TABLE V. Factors Associated With Sustained Virological Response (SVR) to Pegylated Interferon Plus Ribavirin Therapy in Patients With rs8099917 TG or GG Genotype

Factor	SVR (n = 16)	Non-SVR (n = 63)	*P* value
(a) Univariate analysis			
Age (years)	54.4 ± 12.4	61.7 ± 109	0.04
Male (%)	9/16 (56)	25/63 (40)	0.24
Histological fibrosis: 0–2/3–4	10/5	37/25	0.62
Histological activity: 0–1/2–3	8/7	31/30	0.86
BMI (kg/m^2)	24.7 ± 5.5	23.4 ± 3.5	0.41
AST (IU/L)	44.4 ± 29.5	53.0 ± 23.0	0.05
ALT (IU/L)	53.8 ± 31.0	54.0 ± 33.6	0.86
GGT (IU/L)	57.6 ± 37.1	62.7 ± 49.8	0.80
Albumin (g/dl)	4.1 ± 0.3	3.9 ± 0.4	0.03
Total cholesterol (mg/dl)	173.1 ± 33.3	159.8 ± 32.9	0.13
Triglyceride (mg/dl)	97.3 ± 70.0	91.7 ± 39.5	0.53
LDL cholesterol (mg/dl)	95.1 ± 20.4	83.6 ± 24.9	0.07
Apolipoprotein B-100 (mg/dl)	73.5 ± 16.5	62.4 ± 16.2	0.02
HOMA-IR	2.9 ± 2.5	2.3 ± 1.7	0.60
AFP (ng/ml)	5.2 ± 3.1	17.9 ± 30.7	0.002
White blood cells ($\times 10^2/mm^3$)	56.7 ± 16.2	48.9 ± 17.0	0.05
Hemoglobin (g/dl)	14.1 ± 1.4	13.3 ± 1.5	0.06
Platelets ($\times 10^4/mm^3$)	21.5 ± 5.8	16.3 ± 6.1	0.002
HCV RNA (log IU/ml)	5.6 ± 0.9	6.2 ± 0.5	0.003
Peg-IFN dose (μg/kg/week)	1.5 ± 0.1	1.5 ± 0.2	0.97
Ribavirin dose (mg/kg/day)	11.3 ± 2.7	11.4 ± 2.0	0.89
ISDR in NS5A wild-type (%)	14/14 (100)	57/62 (92)	0.27
Substitution at aa 70 Arg (%)	8/15 (53)	28/62 (45)	0.57
Substitution at aa 91 Leu (%)	7/15 (47)	36/62 (58)	0.43

Factor	Category	Odds ratio (95% CI)	*P* value
(b) Multivariate analysis			
Apolipoprotein B-100	By 10 (mg/dl)	1.558 (1.004–2.418)	0.044
HCV RNA (log IU/ml)	By 1 (log IU/ml)	0.263 (0.087–0.796)	0.016

Peg-IFN, Pegylated interferon; CI, confidence interval; BMI, body mass index; AST, aspartate:2-oxoglutarate aminotransferase; ALT, alanine:2-oxoglutarate aminotransferase; GGT, gamma-glutamyl transferase; LDL, low density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; AFP, alpha fetoprotein; HCV, hepatitis C virus; ISDR, interferon sensitivity-determining region.

therapy (protease inhibitor, together with Peg-IFN plus RBV), the sustained virological response rate was reported to be only about 30% [Welsch et al., 2012]. Similarly, with Peg-IFN plus RBV combination therapy, the sustained virological response rate in this cohort study was only 20.3%. Therefore, selecting patients who are most likely to respond to antiviral therapy among those with the hetero/minor IL28B genotype is a matter of urgency. More precise prediction of sustained virological response in patients with the IL28B hetero/minor genotype may improve the cost benefit and reduce adverse effects that accompany antiviral therapy.

Akuta et al. [2010] has suggested that amino acid substitution at Arg70 residue of the HCV core region may be a candidate predictor of sustained virological response to triple therapy. However, as shown in this study, neither substitution at Arg70 in the core nor the number of aa substitutions in NS5A ISDR were significant factors in HCV G1b-infected patients who carried the IL28B hetero/minor genotype and were treated with Peg-IFN plus RBV. In contrast, the serum apoB-100 level was extracted as an independent factor, suggesting that measurement of apoB-100 may be useful as an indicator of virological response to Peg-IFN plus RBV combination therapy in this patient group.

TABLE VI. Correlation of Serum Apolipoprotein B-100 Concentrations With Other Metabolic Factors

	r^2	*P* value
BMI	0.0060	0.216
HOMA-IR	0.0066	0.204
Albumin	0.0728	<0.001
Total cholesterol	0.4867	<0.001
Triglyceride	0.0484	<0.001
LDL cholesterol	0.7006	<0.001

BMI, body mass index; HOMA-IR, homeostasis model assessment of insulin resistance; LDL, low-density lipoprotein.

In addition, it was shown that the serum apoB-100 level was influenced by the aa substitution at residue 70 of the HCV core in patients carrying the IL28B major, but not the IL28B hetero/minor, genotype [Aizawa et al., 2012]. Therefore, aa substitution at residue 70 and serum apoB-100 may independently affect the virological effect of newly developed triple therapy in HCV G1b-infected patients with the IL28B hetero/minor genotype. However, the precise mechanisms of such a peculiarity of lipid metabolisms that the serum apoB-100 level independently affects sustained virological response exclusively in patients with the IL28B hetero/minor genotype are still unknown. While serum apoB-100 may be worth considering as a predictor, only a relatively small number

of IL28B hetero/minor patients were examined in this study. Therefore, a future large-scale study is desired to establish the critical role of serum apoB-100 in the efficacy of antiviral therapy for patients infected with HCV G1b and carrying the IL28B hetero/minor genotype.

In conclusion, the serum apoB-100 level is a predictor of rapid initial virological response in chronic HCV G1b-infected patients treated with Peg-IFN plus RBV, whereas apoB-100 may be a useful marker for predicting sustained virological response in particularly those who carry the IL28B hetero/minor genotype.

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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)

Abbreviations: AHB, acute hepatitis B; ALT, alanine aminotransferase; anti-HBc, antibody to hepatitis B core antigen; anti-HBs, antibody to HBsAg; HBeAg, hepatitis B e-antigen; CLIA, chemiluminescent enzyme immunoassay; EIA, enzyme immunoassay; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HIV, human immunodeficiency virus; IgM, immunoglobulin M; anti-HBe, antibody to HBeAg; NAs, nucleotide analogs; RPHA, reverse passive hemagglutination.

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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

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Additional Supporting Information may be found in the online version of this article.

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.

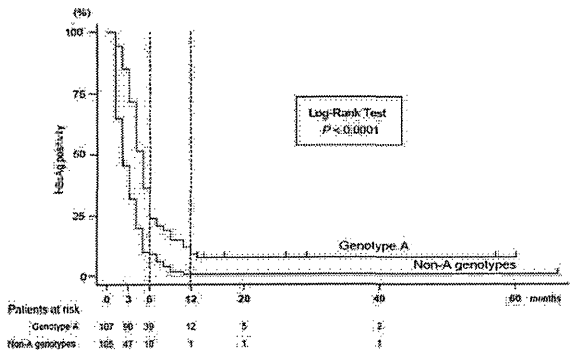

Fig. 1. Comparison of the cumulative proportion of AHB patients maintaining HBsAg positivity between genotype A and non-A genotypes, analyzed using the Kaplan-Meier test. $P < 0.0001$, genotype A: red line, non-A genotypes: blue line.

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

	Persistence of HBsAg More Than 6 Months From AHB		
Factors	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.

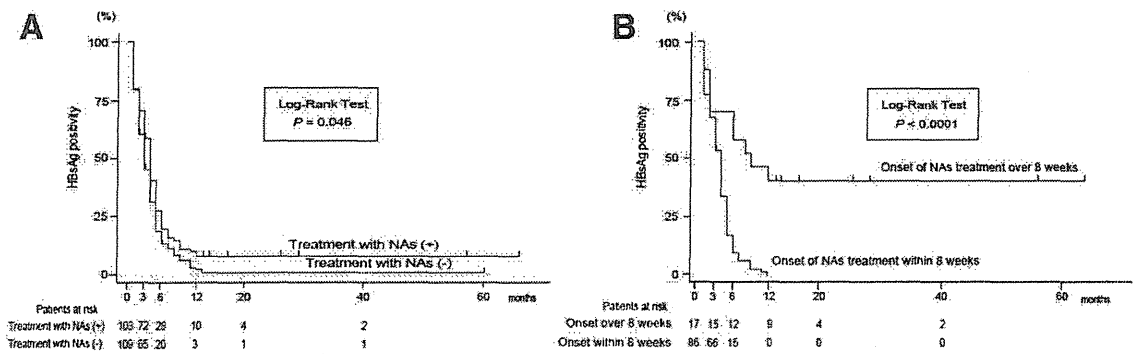

Fig. 2. (A) Comparison of the cumulative proportion of AHB patients maintaining HBsAg positivity between treatment with NAs (+) and treatment with NAs (−), as analyzed using the Kaplan-Meier test. $P = 0.046$, treatment with NAs (+): red line, treatment with NAs (−): blue line. (B) Comparison of the 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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VIRAL HEPATITIS

Hepatic stellate cells that coexpress LRAT and CRBP-1 partially contribute to portal fibrogenesis in patients with human viral hepatitis

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Keywords
Cellular retinol-binding protein-1 (CRBP-1) – hepatic stellate (Ito) cell (HSC) – lecithin retinol acyltransferase (LRAT) – portal fibrosis – retinoid(vitamin A)

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Abstract

Background & Aims: Precisely what type of cells mainly contributes to portal fibrosis, especially in chronic viral hepatitis, such as hepatic stellate cells (HSCs) in the parenchyma or myofibroblasts in the portal area, still remains unclear. It is necessary to clarify the characteristics of cells that contribute to portal fibrosis in order to determine the mechanism of portal fibrogenesis and to develop a therapeutic target for portal fibrosis. This study was undertaken to examine whether LRAT+/CRBP-1+ HSCs contribute to portal fibrosis on viral hepatitis. *Methods:* Antibodies to lecithin:retinol acyltransferase (LRAT), cellular retinol-binding protein-1 (CRBP-1) and widely ascertained antibodies to HSCs (alpha-smooth muscle actin, neurotrophin-3) and endothelial cells (CD31) were used for immunohistochemical studies to assess the distribution of cells that contribute to the development of portal fibrosis with the aid of fluorescence microscopy. A quantitative analysis of LRAT+/CRBP-1+ HSCs was performed. *Results:* The number of LRAT+/CRBP-1+ HSCs was increased in fibrotic liver in comparison with normal liver in the portal area and fibrous septa. The number of double positive cells was less than 20% of all cells/field in maximum. *Conclusion:* This study provides evidence that functional HSCs coexpressing both LRAT and CRBP-1 that continue to maintain the ability to store vitamin A contribute in part to the development of portal fibrogenesis in addition to parenchymal fibrogenesis in patients with viral hepatitis.

Hepatic stellate cells (HSCs), also referred to as Ito cells or fat-storing cells, have been regarded as essential cells for liver fibrogenesis. In a normal liver, HSCs store 80–90% of the hepatic retinoid in characteristic lipid droplets as *fat-storing cells*. When activated in the presence of liver damage, HSCs release cytokines, primarily TGF-β, and transform into myofibroblasts lacking fat droplets. They then produce excessive extracellular matrix and disrupt the liver cytoarchitecture, eventually leading to cirrhosis and liver failure (1).

Hepatic myofibroblasts are transdifferentiated from heterogeneous cell populations in response to a variety of fibrogenic stimuli. Recently, there have been reports that the cellular origin of fibrogenic myofibroblasts is HSCs, portal fibroblasts (6), other mesenchymal cells (6, 7), bone marrow cells (2–5), epithelial-like cells such as hepatocytes or cholangiocytes (8–11) and endothelial cells (12). Although these reports are credible, it is unclear whether this cellular transformation occurs in actual human liver diseases. The current consensus is that hepatic myofibroblasts, myofibroblasts generated in parenchymal injury appear to originate from HSCs and myofibroblasts generated in portal injury may originate from portal fibroblasts (13). However, most of these results were obtained using cultured cells or rodent models. There are few reports that examined the origin

of myofibroblasts in actual human liver diseases, especially in portal injury. Therefore, it is important to determine which type of cells contributes to portal fibrosis in human diseased livers.

One of the candidates that can serve as a marker of quiescent HSCs, which store retinoids in the space of Disse, is lecithin:retinol acyltransferase (LRAT). LRAT activity is strongly expressed in the liver, retinal pigment epithelial (RPE) cells, intestinal mucosa, basal keratinocytes, testis, lungs, *etc.* LRAT has been defined to play the following roles: storing systemic retinoid in the liver, incorporating retinol into the retina and adjusting its concentration in the retinal pigment epithelium to maintain visual function, adjusting the regional concentration of retinoid to differentiate the epithelium in the skin and lungs and regulating the concentration of retinoid to maintain a level optimal for maturation of spermatozoa in the testis (14–20).

Lecithin retinol acyltransferase is also the physiological retinol esterification enzyme which stores retinoid in the liver. Retinyl esters are biosynthesized by removing the fatty acid at position sn-1 of lecithin, using cellular retinol-binding protein-1 (CRBP-1)-bound retinol as a substrate. The expression of LRAT in the liver is regulated by the vitamin A status. Whereas hepatic LRAT activity is low in the livers of vitamin A deficient rats, the decreased LRAT activity is rapidly elevated by repletion with retinol, retinoic acid or RAR-agonists (21–23). Furthermore, LRAT activity is higher in the non-parenchymal cell fraction than in the hepatocyte fraction, and it is estimated that in the liver, the LRAT activity is specifically distributed in HSCs (15). We previously generated antimouse and antihuman LRAT antibodies using peptides of the amino acid sequence of mouse hepatic LRAT and human RPE LRAT, respectively, and reported that these proteins were expressed in quiescent HSCs and endothelial cells of rodent and human normal livers (24).

Cellular retinol-binding protein-1, one of retinol-binding proteins, is present in a variety of tissues, and is most highly expressed in the liver, kidneys and proximal epididymis (25, 26). CRBP-1 is highly expressed in the liver: hepatocytes account for more than 90% of hepatic CRBP-1, while the concentration of the protein (per protein unit) in HSCs is 22 times greater than in hepatocytes (27). In the liver, CRBP-1 mediates retinol esterification to retinyl esters (28). CRBP1-bound retinol is also the substrate of LRAT (29) and the interaction between LRAT and CRBP-1 is required for this enzymatic reaction (30). In an immunohistochemical study, it was shown that quiescent rat HSCs express CRBP-1 (31–33) and quiescent HSCs and myofibroblasts in human normal and diseased livers also express CRBP-1, in addition to hepatocytes and cholangiocytes express CRBP-1 (34, 35).

As described above, both LRAT and CRBP-1 are key molecules involved in the retinoid metabolism in the liver, and in this work we regarded cells coexpressing LRAT and CRBP-1 as functional HSCs that have the ability to store vitamin A.

In this study, the *in situ* distribution of functional HSCs that expressed both LRAT and CRBP-1 was examined in human normal and pathological livers, particularly livers of patients with hepatitis C and B, in order to clarify the contribution of HSCs to portal fibrosis.

Materials and methods

Human liver specimens

Human liver samples were obtained from 24 patients. They corresponded either to percutaneous liver biopsies (n = 20) or large surgical specimens (n = 4). Four corresponded to histologically normal livers and 20 to pathological specimens (Table. 1) with various fibrotic stages. Non-tumourous areas in specimens resected for liver metastasis of colon cancer and in specimens of haemangioma of the liver were studied as histologically 'normal' livers. The stage of fibrosis and the grade of inflammatory activity were classified according to the METAVIR score (36). This work was performed with the permission of the Ethics Committee for Biomedical Research of the Jikei University School of Medicine.

Table 1. Pathological specimens

	Age	Sex	Fibrosis*.	Activity†	Aetiology
1	43	F	F0	A0	Metastatic liver tumor
2	60	F	F0	A0	Metastatic liver tumor
3	35	F	F0	A0	Liver hemangioma
4	83	F	F0	A0	Metastatic liver tumor
5	55	M	F1	A1	HCV
6	63	F	F1	A2	HCV
7	52	F	F1	A1	HCV
8	25	M	F1	A1	HBV
9	51	F	F1	A2	HBV
10	58	F	F2	A3	HCV
11	51	F	F2	A2	HCV
12	62	M	F2	A2	HBV
13	40	M	F2	A2	HBV
14	63	M	F2	A1	HCV
15	55	M	F3	A1	HCV
16	36	M	F3	A3	HBV
17	40	M	F3	A2	HBV
18	28	M	F3	A2	HBV
19	64	F	F3	A2	HCV
20	39	M	F4	A2	HCV
21	47	M	F4	A3	HCV
22	72	M	F4	A2	HCV
23	57	M	F4	A1	HBV
24	70	M	F4	A2	HCV

HBV, hepatitis B virus; HCV, hepatitis C virus.
*F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis with rare septa; F3, numerous septa without cirrhosis; F4, cirrhosis.
†A0, no activity; A1, mild activity; A2, moderate activity; A3, severe activity. No. 1-4: non-tumourous area in the livers with metastasis or haemangioma.

Tissue sampling and processing

A portion of the fresh tissue samples was routinely utilized to prepare 10% buffered neutral formalin-fixed and paraffin-embedded sections for liver pathology diagnosis; these samples were also used for this study. Three micrometer-thick paraffin sections of each sample were stained with haematoxylin-eosin, Masson's trichrome and silver impregnation for the diagnostic purposes.

Antibodies, immunostaining and observation

An antibody specific for human RPE LRAT (Immuno-Biological Laboratories, Gunma, Japan), a rabbit polyclonal antibody against human cellular retinol-binding protein-1 (CRBP-1) (FL-135) (sc-30106; Santa Cruz Biotechnology, Inc., California, CA, USA), a mouse monoclonal antibody against human smooth muscle actin (SMA) (IgG2a) (Clone 1A4; Dako A/S, Denmark), a rabbit polyclonal antibody against neurotrophin-3 (NT-3) (N-20) (sc-547; Santa Cruz Biotechnology, Inc., California, CA, USA) and a mouse monoclonal antibody against CD31 (IgG1, kappa) (Clone JC70A; Dako A/S, Denmark) were used for the immunohistochemical studies.

The formalin-fixed specimens were embedded in paraffin, and 3 μm-thick sections were cut for immunohistochemical examination. After deparaffinization, the sections of human liver were microwave-treated in 10 mM citrate buffer (pH 6.0) for 10 min at 95°C to activate the antigens, then samples were allowed to cool at room temperature for 15 min. The sections were then rinsed with PBS, and endogeneous peroxidase was inhibited by 0.3% hydrogen peroxide in methanol for 30 min at room temperature. After blocking the samples with 5% goat whole serum (Immuno-Biological Laboratories)/PBS, the sections were incubated with antibodies diluted in PBS against human LRAT (1:20), human CRBP-1 (1:50), human SMA (1:500) and human NT-3 (1:50) for 60 min at room temperature. The sections were then rinsed in PBS, and the epitopes were detected with the Envision + system by horseradish peroxidase detection of antirabbit or antimouse (Dako, Carpinteria, CA, USA) for 60 min at room temperature. The immunoreaction was visualized by using 0.5% 3, 3′-diam-

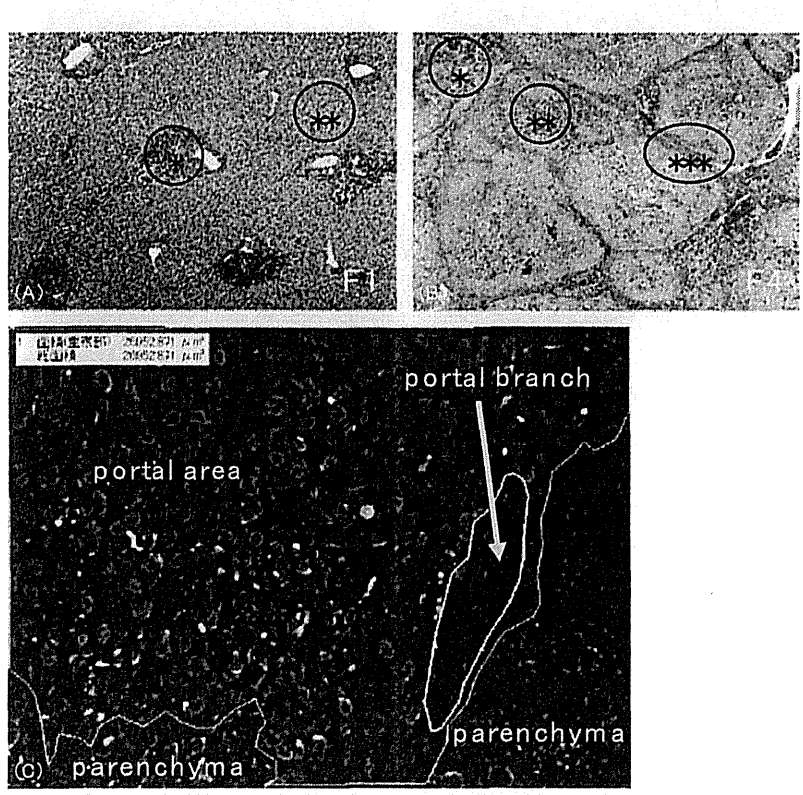

Fig. 1. (A, B): Masson-Trichrome staining of the normal and cirrhotic human liver sections. A total of 3 random fields each in the parenchyma, portal area and fibrous septa (only F3, F4) were used for the quantitative analysis of each case. *portal area, **parenchyma, ***fibrous septa (C) Double immunofluorescence staining for LRAT (green labelling) and CRBP-1 (red labelling) of human liver sections. Each area was automatically measured using a VH analyzer, using the Keyence software program for quantitative analysis, according to the formula: all field - (parenchyma area + portal branch area) = portal area.

inobenzidine tetrahydrochloride (DAB; Dojinkagaku, Japan) and 0.1% hydrogen peroxide in 50 mM Tris buffer (pH 7.6) at room temperature. The sections were lightly washed, counterstained with haematoxylin, dehydrated in ethanol and mounted with Permount.

For double immunostaining of LRAT/SMA, LRAT/CD31 and LRAT/CRBP-1, a Ventana XT System Benchmark (Ventana Medical Systems, Inc., Tucson, AZ, USA) (automated immunohistochemical instrument) was used. Double immunostaining was performed using antibodies at the same dilutions as described above except for CD31 (1:20). The immunoreaction was visualized by using an i-View DAB and RED detection kit (Ventana Medical Systems, Inc.) for each antibody.

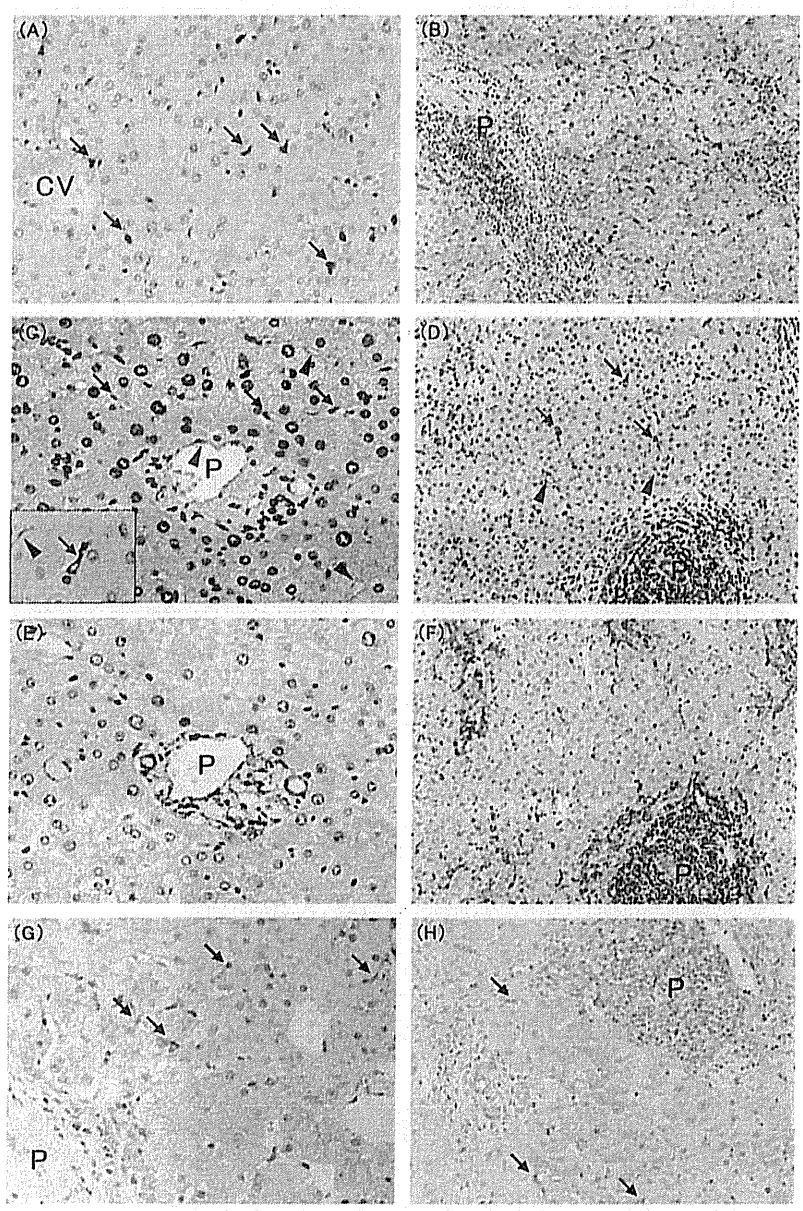

Fig. 2. Immunostaining of normal METAVIR score F0A0 (A, C, E, G) and fibrotic METAVIR score F2A2 (B, D, F, H) human liver sections. (A, B): Liver sections stained for CRBP-1 showed that HSCs (arrows) were positively stained in normal and fibrotic liver. (C, D): Liver sections stained for LRAT showed that both HSCs (arrows) and endothelial cells (arrowheads) were positively stained in the normal and fibrotic liver. The inset shows a higher magnification view of a HSC. (E, F): Liver sections stained for smooth muscle actin (SMA) showed that the HSCs in the space of Disse were hardly stained, while the myofibroblasts around the portal area and smooth muscle cells in small arteries in the portal area were positively stained. (G, H): Liver sections stained for neurotrophin-3 (NT-3) showed that HSCs (arrows) were positively stained in the normal and fibrotic liver. (CV; central vein, P; portal vein) magnification: A, C, E, G; 9 200, B, D, F, H; 9 100, C (inset); 9 1,000.

Subsequently, double immunofluorescence staining was evaluated. After antigen retrieval using a microwave, the sections were incubated with the mixture of anti-LRAT antibody labelled with FITC (Invitrogen Corporation, Carlsbad, CA, USA) and anti-CRBP-1 antibody labelled with R-Phycoerythrin (Seikagaku Biobusiness Corporation, Tokyo, Japan) at the same dilutions as described previously for 60 min at room temperature. The sections were lightly washed and mounted with ProLong® Gold antifade reagent (Molecular Probes, Eugene, OR, USA). As a negative control, we used Negative Control Rabbit IgG (Biocare Medical, Concord, CA, USA) at the same concentration as the respective antibodies.

Finally, sections were examined by fluorescence microscopy using a Biozero BZ-8000 microscope (Keyence Corporation, Osaka, Japan).

Quantitative evaluation of LRAT and CRBP-1 double positive hepatic stellate cells

Quantitative evaluation of double immunofluorescence was performed using a fluorescence microscope (Biozero BZ-8000; Keyence Corporation, Osaka, Japan). After double immunofluorescence staining, the number of LRAT+/CRBP-1+ cells/field was counted in 3 random fields each in the parenchyma, portal area and fibrous septa (only F3, F4) in each case. Each area was automatically measured using a VH analyzer (Keyence Corporation, Osaka, Japan). The average number of LRAT+/CRBP-1+ cells/field was calculated in F0-F4 respectively (Fig. 1).

Statistical analysis

Comparisons between the two groups were performed using Student's *t*-test. The difference between groups was considered statistically significant at $P < 0.05$.

Results

Immunohistochemical examination of liver specimens

Human liver samples obtained from 24 patients were studied. The samples were from 10 females and 14 males aged 25–83 years (average: 52 years). Livers of various fibrotic stages were classified into F1–F4 according to their METAVIR score, excluding normal livers. The cases of viral hepatitis or cirrhosis were used as pathological liver specimens in order to demonstrate whether HSCs were present in the fibrotic areas around the central vein but also in portal fibrosis.

Human normal and fibrotic liver specimens were immunostained with anti-CRBP-1, anti-LRAT, anti-SMA and anti-NT-3 antibodies. HSCs in the space of Disse were positively stained for both CRBP-1 and LRAT in the normal and fibrotic liver specimens (Fig. 2A–D). In addition, the endothelial cells of the portal vein and sinusoids were stained with the anti-LRAT antibody (Fig. 2C,D). Smooth muscle actin (SMA), an actin isoform, can be regarded as a good marker for identification of activated HSCs (37). HSCs in the space of Disse were barely stained for SMA, in contrast to the CRBP-1 and LRAT staining, although the myofibroblasts in the portal area and fibrous fascicles were positively stained (Fig. 2E,F). NT-3, a neurotrophic factor, is also regarded to be a marker identifying HSCs (38, 39). In this study, HSCs in the space of Disse were positively stained for NT-3, as well as LRAT and CRBP-1, in the normal and fibrotic liver specimens (Fig. 2G,H).

Double immunostaining for LRAT and SMA in human cirrhotic liver specimens showed that SMA was

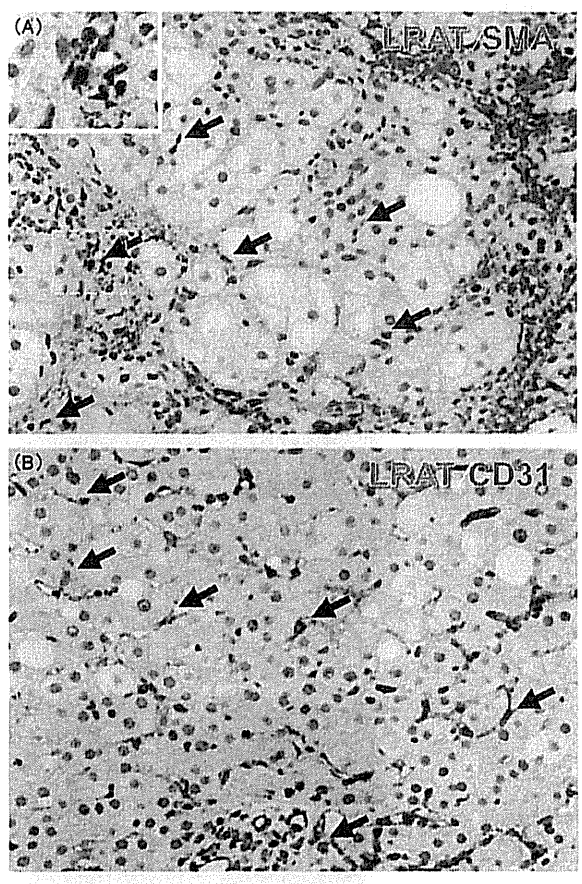

Fig. 3. (A) Double immunostaining for LRAT (brown labelling) and SMA (red labelling) of human cirrhotic liver (METAVIR score of F4A2). SMA was positive in the fibrous fascicles. On the other hand, a few LRAT positive cells (arrows) were observed in the parenchyma and fibrous septa. The inset shows a higher magnification view of a HSC which colocalizated with LRAT and SMA in fibrous fascicles. (B) Double immunostaining for LRAT (brown labelling) and CD31 (red labelling) of the human liver (METAVIR score of F1A1). CD31 was positive in endothelial cells in the sinusoid and portal area. LRAT was positive in both HSCs (arrows) and endothelial cells in the sinusoid. magnification: 9 200, inset9 1000.

strongly positive in fibrous fascicles, while LRAT was mainly positive in HSCs in the space of Disse. Some of the LRAT positive cells in the fibrous fascicles were co-localizated with SMA positive cells (Fig. 3A). Double immunostaining for LRAT and CD31 in the human liver specimens revealed positivity for CD31 in endothelial cells in the sinusoid and portal area and positivity for LRAT in both HSCs (arrows) and endothelial cells in the sinusoid (Fig. 3B).

Double immunostaining for LRAT and CRBP-1 in the human liver specimens showed LRAT+/CRBP-1+ cells in HSCs in the parenchyma (arrows) and myofibroblasts in the portal area (arrowheads) (Fig. 4A). The double immunofluorescence staining for LRAT and CRBP-1 in the human fibrotic liver detected by fluorescence microscopy showed colocalization of both markers in the HSCs in the parenchyma (Fig. 4B–D).

Subsequently, to investigate the relationship between the distribution of LRAT+/CRBP-1+ cells and liver fibrosis, double immunofluorescence staining for LRAT and CRBP-1 was performed for all 24 cases. The number of LRAT+/CRBP-1+ cells/field was counted in 3 random fields each in the parenchyma, portal area and fibrous septa (only F3 and F4) in each case. The numbers in the upper left corner of each image indicate the average number of LRAT+/CRBP-1+ cells/mm^2. In the parenchyma, the number of LRAT+/CRBP-1+ cells in the space of Disse was not significantly different among F0-F4 (Fig. 5A–E). In the portal area, LRAT+/CRBP-1+ cells were hardly seen in normal liver (Fig. 5F). In F1 and F2, the LRAT+/CRBP-1+ cells were observed mainly at the periphery of the portal area (arrows) (Fig. 5G,H). The number of both positive cells was almost same in F1 and F2. In F3, F4, a large number of double positive cells were seen more than in F1 or F2 (Fig. 5I,J). In addition, in the fibrous septa in F3 and F4, a larger number of positive cells were seen than in the portal area of F3 or F4 (Fig. 5K,L).

Statistical analysis (Fig. 6) showed that the average number of positive cells in the parenchyma was not significantly different. The number of F0 in the portal area was statistically different from the number of F1 to F4 (a-b: $P < 0.005$; a-c: $P < 0.0002$; a-d: $P < 0.03$; a-e: $P < 0.04$). In addition, the average number of double positive cells was increased with the progression of fibrosis. Moreover, the average number of fibrous septa in F3 and F4 was statistically different from that of the portal area in F1 and F2 (b-f: $P < 0.005$; c-f: $P < 0.003$; b-g: $P < 0.0001$).

Discussion

HSCs in the space of Disse definitively contribute to fibrogenesis in parenchymal injury, such as alcoholic liver disease and non-alcoholic steatohepatitis, by transdifferentiation to activated HSCs (myofibroblasts). On the other hand, the origin of fibrogenic cells associated with portal fibrosis was unclear. Some agree that the

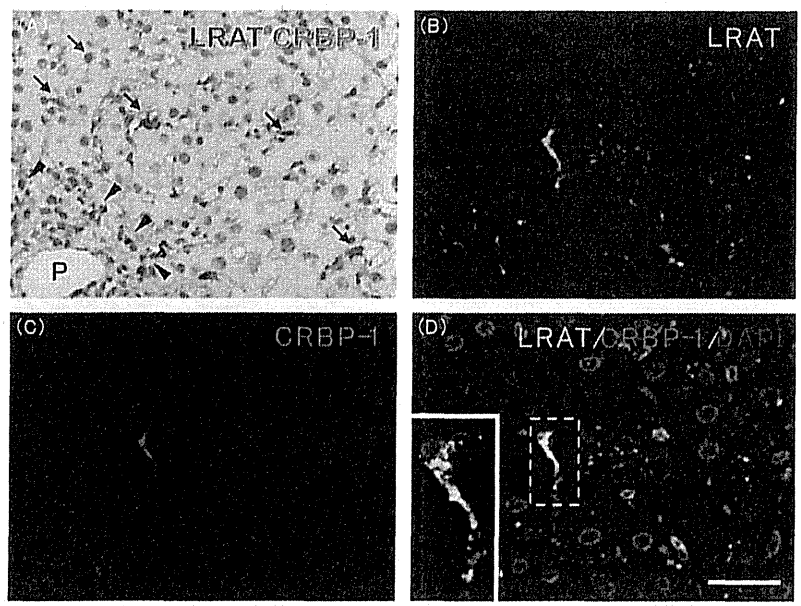

Fig. 4. (A) Double immunostaining for LRAT (red labelling) and CRBP-1 (brown labelling) of the human liver (METAVIR score of F2A2). LRAT+/CRBP-1+ cells were observed in HSCs in the parenchyma (arrows) and myofibroblasts in the portal area (arrowheads). (B–D) Double immunofluorescence staining for LRAT (green labelling) and CRBP-1 (red labelling) of human fibrotic liver with a METAVIR score of F2A2. The HSC in the space of Disse showed reactivity for LRAT (B) and CRBP-1 (C), and colocalization of LRAT and CRBP-1 (yellow double labelling) (D). Bar 20 μm.

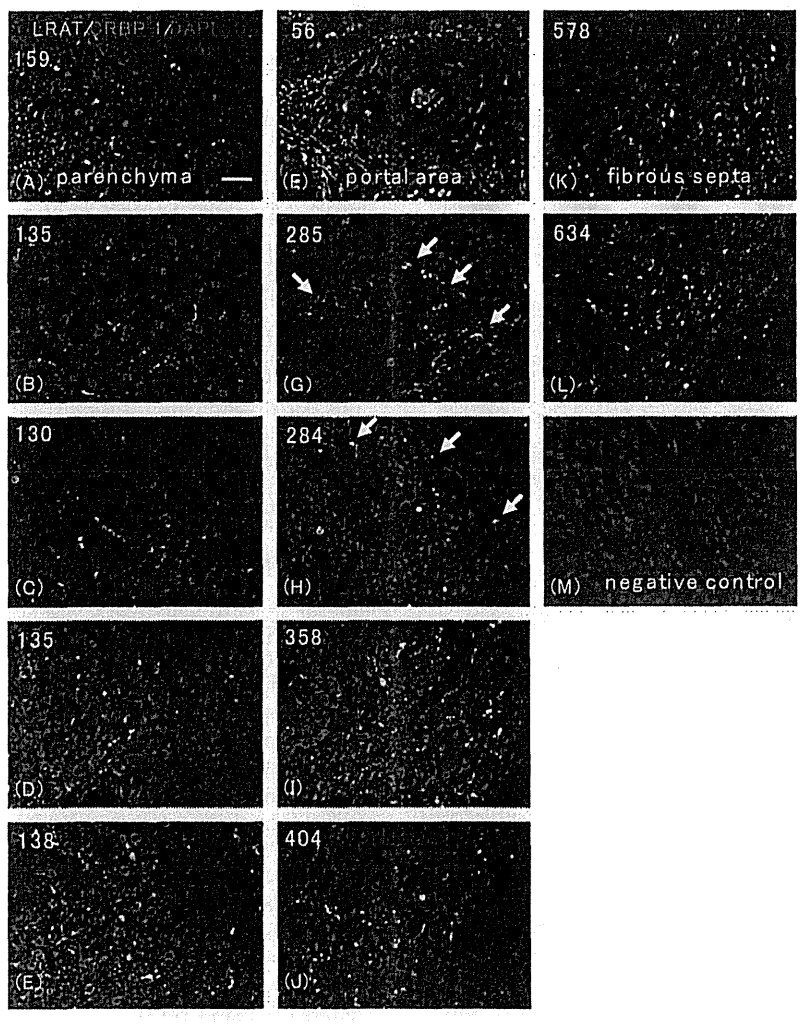

Fig. 5. Double immunofluorescence staining for LRAT and CRBP-1 in the parenchyma (A–E), portal area (F–J), and fibrous septa (K, L). The numbers in the upper left corner of each image indicate the average number of LRAT+/CRBP-1+ cells/mm^2. In the parenchyma, the number of LRAT+/CRBP-1+ cells in the space of Disse was not significantly different among the F0–F4 tissues (A–E). In the portal area, LRAT+/CRBP-1+ cells were hardly seen in the normal liver (F). In the F1 (G) and F2 (H) samples, LRAT+/CRBP-1+ cells were seen mainly at the periphery of the portal area (arrow). The number of both positive cells was almost the same in F1 and F2 tissues. In the F3 (I) and F4 (J) tissues, a large number of double positive cells were seen than in the F1 (G) or F2 (H) tissues. In addition, in the fibrous septa in F3 (K) and F4 (L) tissues, a larger number of positive cells were seen than in the portal area of F3 (I) or F4 (J) tissues. Negative control studies were also done using a Negative Control Rabbit IgG (M). Bar; 20 μm.

fibrogenic cells associated with portal fibrosis are portal fibroblasts (6, 40), or the cells derived from bone marrow (2–5); however, a few people believe that the fibrogenic cells are HSCs (41, 42). This study examined the *in situ* distribution of HSCs that expressed LRAT and CRBP-1 proteins was in human normal and pathological livers, particularly those with hepatitis B and C, to determine their contribution to HSCs on portal fibrosis. Some of the cells that coexpressed both LRAT and CRBP-1 were seen in the portal area and fibrous septa, especially in diseased liver. This result suggests that the cells that have the characteristic profile of HSCs can exist in the fibrous area. This suggests that HSCs in the space of Disse migrate from lobules to the fibrous area or are involved with the fibrous area, since these cells were particularly seen in fibrous expansion of the portal area and fibrous septa than in the portal tracts. These cells represented less than 20% of all cells/field (data not shown). Therefore, HSCs seem to partially contribute to a portal fibrosis. It is possible that cells other that HSCs mainly contributed to portal fibrosis. Ramadori *et al.* reported that the myofibroblasts in the fibrous septa closely resembled the (myo)fibroblasts in the portal field according to an immunohistochemical study using

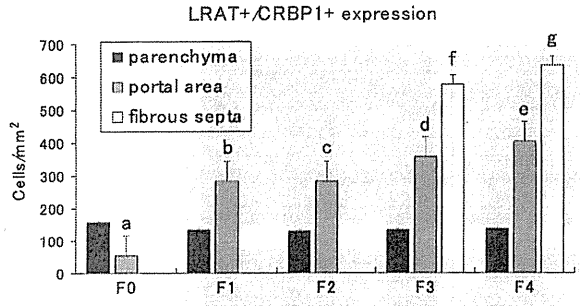

Fig. 6. The average ratio of the number of LRAT+/CRBP-1+ cells. The average number of positive cells in the parenchyma (black bars) was not significantly different. The number of F0 in the portal area (grey bars) was statistically different from the number of F1 to F4 (a-b: $P < 0.005$; a-c: $P < 0.0002$; a-d: $P < 0.03$; a-e: $P < 0.04$). In addition, the average number of double positive cells was increased with the progression of fibrosis. Moreover, the average number of fibrous septa (white bar) in F3 and F4 was statistically different from that of the portal area in F1 and F2 respectively (b–f: $P < 0.005$; c–f: $P < 0.003$; b–g: $P < 0.0001$).

in vivo HSC markers (6). Portal fibrogenesis may involve HSCs as well as portal fibroblasts.

We observed that the LRAT+/CRBP-1+ cells in the fibrous area did not contain lipid droplets in the cytoplasm, in contrast to the HSCs in the space of Disse. These cells apparently do not store vitamin A. Vitamin A from food absorbed from the small intestine is taken up by hepatocytes (liver parenchymal cells) in the form of retinyl ester through several metabolic steps (20). Retinyl ester is hydrolyzed to retinol again and bound to RBP4 in hepatocytes (43). A high concentration of retinol-bound RBP4 (holo-RBP4) in hepatocytes is secreted into the space of Disse. Excessive retinol likely diffuses into HSCs and becomes bound to CRBP-1. LRAT consists of esterified retinol to retinyl esters packed in lipid droplets in the cytoplasm. This vitamin A storing system is useful for maintaining the plasma retinol level at a nearly constant concentration (~1–3 9 10^{-6} mol/L) (43) to prevent toxicity because of hypervitaminosis A. The concentration of retinol in the space of Disse is apparently higher than that observed in the plasma following its release from hepatocytes after the ingestion of food. However, the concentration of retinol in the fibrous area seems to be similar to the plasma retinol level, since the blood supply in the fibrous area is from the portal veins or hepatic arteries. Since the retinol level as a substrate of LRAT is lower in the fibrous area than in the space of Disse, retinyl ester is not synthesized, even in LRAT+/CRBP-1+ cells. There are vitamin A storing-cells in other organs such as the lung, kidney, intestine and pancreas (7, 44–46). Definite lipid droplets in these sites are never seen under normal conditions, though lipid droplets increase in a rat hypervitaminosis A experimental model (44). In particular, pancreatic stellate cells are nearly identical to HSCs and both are presumed to share a common origin (47, 48). Stellate cells in other organs do not store vitamin A because of the lack of sufficient retinol as a substrate of LRAT as there is no microenvironment like the space of Disse which presents a high concentration of retinol . This condition is similar to the fibrous area in the human diseased liver under normal vitamin A conditions (normal nutritive status). Moreover, Fonseca *et al.* reported that fat-storing cells appear in the portal area and fibrous septa in chronic hepatitis model using C. hepatica-infected rats with hypervitaminosis A (42). This finding is consistent with the hypothesis that stellate cells in lobules migrate to the fibrous area including the portal area and contribute to fibrogenesis when liver damage occurs.

In conclusion, the present results provide direct evidence that the cells coexpressing both LRAT and CRBP-1 proteins contribute in part to the development of portal fibrogenesis in addition to parenchymal fibrogenesis in patients with viral hepatitis.

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Conflicts of interest: The authors do not have any disclosures to report.

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