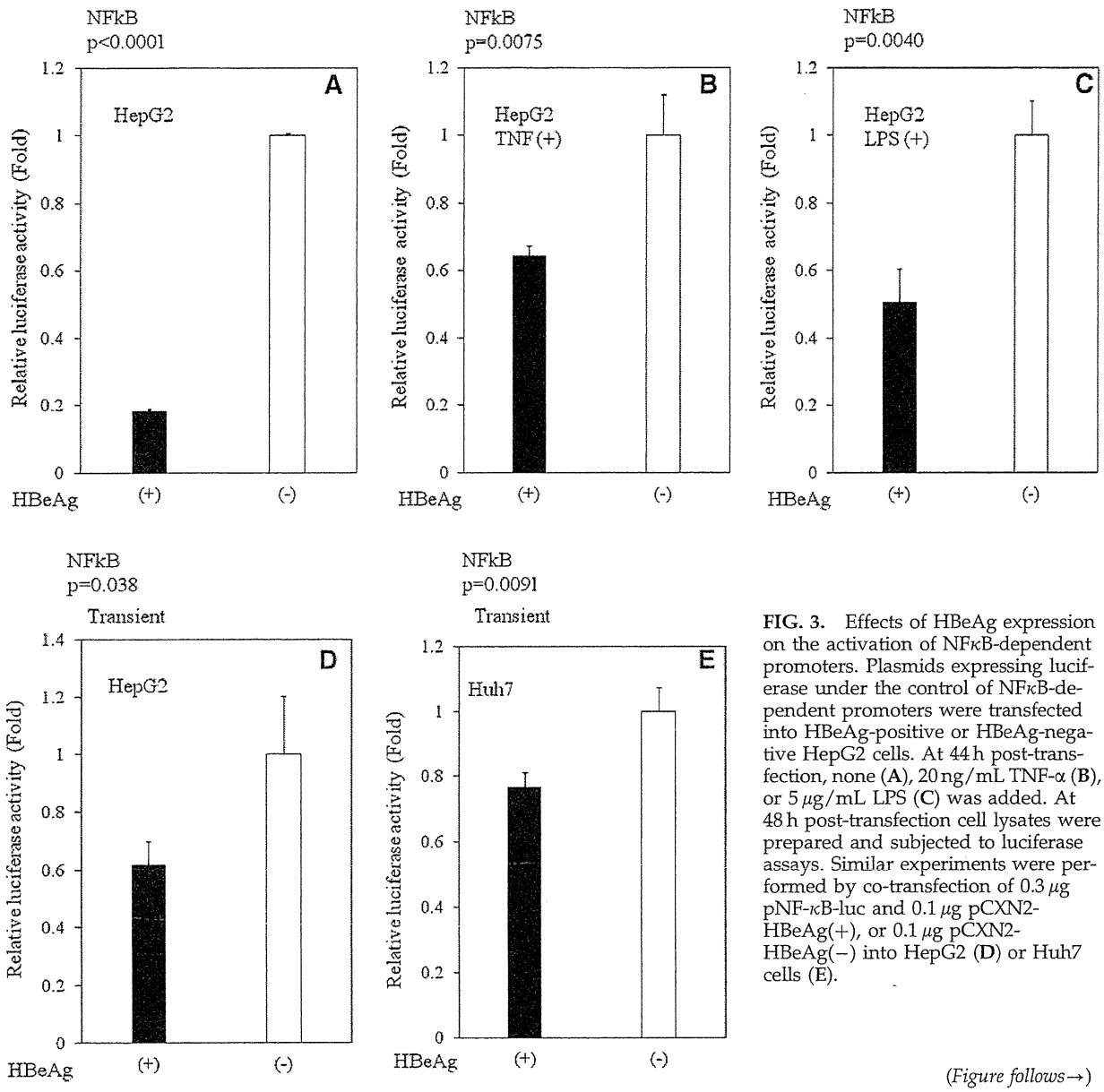

FIG. 3. Effects of HBeAg expression on the activation of NFκB-dependent promoters. Plasmids expressing luciferase under the control of NFκB-dependent promoters were transfected into HBeAg-positive or HBeAg-negative HepG2 cells. At 44 h post-transfection, none (**A**), 20 ng/mL TNF-α (**B**), or 5 μg/mL 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

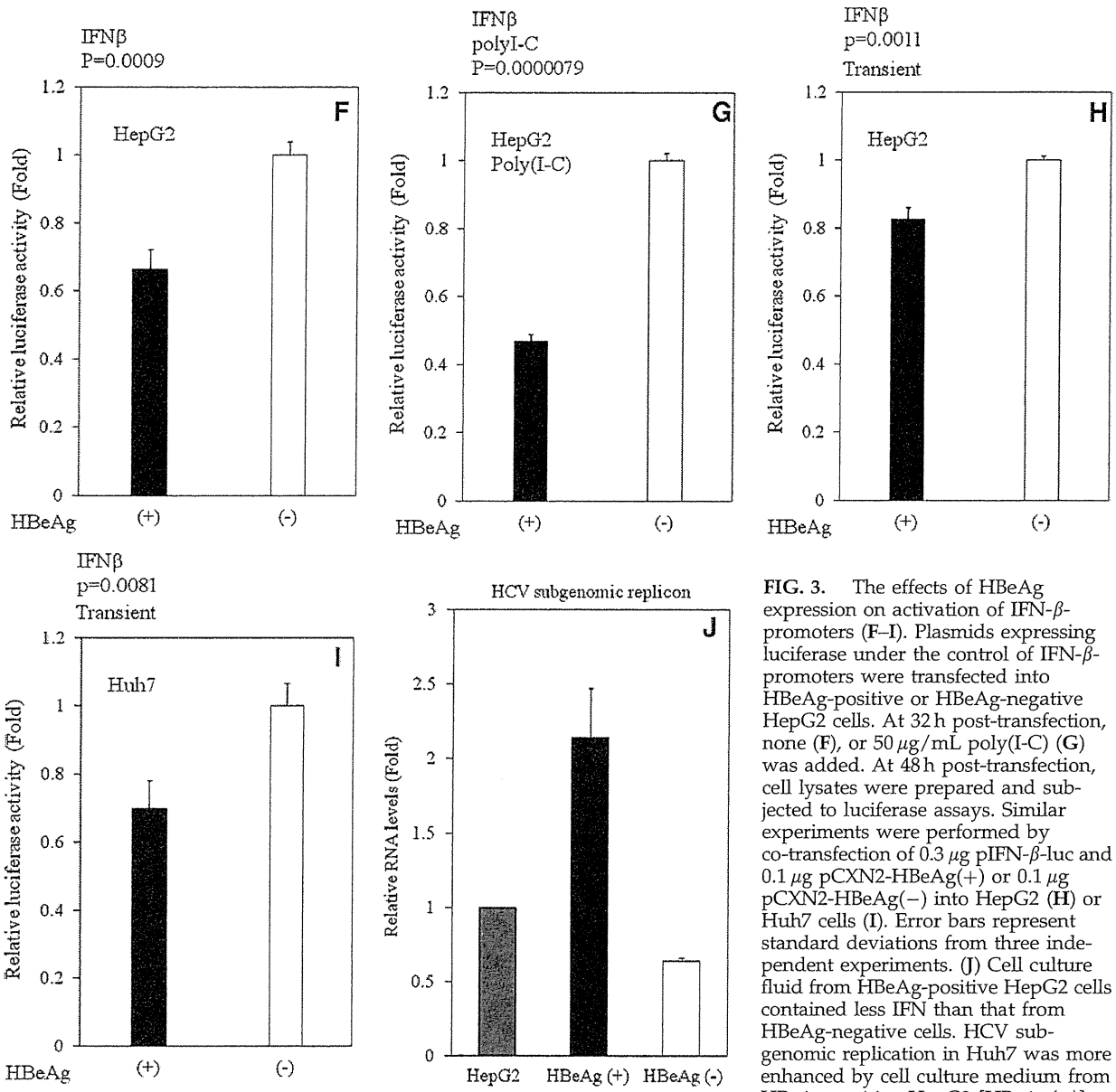

FIG. 3. The effects of HBeAg expression on activation of IFN-β-promoters (**F–I**). Plasmids expressing luciferase under the control of IFN-β-promoters were transfected into HBeAg-positive or HBeAg-negative HepG2 cells. At 32 h post-transfection, none (**F**), or 50 μg/mL poly(I-C) (**G**) was added. 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

TLR-signaling, from HBeAg-positive HepG2. Expression profiling showed more than twofold inhibition of nine genes compared to that of HBeAg-negative HepG2 cells (TLR7, LY96, RIPK2, NFκB1, TNF, IL-6, IL-8, IFN-β, and MAP3K1) (Fig. 2A). To confirm these results, real-time PCR was performed. All of these genes except NF-κB1 were significantly downregulated in HBeAg-positive cells compared to HBeAg-negative cells. All of these genes have important roles in the immune response and activation of transcription (Fig. 2B).

Effects of HBeAg on NF-κB activation

Next, we assessed the mechanisms by which HBeAg affects cytokine and IFN production. HBV activates NF-κB, a major player in innate immune responses to viral infections (19). Therefore, we postulated that HBeAg inhibition of the activation of NF-κB might result in the inhibition of cytokine and IFN production, and the subsequent escape of an antiviral response. To test this assumption, we expressed luciferase reporter protein under the control of an NF-κB-dependent promoter in HBeAg-positive or HBeAg-negative HepG2 with or without TNF-α or LPS stimulation (Fig. 3A–E). As expected, HBeAg inhibited NF-κB promoter activity in HBeAg-positive HepG2 cells (Fig. 3A–C; $p < 0.001$ with no drug [n = 3]; $p = 0.004$ with LPS [n = 3]; $p = 0.0075$ with TNF [n = 3]). These results were also confirmed by the transient HBeAg-expression assay in HepG2 ($p = 0.038$, n = 3) and Huh7 cells ($p = 0.0091$, n = 3) (Fig. 3D and E). These findings suggest that HBeAg may affect cytokine production, at least in part, through NF-κB.

Effects of HBeAg on IFN-β activation

NF-κB stimulation leads to the expression of multiple cellular factors, including IFN-β, a central player in the innate immune response that is activated upon virus infection. In order to ascertain whether HBeAg inhibits IFN-β-promoters, we performed experiments using IFN-β-promoter luciferase reporter, essentially as described in the previous section. That is, we used the luciferase gene under the control of an IFN-β-stimulated promoter, and examined its expression in HBeAg-positive and HBeAg-negative HepG2 cells. HBeAg inhibited IFN-β-stimulated promoter activity in HBeAg-positive HepG2 cells with ($p < 0.001$, n = 3), or without poly(I-C) ($p < 0.001$, n = 3) (Fig. 3F and G). We also confirmed these results by transient transfection experiments with HepG2 ($p = 0.0011$, n = 3), and with Huh7 ($p = 0.0081$, n = 3) (Fig. 3H and I). These results demonstrated that HBeAg inhibits both NF-κB- and IFN-β-signaling pathways in hepatocytes.

Cell culture fluid from HBeAg-positive HepG2 enhanced HCV subgenomic RNA replication

To confirm the function of IFN production of these cell lines, we examined whether conditioned media from HBeAg-positive or HBeAg-negative HepG2 cells would cause any differences in HCV subgenomic RNA replication, which is IFN-sensitive replication (14), as it has been reported that there are no direct interactions between HBV and HCV replication in cell culture models and in a mouse study (1,9,11). Cell culture fluid from HBeAg-positive HepG2 cells enhanced HCV subgenomic RNA replication, more than that from HBeAg-negative HepG2 cells (Fig. 3J; $p = 0.0014$, n = 3), suggesting that HBeAg-expressing HepG2 cells contain less IFN than do HBeAg-negative cells, and that conditioned medium from HBeAg-positive HepG2 cells contains less IFN than that from HBeAg-negative cells. In this system, when we treated cells with 0, 1, 10, 100, and 1000 U/mL IFN-α, HCV subgenomic RNA levels were 100%, 57%, 39%, 28%, and 25%, respectively. We estimated that conditioned medium from HBeAg-negative HepG2 cells was equal to $\sim$10 IU/mL IFN-α. Our results showed that HBeAg inhibits IFN production in cell culture medium.

Since the NF-κB target gene IL-6 has also been implicated in hepatitis B pathogenesis (30), the modulation of IL-6 involved in innate signaling by HBeAg was also verified at the protein level by ELISA. Our results demonstrated that IL-6 expression was downregulated in HBeAg-positive HepG2 cells (36.6 ± 30.1 pg/mL; 0 ± 0 pg/mL in conditioned medium from HepG2 control cells; 324.2 ± 15 pg/mL in conditioned medium from HBeAg-negative HepG2 cells). The concentration of IL-6 from HBeAg-positive HepG2 cells was significantly lower than that from HBeAg-negative HepG2 cells ($p = 0.00012$, n = 3).

Discussion

In this study, we investigated the regulation of HBeAg-induced suppression of IFN and cytokines in HepG2 stably expressing HBeAg protein as a model cell line. Our results demonstrated that HBeAg expression inhibits IFN and cytokine production. Transient expression of HBeAg also downregulated both NF-κB- and IFN-β-promoter activity in HepG2 or Huh7, although the mechanisms for this downregulation are unknown. In contrast to our findings, Yang *et al.* (46) observed that HBeAg activates NF-κB through IκBα degradation, and produces TNF-α and GM-CSF in the human hepatoma cell HA22T/VGH. These differences between their findings and ours may have been caused by the differences in the cell lines, and/or promoters (33). Extensive immunological studies by the Milich group (3,4,27) demonstrated that HBeAg appears more efficient at eliciting T-cell tolerance, including production of its specific cytokines IL-2 and IFN-γ, than HBV core antigen. Our observations support the immune-modulating role of HBeAg.

Locarnini *et al.* (23) used the Tet-off tetracycline gene expression system in Huh7, and revealed that core/precore expression affected gene expression, including cytokines. The system used in our present study, with HepG2 stably expressing HBeAg, supports these findings. Our results provide further direct evidence that hepatocytes exposed to HBeAg have enhanced HCV subgenomic RNA replication, and are significantly influenced in their ability to replicate. Several recent reports have also suggested that there was no evidence of direct interaction between HBV and HCV (1,9,11), although clinical studies showed interaction between HBV and HCV replication (24). It is possible that HBV might interfere with another virus by IFN or another cytokine. A cytokine response is critical for clearance of HCV, as failure to mount a potent and broad T-cell-repertoire response results in persistent HCV replication. This would explain how patients dual-infected with HBV and HCV exhibit a selective deficit of anti-HCV immunity, while demonstrating preservation of a normal immune response to unrelated antigens.

We used RT-PCR to observe the expression of TLRs 1, 3, 4, 5, 6, and 7 in HepG2 cells. We also confirmed in the present study that HepG2 has functional TLRs 3 and 4. Preiss *et al.*

(32) could not detect an NF-κB response to 1 ng/mL–1 μg/mL LPS in HepG2, whereas we could detect such a response to 10–50 μg/mL LPS (Table 2). Downregulation of TLR2 mRNA by genotype C HBV-derived HBeAg was not observed in our study, in contrast to the results of a previous study (43), in which genotype D HBV-derived clone (23) was used. Xu *et al.* (45) reported that TLR7 was suppressed in HBV infection, supporting our results. We do not know why LY96, an important molecule for TLR4, is downregulated (Fig. 2). Viruses encode proteins that target various intracellular signaling pathways, causing their constitutive or prolonged activation, resulting in increased cell proliferation and survival (41). It is well known that HBV activates the MAPK pathway (5). It is also known that RIPK2 activates the NF-κB- and IFN-β-dependent antiviral responses (8). These findings were in accordance with HBeAg's inhibition of the production of IFNs and cytokines (Fig. 2).

What is the mechanism of the downregulation of cytokine production by HBeAg? From our results (Fig. 2), HBeAg appears to interact with the TLR signaling pathway upstream of NF-κB. In LPS stimulation, we observed downregulated TLR4 in HBeAg-positive HepG2 cells (data not shown). Although we are currently investigating this issue, TLR4 might be one of the more important molecules. Precore protein also may affect intracellular signal transduction pathways. Further studies will be needed to clear up these issues.

Many viruses have evolved strategies that block the effector mechanisms induced through IFN- and/or cytokine-signaling pathways (17). Although multiple mechanisms contribute to viral persistence, the ability of the virus to evade innate immune responses is likely to be particularly important. In this report, we have demonstrated that HBeAg suppresses IFN and cytokine mRNA expression. Exploration of the novel HBeAg-inhibiting signaling pathways could lead to the development of new therapeutic strategies for persistent HBV infection.

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Author Disclosure Statement

The authors have no conflicts with regard to financial interests. This material has not been previously reported and is not under consideration for publication elsewhere.

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ORIGINAL ARTICLE

Risk of Hepatocellular Carcinoma in Patients with Chronic Hepatitis B Virus Infection

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Abstract

Objective. To determine the risk factors for the occurrence of hepatocellular carcinoma (HCC) in patients with hepatitis B virus (HBV) infection. ***Material and methods.*** A total of 620 patients who tested positive for hepatitis B surface antigen and were referred to Chiba University Hospital between February 1985 and March 2008 were included in the study and the following characteristics were analyzed: age, gender, status of hepatitis B e antigen, alanine aminotransferase level, HBV DNA level, and number of platelets (PLTs). ***Results.*** HCC was detected in 30 cases during the follow-up period (5.4 ± 5.1 years). Multivariate analysis revealed that age >40 years [compared with patients aged <40 years; odds ratio (OR) = 4.28; 95% confidence interval (CI) = 1.68–10.9] and PLT level <206,000/μl (compared with patients with a higher PLT level; OR = 8.50; 95% CI = 1.98–36.2) were predictive factors for HCC occurrence. In patients aged >40 years, the HBV DNA level (compared with <5.0 log copies/ml; OR = 4.22, 95% CI = 1.13–15.8) and PLT level (compared with patients with >196,000/μl PLTs; OR = 15.6, 95% CI = 2.06–118.3) were predictive factors for HCC occurrence. ***Conclusions.*** Advanced age and low PLT level were risk factors for HCC occurrence in patients with HBV infection. In patients aged >40 years, viral load was also a risk factor for HCC.

Key Words: *Hepatitis B virus, hepatocellular carcinoma*

Introduction

The clinical course of patients with hepatitis B virus (HBV) infection varies considerably [1]. Therefore, long-term follow-up studies of patients with HBV infection are quite complex and difficult. In most of the patients, the disease is either non-progressive or shows a slow progression and is usually accompanied by the loss of serum HBV DNA after seroconversion of hepatitis B e antigen (HBeAg) [2]. Some patients show continuous elevation of the alanine aminotransferase (ALT) level, which leads to cirrhosis [3]. HBV infection is also associated with an increased risk of developing hepatocellular carcinoma (HCC), which is one of the most common human cancers and causes of death. Although previous studies have attempted to determine factors influencing the prognosis of patients with HBV infection, the key factors remain to be identified. Recent studies have indicated that the serum level of HBV DNA correlates with the progression of liver diseases [1,4–6]. However, viral load alone cannot predict the occurrence of HCC in the future [7]. In this study, multivariate analyses of the risk factors for HCC occurrence were performed for data obtained from 620 patients with HBV infection who were referred to a single institute in Japan.

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Material and methods

Patients

This was a retrospective analysis. The study was approved by the ethical committee of Chiba University and written informed consent was obtained from all the patients. Of the hepatitis B surface antigen (HBsAg)-positive carriers ($n = 676$) who were referred to Chiba University Hospital between February 1985 and March 2008, those who tested positive for hepatitis C virus (HCV) antibody (anti-HCV) or had autoimmune liver disease and those who had another potential cause of chronic liver disease were excluded. The characteristics of the excluded HBsAg-positive carriers were as follows: anti-HCV positivity in 12, autoimmune liver disease in four and primary biliary cirrhosis in one. Five patients who had previously received lamivudine treatment were also excluded. Thirty-nine patients consulted a physician only once and were excluded from further analysis. Thus, a total of 620 patients were further analyzed. Serum samples were collected during diagnosis and stored at –20°C until analysis.

Serologic markers, HBV DNA quantitative assay, and genotyping

HBsAg, HBeAg, and anti-HBe levels were determined by enzyme-linked immunosorbent assay (ELISA; Abbott Laboratories, Chicago, IL) and anti-HCV was also measured by ELISA (Ortho Diagnostics, Tokyo, Japan). Serum HBV DNA levels were quantified by polymerase chain reaction (PCR) assay (Amplicor HBV Monitor; Roche Diagnostics, Basle, Switzerland); the linear range of this assay was 2.6–7.6 log copies (LC)/ml. The six major genotypes of HBV (A–F) were determined by EIA (HBV Genotype EIA; Institute of Immunology Co., Ltd., Tokyo, Japan). Aspartate aminotransferase (AST), ALT, and the number of platelets were determined and the aminotransferase to platelet ratio index (APRI) was calculated [8].

Statistical analysis

The baseline data are presented as mean ± SD. The difference in the values of clinical parameters between the two groups was analyzed by unpaired *t*-test, Welch's *t*-test, and chi-square test. The Cox proportional hazards model was used to identify factors predictive of HCC occurrence using the SPSS version 16.1 software package (SPSS Inc., Chicago, IL).

Results

Demographic characteristics of HCC and control patients

None of the study participants had HCC at entry. In total, 30 incident HCC cases (HCC group) occurred during the follow-up period. During the follow-up period, most of the patients were re-evaluated at least once a year for liver function and detection of HCC. Screening for detection of HCC was performed on the basis of typical findings of abdominal ultrasonography, dynamic CT, angiography, and/or MRI. For all patients suspected of having HCC by imaging analysis, the diagnosis of HCC was confirmed by pathological analysis. If the patient had HCC or was being treated with an antiviral drug (lamivudine or entecavir), we terminated the follow-up. At baseline, significant differences were observed in age, gender, status of HBeAg, ALT and HBV DNA levels, number of platelets (PLTs), and APRI between the HCC ($n = 30$) and control ($n = 590$) groups (Table I). The 590 patients in whom HCC was not detected during the follow-up period constituted the control group. The average follow-up period was 5.1 ± 4.1 and 5.4 ± 5.2 years in the HCC and control groups, respectively, and this difference was not significant.

Patients with HBV

The differences in age, sex, PLT and ALT levels, status of HBeAg, and HBV DNA level between the HCC and control groups were investigated. We defined threshold levels as age 40 years, HBV DNA 5.3 LC/ml, ALT 72.9 IU/l, and PLTs 206,000/μl according to the average data of all patients. Univariate analysis revealed that age, number of PLTs, and HBV DNA level at baseline were predictive factors for HCC occurrence. Multivariate analysis revealed that age >40 years [compared with patients aged <40 years; odds ratio (OR) = 4.28; 95% confidence interval (CI) = 1.68–10.9] and PLT level <206,000/μl (compared with patients with a higher PLT level; OR = 8.50, 95% CI = 1.98–36.2) were predictive factors for HCC occurrence (Table II). Thus, these analyses revealed that age and PLT level were the most important factors influencing future occurrence of HCC. Kaplan–Meier curves were constructed for age ($P < 0.0001$; log-rank test; Figure 1a), PLT level ($P < 0.0001$; log-rank test; Figure 1b), and HBV DNA (P = NS; log-rank test; Figure 1c). Next, we categorized the HBV patients into two subgroups according to the thresholds of age and PLT level based on the average data, and performed further analysis. Because there was only one HCC patient aged <40 years and

Table I. Characteristics of study subjects and their association with HCC.

Parameter	Group			*P*
	Total	HCC	Controls	
No. of patients	620	30	590	
Gender; *n* (%)				<0.001[a]
Male	364 (59)	20 (67)	344 (58)	
Female	256 (41)	10 (33)	246 (42)	
Age (years); mean ± SD	40.0 ± 14.2	50.0 ± 11.6	40.0 ± 14.2	<0.001[b]
HBeAg status; *n* (%)				<0.001[a]
Positive	269 (43)	17 (57)	252 (43)	
Negative	351 (57)	13 (43)	338 (57)	
HBV DNA (LC/mL); mean ± SD	5.3 ± 2.0	6.4 ± 1.3	5.3 ± 2.0	0.002[b]
ALT (IU/l); mean ± SD	72.9 ± 89.3	105.0 ± 129.3	71.0 ± 86.6	0.041[c]
PLTs (/μl); mean ± SD	206,000 ± 66,000	130,000 ± 51,160	210,000 ± 64,410	<0.001[c]
APRI >0.5; *n* (%)	294 (47.4)	27 (90)	267 (45.3)	<0.001[a]
Interval between two consecutive visits (years); mean ± SD	5.4 ± 5.1	5.1 ± 4.1	5.4 ± 5.2	NS[c]
Genotype A/B/C/D/not determined; *n*	7/38/333/0/242	1/0/24/0/5	6/38/309/0/237	NS[a]

[a]Chi-square test.
[b]Welch's *t*-test.
[c]Unpaired *t*-test.

only two cases had a PLT level >206,000/μl, we did not analyze these groups.

Analysis of the subgroup of HBV patients aged >40 years

HCC was detected in 29 patients in the group aged >40 years (n = 372). Significant differences were observed in the status of HBeAg, HBV DNA, and PLT levels at baseline between the HCC (n = 29) and control groups (n = 343). The average follow-up period was 5.1 ± 4.1 and 5.0 ± 4.7 years in the HCC and control groups, respectively, and this difference was not significant. We defined thresholds as age 49 years, HBV DNA 5.0 LC/ml, ALT 66.0 IU/l, and PLTs 196,000/μl, according to the average data for the patients aged >40 years. The risk factors for HCC occurrence in patients aged >40 years were analyzed by Cox regression analysis. Univariate analysis revealed that ALT, PLT, and HBV DNA levels at baseline were predictive factors for HCC occurrence. Multivariate analysis revealed that the HBV DNA

Table II. Multivariate analysis of risk factors associated with HCC in patients with HBV infection.

Risk factor	All patients[a]		Patients aged >40 years[b]		Patients with PLTs <206,000 /μl[c]	
	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
Age	4.28 (1.68–10.9)	0.002	2.16 (0.88–5.29)	NS	1.75 (0.71–4.34)	NS
Male gender	1.48 (0.67–3.26)	NS	2.25 (0.86–5.90)	NS	1.43 (0.61–3.35)	NS
HBeAg-positive	1.34 (0.59–3.06)	NS	0.98 (0.41–2.33)	NS	1.06 (0.45–2.51)	NS
HBV-DNA	1.59 (0.62–4.13)	NS	4.22 (1.13–15.8)	0.032	1.20 (0.49–2.94)	NS
ALT	0.86 (0.40–1.87)	NS	1.44 (0.61–3.44)	NS	0.923 (0.40–2.11)	NS
PLTs	8.50 (1.98–36.2)	0.004	15.6 (2.06–118.3)	0.008	4.49 (1.62–12.5)	0.004

[a]The thresholds of age, HBV-DNA, ALT, and PLTs were defined as 40 years, 5.3 LC/ml, 72.9 IU/l, and 206,000 /μl, respectively.
[b]The thresholds of age, HBV-DNA, ALT, and PLTs were defined as 49 years, 5.0 LC /ml, 66.0 IU/l, and 196,000 /μl, respectively.
[c]The thresholds of age, HBV-DNA, ALT, and PLTs were defined as 42 years, 5.8 LC /ml, 84 IU/l, and 159,000 /μl, respectively.
HR = hazard ratio.

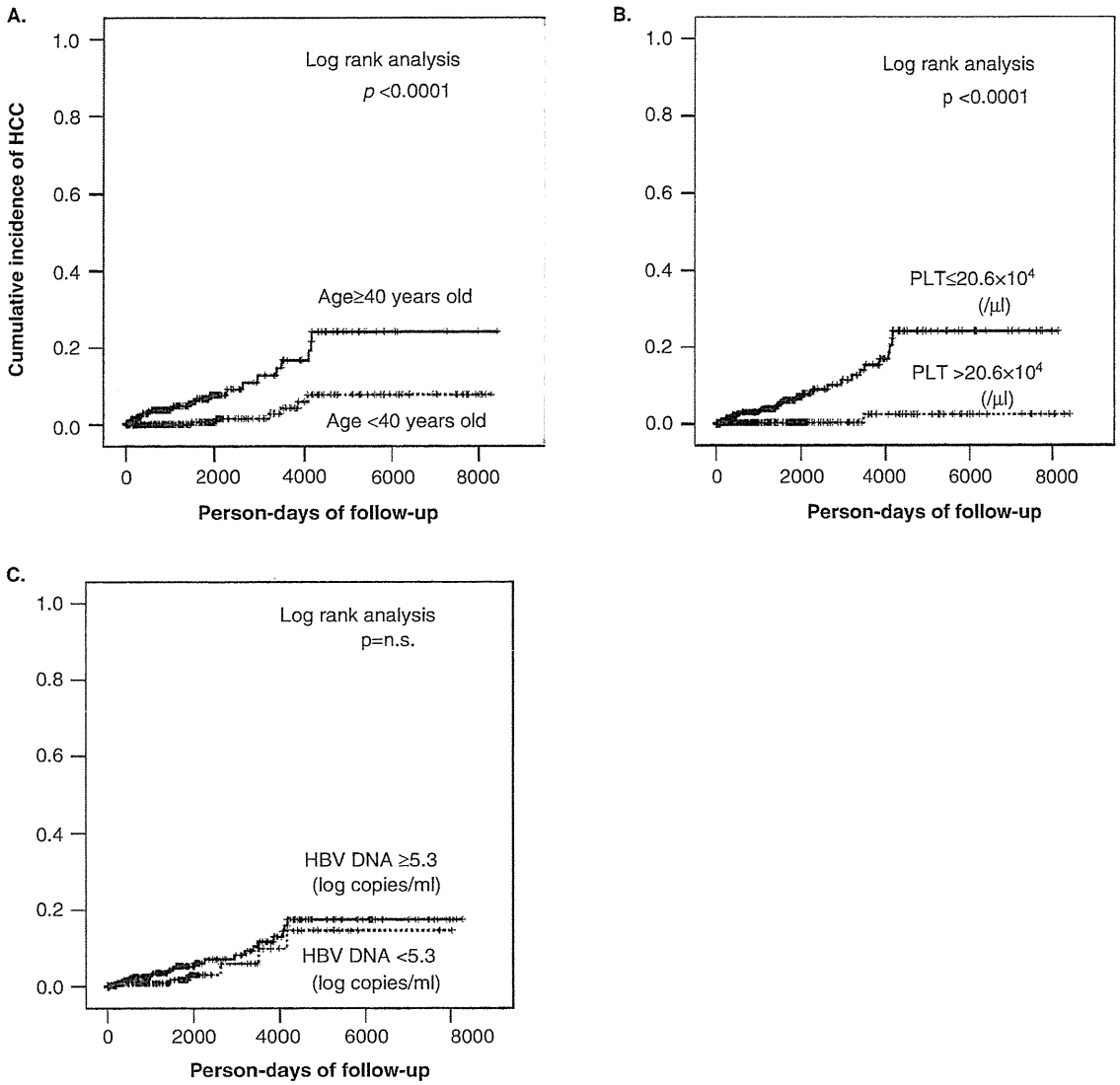

Figure 1. Cumulative occurrence of HCC based on (a) number of PLTs, (b) age, and (c) HBV DNA level. Thresholds for age, number of PLTs, and HBV DNA level were defined according to the average data for all patients. Dotted lines indicate the control group (high number of PLTs, younger age, and low HBV DNA level).

level (compared with < 5.0 LC/ml; OR = 4.22; 95% CI = 1.13–15.8) and PLT level (compared with > 196,000/μl; OR = 15.6; 95% CI = 2.06–118.3) were predictive factors for HCC occurrence (Table II). Kaplan–Meier curves were constructed for HBV DNA (P = 0.001; log-rank test; Figure 2).

Analysis of the subgroup of HBV patients with PLTs < 206,000/μl

HCC was detected in 28 patients in the group with PLTs < 206,000/μl (n = 329). The risk factors for HCC occurrence in the group with < 206,000/μl PLTs were analyzed by Cox regression analysis. Univariate analysis revealed that age and PLT level at baseline were predictive factors for HCC occurrence. Multivariate analysis revealed that PLT level (compared with patients with > 159,000/μl; OR = 4.49; 95% CI = 1.62–12.5) was the only predictive factor for HCC occurrence (Table II).

Discussion

In Japan, HBV infection is one of the most important factors determining HCC occurrence [9]. Moreover, HCC is one of the most important determinants for

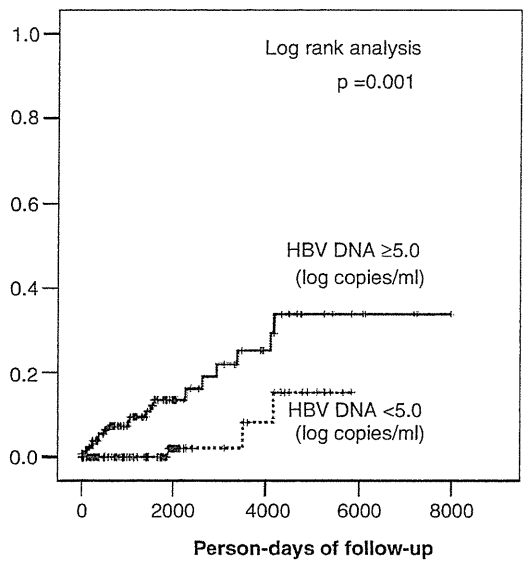

Figure 2. Cumulative occurrence of HCC based on the HBV DNA level in patients aged > 40 years. The threshold for the HBV DNA level was defined according to the average data for the patients aged > 40 years. A significant difference was observed by log-rank test. The dotted line indicates the control group (low HBV DNA level).

the prognosis of patients with HBV infection. In previous studies, factors associated with an increased risk of HCC among people with chronic HBV infection included demographic characteristics, lifestyle, and environmental, viral and clinical factors. Among these, male gender, older age, HBV genotype, cirrhosis, elevated ALT, and high viral load were found to be factors associated with HCC [6,10–19]. We focused on clinical factors which may be tested easily and for which tests are available all over the world. This report clarifies the relative risk for HCC in all patients with HBV who were referred to a single institute in Japan and provides important information for physicians.

In this study, the relative risk of HCC was found to be increased to 4.28 (95% CI 1.68–10.9) times higher for patients aged > 40 years compared with those aged < 40 years. In addition, a low PLT level, which indicates advanced fibrosis in the liver, including cirrhosis, was a risk factor for HCC: the relative risk was found to be increased to 8.50 (95% CI 1.98–36.2) times higher for patients with a PLT level < 206,000/μl compared with higher levels. The HBV DNA level was not selected as a risk factor for HCC occurrence in all patients with HBV infection by multivariate analysis. Previous follow-up studies have shown that viral load is an important and independent factor for HCC occurrence [4,5,20]. However, in the present study, although various thresholds of HBV DNA level were used for analysis, none of the thresholds showed statistical significance in multivariate analysis (data not shown). In contrast, the analysis intended for patients aged > 40 years revealed that high HBV viral load was added as a risk factor for HCC. By changing the threshold of HBV DNA from 4.5 to 5.3 LC/ml in 0.1-log increments, 5.0 or 5.1 LC/ml were found to be the best (data not shown); therefore we designated the threshold of HBV DNA level as > 5.0 LC/ml. In our study, HBV carriers aged > 40 years with HBV DNA levels > 5.0 LC/ml had a 4.22-times higher risk of HCC compared to HBV carriers with lower viral loads. In previous studies in Japan regarding predictive factors for HCC, Ohata et al. [5] reported that age, HBV DNA, and staging of fibrosis were the important factors, while Murata et al. [21] reported that the number of PLTs was the only factor after HBeAg seroconversion. On the other hand, in an analysis of patients with liver cirrhosis in Japan, levels of HBV DNA and/or ALT were the predictive factors for HCC [12,19]. Taken together with the present study, these reports suggest that the HBV DNA level may not be an absolute factor for predicting HCC in the analysis, irrespective of the age of the patients and the number of PLTs, but that in patients with advanced age or low numbers of PLTs, indicating advanced fibrosis of the liver, HBV DNA could be a predictive factor for the occurrence of HCC. The PLT level negatively reflects the extent of liver fibrosis [22], therefore it is very difficult to achieve an improvement in liver fibrosis and to recover the PLT level concomitantly, but a high viral load can be lowered by antiviral drug treatment. Therefore, in patients aged > 40 years, lowering the viral load using an antiviral drug might be an important way to avoid the occurrence of HCC but, in younger patients, lowering the HBV DNA level may not result in direct inhibition of HCC occurrence, although the activity of hepatitis could be suppressed.

The decrease in the number of PLTs in patients with liver disease reflects advanced fibrosis of the liver, which is strongly related to HCC occurrence. In fact, the patients in the HCC group of our study were suggested to show advanced fibrosis because they had higher values of APRI than the controls. In addition to being a marker of liver fibrosis, the influence of PLTs on cytotoxic T lymphocytes (CTLs) has been studied with keen interest. Chronic HBV infection is characterized by an inefficient CTL response, which often results in continuous destruction of hepatocytes. A recent study indicated that PLTs are required for virus-specific CTLs to accumulate within the liver and perform pathogenetic and/or antiviral roles [23]. In our study, low PLT number was a strong risk factor for HCC in all the HBV carriers, irrespective of age or PLT number at baseline. Especially in the HBV

carriers aged >40 years, low PLT number has the strongest association with HCC occurrence. Therefore, older HBV carriers with low PLT levels should be followed closely because of a high possibility of HCC occurrence, as for HCV carriers with low PLT levels [24].

The presence of HBeAg is often associated with active liver disease, whereas HBeAg seroconversion often coincides with loss of HBV DNA in serum, normalization of the ALT level, and clinical remission [25]. Spontaneous HBeAg seroconversion confers a good long-term outcome on most patients. In this study, the status of HBeAg at baseline differed significantly between the HCC and control groups; however, the status of HBeAg was not identified by univariate analysis as a predictive factor for HCC occurrence. From these results, we speculated that the HBe protein was not the direct precursor of HCC, although the HBe antigen status often reflects the replication of HBV DNA.

In this study, we evaluated parameters for predicting HCC only at first admission. A previous study reported that changes in ALT or HBV DNA levels during the follow-up period were important for predicting advanced liver disease and HCC [26]. We need to evaluate the importance of following changes in these parameters.

There was only one HCC patient aged <40 years. This patient was male and was followed up from the age of 27 years; his ALT, HBV DNA, and PLT levels and the status of HBeAg at baseline were 34 IU/l, 7.7 LC/ml, 203,000/μl, and positive, respectively. It was difficult to predict the occurrence of HCC in this case only on the basis of the risk factors for HCC indicated in this study. Hence, we need to find an adequate risk factor to predict HCC in such a case.

In conclusion, advanced age and low PLT level were the risk factors for HCC in patients with HBV infection, irrespective of the PLT level at baseline. In patients aged >40 years, viral load was added as a risk factor for HCC.

Declaration of interests: The authors indicated no potential conflict of interest.

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HEPATOLOGY

Efficacy and safety of entecavir in nucleoside-naive, chronic hepatitis B patients: Phase II clinical study in Japan

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Abstract

Background and Aim: Entecavir has demonstrated clinical efficacy for chronic hepatitis B. This study evaluated the efficacy and safety of entecavir in nucleoside-naive Japanese chronic hepatitis B patients.

Methods: In this multicenter, double-blind study, 66 nucleoside-naive Japanese chronic hepatitis B patients were randomized to 0.1 mg entecavir ($n = 32$) or 0.5 mg entecavir ($n = 34$) daily for 52 weeks. The primary endpoint was the proportion of patients whose serum hepatitis B virus (HBV) DNA decreased from baseline by ≥ 2 $\log_{10}$ copies/mL or became undetectable (<400 copies/mL by polymerase chain reaction assay) at week 48.

Results: One hundred percent of patients in both treatment groups achieved the primary efficacy endpoint, with 81% and 68% of patients achieving undetectable HBV DNA in the 0.1 mg and 0.5 mg treatment groups, respectively. Mean changes from baseline in HBV DNA were -4.49 $\log_{10}$ and -4.84 $\log_{10}$ copies/mL for the 0.1 mg and 0.5 mg groups, respectively. Significant improvements in necroinflammation were seen in both groups, as assessed by Knodell and New Inuyama classifications. Most adverse events were transient and classified as grade 1 or 2. There were no clinically significant differences in adverse events across the two treatment groups and no discontinuations due to adverse events in either group.

Conclusions: In Japanese nucleoside-naive patients with chronic hepatitis B, 0.1 mg or 0.5 mg entecavir daily provided excellent efficacy and was well tolerated. The 0.5 mg dose was selected for the treatment of nucleoside-naive patients.

Introduction

It has been reported that 350–400 million people worldwide are chronically infected with hepatitis B virus (HBV)[1,2] despite the widespread use of HBV vaccination for prevention of this disease. HBV infection is particularly prevalent in Asia–Pacific countries, with an estimated 75% of all chronically infected patients living in the region.[3] Prevalence rates reported in 2000 indicated that 0.8% of the Japanese population were hepatitis B virus surface antigen (HBsAg) positive with 36% of infected individuals being chronically infected.[4] Among those chronically infected, 20–40% will develop cirrhosis, decompensated liver disease or hepatocellular carcinoma.[5] In Asia, HBV is the leading cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma.[4]

Treatment for chronic hepatitis B has evolved markedly over the last decade. Interferon-α was the only available treatment for many years, but this cytokine is efficacious in only approximately 35% of patients,[6] and is poorly tolerated by many patients due to adverse effects. Lamivudine, a cytosine analog, was the first oral anti-HBV nucleoside analog developed, and has demonstrated efficacy for treatment of chronic hepatitis B during short-term administration.[7,8] However, viral breakthrough due to emergence of lamivudine-resistant strains of HBV with amino acid substitutions in the YMDD (tyrosine–methionine–aspartate–aspartate) motif of reverse transcriptase result in loss of clinical benefit.[9–11] Long-term follow up of hepatitis B e antigen (HBeAg)-negative patients treated with adefovir, the second approved oral anti-HBV, have shown cumulative probabilities of genotypic

resistance of 29% at 5 years[12] and some studies have reported that 20–50% of patients receiving a 10 mg dose of adefovir have primary non-response[13] indicating that the approved dose of adefovir may be suboptimal.[14]

Entecavir is a deoxyguanosine analog that has more than 300 times greater potency than lamivudine *in vitro*.[15,16] Entecavir inhibits all three steps of HBV DNA replication: (i) priming of the HBV DNA polymerase; (ii) reverse transcription of negative-strand HBV DNA from pre-genomic messenger RNA; and (iii) synthesis of positive-strand HBV DNA.[17] In woodchuck models of HBV infection, entecavir reduced viral loads by up to 9 $\log_{10}$ copies/mL and prevented the onset of hepatocellular carcinoma.[18] In early clinical studies, entecavir was demonstrated to be safe and efficacious when given for 28 days.[19] In a 24-week, phase II international clinical trial, Lai *et al.* demonstrated a dose–response relationship for entecavir, and showed that entecavir was superior to lamivudine at doses of 0.1 mg and 0.5 mg for HBV DNA reduction.[20] Subsequently, two phase III international trials showed that 0.5 mg entecavir daily for 48 weeks achieved superior histological, virological and biochemical improvement in HBeAg-positive and HBeAg-negative nucleoside-naive patients compared with lamivudine, with comparable safety and no emergence of resistance without prior existence of the amino acid substitutions rtL180M and rtM204V/I/S which are associated with lamivudine resistance.[21,22] Entecavir was approved by the US regulatory authorities in March 2005. Study AI463047 evaluated 0.01 mg, 0.1 mg and 0.5 mg entecavir and 100 mg lamivudine in nucleoside-naive Japanese patients and established the 0.5 mg dose of entecavir as the optimal dose in this patient population. The current phase II dose-ranging trial, which commenced before the completion of AI463047, evaluated the efficacy and safety of 0.1 mg and 0.5 mg entecavir daily for 52 weeks in nucleoside-naive chronic hepatitis B patients in Japan. This study's primary objective was to demonstrate that entecavir has antiviral activity as indicated by the proportion of subjects who achieve a reduction from baseline in HBV DNA by ≥ 2 $\log_{10}$ copies/mL or to <400 copies/ml at week 48.

Methods

Study design

This was a randomized, double-blind, multicenter trial of 0.1 mg entecavir once daily and 0.5 mg entecavir once daily for 52 weeks in nucleoside-naive patients with HBeAg-positive or -negative chronic hepatitis B. Patients were randomized via a central registration procedure. A total of 66 patients were enrolled in this study, including men and women ranging in age from 27–68 years who were determined to be eligible for the study during the 6-week screening period. Following randomization, patients received either a 0.1 mg entecavir tablet plus a 0.5 mg placebo tablet ($n = 32$) or a 0.5 mg entecavir tablet plus a 0.1 mg placebo tablet ($n = 34$) orally once daily for 52 weeks. After 52 weeks of blinded dosing, patients were given the option of enrolling in an entecavir rollover study. All patients who discontinued blinded dosing early, or who completed the protocol but did not enroll in the entecavir rollover study, were followed for 24 weeks post-dosing, and could receive marketed anti-HBV therapy as recommended by their physician.

The study was conducted in compliance with the ethical principles of the Declaration of Helsinki, Good Clinical Practice guidelines, and Articles/Notifications of the Ministry of Health, Labor and Welfare in Japan. Written informed consent was obtained from all patients.

The study's primary efficacy objective was to demonstrate that 0.1 mg and 0.5 mg doses of entecavir had antiviral activity as indicated by the proportion of patients who achieve a reduction in HBV DNA of ≥ 2 $\log_{10}$ copies/mL or to below the limit of quantification (LOQ, 400 copies/mL) by polymerase chain reaction (PCR) assay (Roche Amplicor, Hoffmann-La Roche Ltd, Basel, Switzerland) at week 48. Secondary endpoints included the mean change from baseline in HBV DNA by PCR assay at week 48, and proportions of patients who achieved the following at week 48: (i) HBV DNA less than 400 copies/mL; (ii) serum alanine aminotransferase (ALT) normalization (<1.25 times the upper limit of normal [ULN], World Health Organization [WHO] toxicity grade 0); (iii) HBeAg loss and HBeAg seroconversion (HBeAg loss and appearance of anti-HBe) among patients who were HBeAg-positive at baseline; and (iv) complete response, defined as HBV DNA less than 400 copies/mL by PCR assay plus ALT less than $1.25 \times$ ULN plus HBeAg negativity for those who were HBeAg-positive at baseline. The incidence of amino acid substitutions associated with entecavir resistance in patients who experienced a virological breakthrough, defined as an increase in HBV DNA of ≥ 1 $\log_{10}$ copies/mL from nadir, was also determined. Among patients with evaluable baseline and week 48 liver biopsies, the proportion of patients with histological improvement was determined. Histological improvement was defined as a $\geq$2-point decrease in the Knodell necroinflammatory score and no worsening of fibrosis (worsening was defined as a $\geq$1-point increase in the Knodell fibrosis score) from baseline to week 48. Liver biopsies were also evaluated using the New Inuyama classification system. The biopsy reading committee was blinded to treatment and sequence.

The primary safety endpoint was the proportion of patients in each treatment group who discontinued study medication due to adverse events. Secondary safety endpoints included incidence of adverse events, serious adverse events, laboratory abnormalities, grade 3–4 clinical adverse events and grade 3–4 laboratory abnormalities. ALT flares were defined as ALT $>2 \times$ baseline and $>10 \times$ ULN.

Study population

Patients were eligible for enrollment if they met the following inclusion criteria: (i) hepatitis B surface antigen (HBsAg)-positive for 24 weeks or more prior to screening or HBsAg-positive for less than 24 weeks prior to screening, negative for immunoglobulin M anti-hepatitis B core antibody and confirmation of chronic hepatitis on liver biopsy; (ii) HBeAg-positive for more than 12 weeks prior to screening or HBeAg-negative and positive for anti-HBe; (iii) active viral replication as evidenced by HBV DNA of $\geq 10^5$ copies/mL by PCR assay at screening; (iv) serum ALT ranging 1.3–10 times the ULN; and (v) compensated liver disease, as indicated by a prothrombin time $\leq$3 s longer than the normal control value or international normalized ratio of $\leq$1.5, serum albumin of $\geq$3.0 g/dL (30 g/L) and total bilirubin of $\leq$2.5 mg/dL (42.75 μmol/L). Women of childbearing potential underwent

contraceptive procedures as appropriate to avoid pregnancy during the trial period and for up to 8 weeks after completion of the trial.

The following patients were excluded from the study: pregnant and nursing women; patients diagnosed with cirrhosis, or with a history or evidence of variceal bleeding, encephalopathy or ascites requiring diuretics or paracentesis; patients with other forms of liver disease or suspected hepatic tumors; patients diagnosed with HIV infection; patients with a history of pancreatitis within 24 weeks prior to initiation of protocol therapy; and patients with an increased risk of hepatic toxicity or pancreatitis. In addition, patients who received immunosuppressive therapy (including systemic administration of corticosteroid-derivative agents) or who were treated with interferon-α or -β, within 24 weeks prior to initiation of protocol therapy, were excluded from the study. Patients treated with anti-HBV nucleoside analogs for more than 12 weeks were also excluded.

Assay methods

Serum HBV DNA was determined by Roche Amplicor PCR assay (LOQ, 400 copies/mL; Roche Diagnostics, Tokyo, Japan)[23] in a central laboratory. Clinical laboratory tests, PCR assays for HBV DNA, and serological tests for HBV were performed at SRL Inc. (Tokyo, Japan), the central clinical laboratory designated by the trial sponsor. Liver biopsy was performed within 6 weeks of initiation of study therapy; or, if a liver biopsy had been previously obtained within 52 weeks before initiation of protocol therapy, it was used as the baseline specimen for histological evaluation. Baseline biopsies were evaluated using the Knodell Histological Activity Index (HAI) and Knodell fibrosis scores,[24] and the New Inuyama classifications.[25] Genotype analysis of HBV strains was performed on samples from all patients at baseline using a PCR-restriction fragment length polymorphism assay (SRL). All samples were also analyzed at baseline for evidence of amino acid substitutions associated with lamivudine resistance (rtM204V/I) using a PCR-enzyme-linked minisequence assay (Medical & Biological Laboratories, Aichi, Japan). In addition, patients who experienced virological breakthrough (increase in HBV DNA of $\geq$1 $\log_{10}$ copies/mL from nadir of treatment) had baseline and on-treatment samples analyzed for amino acid substitutions associated with entecavir resistance (rtT184, rtS202 and rtM250) using a HBV DNA polymerase sequence assay at SRL.

Statistical analysis

Analyses of efficacy endpoints were based on treated patients. The primary objective would be demonstrated if the lower limit of the 95% confidence interval for the proportion of patients who achieved a reduction in HBV DNA from baseline by $\geq$2 $\log_{10}$ copies/mL or to less than 400 copies/mL by PCR assay at week 48 in either treatment arm was at least 60%. Parameters represented by continuous variables were summarized by the mean and standard error. In the analysis of binary endpoints, patients with missing week 48 measurements were treated as missing (non-completer = missing). All reported *P*-values are two-sided. For comparison of liver biopsy specimens before and after treatment, a Wilcoxin signed-rank test was performed.

Table 1 Baseline demographics and characteristics

	0.1 mg entecavir $n=32$	0.5 mg entecavir $n=34$
Men, *n* (%)	24 (75)	26 (76)
Women, *n* (%)	8 (25)	8 (24)
Age (years), mean ± SD	44.1 ± 11.4	46.6 ± 10.1
Weight (kg), mean ± SD	67.6 ± 18.2	65.6 ± 13.1
Ethnicity		
Japanese, *n* (%)	32 (100)	34 (100)
HBV DNA, mean ± SD		
$\log_{10}$ copies/mL by PCR	7.26 ± 1.08	7.68 ± 0.97
HBeAg-positive, *n* (%)	26 (81)	27 (79)
ALT (IU/L), mean ± SD	159.6 ± 210.4	141.0 ± 91.9
AST (IU/L), mean ± SD	97.7 ± 103.2	93.1 ± 60.6
Total bilirubin (mg/dL), mean ± SD	0.58 ± 0.22	0.62 ± 0.25
Knodell HAI score, no. of biopsy	31	31
pairs performed, mean ± SE	8.5 ± 0.5	8.7 ± 0.5
Prior treatment		
Interferon, *n* (%)	0	0
Lamivudine, *n* (%)	0	1†
Nucleside/nucleotide analogs, *n* (%)	0	0
HBV genotype *n* (%)		
C	29 (91)	33 (97)
B	2 (6.3)	0
Unknown	1 (3)	1 (3)

†Less than 12 weeks of therapy, consistent with protocol.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; HAI, Histological Activity Index; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; PCR, polymerase chain reaction; SD, standard deviation; SE, standard error.

Results

Study population

Of 102 patients enrolled and screened, 66 were randomized and treated. Thirty-two patients were assigned 0.1 mg entecavir and 34 patients 0.5 mg entecavir. The two treatment groups were well balanced at baseline for demographic and disease-related characteristics (Table 1). Approximately 80% of patients in both groups were HBeAg-positive, and mean HBV DNA at baseline were 7.26 and 7.68 $\log_{10}$ copies/mL for the 0.1 mg and 0.5 mg groups, respectively. Overall, 62 patients were infected with HBV genotype C, and two patients were infected with HBV genotype B. In two patients, HBV genotype was not identified. All patients completed protocol therapy for 52 weeks and were assessed for efficacy and safety. Compliance, measured by the volume of unused product returned from subjects to the institution, was reported to be between 95% and 100%. After completion of the protocol therapy, all patients entered an entecavir rollover study.

Virological response

One hundred percent of patients in both treatment groups achieved the primary efficacy endpoint (a reduction from baseline in HBV DNA of $\geq$2 $\log_{10}$ copies/mL or to <400 copies/mL by PCR assay

Table 2 Virological, biochemical and serological responses at weeks 24 and 48

Response	0.1 mg entecavir $n = 32$	0.5 mg entecavir $n = 34$
Primary efficacy endpoint		
Reduction in HBV DNA ≥2.0 $\log_{10}$ copies/mL or to >400 copies/mL by PCR assay at week 48	32 (100)	34 (100)
Other virological endpoints		
Mean change from baseline by PCR ($\log_{10}$ copies/mL), mean ± SE		
Week 24	−4.43 ± 0.16	−4.79 ± 0.14
Week 48	−4.49 ± 0.16	−4.84 ± 0.14
HBV DNA <400 copies/mL by PCR, *n* (%)		
Week 24	20 (63)	19 (56)
Week 48	26 (81)	23 (68)
Normalization of ALT†		
Week 24, *n/n* with abnormal baseline (%)	24/28 (86)	27/32 (84)
Week 48, *n/n* with abnormal baseline (%)	27/28 (96)	30/32 (94)
HBeAg seroconversion at week 48‡	8/26 (31)	8/27 (30)
Complete response§ at week 48, *n* (%)	12 (38)	13 (38)

†World Health Organization grade 0: ALT of <1.25 × ULN.
‡Loss of HBeAg and gain of anti-HBe.
§HBV DNA <400 copies/mL, HBeAg-negative and ALT of <1.25 × ULN.
ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; PCR, polymerase chain reaction; SE, standard error.

at week 48; Table 2). By week 4, 94% and 91% of patients in the 0.1 mg and 0.5 mg groups, respectively, achieved the primary endpoint; 100% of patients in both groups had achieved it by week 8 and that proportion was maintained through to the end of treatment (week 48). Mean serum HBV DNA declined rapidly in both groups through week 4, and thereafter declined more slowly (Fig. 1). Mean change from baseline in HBV DNA at week 24 was −4.43 $\log_{10}$ copies/mL for the 0.1 mg group and −4.79 $\log_{10}$ copies/mL for the 0.5 mg group. Between weeks 24 and 48, across both groups, only slight decreases in mean HBV DNA occurred. At week 48, mean change from baseline in HBV DNA was −4.49 $\log_{10}$ copies/mL for the 0.1 mg group and −4.84 $\log_{10}$ copies/mL for the 0.5 mg group (P = non-significant [NS]; Fig. 1, Table 2). Eighty-one percent of patients receiving 0.1 mg entecavir and 68% of patients receiving 0.5 mg entecavir achieved HBV DNA of less than 400 copies/mL by PCR assay at week 48 (P = NS, Table 2).

Biochemical response

Approximately 90% of patients demonstrated abnormal ALT (≥1.25 × ULN) at baseline. Among patients with abnormal baseline ALT, the proportions achieving ALT normalization (<1.25 × ULN; WHO toxicity grade 0) at week 48 were 96% (27/28) for patients receiving 0.1 mg entecavir and 94% (30/32) for patients receiving 0.5 mg entecavir (P = NS, Table 2).

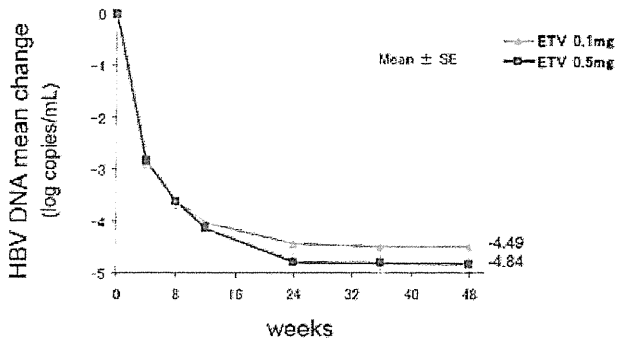

Figure 1 Mean change from baseline in hepatitis B virus (HBV) DNA through week 48 by polymerase chain reaction assay ($\log_{10}$ copies/mL) in patients treated with 0.1 mg and 0.5 mg entecavir (ETV). Data expressed as mean ± standard error.

Serological response

Among patients who were HBeAg-positive at baseline, the proportions achieving HBeAg loss at week 48 were 31% (8/26) in the 0.1 mg group and 30% (8/27) in the 0.5 mg group (Table 2). All patients who demonstrated HBeAg loss also showed acquisition of anti-HBe, thus rates of HBeAg seroconversion at week 48 were also 31% and 30% for the 0.1 mg group and 0.5 mg groups, respectively (Table 2).

Complete response

At week 48, the proportions of patients achieving complete response (defined as HBV DNA <400 copies/mL by PCR assay plus ALT <1.25 × ULN plus HBeAg negativity if they were HBeAg-positive at baseline) were 38% (12/32) for patients receiving 0.1 mg entecavir and 38% (13/34) for patients receiving 0.5 mg entecavir (P = NS, Table 2).

Histological response

Ninety-one percent (29/32) of patients in the 0.1 mg entecavir group and 88% (30/34) of patients in the 0.5 mg entecavir group had evaluable biopsy pairs from baseline and week 48 (Table 3). Histological improvement, defined using the Knodell classification system, occurred in 72% (21/29) and 80% (24/30) of patients in the 0.1 mg and the 0.5 mg groups, respectively. Mean change in Knodell HAI scores were −3.2 and −4.6 for the 0.1 mg and the 0.5 mg groups, respectively. For both groups, the change from baseline in HAI score was significant ($P < 0.0001$ for both groups). In patients who received 0.5 mg entecavir, 29% (9/31) of patients experienced an improvement or no worsening of Knodell fibrosis score, and the mean change from baseline in Knodell fibrosis score from baseline was significant ($P = 0.004$). According to New Inuyama classification, grading of necrotic/inflammatory findings improved for 64% (20/31) of patients in the 0.1 mg group and 74% (23/31) of patients in the 0.5 mg group, while no patient demonstrated worsening. For both groups, the improvement from baseline was significant ($P < 0.0001$ for both comparisons)

Table 3 Histological improvement at week 48 by Knodell scores and New Inuyama classification, relative to baseline

	0.1 mg entecavir $n = 32$	0.5 mg entecavir $n = 34$
Performed biopsy pairs, *n* (%)	31 (97)	31 (91)
Biopsy pairs evaluable for fibrosis, *n* (%)	29 (91)	30 (88)
Histological improvement (Knodell scores), *n* (%)†	21/29 (72)	24/30 (80)
Knodell HAI scores, reduction from baseline at Week 48, mean ± SE	−3.2 ± 0.5*	−4.6 ± 0.5*
New Inuyama classification, *n* (%)		
Grading (necroinflammation)‡	20/31 (64)*	23/31 (74)*
Staging (fibrosis)		
Improvement	7/29 (24)**	12/30 (40)***
No change	18/29 (62)**	17/30 (57)***
Worsening	4/29 (14)**	1/30 (3)***

Statistical significance relative to baseline. *$P < 0.0001$, Wilcoxin signed-rank test (two patients in the 0.1 mg group and one in the 0.5 mg group are evaluable for necroinflammation but not fibrosis). **$P = 0.432$, Wilcoxin signed-rank test. ***$P = 0.003$, Wilcoxin signed-rank test.

†≥2-point decrease in Knodell necroinflammatory score with no worsening of Knodell fibrosis score.

‡Proportions with improvement.

HAI, Histological Activity Index; SE, standard error.

Table 4 Summary of safety

	No. of subjects (%)	
	0.1 mg entecavir $n = 32$	0.5 mg entecavir $n = 34$
Any adverse event *n* (%)	32 (100)	34 (100)
Clinical adverse events *n* (%)	31 (97)	34 (100)
Most frequent clinical adverse events,† *n* (%)		
Nasopharyngitis	15 (47)	17 (50)
Headache	9 (28)‡	10 (29)§
Abdominal pain upper	0	7 (21)
Tachycardia	0	5 (15)
Diarrhea	3 (9)	5 (15)
Laboratory adverse events *n* (%)	30 (94)	28 (82)
Grade 3–4 clinical adverse events *n* (%)	2 (6)	2 (6)
Grade 3–4 laboratory adverse events *n* (%)	5 (16)	6 (18)
Serious adverse events *n* (%)	3 (9.4)	3 (8.8)
Discontinuations due to adverse events *n* (%)	0	0¶
Death *n* (%)	0	0
ALT flare *n* (%)	2 (6)	2 (6)

†Occurring in at least 15% of patients of either treatment group.

‡Five cases related to study drug.

§Eight cases related to study drug.

¶One patient temporarily discontinued, but treatment resumed after 5 days when adverse event was judged unrelated to study drug.

ALT, alanine aminotransferase.

(Table 3). According to the New Inuyama fibrosis staging system, improvement in fibrosis occurred in 24% (7/29) of patients in the 0.1 mg group (P = NS) and in 40% (12/30) of patients in the 0.5 mg group ($P = 0.003$, Table 3).

Resistance analysis

During the treatment period, two patients who received 0.5 mg entecavir experienced virological breakthrough at week 36. Both patients achieved undetectable HBV DNA. The first patient, with a baseline HBV DNA of 8.4 $\log_{10}$ copies/mL, experienced an increase in HBV DNA to 3.6 $\log_{10}$ copies/mL which was maintained at 48 weeks. The second patient, with a baseline HBV DNA of 8.5 $\log_{10}$ copies/mL, experienced an increase in HBV DNA to 4.5 $\log_{10}$ copies/mL and a subsequent decrease to 3.1 $\log_{10}$ copies/mL at week 48. Neither of the patients experienced ALT flares or other clinically relevant events. Genotypic analysis of HBV DNA polymerase was performed on samples from these two patients. Neither L180M nor M204V/I/S (which are associated with lamivudine resistance)[17,26,27] nor amino acid substitutions associated with entecavir resistance (at positions T184, S202 and M250) were detected.[28] Genotypic analysis of virus from all patients was carried out at baseline using methods with a sensitivity cut-off of 25%. Neither rtL180M nor rtM204V/I/S was detected in any patient. At week 48, the amino acid substitution rtM204I (associated with lamivudine resistance) was detected in two patients, one in the 0.1 mg group and one in the 0.5 mg group. However, entecavir was efficacious in the presence of the rtM204I variants and neither patient demonstrated virological breakthrough (HBV DNA decreased by 3.1 $\log_{10}$ copies/mL in the patient in the 0.1 mg group, and reduced to <2.6 $\log_{10}$ copies/mL in one patient in the 0.5 mg group at week 48) or elevation of ALT.

Safety

All 66 patients treated with the study drug completed 52 weeks of dosing. Adverse events were reported for all patients, but most were transient and mild or moderate (grade 1–2) in severity (Table 4). Adverse events were not considered to be related to the study drug, except for a number of cases of headache (five in the 0.1 mg arm and eight in the 0.5 mg arm), all of which were grade 1 or 2. Grade 3–4 clinical adverse events were observed in two patients (6%) in each treatment group, none of which was related to the study drug (one case of enteritis and one of spondylolisthesis in the 0.1 mg arm; one case of enteritis and one of intervertebral disc herniation in the 0.5 mg arm). Grade 3–4 laboratory adverse events occurred in five (16%) and six (18%) of patients in the 0.1 mg and 0.5 mg groups, respectively (two cases of ALT and AST elevations, two of lipase elevations and one of blood glucose elevation in the 0.1 mg arm; two cases of ALT and AST elevations, one of ALT elevation, one of lipase elevation, one of blood glucose elevation and one of amylase elevation in the 0.5 mg arm). There were no deaths in the study.

Serious adverse events occurred in three (9%) of patients in each treatment group (one case of infectious enterocolitis, one of acquired spondylolisthesis and one of ALT elevation in the 0.1 mg arm; one case of duodenal ulcer hemorrhage, one of ligament damage and one of intervertebral disc protrusion in the 0.5 mg arm). No serious adverse event was judged by the investigator

to be related to study medication. The protocol therapy was temporarily discontinued for one patient who developed duodenal ulcer hemorrhage, but therapy was restarted after 5 days when causal relationship with the test medication was ruled out. Except for this temporary interruption, no patient discontinued therapy for adverse events.

Alanine aminotransferase flares (defined as ALT of >2 times baseline and of >10 × ULN: grade 4) occurred in two patients (6%) in the 0.1 mg entecavir group and two patients (6%) in the 0.5 mg entecavir group. All ALT flares were transient, resolved on treatment, and were associated with a ≥2 $\log_{10}$ copies/mL reduction in HBV DNA. No ALT flare was associated with signs or symptoms of hepatic decompensation.

Discussion

The current study demonstrates that entecavir dosing for 52 weeks in Japanese patients was highly effective in reducing HBV DNA and normalizing ALT. The primary objective of this study was to demonstrate that entecavir has antiviral activity as indicated by the proportion of subjects who achieve a reduction from baseline in HBV DNA by ≥2 $\log_{10}$ copies/mL or to <400 copies/mL at week 48. All patients in both treatment groups achieved this primary efficacy endpoint, underscoring entecavir's potent anti-HBV efficacy. In both treatment groups, serum HBV DNA declined rapidly through week 4, then declined more slowly through week 24, and continued to decline through week 48 (mean change from baseline of −4.49 ± 0.16 and −4.84 ± 0.14 $\log_{10}$ copies/mL for 0.1 mg and 0.5 mg entecavir, respectively). This profile confirms the typical multiphasic pattern of antiviral action against HBV, similar to that observed in an international phase II 24-week trial of entecavir.[20]

Entecavir treatment also resulted in ALT normalization (ALT of <1.25 × ULN: WHO grade 0 toxicity) and HBeAg seroconversion concurrent with the observed declines in HBV DNA. Overall (across both treatment groups), approximately 95% of patients achieved ALT normalization, and approximately 30% of HBeAg-positive patients achieved HBeAg seroconversion. These results are similar to those reported in other international clinical trials of entecavir.[21,22] Specifically, Chang *et al.* reported that 48 weeks of entecavir 0.5 mg daily in nucleoside-naive, HBeAg-positive patients resulted in ALT normalization (ALT of ≤1.0 × ULN) in 68% and HBeAg seroconversion in 21% of these patients.[21] In the same study, 67% of patients achieved undetectable HBV DNA at week 48, which is comparable to the results of the present study. However, the mean change from baseline in HBV DNA in the study by Chang *et al.* (−6.9 $\log_{10}$ copies/mL) was greater than was observed in the current study (−4.84 $\log_{10}$ copies/mL for the entecavir 0.5 mg group). This difference is accounted for by the higher baseline HBV DNA of patients in the international trial (9.6 $\log_{10}$ copies/mL vs 7.7 $\log_{10}$ copies/mL for the 0.5 mg group in the present study).

The ultimate goal of chronic hepatitis B treatment is to arrest or reverse liver disease progression associated with HBV infection. This parameter is most directly and reliably measured by histological evaluation. In the current study, entecavir at doses of both 0.1 mg and 0.5 mg daily resulted in high rates of histological improvement (72% and 80%, respectively) when assessed by the Knodell scoring system. As the New Inuyama classification system is most often used to grade and stage liver disease progression in Japan, we also employed this method of histological evaluation, and obtained results consistent with the results of the Knodell evaluations; that is, there was significant improvement in necrosis/inflammation across both entecavir treatment groups and significant improvement in fibrosis for the 0.5 mg entecavir group. Entecavir's demonstrated histological benefit after 1 year of treatment suggests that its potent viral suppression might also reduce the risk of progression to cirrhosis and end-stage liver disease among chronic hepatitis B patients.

A high barrier to resistance among nucleoside-naive patients has been demonstrated with entecavir.[28,29] The combination of potent viral suppression and the requirement for multiple amino acid substitutions in the HBV reverse transcriptase to confer resistance to entecavir suggests that resistance emergence will be a rare event during long-term administration of entecavir. In phase III international clinical trials, less than 1% of patients treated with entecavir through 2 years experienced a virological breakthrough due to the emergence of entecavir resistance.[29] Phenotypic analyses have demonstrated that entecavir-resistant strains do not emerge in the absence of amino acid substitutions associated with lamivudine resistance (rtL180M and/or rtM204V/I/S).[29] In the present study, no amino acid substitutions at T184, S202 or M250 (all of which can mediate resistance in the presence of rtL180M + rtM204V/I/S) were detected. rtM204I emerged in two patients, one in the 0.1 mg entecavir group and one in the 0.5 mg entecavir group. In both patients, entecavir continued to suppress HBV replication and virological breakthroughs were not observed.

Entecavir was generally well tolerated in the current study. There were no clinically significant differences in the incidence or severity of adverse events between the two treatment groups, indicating that entecavir was well tolerated at a daily dose of 0.5 mg. ALT flares were infrequent, and those flares that did occur were associated with reductions in HBV DNA, and resolved without treatment interruption. These results are consistent with the safety and tolerability profile of entecavir reported in international trials.[20–22]

Entecavir's potent antiviral efficacy, good tolerability and high barrier to resistance offer the potential for long-term treatment of chronic hepatitis B with the objective of halting or reversing liver disease progression. The mean reduction in HBV DNA from baseline at week 48 and histological improvement observed in this trial, together with the results of previously published international trials, support the selection of the 0.5 mg dose of entecavir as an appropriate choice of primary therapy for treatment of nucleoside-naive Japanese patients with chronic hepatitis B infection.

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検査値の読み方　連載

HBs抗原測定—新たな臨床応用の話題

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はじめに

HBs抗原は，B型肝炎ウイルス(HBV)感染を診断するもっとも基本的な測定項目であり，1964年に免疫学的に測定可能となって以降，おもにHBV感染の診断根拠としての測定意義を示してきた．近年，高感度で定量性を有するHBs抗原測定系の導入やHBV陽性患者の長期間観察の評価などから，新たな視点で再評価が進んでおり，Gastroenterology誌でも“a 40-year-old hepatitis B virus seromarker gets new life”と表された[1]．

本稿では，われわれのデータを含めて，HBs抗原測定の新しい視点について概説する．

Ⅰ．HBs抗原と測定

1．HBs抗原蛋白

感染した肝細胞の中でHBVが増殖する際に，HBs抗原蛋白は過剰に作られ，感染性を有するDane粒子とは別に，小型球形粒子，棹状粒子としても血液中に流出する．これらは，large，middle，smallの3種類のHBs蛋白で構成されている．また，肝細胞内に組み込まれたHBVからもHBs抗原が産生される[2]．HBV粒子1個に対して，小型球形粒子は500～1,000個，棹状粒子は50～100個存在している．通常のHBs抗原測定系では，これらの粒子のすべてを合わせて検出している．

2．HBs抗原の測定系

HBs抗原の測定は，以前は，micro-Ouchterlony (MO)法，逆受身赤血球凝集反応(R-PHA)法で行われ，その後，radioimmunoassay (RIA)法やenzyme immunoassay (EIA)法による測定法が，さらに，最近では，chemiluminescent immunoassay (CLIA)法が導入されてきた．以前は約40種類のHBs抗原検査薬が販売されていたが，見直しの結果から2001年に安全性情報が出され，2006年の段階では27種類に整理された．

測定原理は，凝集法では，抗HBs抗体を結合させたビーズなどの担体と反応させる．イムノ

Key words: HBs抗原，HBコア関連抗原，cccDNA，HBV遺伝子型，核酸アナログ薬中止

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