Figure 2 Relationship between incidence of hepatocellular carcinoma (HCC) and various virological responses. (a) Comparison between those with alanine aminotransferase (ALT) of ≥40 IU/L and <40 IU/L. (b) Comparison between positive and negative hepatitis B virus (HBV) DNA groups by real-time polymerase chain reaction (PCR). (c) Comparison between the positive hepatitis B e-antigen (HBeAg) group and the HBeAg seroconversion group. (d) Comparison between the positive hepatitis B surface antigen (HBsAg) group and HBsAg seroclearance group (HBsAg <0.05 IU/mL by chemiluminescent immunoassay or <0.03 IU/mL by chemiluminescence enzyme immunoassay). In these comparisons, in chronic hepatitis (CH) patients, a very low risk of HCC was observed, compared with liver cirrhosis (LC) patients, irrespective of conventional virological responses. However, none of the patients who achieved HBsAg seroclearance developed HCC during the therapy.

were demonstrated to be in good correlation with each other in previous studies.[25] In this study, HBsAg seroclearance was defined as less than 0.05 IU/mL by CLIA or less than 0.03 IU/mL by CLEIA. Only 13 out of 602 patients achieved HBsAg seroclearance in this study. While HBsAg seroclearance was not common, none of the 13 patients developed HCC during the therapy. Thus, HBsAg seroclearance was indicated to be the ultimate goal of the therapy.

In this study, a short duration of NA therapy, especially if less than 57 months, was revealed to carry a high risk of HCC in both CH and LC patients. In the early duration of therapy, inflammation in liver still may be active. It was supposed that a long enough duration of suppression of HBV and ALT by NA therapy was needed for suppression of development of HCC. However, because this study was retrospective, some selection bias of the patient data might not have been excluded. It was a concern that rather more patients who did not develop HCC during long-term therapy were collected in this study. However, careful observation for risk of development of HCC is necessary in the early stage of therapy.

In summary, we demonstrated that during NA therapy for chronic HBV infection, cirrhotic status was a significant risk factor of development of HCC. In such a scenario, careful observation is necessary irrespective of various virological responses. Finally, the ultimate goal of NA therapy, as well as other antiviral therapy, should be HBsAg seroclearance.

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Risk Factors for Long-Term Persistence of Serum Hepatitis B Surface Antigen Following Acute Hepatitis B Virus Infection in Japanese Adults

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The proportion of patients who progress to chronicity following acute hepatitis B (AHB) varies widely worldwide. Moreover, the association between viral persistence after AHB and hepatitis B virus (HBV) genotypes in adults remains unclear. A nationwide multicenter study was conducted throughout Japan to evaluate the influence of clinical and virological factors on chronic outcomes in patients with AHB. For comparing factors between AHB patients with viral persistence and those with self-limited infection, 212 AHB patients without human immunodeficiency virus (HIV) coinfection were observed in 38 liver centers until serum hepatitis B surface antigen (HBsAg) disappeared or a minimum of 6 months in cases where HBsAg persisted. The time to disappearance of HBsAg was significantly longer for genotype A patients than that of patients infected with non-A genotypes. When chronicity was defined as the persistence of HBsAg positivity for more than 6 or 12 months, the rate of progression to chronicity was higher in patients with genotype A, although many cases caused by genotype A were prolonged cases of AHB, rather than chronic infection. Multivariate logistic regression analysis revealed only genotype A was independently associated with viral persistence following AHB. A higher peak level of HBV DNA and a lower peak of alanine aminotransferase (ALT) levels were characteristics of AHB caused by genotype A. Treatment with nucleotide analogs (NAs) did not prevent progression to chronic infection following AHB overall. Subanalysis suggested early NA initiation may enhance the viral clearance. *Conclusion*: Genotype A was an independent risk factor for progression to chronic infection following AHB. Our data will be useful in elucidating the association between viral persistence after AHB, host genetic factors, and treatment with NAs in future studies. (HEPATOLOGY 2014;59:89-97)

89

Hepatitis B virus (HBV) infection is one of the most common persistent viral infections in humans. Approximately 2 billion people worldwide have been exposed to HBV and 350 million of them remain persistently infected.[1] The incidence of HBV infection and the patterns of transmission vary greatly worldwide among different population subgroups.[2] In Western countries, chronic HBV infection is relatively rare and acquired primarily in adulthood by way of sexual transmission or the use of injectable drugs. Meanwhile, in Asia and most of Africa, the majority of infections are the result of transmission from an infected mother to her newborn. However, very few studies of acute hepatitis B (AHB) in adults have been reported.

HBV genomic sequences vary worldwide and have been classified into at least eight genotypes (A through H) based on an intergroup divergence of 8% or more over the complete nucleotide sequence.[3,4] These genotypes have distinct geographic distributions.[5-7] In particular, genotype A is predominant in Northwestern Europe, the United States, Central Africa, and India.[8,9] The Japanese have been infected with genotypes B and C since prehistoric times.[10] Recently, many lines of evidence have revealed among the Japanese an increase in acute infection with HBV genotype A following sexual transmission.[11,12] As a result of this increasing transmission of genotype A, the distribution of HBV genotypes in Japan clearly differs among patients with acute and chronic infections.[13] Moreover, recent studies suggest that acute infection with HBV genotype A may be associated with an increased risk of progression to persistent infection.[15] Indeed, the prevalence of HBV genotype A in chronic hepatitis B patients doubled in Japan between 2000-2001 and 2005-2006 (1.7% versus 3.5%).[11]

Human immunodeficiency virus (HIV)-1 infection results in an immunodeficient state, with the virus sharing routes of transmission with HBV. HIV-related immunodepletion influences the natural history of HBV infection, and epidemiological studies have revealed that HIV-positive patients are more likely to have a prolonged acute illness following HBV infection and lower rates of hepatitis B e-antigen (HBeAg) clearance.[16] Therefore, in this study patients with coinfection of HIV were excluded to examine the influence of HBV genotype directly without the confounding influence of HIV.

From 2005 to 2010, a multicenter cohort study was conducted throughout Japan on 212 patients with AHB. The aim of this cohort study was to assess the influence of clinical and virological factors, including HBV genotypes and treatment with nucleotide analogs (NAs), on AHB patients who became persistently infected.

Patients and Methods

Patients With AHB. The multiple-source cohort included 212 randomly selected AHB patients without coinfection of HIV. From 2005 through 2010, the study participants were recruited from 38 liver centers throughout Japan. The cohort included patients who were admitted to the hospital because of AHB and who visited the hospital every month after being discharged. The diagnosis of AHB was contingent on the rapid onset of clinical symptoms accompanied by elevated serum alanine aminotransferase (ALT) levels, the detection of serum hepatitis B surface antigen (HBsAg), and a high-titer antibody to hepatitis B core antigen (anti-HBc) of the immunoglobulin M (IgM) class. Patients with initial high-titer anti-HBc (>10.0 S/CO) were diagnosed as having an exacerbation of chronic hepatitis B and were excluded. If the patient had been tested previously, the absence of serum HBsAg and anti-HBc before admission was verified from the medical record to discriminate a new infection from an acute exacerbation of a persistent infection. Patients with acute hepatitis A, hepatitis C, and drug- or alcohol-induced acute hepatitis were also excluded; hepatitis D virus infection was not determined because of its extreme rarity in Japan. The study protocol conformed to the 1975 Declaration of

Table 1. Characteristics of Patients With Genotype A or a Non-A Genotype Acutely Infected With Hepatitis B Virus

Features	Genotype A (n = 107)	Non-A Genotypes (n = 105)*	P Value
Age (years)	36.3 ± 12.0	40.7 ± 14.3	0.032
Male sex	102 (95.3)	75 (71.4)	<0.001
HBeAg positive	104 (97.2)	79 (75.2)	<0.001
ALT (IU/L)	1210 ± 646	2225 ± 2851	0.045
Total bilirubin (mg/dL)	9.9 ± 9.4	7.5 ± 6.7	0.115
HBV DNA (log copies/mL)	7.0 ± 1.5	5.8 ± 1.5	<0.0001
Duration until disappearance of HBsAg (month)	6.7 ± 8.5	3.4 ± 6.5	<0.0001
Persistence of HBsAg positivity more than 6 months	25 (23.4)	9 (8.6)	0.003
Persistence of HBsAg positivity more than 12 months	8 (7.5)	1[†] (0.9)	0.018
Sexual transmission	81/84 (96.4)[‡]	71/79 (89.9)[§]	0.095
Treatment with NAs	61 (57.0)	42 (40.0)	0.013

Data are presented as n (%), mean ± standard deviation. HBV, hepatitis B virus; HBeAg, hepatitis B e-antigen; ALT, alanine aminotransferase; NAs, nucleotide analogs.
*Non-A genotypes include genotypes B, C, D, F and H (n = 25, 77, 1, 1, and 1, respectively).
[†]One patient had genotype C.
[‡]Transmission routes were unknown for 23 patients.
[§]Transmission routes were unknown for 26 patients.

Helsinki and was approved by the Ethics Committees of the institutions involved. Every patient gave informed consent for this study.

Serological Markers of HBV Infection. HBsAg, HBeAg, antibodies to HBsAg (anti-HBs), HBeAg (anti-HBe), and HBcAg, and anti-HBc of the IgM class were tested by a chemiluminescent enzyme immunoassay (CLIA) by ARCHITECT (Abbott Japan, Tokyo, Japan). HBV DNA measurements were performed using a real-time polymerase chain reaction (PCR) assay (Cobas TaqMan HBV Auto; Roche Diagnostics, Tokyo, Japan).

Genotyping of HBV. The six major HBV genotypes (A through F) were determined serologically by enzyme immunoassay (EIA) using commercial kits (HBV GENOTYPE EIA; Institute of Immunology, Tokyo, Japan). This method is based on the pattern of detection by monoclonal antibodies of a combination of epitopes on preS2-region products, which is specific for each genotype.[17,18] Samples for which EIA could not determine the genotype were examined by direct sequencing of the pre-S2/S gene, followed by phylogenetic analysis.

Treatment With NAs. Treatments with NAs were performed using lamivudine or entecavir for more than 3 months. The individual clinicians determined if NAs were administered to patients, and when the treatment was to be started. The time to onset of treatment with NAs was measured in days from onset of AHB.

Statistical Analysis. Categorical variables were compared between groups by the chi-squared test and noncategorical variables by the Mann-Whitney U test.

A P value less than 0.05 was considered significant. Multivariate analysis was performed using a backward stepwise logistic regression model to determine independent factors for viral persistence following AHB. Variables in the multivariate analysis were selected based on variables that were marginally significant with $P < 0.1$ in univariate analysis. Maintenance of HBsAg positivity was analyzed using the Kaplan-Meier method and significance was tested with the log-rank test. STATA Software (StataCorp, College Station, TX) v. 11.0 was used for analyses.

Results

Comparison of Characteristics Between Genotype A and Non-A Genotype AHB Patients. A total of 107 AHB patients (50.5%) were infected with genotype A while 105 AHB patients (49.5%) were infected with non-A genotypes, including genotypes B (25 [11.8%]), C (76 [35.8%]), D (1 [0.5%]), F (1 [0.5%]), and H (1 [0.5%]). Compared to those infected with non-A genotypes, genotype A patients were significantly younger (36.3 ± 12.0 versus 40.7 ± 14.3 years, P = 0.032), predominantly men (95.3% versus 71.4%, P < 0.001), and more frequently positive for HBeAg (97.2% versus 75.2%, P < 0.001). Moreover, genotype A patients had a lower peak ALT levels (1,210 ± 646 versus 2,225 ± 2,851 IU/L, P = 0.045) and a higher peak level of HBV DNA (6.7 ± 8.5 versus 3.4 ± 6.5 log copies/mL, P < 0.0001). A significantly higher percentage of genotype A patients were treated with NAs (57% versus 40%, P = 0.013). These data are summarized in Table 1.
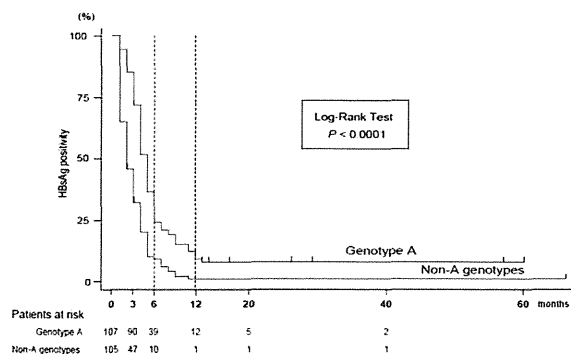
Fig. 1. Comparison of the cumulative proportion of AHB patients maintaining HBsAg positivity between genotype A and non-A genotypes, analyzed using the Kaplan-Meier test. $P < 0.0001$, genotype A: red line, non-A genotypes: blue line.

Cumulative Maintenance of HBsAg Positivity During Follow-up in Patients With Genotype A and Non-A Genotypes.

In the patients infected with genotype A and non-A genotypes, the mean durations of HBsAg positivity maintenance were 6.7 ± 8.5 and 3.4 ± 6.5 months, respectively ($P < 0.0001$; Table 1, Fig. 1). For 6 months after AHB onset, the number of patients with genotype A and non-A genotypes maintaining HBsAg positivity were 39/107 (36.4%) and 10/105 (9.5%), respectively ($P < 0.001$). However, in many patients HBsAg disappeared between 7 and 12 months after AHB onset; that is, HBsAg disappeared in 31/107 (29.0%) of patients with genotype A and in 9/105 (8.6%) of patients with non-A genotypes during this time period. However, in some patients HBsAg never disappeared after persisting for more than 12

months following AHB onset. When chronicity after AHB was defined as the persistence of HBsAg for more than 12 months, chronicity developed in 7.5% (8/107) of patients with genotype A and in 0.9% (1/105) of patients with non-A genotypes ($P = 0.018$).

Comparison of Characteristics Between Patients in Whom HBsAg Persisted More Than 6 or 12 Months and Those With Self-Limited AHB Infection.

Table 2 compares the demographic and clinical characteristics between patients in whom HBsAg disappeared within 6 months and those in whom HBsAg persisted for more than 6 months from AHB. The peak ALT levels ($1,882 \pm 2,331$ versus $1,018 \pm 696$ IU/L, $P = 0.0024$) and peak HBV DNA levels (6.3 ± 1.6 versus 7.4 ± 1.6 mg/dL, $P = 0.0004$) were significantly higher and lower in the former group than in the latter group, respectively. Moreover, marked differences were present in the distribution of genotypes between the two groups. The percentage of the HBV genotype A (46.1% versus 73.5%, $P = 0.003$) was significantly higher among patients in whom HBsAg was persistent for more than 6 months. In addition, we compared the demographic and clinical characteristics between patients in whom HBsAg disappeared within 12 months and those in whom HBsAg persisted for more than 12 months from AHB. Peak ALT ($1,787 \pm 2,118$ versus 775 ± 513 IU/L, $P = 0.0089$) and peak total bilirubin (8.7 ± 8.2 versus 3.8 ± 6.6 mg/dL, $P = 0.0039$) levels were significantly higher in the former group than in the latter group. In contrast, the peak HBV DNA levels (6.4 ± 1.6 versus 7.9 ± 1.4 mg/dL, $P = 0.0046$) were significantly lower

Table 2. Comparison Between Patients With Chronicity Following Acute Hepatitis B and Those With Self-Limited Acute Infections Determined by the Persistence of HBsAg for More Than 6 or 12 Months

Features	Disappearance of HBsAg Within 6 Months (n = 178)	Persistence of HBsAg for More Than 6 Months From AHB (n = 34)	P Value	Disappearance of HBsAg Within 12 Months (n = 203)	persistence of HBsAg for More Than 12 Months From AHB (n = 9)	P Value
Age (years)	38.2 ± 13.1	40.0 ± 14.5	0.454	38.1 ± 13.2	46.7 ± 14.0	0.061
Male sex	147 (82.6)	30 (88.2)	0.416	169 (83.3)	8 (88.9)	0.677
HBeAg positive	150 (84.3)	32 (94.1)	0.131	175 (86.2)	8 (88.9)	0.815
ALT (IU/L)	1882 ± 2331	1018 ± 696	0.0024	1787 ± 2118	775 ± 513	0.0089
Total bilirubin (mg/dL)	8.6 ± 7.5	8.7 ± 11.3	0.137	8.7 ± 8.2	3.8 ± 6.6	0.0039
HBV DNA (log copies/mL)	6.3 ± 1.6	7.4 ± 1.6	0.0004	6.4 ± 1.6	7.9 ± 1.4	0.0046
HBV genotype						
Non-A	96 (53.9)	9 (26.5)		104 (51.2)	1 (11.1)	
A	82 (46.1)	25 (73.5)	0.003	99 (48.8)	8 (88.9)	0.018
Sexual transmission	128/137 (93.4)*	24/26 (92.3)†	0.711	146/157 (93.0)‡	6/6 (100.0)§	0.356
NAs treatment (+)	82 (46.1)	21 (61.8)	0.093	98 (48.3)	8 (88.9)	0.017

Data are presented as n (%) and mean ± SD. HBsAg, hepatitis B surface antigen; AHB, acute hepatitis B; HBeAg, hepatitis B e-antigen; ALT, alanine aminotransferase; HBV, hepatitis B virus; NAs, nucleotide analogs.
*Transmission routes of 41 patients were unknown.
†Transmission routes of 8 patients were unknown.
‡Transmission routes of 46 patients were unknown.
§Transmission routes of 3 patients were unknown.

Table 3. Multivariate Analysis of Factors Independently Associated With Persistence of HBsAg Positivity Following Acute Hepatitis B

Factors	Persistence of HBsAg More Than 6 Months From AHB		
	Odds Ratio	95% CI	P Value
ALT (per 1 IU/L increase)	1.000	0.999-1.000	0.035
HBV DNA (per 1 log copy/mL increase)	1.176	0.931-1.484	0.173
Genotypes			
Non-A	1.00		
A	4.224	1.853-9.631	0.001

95% CI, 95% confidence interval; ALT, alanine aminotransferase; HBV, hepatitis B virus.

in the former group than in the latter group. The percentages of HBV genotype A (48.8% versus 88.9%, $P = 0.018$) and NAs treatment (+) (48.3% versus 88.9%, $P = 0.017$) were significantly higher among patients in whom the HBsAg persisted for more than 12 months.

Factors Independently Associated With Viral Persistence Following AHB. A stepwise logistic regression model was used to perform multivariate analysis which explains relationships between some factors and persistence of HBsAg positivity more than 6 months following AHB. Peak ALT level, peak HBV DNA level, genotype A, and treatment with NAs were retained in the final multivariate logistic model in a backward stepwise manner ($P < 0.1$). For predicting the persistence of HBsAg for more than 6 months, only genotype A was independently associated with progression of AHB to the persistence of HBsAg (odds ratio [OR]: 4.224, $P = 0.001$, Table 3).

Characteristics of Patients Who Progressed to Chronicity That Was Defined as the Persistence of HBsAg for More Than 12 Months Following Acute Hepatitis B. Table 4 shows the clinical and virological characteristics of nine patients who progressed to

chronicity defined as the persistence of HBsAg for more than 12 months following AHB. Among the nine patients who progressed to chronicity from AHB, eight (88.9%) were men and eight (88.9%) were HBeAg-positive. In general, among the patients who progressed to chronicity following AHB, the peak HBV DNA levels were high, and the peak total bilirubin and ALT levels were low. In eight (88.9%) patients, entecavir was administered; however, the duration until the onset of NA treatment from AHB onset was long (75-570 days).

Early Onset of Treatment With NAs Was Able to Prevent Viral Persistence After AHB Caused by Genotype A. The cumulative proportion maintaining HBsAg positivity during follow-up, expressed in terms of time after AHB onset, were significantly longer in patients with NAs treatment than in those without NAs treatment ($P = 0.046$, Fig. 2A). Table 5 shows the percentages of patients in whom HBsAg persisted for more than 6 or 12 months among patients categorized based on the period of time (i.e., duration) until the onset of NAs treatment. For patients in whom the onset of NAs treatment was less than 4 weeks from the onset of AHB, 12.7% of the patients showed persistent HBsAg for more than 6 months, while none showed HBsAg positivity for more than 12 months. For patients in whom the onset of NAs treatment was at 5-8 weeks, 37.5% of the patients showed persistent HBsAg for more than 6 months, whereas none showed persistent HBsAg for more than 12 months. For all groups, the period of HBsAg positivity in patients starting NAs treatment within 8 weeks from AHB onset was significantly shorter than that in patients beginning NAs treatment after more than 8 weeks from AHB onset ($P < 0.0001$, Fig. 2B). Patients starting NAs treatment within 8 weeks from AHB onset never progressed to chronicity after AHB caused by genotype A.

Table 4. Characteristics of Patients Who Progressed to Chronicity Following Acute Hepatitis B

Case	Age	Gender	HIV	HBeAg	HBV DNA (log copies/mL)	Total Bilirubin (mg/dL)	ALT (IU/L)	Observation Period (Months)	NAs Treatment	Duration Until NAs Treatment (Days)	Transmission Routes	Genotype
1	23	Male	(−)	(+)	7.6	1.7	1271	26	ETV	570	Heterosexual	A
2	40	Male	(−)	(−)	8.8	1.4	568	13	ETV	240	Heterosexual	A
3	45	Male	(−)	(+)	7.7	0.9	867	57	ETV	135	Heterosexual	A
4	37	Male	(−)	(+)	7.6	3.4	384	29	ETV	75	Unknown	A
5	54	Male	(−)	(+)	9	2	455	17	ETV	155	Homosexual	A
6	45	Male	(−)	(+)	4.8	21.2	512	60	(−)	(−)	Homosexual	A
7	61	Male	(−)	(+)	9.1	1.5	804	17	ETV	88	Unknown	A
8	56	Male	(−)	(+)	9.0	1.1	1820	14	ETV	118	Unknown	A
9	31	Female	(−)	(+)	7.4	0.8	296	66	ETV	150	Blood transfusion	C

HIV, human immunodeficiency virus; HBeAg, hepatitis B e-antigen; HBV, hepatitis B virus; ALT, alanine aminotransferase; NAs, nucleotide analogs; ETV, entecavir.

Table 5. Proportion of Patients in Whom HBsAg Persisted for More Than 6 or 12 Months Among Patients Categorized Based on the Number of Weeks Until the Onset of NAs Treatment

Duration Until Onset of NAs Treatment (Weeks)	Persistence of HBsAg for More Than 6 Months	Persistence of HBsAg for More Than 12 Months	Total Patients
<4 weeks (n, %)	9 (12.7)	0 (0)	71
5-8 weeks (n, %)	6 (37.5)	0 (0)	16
9-12 weeks (n, %)	1 (33.3)	1 (33.3)	3
13-16 weeks (n, %)	4 (100)	1 (25.0)	4
>17 weeks (n, %)	9 (100)	6 (66.7)	9
Total	29	8	103

HBsAg, hepatitis B surface antigen; NAs, nucleotide analogs.

Discussion

A multicenter nationwide study was conducted throughout Japan to evaluate the influence of clinical and virological factors on chronic outcomes in Japanese patients who contracted AHB in adulthood. The study was feasible in Japan, where a universal vaccination program for HBV has not been implemented because of the extremely high efficacy of the immunoprophylaxis that is given to babies born to carrier mothers. The implementation of this program has resulted in a decrease in the persistent HBV carrier rate from 1.4% to 0.3%.[19] Selective vaccination means that Japanese are more likely to be infected with HBV by way of horizontal transmission since the percentage of the population possessing anti-HBs is much lower than that in countries in which universal vaccination programs have been established.[20] In addition, Japan is faced with the ever-increasing impacts of globalization: as many as 17 million Japanese travel abroad and over 7 million people visit Japan from overseas each year. This "population mixing" may help to explain the increased prevalence in Japan of AHB due to genotype A, which is transmitted through indiscriminate sexual contact. Consequently, Japan may be the only country in the world where the influences of HBV genotypes, including genotype A (as is predominant in Western countries) and genotypes B and C (as are predominant in Asian countries), on chronic outcomes after AHB can be compared.

Currently, the persistence of HBsAg in serum for more than 6 months is considered to represent a progression to chronic infection.[21] However, our data showed that HBsAg frequently disappeared between 7 to 12 months after the onset of AHB in patients with genotype A (31/107 [29.0%]) and non-A genotypes (9/105 [8.6%]) (Fig. 1). These patients were considered to exhibit prolonged cases of AHB, rather than persistent infection. This finding reflects the higher sensitivity of the most up-to-date assays for HBsAg as compared with previous methods. In the present study, HBsAg was measured by CLIA, which has been reported to be about 150 times more sensitive in the detection of HBsAg than reverse passive hemagglutination (RPHA)-HBsAg, which has been used for the last 30 years in Japan.[22] The use of a more sensitive assay for HBsAg results in a longer period during which HBsAg may be detected. In this study, HBsAg did not disappear in nine patients after remaining continuously detectable for more than 12 months. Therefore, the persistence of HBsAg for more than 12 months, as measured with a highly sensitive method for detecting HBsAg, may be suitable for defining the progression of AHB to chronicity; however, further study is necessary to determine whether this definition is appropriate worldwide.
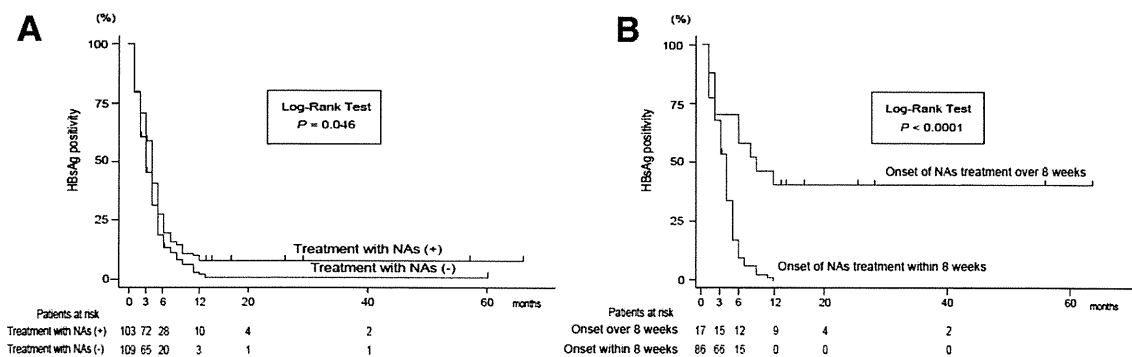
Fig. 2. (A) Comparison of the cumulative proportion of AHB patients maintaining HBsAg positivity between treatment with NAs (+) and treatment with NAs (−), as analyzed using the Kaplan-Meier test. P = 0.046, treatment with NAs (+): red line, treatment with NAs (−): blue line. (B) Comparison of the cumulative proportion of AHB patients in genotype A maintaining HBsAg positivity between treatment onset with NAs within 8 weeks and treatment onset with NAs over 8 weeks after onset of AHB, as analyzed using the Kaplan-Meier test. P < 0.0001, treatment onset with NAs over 8 weeks: red line, treatment onset with NAs within 8 weeks: blue line.

It has been reported that ~10% of patients who contract HBV as adults do not clear HBsAg from their serum and become carriers.[23] Meanwhile, a wide variation has been seen in the rate of persistence after AHB infection in adults. For example, viral persistence following AHB was seen in 0.2% (1/507) of adults in Greece,[24] 7.7% (5/65) of adult Alaskan Eskimos, and 12.1% (7/58) of adults in Germany.[25] The difference in the proportion of patients progressing from AHB to chronicity in different regions may be attributable to virological and host factors. In this study, 4.2% (9/212) of patients progressed to chronicity after AHB: 7.5% (8/107) of those infected with genotype A and 0.9% (1/105) of those infected with non-A genotypes. The non-A genotypes included genotypes B, C, D, F, and H (n = 25, 77, 1, 1, and 1, respectively). Genotypes B and C are predominant in eastern Asian countries, where the majority of those infected with HBV acquired the virus during the perinatal period by way of vertical transmission.[26] On the other hand, genotype A is predominant in Western countries, where the main route is horizontal transmission later in life.[26,27] Because HBeAg persists long after the infection in the genotype C as compared to other genotypes, this genotype has been shown to be a risk factor for perinatal and horizontal transmission in newborns and children.[28] The predominance of genotype A in Western countries may be attributable to a higher chronicity rate following AHB by way of horizontal transmission in adults.

In this study the characteristics of AHB associated with genotype A were a higher peak level of HBV DNA and a lower peak level of ALT. These findings were similar to those for patients with HBV-HIV coinfection.[29] Such characteristics of genotype A or coinfection with HIV are assumed to be attributable to milder hepatitis associated with weaker cellular immune responses. More slowly replicating viruses have been reported to evoke weaker cellular responses, enhancing the likelihood of persistence.[30] Indeed, our prior study showed that the replication of genotype A was significantly slower than that of genotype C in immunodeficient, human hepatocyte chimeric mice.[31] Moreover, variation among genotypes in the expression pattern of HBeAg may affect the progression of AHB to chronicity. Another previous study of ours revealed that a single form of HBeAg was detected by western blot analysis in serum samples from patients infected with genotypes B through D, but that two additional larger forms of HBeAg were detected in patients with genotype A.[32] Milich and Liang[33] reported that HBeAg may modulate the host immune response as a

tolerogen to promote chronicity. Therefore, the different expression pattern of HBeAg by genotype A HBV may contribute to chronicity following AHB.

Early NAs initiation appeared to enhance the viral clearance across genotypes, although treatment with NAs did not show any overall benefit in duration of HBsAg. Previous studies examining the efficacies of NAs for preventing progression to chronic infection after AHB have reported conflicting results. Some small-scale studies have suggested the efficacy of lamivudine and entecavir in preventing the progression of AHB to chronic hepatitis.[34,35] Another study showed a lower seroconversion rate of HBsAg in lamivudine users.[36] Further, a randomized placebo-controlled trial showed no significant difference in clinical outcomes.[37] However, these previous studies did not mention the prevalence of HBV genotypes in the respective study populations. Although this was a retrospective study, our study included data on the prevalence of HBV genotypes. Additionally, our findings suggested that larger prospective randomized studies for every HBV genotype should be performed to determine whether early treatment with NAs prevented the progression of AHB to a chronic state.

In conclusion, in Japan genotype A was an independent risk factor for progression to chronic infection following AHB in adults. Confirmation of this association in patients with AHB in other countries is desirable and may provide insight into the pathogenetic mechanisms underlying this association. Early NA treatment appeared to reduce the likelihood of chronicity but this potentially important intervention needs to be prospectively studied before recommendations can be made.

Appendix

Members of the Japanese AHB Study Group include Yasuharu Imai (Ikeda Municipal Hospital), Norie Yamada, Hideaki Takahashi (St. Marianna University School of Medicine), Koji Ishii (Toho University School of Medicine), Hideyuki Nomura (Shin-Kokura Hospital), Jiro Nishida (Tokyo Dental Collage Ichikawa General Hospital), Shigeru Mikami (Kikkoman Hospital), Tsuneo Kitamura (Juntendo University Urayasu Hospital), Akihito Tsubota (Kashiwa Hospital Jikei University School of Medicine), Noritomo Shimada (Shinmatsudo Central General Hospital), Tetsuya Ishikawa (Nagoya University Graduate School of Medicine), Yoshiyuki Ueno (Tohoku University Graduate School of Medicine), Tomoyoshi Ohno (Social Insurance Chukyo Hospital), Etsuro Orito (Nagoya

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LETTER TO THE EDITOR

Serum HBV RNA as a possible marker of HBV replication in the liver during nucleot(s)ide analogue therapy

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Hiroyuki Nakanishi · Jun Itakura · Namiki Izumi

We read with interest the article by Tsuge et al. [1] published in the recent issue of the Journal of Gastroenterology. Treatment with nucleot(s)ide analogue (NUC) strongly suppresses the replication of hepatitis B virus (HBV) leading to a high rate of serum HBV DNA negativity. However, the incidence of relapse after the cessation of NUCs is high. Criterion for safe discontinuation of NUC therapy after long term therapy is not established to date. In HBe antigen positive patients, seroconversion, HBV DNA negativity and consolidation therapy of >6 months may be a consensus criteria but 30–50 % of patients fulfilling this criteria experience a relapse. In HBe antigen negative patients, NUC therapy is generally recommended until HBs antigen becomes undetected. Tsuge et al. [1] measured serum HBV RNA plus DNA by real time PCR and showed that the serum HBV DNA + RNA titer following 3 months of NUC treatment was a significant predictor of early (within 24 weeks) HBV DNA rebound after discontinuation of NUC. The serum HBV DNA + RNA titer was also associated with ALT rebound in HBe antigen positive patients. The results of the study by Tsuge et al. indicate that serum HBV DNA + RNA titer may serve as predictor of relapse after discontinuation of NUC.

The high rate of relapse after discontinuation of NUC is due to the persistence of HBV replication in the liver even during the NUC therapy. The replicative intermediate form

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of HBV, covalently closed circular DNA (cccDNA), may not be eliminated by NUC therapy and serves as a template for viral pre-genomic messenger RNA [2]. This concept was proved by a study showing that quantification of intrahepatic HBV cccDNA had a high accuracy of predicting sustained virological response after NUC discontinuation [3]. Still, we need a non-invasive and clinically usable marker for the assessment of HBV replication in the liver during NUC therapy. The measurement of HBV core related antigen may be an alternative [4]. The rationale of measuring HBV RNA in serum was that immature HBV particles including HBV RNA are released from hepatocytes during NUC treatment under the circumstances that pre-genomic HBV RNA are transcribed from HBV cccDNA, packaged into HBV core particles, but not reverse transcribed into plus-strand HBV DNA due to strong interference by NUC, and the excessive amounts of these immature particles are accumulated in hepatocytes [5, 6]. Tsuge et al. showed that serum HBV DNA + RNA titer following 3 months of NUC treatment was significantly lower in patients with no rebound of HBV DNA. By using a cut-off value of 4.8 log copies/mL, the cumulative incidence of HBV DNA rebound was significantly lower in patients with serum HBV DNA + RNA titer < 4.8 log at 3 months of NUC treatment. The same groups previously showed that HBV RNA levels at 3 months of lamivudine treatment were predictor of early emergence of resistant mutations [7]. Taken together, serum HBV DNA + RNA titer may be linked to the level of HBV replication in the liver during NUC therapy. Monitoring of serum HBV DNA + RNA response may be utilized in various decision makings in treatment of HBV patients with NUC therapy.

Based on these important findings, several questions may remain for future elucidation. Commercially available transcription-mediated amplification and hybridization assay

(TMA) detects both HBV DNA and RNA. We do recognize that detection sensitivity of this assay is not sensitive but could this assay be used in alternative to real time PCR? In the present study, duration of therapy was 36 weeks in average. The question is whether serum HBV DNA + RNA decrease further by a longer duration of therapy and whether monitoring of serum HBV DNA + RNA (at the end of treatment) serve as a predictor of safe discontinuation after long term NUC therapy. Various protocols of sequential interferon therapy starting with NUC are reported in an attempt to enhance the antiviral activity or to achieve drug-free status [8]. However, their outcome varies considerably and negative HBe antigen at the start of interferon is the only predictor of response [9]. Since 26 out of 36 patients in the study by Tsuge et al. received sequential interferon therapy, serum HBV DNA + RNA titer may be an alternative predictor of favorable response to sequential interferon therapy. Further investigation may be necessary to solve these issues but readers of the journal may be interested if comments can be made by the authors.

Conflict of interest The authors declare that they have no conflict of interest.

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ORIGINAL PAPER

Restoration of albumin production by nucleoside analogue therapy in patients with chronic hepatitis B

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Abstract The clinical course of patients with chronic hepatitis B (CH-B) was greatly changed by the introduction of nucleoside analogues. We often encounter patients where the serum level of albumin recovers quickly following the treatment. In this study, we focused carefully on the changes in serum albumin level noted during nucleoside analogue therapy, in an effort to clarify the mechanism behind the restoration of albumin production. We observed changes in serum albumin levels during nucleoside analogue therapy in 12 patients with CH-B and studied the mechanism behind the restoration of albumin production following the therapy. The serum level of albumin was significantly increased very soon after the treatment was started. Prior to treatment with nucleoside analogues, the albumin signal for mRNA was only slightly seen in the peri-portal area, whereas 12 months after the treatment, the liver tissue presented an obvious signal of albumin mRNA. Serum levels of hepatocyte growth factor (HGF) were significantly decreased 12 months after the treatment. In this study, we demonstrated that nucleoside analogues decrease HGF through the suppression of hepatocyte damage, leading to the restoration of albumin production in patients with CH-B.

Keywords Chronic hepatitis B · Nucleotide analogues · Albumin · Hepatocyte growth factor · In situ hybridization

Introduction

Hepatitis B virus (HBV) infection has been a major public health problem, with 350–400 million patients worldwide. Chronic hepatitis B (CH-B) can lead to progression of liver diseases with increased risk of cirrhosis and hepatocellular carcinoma (HCC) [1].

The clinical course of CH-B was greatly changed by the introduction of nucleoside analogues. Long-term treatment can reverse, and thereby lead to the recovery of, fibrosis of the liver, even if the liver disease was progressing toward cirrhosis [2, 3].

Since albumin is a ubiquitous protein that is synthesized only by hepatocytes, the serum albumin level is an important factor in the evaluation of liver function. We often encounter patients where the serum level of albumin recovers quickly following treatment with nucleoside analogues.

In this study, we focused carefully on the changes in serum albumin level noted during nucleoside analogue therapy, in an effort to clarify the mechanism behind the restoration of albumin production. Moreover, we attempted to demonstrate the restoration of albumin expression, morphologically.

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Patients and methods

Patients

We investigated the changes in serum albumin level noted in patients with CH-B who were started treatment with lamivudine or entecavir in Fukuoka University Hospital. Twelve of the patients who met the following 5 requirements comprised the object of this study: (1) did not have HCC; (2) did not have esophago-gastric varices requiring treatment; (3) were followed up for more than 12 months; (4) suffered no recurrence of hepatitis within the 12 months period; (5) serum samples are saved. The characteristics of the patients are shown in Table 1.

Quantification of serum levels of cytokines

We investigated the changes in serum levels of human hepatocyte growth factor (HGF) and transforming growth factor beta 1 (TGF-β1). Serum samples before and 1 year after beginning the treatment with nucleoside analogues were obtained from the patients. Quantification of HGF and TGF-β1 in serum was performed using the Quantikine ELISA kit (R&D Systems Co. Ltd., USA) and the procedures were carried out according to the manufacturer's protocol.

Liver biopsy specimens

Liver specimens were obtained from four patients who received needle biopsy both before and 1 year after beginning the treatment with nucleoside analogues. The liver specimens were immersion fixed in 10 % neutral buffered formalin at room temperature for 24 h, and then samples were processed for paraffin embedding. Five-micrometre thick sections were cut and mounted on glass slides that had been previously covered with 3-aminopropyltriethoxysilane (Matsunami Glass Ind., Ltd., Japan). The samples were then used for in situ hybridization.

Table 1 Characteristics of the patients

	Median (min–max)
Age (years)	42.0 (21–68)
Gender (M/F)	10/2
Platelet ($\times 10^4/\mu L$)	14.8 (3.6–27.5)
Albumin (g/dL)	3.70 (2.9–4.3)
AST (IU/L)	50.0 (25–256)
ALT (IU/L)	71.5 (38–380)
GGT (IU/L)	71.0 (22–180)
HBeAg (±)	11/1
HBV DNA (log copy/mL)	7.3 (5.9–8.7)

Albumin probe

Five oligonucleotides labelled with FITC were prepared based on the published sequence of albumin complementary DNA (cDNA) [4]. These included the regions of the cDNA coding for amino acids −17 to −8, −2 to 8, 111 to 121, 291 to 300, and 561 to 570 [5].

In situ hybridization

Liver sections were dewaxed and incubated in 0.2 N HCl for 10 min at room temperature, and washed with PBS 3 times, each time for 5 min. The sections were digested with proteinase K (final concentration, 2.5 μg/ml, SIGMA, St. Louis, MO, USA) for 30 min at 37 °C. Sections were fixed with 4 % paraformaldehyde (Wako, Japan) for 5 min, and then washed 3 times with PBS for 5 min. Sections were dehydrated and dried with cool air. Nonspecific probe binding was blocked using a prehybridization buffer [20 × SSC, dextran sulphate sodium, formamide (deionized), mix well all components, vortex and then add poly A (10 mg/ml), ssDNA (10 mg/ml), tRNA (5 mg/ml), 1 M DTT and 50 × Denhardt's solution were added], and this was incubated for 30 min at 37 °C. A labelled probe was added to the hybridization buffer with a final concentration of 2.5 ng/μl, and the sections were hybridized overnight at 37 °C in a humid chamber.

Visualization of albumin mRNA

After hybridization, slides were incubated twice for 15 min at 37 °C in a solution of formamide 50 % in buffer 2 × SSC, then washed twice with 2 × SSC in 2-mercaptoethanol (Wako, Japan) for 5 min. Sections were washed and blocked with 5 % skim milk for 30 min. Alkaline phosphatase-conjugated polyclonal rabbit anti-FITC (Dako Cytomation, Japan) antibody diluted 1:50 was applied for 1 h at room temperature. To enable the sections to develop, they were incubated overnight at 4 °C with BCIP/NBT substrate system (Dako Cytomation). Sections were washed with distilled water and counterstained with methyl green solution (Wako, Japan) for 30 min at room temperature, washed with distilled water and covered with Aquatex mounting media (Merck, Germany). For a negative control, we used one slide cover only with hybridization buffer omitting the probe. For a positive control, we used normal liver tissue.

Statistical analyses

Statistical analyses were performed with the statistical software package JMP version 5.1.2. Values are expressed as mean ± S.D. The changes in biochemical parameters

and cytokines were evaluated using a paired t test. A p value of <0.05 was considered to be statistically significant.

Results

Biochemical parameters and platelet count

The serum level of albumin before treatment was 3.70 ± 0.44 g/dl and was significantly increased very soon after the treatment was started. The levels after treatment for 3, 6, 9, and 12 months were 3.88 ± 0.29, 4.07 ± 0.27, 4.08 ± 0.31 and 4.18 ± 0.28 g/dl, respectively. In contrast, the serum level of ALT was significantly decreased very soon after the treatment was started.

As for, platelet count, this increased only gradually. The level of platelet count before treatment was $15.5 \pm 7.1 \times 10^4/\mu$l and the levels after treatment for 3, 6, 9, and 12 months were 15.2 ± 6.7, 15.5 ± 7.8, 15.7 ± 7.3 and $17.0 \pm 8.4 \times 10^4/\mu$l, respectively (Fig. 1).

In situ hybridization

Staining for albumin mRNA was considered to be positive when a distinct fine granular cytoplasmic purple staining was observed. In the positive control liver sections, we observed abundant cords of hepatocytes stained for albumin mRNA. These were close to the portal areas. However, in the negative controls where the albumin probe was omitted, the signal for albumin mRNA was absent in the hepatocytes (Fig. 2).

In patients prior to treatment with nucleoside analogues, important areas of fibrosis and hepatic necrosis with inflammatory infiltrate were seen surrounding the portal areas and the albumin signal for mRNA was only slightly seen. Twelve months after the treatment, the liver tissue presented an obvious signal of albumin mRNA extensively in the hepatic lobes (Fig. 3).
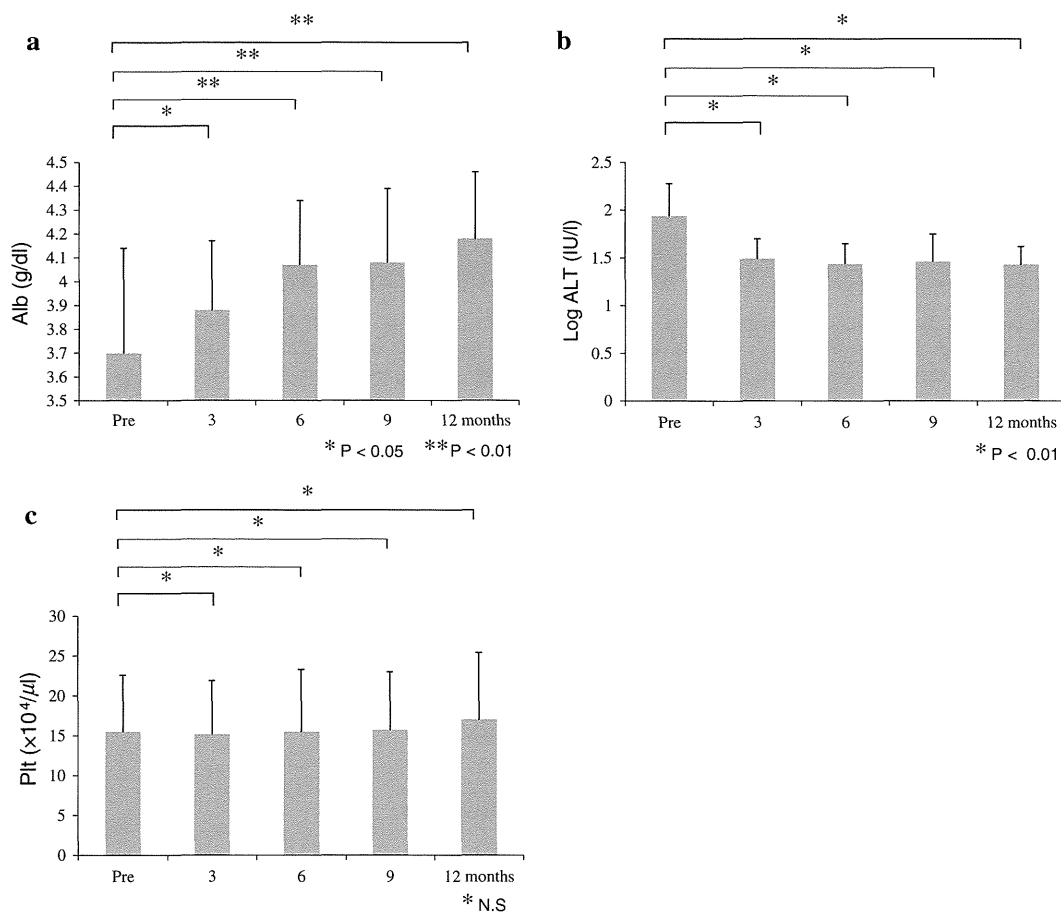

Fig. 1 **a** The serum level of albumin was significantly increased very soon after the treatment was started. **b** In contrast, the serum level of ALT was significantly decreased very soon after the treatment was started. **c** Platelet count increased only gradually
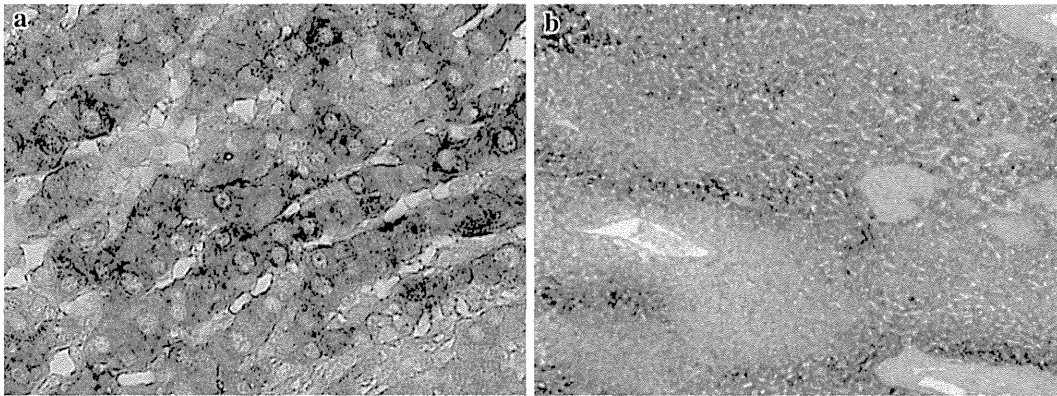
Fig. 2 In the positive control liver sections, we observed abundant cords of hepatocytes stained for albumin mRNA (**a**) (×400). These were close to the portal areas (**b**) (×100)
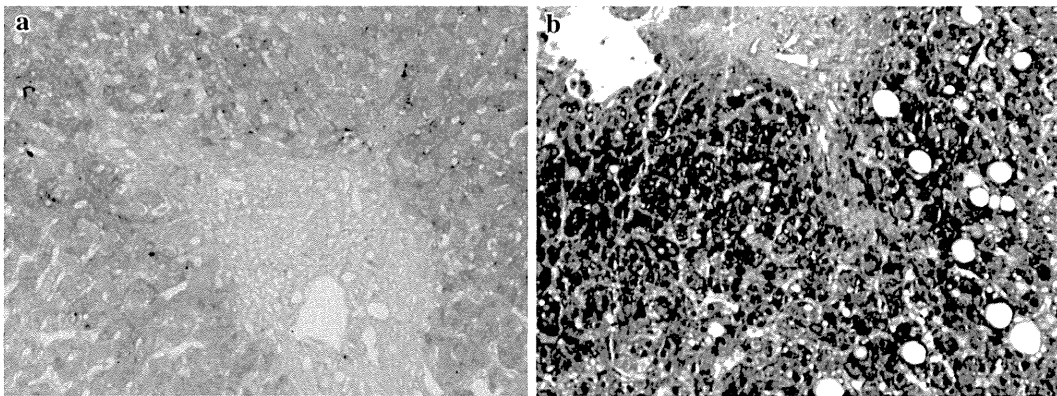
Fig. 3 In a patient (moderate fibrosis and moderate activity) prior to treatment with nucleoside analogue, fibrosis and hepatic necrosis with inflammatory infiltrate were seen surrounding the portal area and the albumin signal for mRNA was only slightly seen (**a**). After the treatment, the liver tissue presented an obvious signal of albumin mRNA extensively in the hepatic lobes (**b**) (×200)

Fig. 4 **a** The serum level of HGF was significantly decreased 12 months after the treatment, **b** whereas the TGF-β1 level did not show any obvious change
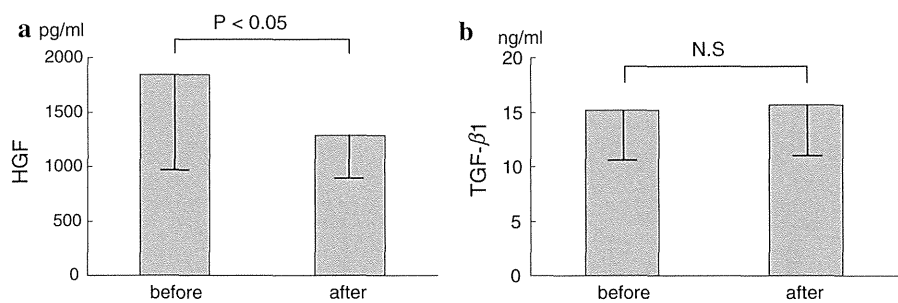
Changes in serum levels of HGF and TGF-β1

The serum level of HGF before treatment was $1{,}845.3 \pm 930.6$ pg/ml. This was significantly decreased to $1{,}287.3 \pm 443.7$ pg/ml 12 months after the treatment, whereas the TGF-β1 level did not show any obvious change (Fig. 4).

Discussion

We successfully showed that albumin production is quickly restored following treatment with nucleoside analogues in both serum and liver tissue. We speculate that the decrease in HGF level brought about by the treatment directly led to

the restoration of albumin production. Although there were few cases, it has been proved with the significant difference statistically and the result of in situ hybridization was almost same in all the examples.

Nucleoside analogues block the reverse transcription of the HBV gene in hepatocytes, leading to a decrease in HBV replication. In most patients with CH-B who receive treatment with nucleoside analogues, serum levels of transaminase are normalized within a few months. Moreover, long-term treatment has been reported to lead to the recovery of fibrosis in the liver [6, 7]. The fibrosis stage in CH-B was reported to recover after a few years from the commencement of treatment, but it should be noted that the restoration of the serum level of albumin was seen much earlier than that.

Albumin synthesis is reported to be regulated by several cytokines, such as IL-6, HGF and TGFβ [8–13]. IL-6, an inflammatory cytokine which is released from lymphocytes or macrophages, increases serum C-reactive protein especially in case of bacterial infection. Under such conditions, albumin synthesis in the liver is suppressed. HGF and TGFβ are related to hepatocyte regeneration following liver damage and hepatic fibrosis, respectively.

Among the above cytokines, HGF is the most potent in regulating albumin synthesis. In general, HGF stimulates the albumin synthesis of hepatocytes, as well as DNA synthesis. However, HGF stimulates both albumin and DNA syntheses of hepatocytes, in a reciprocal relationship. Namely, when the DNA synthesis of hepatocytes is vigorous, HGF suppresses albumin synthesis [11]. In patients with viral hepatitis, serum level of HGF increases at the stage of exacerbation and reduces with recovering of serum level of ALT. A serum level of alpha-fetoprotein (AFP), one of proliferation markers of hepatocytes, is variable in parallel to serum level of HGF, and serum level of albumin rises contrary to the reduction of the AFP level at the healing stage.

In the present study, serum levels of HGF before treatment were high, suggesting that HGF may act to stimulate the DNA synthesis of hepatocytes for liver regeneration, resulting in the albumin synthesis of hepatocytes being suppressed. After treatment for hepatitis was initiated, the serum level of HGF decreased and albumin production was recovered. Therefore, we speculate that nucleoside analogues decrease HGF through the suppression of hepatocyte damage, leading to the rapid restoration of albumin production.

TGF-β1 is also an important cytokine in the albumin synthesis. Flisiak et al. [14] reported that lamivudine treatment for 6 months decreases plasma level of TGF-β1 in patients with CH-B. However, our results showed that TGF-β1 is not changed during treatment for 6 months. In our study, patient's age and TGF-β1 levels before treatment were different than the previous report. Thus, our cases were older and the liver fibrosis was more severe. We

speculate that longer period of treatment with nucleotide analogues is required for reduction of TGF-β1 level in patients with CH-B with severe fibrosis.

There have been several studies using albumin in situ hybridization in HCC and also showing its use as a diagnostic method [15]. However, there have been only a few studies regarding albumin in situ production in hepatitis B and C which have mentioned the correlation between albumin serum level and the prognosis of the disease. However, the precise mechanism behind the production of albumin protein by hepatocytes during viral liver disease and after treatment still needs further clarification.

We showed that the mRNA of albumin was present in hepatocytes close to the portal area. Since inflammation mainly occurs at the portal area, we speculate that the HGF induced by inflammation was able to suppress albumin production in non-treated patients with CH-B.

In conclusion, our results lead us to believe that nucleoside analogues decrease HGF through the suppression of hepatocyte damage, thereby leading to the rapid restoration of albumin production in patients with CH-B. The significance of albumin production in regenerated liver tissue requires further investigation.

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