[14] With increasing numbers of foreigners living in Japan, we will have more opportunities to see them as patients in clinical practice. It is important for us to know, among other things, their prevalence of hepatitis virus markers, as vaccinations and appropriate medical practices should be provided when seeing them and their offspring.

Therefore, in the present study, we examined the relationship between the prevalence of hepatitis virus markers and background such as age, birthplace and their duration of domicile in Japan among Chinese living in Tokyo, Japan.

Correspondence: Associate Professor Tatsuo Kanda, Department of Medicine and Clinical Oncology, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan. Email: kandat-cib@umin.ac.jp

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METHODS

Study subjects and serum collection

THE SUBJECTS IN this study were 623 consecutive outpatients attending the Kyowa Clinic in Tokyo. Of these patients, 568 (80%) were Chinese who were staying in Japan. The others were 55 indigenous Japanese, and all patients were seen between August 2010 and January 2011 (Table 1). The duration of the Chinese subjects' stay in Japan was 103 ± 76 days. There were no differences in age, sex or alanine aminotransferase (ALT) levels between the two groups, but the platelet counts of the Chinese were lower than those of the Japanese subjects (Table 1). Chinese patients were divided into eight groups according to their birthplace in China, as follows: 32, nine, one, 180, 331, 10, zero and five were from North China (Beijing, Tianjing, Hebei, Shanxi and Inner Mongolia), Central China (Henan, Hunan and Hubei), South China (Guangdong, Guangxi and Hainan), East China (Shanghai, Jiangsu, Zhejiang, Fujian, Shandong, Jiangxi and Anhui), North-East China (Heilongjiang, Liaoning and Jilin), South-West China (Sichuan, Chongqing, Yunnan, Guizhou and Tibet), North-West China (Xinjiang, Shanxi, Gansu, Ningxia and Qinghai) and Hong Kong, Macao and Taiwan, respectively. All patients were adults and the most common symptoms were other than liver diseases. Family history of liver diseases, history of surgeries, blood transfusion, drug abuse and tattoo were investigated from patients' interviews and medical records.

Serological diagnosis

All patients were screened by serological tools for hepatitis A, B, C and E virus infections. HBsAg, anti-HBs, anti-HCV, anti-HAV and anti-HEV IgG were tested in each sample by magnetizing particle aggregation (MAT; Shino-Test Tokyo, Japan), particle agglutination (PA; Fujirebio, Tokyo, Japan), chemiluminescent enzyme immunoassay (CLEIA; Fujirebio), chemiluminescent immunoassay (CLIA; Abbott Laboratories, North Chicago, IL, USA) and enzyme immune assay (EIA; Institute of Immunology, Tokyo, Japan), respectively. A positive reaction was indicated when the cut-off index (COI) exceeded 1.0 in anti-HCV, anti-HAV and anti-HEV IgG. The lower detection limit for HBsAg tested by MAT was 8 IU/mL, corresponding to approximately 10 COI measured by CLEIA method. The lower detection limit for anti-HBs examined by PA corresponded to 30 mIU/mL measured by CLEIA.

Hepatitis B virus genotype of patient sera was determined by ELISA (Institute of Immunology) based on the methodology described by Usuda *et al.*[7,15] Informed consent was obtained at the time of blood sampling from each patient included in the study. This study was approved by the ethics committee of Chiba University, Japan, and that of Kyowa Clinic, and conformed to the Declaration of Helsinki. Sera were collected as part of clinical practice and stored at −20°C until laboratory testing was performed.

Table 1 Background of study patients and hepatitis virus markers

	Total subjects	Chinese staying in Japan	Indigenous Japanese	*P*-value*
No. of patients	623	568	55	
Age, years	47 ± 14	47 ± 14	45 ± 15	NS
Sex (M/F)	292/331	264/304	28/27	NS
ALT (IU/L)	26 ± 44	26 ± 46	25 ± 19	NS
Platelets ($\times 10^4/mm^3$)	22 ± 5.4	22 ± 5.8	24 ± 5.1	0.013
HBsAg (+/−)	63/556	62/502	1/54	0.031
Anti-HBs (+/−)	258/362	259/305	5/50	<0.0001
Anti-HCV (+/−)	11/607	10/553	1/54	NS
Anti-HAV (+/−)	518/100	510/53	8/47	<0.0001
Anti-HEV IgG (+/−)	128/493	120/446	8/47	NS

**P*-value between Chinese subjects staying in Japan and indigenous Japanese subjects.
+, Positive; −, negative; ALT, alanine aminotransferase; HAV, hepatitis A virus; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; HEV, hepatitis E virus; IgG, immunoglobulin G; NS, not significant.

Data analysis

Data were expressed as mean ± standard deviation. Differences were evaluated by Student *t*-test or χ^2-test. $P < 0.05$ was considered statistically significant. 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 ver. 7 (SSRI, Tokyo, Japan) and DA Stats software (O. Nagata, Nifty Serve: PAF01644).

RESULTS

Chinese subjects staying in Tokyo have more immunity against HAV and HBV

AMONG 623 STUDY subjects, 549 (88%) had normal ALT levels (ALT ≤40 IU/L). HBsAg, anti-HBs, anti-HCV, anti-HAV and anti-HEV IgG were determined in 619 (99%), 620 (99%), 618 (99%), 618 (99%) and 621 (99%), respectively. The overall prevalence of HBsAg, anti-HBs, anti-HCV, anti-HAV and anti-HEV IgG in the present study was 10%, 45%, 1.7%, 90% and 21%, respectively (Table 1). The prevalence of HBV and HAV markers of Chinese staying in Japan was higher than that of indigenous Japanese (HBsAg, 10% vs 1.8%; anti-HBs, 45% vs 9.0%; anti-HAV, 90% vs 14%), but there were no differences in anti-HCV and anti-HEV IgG between the two groups (Table 1). These results suggest that Chinese have more immunity against HAV and HBV than indigenous Japanese. A greater proportion of Chinese subjects was HBsAg positive compared to indigenous Japanese subjects.

Sex differences in hepatitis virus markers

Next, we examined the sex differences in the two groups (Table 2). There were no sex differences concerning HBsAg, anti-HBs, anti-HCV and anti-HAV in each of the two groups. Among Chinese subjects staying in Japan, men with anti-HEV IgG were predominant, but this predominance was not seen in the Japanese group (Table 2).

Age differences in relation to prevalence of hepatitis virus markers

Among Chinese subjects staying in Japan, the HBsAg positive rate under 30 years was higher than in those in their 30s ($P = 0.0018$), 40s ($P < 0.0001$) and over 50 years ($P < 0.0001$), and the HBsAg positive rate of those in their 30s was also higher than those over 50 years ($P < 0.053$) (Fig. 1a). Only one HBsAg positive Japanese subject was a 53-year-old man. There were no differences in each age group between Chinese and Japanese subjects (Fig. 1a).

Positive rates of anti-HBs in those under 30 years, in their 30s, 40s and over 50 years in Chinese subjects staying in Japan were higher than those in indigenous Japanese ($P = 0.0037$, 0.0020, 0.0065 and 0.0034,

Table 2 Background of study patients and hepatitis virus markers according to sex differences

	Chinese staying in Japan			Indigenous Japanese		
	Male ($n = 264$)	*P*	Female ($n = 304$)	Male ($n = 28$)	*P*	Female ($n = 27$)
Age, years	47 ± 14	NS	47 ± 13	46 ± 13	NS	43 ± 18
ALT (IU/L)	29 ± 43	NS	23 ± 49	31 ± 19	0.0083	18 ± 16
Platelets ($\times 10^4$/mm^3)	21 ± 5.7	<0.0001	23 ± 5.9	24 ± 5.4	NS	24 ± 4.9
Length of stay (days)	103 ± 78	NS	104 ± 75			
Family of liver diseases (+/−)	13/248	NS	24/273	2/26	NS	0/27
Transfusion (+/−)	5/259	NS	6/297	1/27	NS	1/26
Surgery (+/−)	24/240	0.017	49/254	4/24		4/23
Drug abuse (+/−)	0/264	NA	0/303	0/28	NA	0/27
Tattoo (+/−)	0/264	NS	1/302	0/28	NA	0/27
HBsAg (+/−)	30/232	NS	32/270	1/27	NS	0/27
Anti-HBs (+/−)	119/144	NS	140/162	2/26	NS	3/24
Anti-HCV (+/−)	3/259	NS	7/292	1/27	NS	0/27
Anti-HAV (+/−)	235/28	NS	275/25	3/25	NS	5/22
Anti-HEV IgG (+/−)	68/195	0.015	52/251	2/26	NS	6/21

+, Positive; −, negative; ALT, alanine aminotransferase; HAV, hepatitis A virus; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; HEV, hepatitis E virus; IgG, immunoglobulin G; NS, not significant.

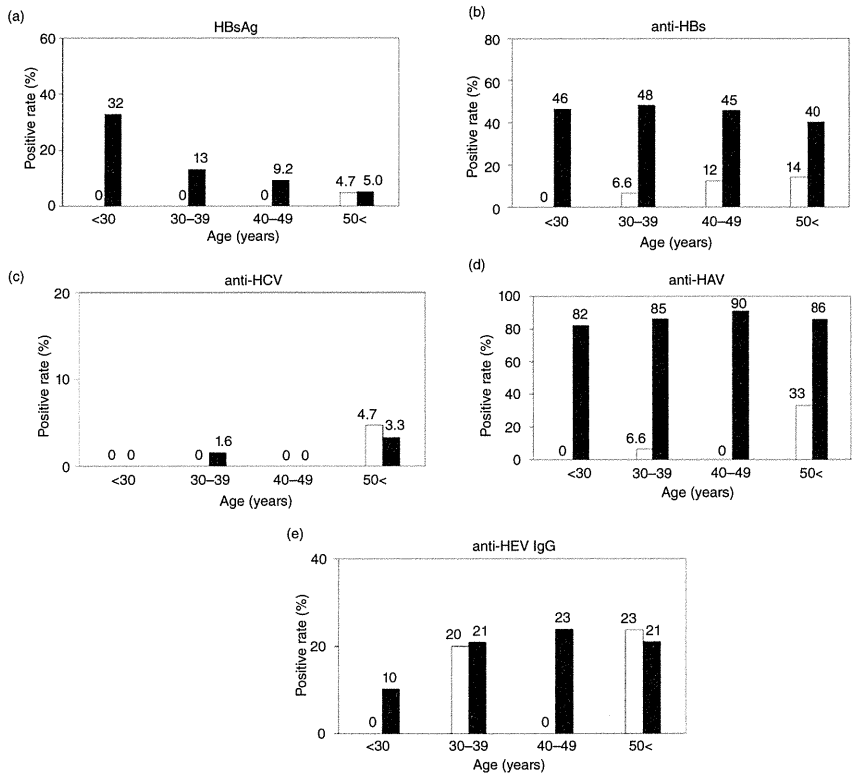

Figure 1 Hepatitis virus markers in study subjects according to age. (a) Hepatitis B surface antigen (HBsAg); (b) anti-HBs antibody; (c) anti-hepatitis C virus (HCV) antibody; (d) anti-hepatitis A virus (HAV) antibody; and (e) anti-hepatitis E virus (HEV) immunoglobulin (Ig)G antibody. White bar, indigenous Japanese; black bar, Chinese staying in Japan. Positive rates (%) are indicated.

respectively). There were no differences between each age group of Chinese subjects and also no differences between each age group of indigenous Japanese subjects in the present study (Fig. 1b).

There were no significant differences in anti-HCV positive rates in each age group of Chinese subjects or in each age group of Japanese indigenous subjects. There were also no significant differences in anti-HCV positive rates of each age group between Chinese and Japanese groups (Fig. 1c).

The positive rate of anti-HAV in subjects under 30 years was lower than in those over 50 years in the indigenous Japanese group ($P = 0.030$) (Fig. 1d). There were no differences among the respective age groups in Chinese subjects. Among the same age groups, the positive rates of anti-HAV in Chinese subjects were higher than those in indigenous Japanese subjects ($P < 0.0001$, each) (Fig. 1d).

There were no significant differences of anti-HEV IgG positive rates in each age group of Japanese indigenous subjects. As for Chinese subjects, there was a difference in anti-HEV IgG positive rate between the groups under 30 years and those in their 40s ($P = 0.029$). There were no significant differences in anti-HEV positive rates of each age group between the Chinese and Japanese groups (Fig. 1e).

Prevalence of hepatitis virus markers in Chinese subjects according to birthplace

Next, we examined the prevalence of hepatitis virus markers in Chinese subjects according to birthplace (Tables 3,4). Although the number of study subjects was limited, the prevalence of anti-HBs, anti-HCV, anti-HAV and anti-HEV IgG was quite similar in Chinese subjects independent of their place of birth. Interestingly, the HBsAg carrier rate was higher in the patients from East China than in those from North-East China (Table 4, $P < 0.0001$). In the background between these two areas (Table 3), young age, male dominance and longer term stays from East China were more than those from North-East China ($P < 0.0001$, $P = 0.029$ and $P < 0.0001$, respectively). As for risk factors of hepatitis virus infection, a history of surgery was seen more frequently in those from North China ($P = 0.023$, Table 3). We determined HBV genotypes in 57 of 63 HBsAg positive subjects and revealed that HBV genotype B was more common in those from East China than in those from North-East China ($P = 0.013$, Table 4). We also

Table 3 Background and risk factors of hepatitis virus infection in Chinese subjects staying in Japan: comparison with indigenous Japanese subjects

Birthplace	Chinese staying in Japan							Japanese
	North China	Central China	South China	East China	North-East China	South-West China	Hong Kong and Taiwan	Indigenous
No. of patients	32	9	1	180	331	10	5	55
Age, years	53 ± 13	40 ± 14	39	41 ± 12	50 ± 13	42 ± 11	60 ± 13	45 ± 15
Sex (M/F)	14/18	8/1	0/1	95/85	140/191	4/6	3/2	28/27
ALT (IU/L)	29 ± 53	22 ± 12	10	26 ± 46	26 ± 47	20 ± 9.8	20 ± 9.8	25 ± 19
Platelets ($\times 10^4/mm^3$)	22 ± 5.2	22 ± 7.3	27	22 ± 5.5	22 ± 6	21 ± 6.5	21 ± 6.5	24 ± 5
Length of their stay (days)	137 ± 90	66 ± 71	46	80 ± 68	113 ± 75	94 ± 76	192 ± 103	
Family history of liver diseases (+/−)	2/30	0/9	0/1	14/162	21/304	0/10	0/5	2/53
History (+/−)								
Transfusion	0/32	1/8	0/1	3/177	6/324	1/9	0/5	2/53
Surgery	7/25	1/8	0/1	14/166	50/280	1/9	0/5	8/43
Drug abuse	0/32	0/9	0/1	0/180	0/330	0/10	0/5	0/54
Tattoo	0/32	0/9	0/1	0/18	1/329	0/10	0/5	0/5

+, Positive; −, negative; ALT, alanine aminotransferase.

Table 4 Hepatitis virus markers in study subjects according to birthplace in Chinese subjects staying in Japan: comparison with indigenous Japanese subjects

Birthplace	Chinese staying in Japan							Japanese
	North China	Central China	South China	East China	North-East China	South-West China	Hong Kong and Taiwan	Indigenous
No. of patients	32	9	1	180	331	10	5	55
Hepatitis virus markers: +/−								
HBsAg	5/27	1/8	0/1	37/142	17/311	1/9	1/4	1/54
HBV genotype (B/C)	1/4	0/1	0/0	12/20	0/16	1/0	0/1	0/1
Anti-HBs	10/22	4/5	0/1	83/95	158/172	5/5	0/5	5/50
Anti-HCV	0/32	0/9	0/1	3/177	7/320	0/10	0/5	1/54
Anti-HAV	25/7	8/1	1/0	147/29	315/15	9/1	5/0	8/47
Anti-HEV IgG	10/22	2/7	0/1	41/138	60/270	5/5	2/3	8/47

+, Positive; −, negative; ALT, alanine aminotransferase; HAV, hepatitis A virus; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; HEV, hepatitis E virus; IgG, immunoglobulin G; NS, not significant.

determined HCV genotype by direct sequencing HCV core region in two cases from North-East China and one indigenous Japanese, with all three showing genotype 2a (data not shown).

DISCUSSION

THE PRESENT STUDY revealed that Chinese subjects staying in Tokyo have more immunity against HAV and HBV than indigenous Japanese subjects and that approximately 10% of Chinese subjects staying in Tokyo are HBsAg carriers. The HBsAg carrier rate seems to be higher in patients from East China than those from North-East China (Table 4). This might be useful to see the Chinese patients from these areas in clinical practices. There have been several reports about the HBsAg carrier rates of East China[16,17] and North-East China.[13,18] Hepatitis B vaccine was first recommended for routine vaccination of infants in China in 1992.[19] Because of high vaccine prices and user fees charged to parents by local health departments for vaccine purchase and administration, until 2002, infant hepatitis vaccination occurred primarily in large cities of wealthier eastern provinces.[19] In the 2004 survey, estimated vaccine coverage was higher in East China than in North-East China.[19] It is a possible reason why the difference in HBsAg prevalence between these areas was observed in the present study. We do not know the exact reason for this difference, and we consider that further studies will be needed.

Several medical institutes at which mostly Chinese gather have existed in Japan. Kyowa Clinic, one such facility, is located in Okachimachi, Tokyo, an important juncture of traffic networks. Because Japanese newspapers advertise this clinic, and the doctor sees the patients using both Chinese and Japanese languages, this outpatient-only clinic is known to Chinese subjects' staying in Japan. The patients of this clinic consist of 90% Chinese and 10% Japanese. Most of the less than 65-year-old male Chinese patients are cooks in Chinese restaurants, interior decorators and students, most of the less than 65-year-old female Chinese patients are housewives and students, and most of the Chinese patients 65 years or older are unemployed. Most of the Japanese patients are employees of small businesses and residents near this clinic. The present study has an authentic potential in terms of the clinical practice being different from previous studies, such as those concerning blood donors, in spite of the population selection of the present study seeming unnatural. Although selection biasness of patients with Japanese and Chinese background might exist, we included these Japanese patients, who come to the same clinic as controls to compare with Chinese in the present study. Although the number of hepatitis cases is decreasing, hepatitis is still a major health problem in Japan.[5,8,10,20] In China as well, hepatitis is a major public health burden.[13] As more foreigners take up residence in Japan, we are likely to see more Chinese patients in clinical practice, as approximately one-third of such foreigners come from China.[14] The present study might provide us with important information.

The number of cases of adult hepatitis A has been decreasing in Japan in accordance with socioeconomic and sanitation improvements.[9,10] In 1986, a national prevention program was launched in Japan with selective vaccination of babies born to carrier mothers with hepatitis B e antigen (HBeAg).[21] In 1995, this was extended to babies born to HBeAg negative carrier mothers. As a result, the prevalence of HBsAg among younger people born since 1986 has decreased dramatically.[21,22] Because there are no universal vaccination programs against HAV or HBV in Japan, HAV and HBV infections are still seen as important issues.[10]

Hepatitis A virus is a single-stranded RNA virus and usually spreads via the fecal–oral route, similarly to HEV. Of interest is the fact that the distribution of anti-HEV IgG among Chinese subjects staying in Tokyo is similar to that of indigenous Japanese subjects, although the prevalence of anti-HAV in Chinese staying in Tokyo is higher than that of indigenous Japanese (Fig. 1d,e). This may be related to differences in infectious routes of transmission of these two viruses or in differences of HAV vaccination between the two countries, as a certain number of HAV-vaccinated young Chinese adults seemed to be included in the present study.[23,24] In any event, a large proportion of Chinese adults seem to be protected by latent infection or immunization against HAV.[13,25]

The positive rate of anti-HEV IgG in the Kanto metropolitan area of Japan was previously reported as 8.6% in qualified blood donors[12] and 6.5% in health checkups.[26] In general, the positive rate of anti-HEV IgG in China has been recognized to be higher than that in Japan,[27] and the same report described a positive rate of anti-HEV IgG of more than 20% in indigenous Japanese aged 70 years or older. In the present study, the mean age of indigenous Japanese was 45 years (Table 1), and anti-HEV IgG positive indigenous Japanese numbered three in their 30s, one in their 50s, three in their 60s and one in their 70s, with the anti-HEV IgG positive rate being higher than in previous reports.[12,25,27] In most areas of

Japan, the positive rate of anti-HEV IgG in males was higher than that in females. We do not know the exact reasons why our anti-HEV IgG patients were not male-dominant (Table 2). The population selection of the present study may not be unbiased. However, as it seems that Japanese females have in recent years developed a taste for broiled pig innards on skewers compared to before, the potential of HEV infection is likely to grow, and greater attention should also be paid to Japanese females.

As HCV is a blood-borne RNA virus, and blood screening for HCV is a standard procedure in Japan, the distribution of anti-HCV of indigenous Japanese subjects is similar to that of Chinese subjects staying in Tokyo. HBV is an incomplete double-stranded DNA virus that infects through blood products and sexual contact as well as mother-to-baby transmission. The differences in the distribution of anti-HBs may be dependent on a different HBV vaccination status or different past HBV infection.

In conclusion, indigenous Japanese subjects have less immunity against HAV and HBV. As the HBV carrier rate is higher in Chinese subjects, this should receive some attention in clinical practice, and it might be important to control hepatitis viruses in Chinese subjects when they are seen by doctors in Japan.

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

Shuang Wu,[1] Tatsuo Kanda,[1] Fumio Imazeki,[1] Shingo Nakamoto,[1,2] Takeshi Tanaka,[1,3] Makoto Arai,[1] Thierry Roger,[5] Hiroshi Shirasawa,[2] Fumio Nomura,[4] and Osamu Yokosuka[1]

[1]Department of Medicine and Clinical Oncology, [2]Department of Molecular Virology, [3]Department of Environment Biochemistry, and [4]Department of Molecular Diagnosis, Graduate School of Medicine, Chiba University, Japan; and [5]Infectious Diseases Service, Department of Medicine, Centre Hospitalier Universitaire Vaudois and University of Lausanne, Lausanne, Switzerland

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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Correspondence: Tatsuo Kanda, MD, PhD, Department of Medicine and Clinical Oncology, Chiba University, Graduate School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan (kandat-cib@umin.ac.jp).

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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

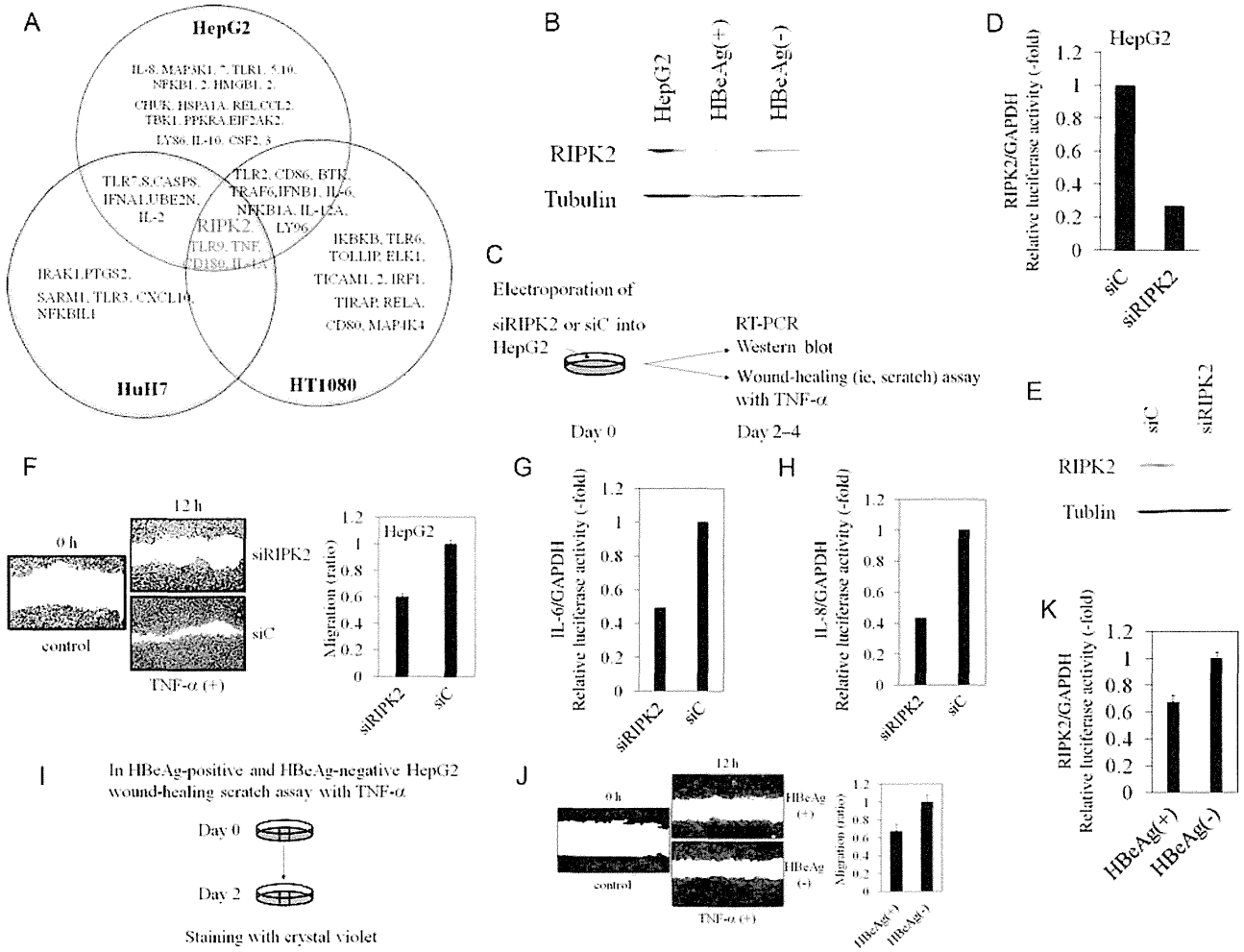

Figure 1. Receptor-interacting serine/threonine protein kinase 2 (RIPK2) expression is downregulated by hepatitis B virus e antigen (HBeAg), and knockdown of RIPK2 and HBeAg impairs hepatic wound repair. *A*, Venn diagram representing Toll-like receptor (TLR)–related genes downregulated ≥1.3-fold in 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

wound healing in the presence of TNF-α in vitro (Figure 1*C*). As shown in Figure 1*D* and 1E, RIPK2 mRNA and protein expression were efficiently decreased in HepG2 cells transfected with RIPK2 siRNA (siRIPK2), but not control (siC). RIPK2 silencing reduced hepatic wound closure 1.8-fold, which was associated with a 2-fold decrease in IL-6 production, known to be an important cytokine for the regeneration of liver [11], and a 2.3-fold decrease in interleukin 8 production (Figure 1*F–H*). Importantly, RIPK2 silencing did not affect cell viability (data not shown).

Given that HBeAg downregulates RIPK2 expression (Figure 1*A* and 1*B*), we examined whether HBeAg has an effect on hepatic wound healing in the presence of TNF-α (Figure 1*I*). As expected, we observed that both cell migration

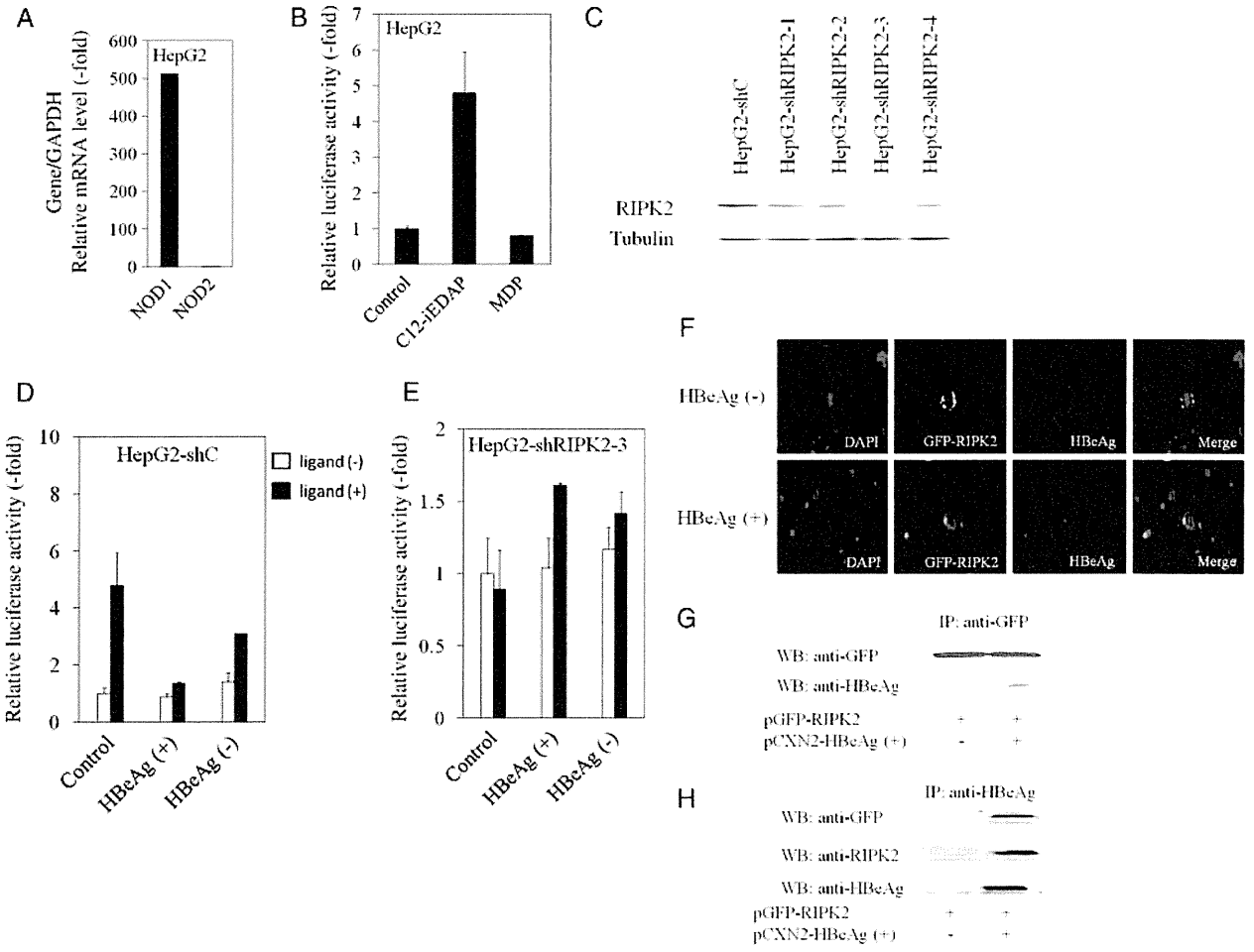

Figure 2. The nucleotide-binding oligomerization domain–containing protein 1 (NOD1) ligand C12-iEDAP induces NF-κB activation, knockdown of receptor-interacting serine/threonine protein kinase 2 (RIPK2) inhibits NOD1 ligand–induced NF-κB activation in HepG2 cells, and hepatitis B virus e antigen (HBeAg) interacts with RIPK2. *A*, Real-time reverse transcription–polymerase chain reaction analysis of NOD1 and NOD2 messenger RNA expression in HepG2. NOD1 and NOD2 expression levels were normalized to GAPDH expression levels. *B*, NF-κB–driven luciferase activity in HepG2 cells stimulated with the NOD1 ligand C12-iEDAP or the NOD2 ligand muramyl dipeptide (MDP) in HepG2. *C*, Western blot analysis of RIPK2 and tubulin expression in HepG2 cells stably transfected with control small hairpin RNA (shRNA; HepG2-shC) or with RIPK2 shRNA (HepG2–shRIPK2-1/2-4) expressing plasmids. *D* and *E*, HepG2-shC (*D*) and HepG2–shRIPK2-3 (*E*) cell lines were transiently transfected with pCXN2, pCXN2-HBeAg(+), or pCXN2-HBeAg(−) plasmids together with pNF-κB–luc. Cells were treated for 4 hours, with or without NOD1 ligand C12-iEDAP (2.5 μg/mL), and luciferase activity was determined. Primers specific for NOD1 (sense primer: 5′-ACTACCTCAAGCTGACCTAC-3′; antisense primer: 5′-CTGGTTTACGCTGAGTCTG-3′), for NOD2 (sense primer: 5′-CCTTGCATGCAGGCAGAAC-3′; antisense primer: 5′-TCTGTTGCCCCAGAATCCC-3′), and for other genes as described previously were purchased from Sigma [4]. *F*, HBeAg specifically colocalizes with RIPK2. COS7 cells were transiently cotransfected with 0.1 μg pCXN2-HBeAg(+) or pCXN2-HBeAg(−) together with 0.3 μg pGFP–human RIPK2. HBeAg was revealed with anti-HBeAg primary antibody and Alexa-Fluor-548 secondary antibody. *G* and *H*, HEK293T cells were transiently transfected with or without GFP-RIPK2 and HBeAg-expressing plasmids. Cellular extracts were precleared with protein G–Sepharose, and interacting complexes were immunoprecipitated (IP) with either anti-GFP (*G*) or anti-HBeAg (*H*) antibodies. Immunocomplexes were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and proteins were visualized by immunoblotting (WB) with indicated antibodies. Results are representative of 3 independent experiments.

and RIPK2 mRNA expression were reduced in HBeAg-positive HepG2 cells, compared with HBeAg-negative cells (1.5-fold decrease; Figure 1*J* and 1*K*). These results suggest that HBeAg impairs hepatic cell migration–dependent RIPK2 expression. Among NF-κB–targeting genes, expression of vimentin mRNA was impaired in HepG2-shRIP2 and in HBeAg-positive HepG2 (data not shown), and vimentin might be one of the candidates for impairment of their migrations [12].

RIPK2 Plays an Important Role in NF-κB Activation Induced by NOD1 Ligand, and HBeAg Blocks This Pathway

HepG2 cells express NOD1 but not NOD2 at the mRNA level (Figure 2*A*). In agreement with this finding, NF-κB was activated in HepG2 cells exposed to NOD1 ligand C12-iEDAP (level of activation, 4.8-fold, compared with untreated control) but not in those exposed to NOD2 ligand MDP (Figure 2*B*). As for Huh7 cells, activation of NF-κB was not detected following exposure to C12-iEDAP or MDP (data not shown). These results suggest that C12-iEDAP triggered NF-κB activation through NOD1 in HepG2 cells, which is consistent with findings from a previous study [9].

We examined whether knockdown of RIPK2 has an effect on NOD1-induced NF-κB activation in HepG2 cells. First, we established HepG2 cell lines that constitutively expressed RIPK2-shRNA (HepG2-shRIPK2-1/2-4) or control-shRNA (HepG2-shC) (Figure 2*C*). The HepG2–shRIPK2-3 cell line, which expresses the lowest levels of RIPK2, and the HepG2-shC cell line were treated for 4 hours, with or without C12-iE-DAP, before measurement by the NF-κB–driven luciferase assay (Figure 2*D* and 2*E*). C12-iEDAP triggered NF-κB activation in HepG2-shC (Figure 2*D*) but not in HepG2–shRIPK2-3 (Figure 2*E*), indicating that RIPK2 plays an important role in NF-κB activation induced through NOD1 triggering.

To assess the influence of HBeAg in that pathway, we measured NOD1-mediated NF-κB activity in HepG2-shC and HepG2–shRIPK2-3 cell lines transiently transfected with HBeAg-expressing plasmids. As shown in Figure 2*D*, HBeAg expression in HepG2-shC abolished C12-iEDAP–induced NF-κB activation.

HBeAg Interacts With RIPK2 and Colocalizes With RIPK2

RIPK2 mediates activation of transcription factors, such as NF-κB, following its activation, which is initiated by membrane-bound or intracytosolic receptors, such as TLR, NOD1, and NOD2 [7, 13, 14]. Confocal microscopy analysis of cells transfected with GFP-RIPK2 revealed subcellular localization of RIPK2 (data not shown). To compare the localization of RIPK2 with that of HBeAg, cells were cotransfected with pGFP–human RIPK2 with pCXN2-HBeAg(+) or pCXN2-HBeAg(−). After 48 hours, cells were stained with mouse monoclonal anti-HBe antibody. Confocal microscopy suggested subcellular colocalization of RIPK2 with HBeAg (Figure 2*F*). Reinforcing this assumption, GFP-RIPK2 coimmunoprecipitated with HBeAg (Figure 2*G*), while HBeAg coimmunoprecipitated with RIPK2 (Figure 2*H*) in transiently transfected cells with RIPK2- and HBeAg-expressing plasmids.

DISCUSSION

In the present study, we have shown the expression of NOD1 and NOD1 ligand–induced NF-κB activation in HepG2 cells and that RIPK2 plays an important role in NOD1 ligand–induced NF-κB activation. NF-κB activation plays an essential role in the production of inflammatory cytokines such as IL-6, which HBeAg could suppress in hepatocytes [4]. We have also shown that HBeAg inhibits RIPK2 expression and interacts with RIPK2, which may represent 2 mechanisms through which HBeAg blocks NOD1 ligand–induced NF-κB activation, thus contributing to the pathogenesis of chronic HBV infection and establishing viral persistence, although further studies including clinical situations might be needed.

HBeAg can be secreted by hepatocytes. Yet, it has been reported that as much as 80% of the precore protein p22 remains localized to the cytoplasm rather than undergoing further cleavage that allows its secretion as mature HBeAg [15]. Our present study showed subcellular colocalization of HBeAg with RIPK2 (Figure 2*F*). In addition to HBeAg protein in cell culture medium, we observed similar inhibition of NF-κB activation (data not shown).

Overall, we provided a novel molecular mechanism whereby HBeAg modulates innate immune signal-transduction pathways through RIPK2. Elsewhere, it was also reported that HBeAg impairs cytotoxic T-lymphocyte activity [2]. HBeAg inhibits RIPK2 expression and interacts with RIPK2, decreasing NF-κB activation and inflammatory cytokine production in hepatocytes. Taken together, HBeAg could impair both innate and adaptive immune responses to promote chronic HBV infection.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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