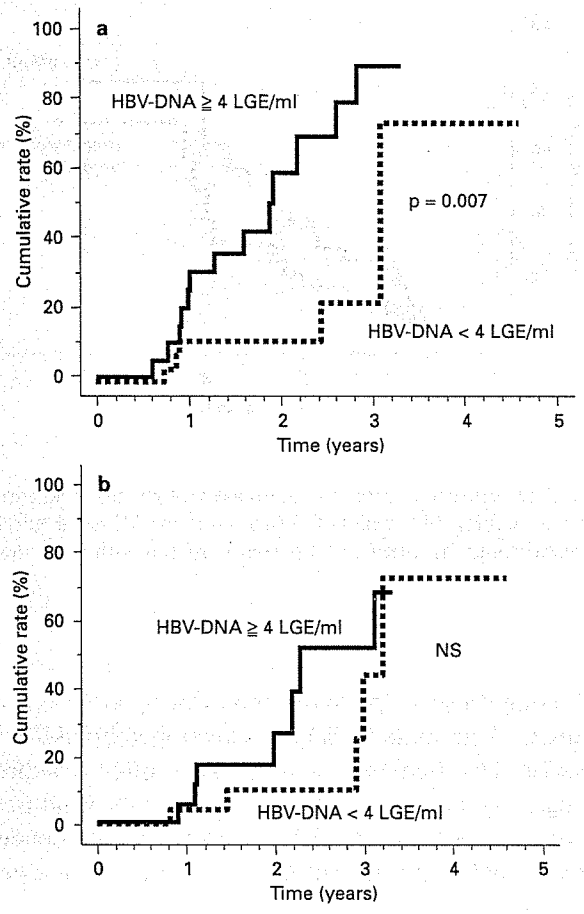

Fig. 2. The cumulative rate of viral breakthrough and breakthrough hepatitis according to HBV-DNA levels after 24 weeks of lamivudine therapy. Patients were divided into 2 groups according to HBV-DNA levels after 24 weeks of lamivudine therapy. Kaplan-Meier plots of time to viral breakthrough (**a**) and time to breakthrough hepatitis (**b**) are shown. Log-rank tests show that higher HBV-DNA levels after 24 weeks of lamivudine therapy are associated with the more rapid development of viral breakthrough (p = 0.007) but not with breakthrough hepatitis.

Resistance-Associated Mutations, HBV-DNA Levels after Viral Breakthrough

At the time of viral breakthrough, a polymerase gene mutation in domain B (rt180M) was detected in 13 patients, and a mutation in domain C was detected in 19 patients (rt204I in 10, rt204V in 6 patients and a mixture of rt204I and rt204V in 3 patients). Six patients had a single rt204I mutation; 4 patients had rt180M/rt204I double mutations; 6 patients had rt180M/rt204V double mutations; and 3 patients had rt180M/rt204I/V (mixture) double mutations. The emergence of the rt180M mutation was significantly associated with breakthrough hepatitis: breakthrough hepatitis occurred in 11 of 13 patients with the rt180M mutation, while only 1 of 6 patients with rt180wt (baseline wild-type) developed significant biochemical changes (p = 0.01, Fisher's exact test; table 2). The positive predictive value of the presence of rt180M was 85%, and the negative predictive value was

Table 1. Pretreatment variables in association with viral breakthrough and breakthrough hepatitis

	Viral breakthrough		Breakthrough hepatitis	
	no (n = 34)	yes (n = 19)	no (n = 41)	yes (n = 12)
Gender (male/female)	22/12	10/9	16/25	5/7
Age, years	49.8 ± 11.9	47.1 ± 11.8	49.2 ± 11.5	47 ± 13.3
ALT, U/l	255 ± 338	141 ± 128	230 ± 314	159 ± 148
HBeAg positive/negative	17/17	6/13	22/19	8/4
HBV-DNA, LGE/ml	7.0 ± 1.1	7.3 ± 1.1	7.1 ± 1.1	7.2 ± 1.2
CP mutations (M/W)	25/6	19/0	32/6	12/0
PC mutation (M/W)	14/17	7/12	15/23	6/6

HBeAg = Hepatitis Be antigen; CP = core promoter; PC = precore; M = mutant type; W = wild type. There were no variables that had a statistically significant association with viral breakthrough or with breakthrough hepatitis.

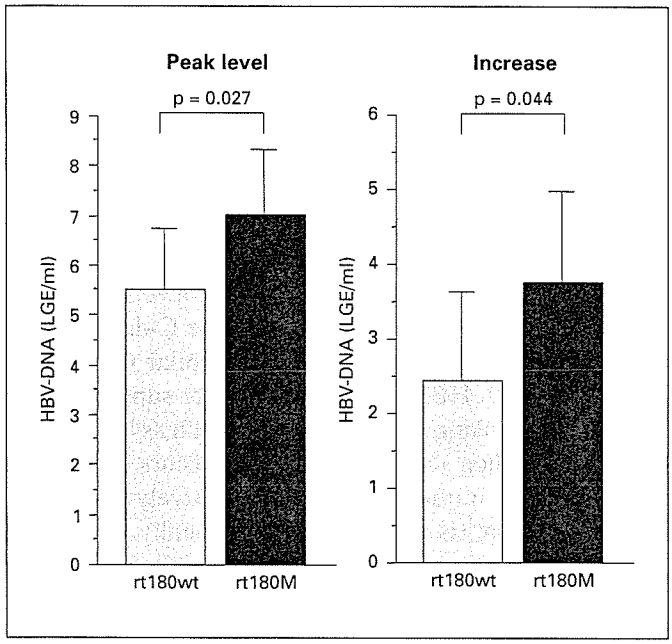

Fig. 3. The association between domain B mutations and HBV-DNA levels after viral breakthrough. After viral breakthrough, patients with the rt180M mutation had higher peak levels of HBV-DNA (p = 0.027) and larger increases of HBV-DNA (p = 0.044) compared to patients with rt180wt.

Table 2. Mutational patterns of the polymerase gene in association with breakthrough hepatitis

Mutational pattern	Breakthrough hepatitis		
	no (n = 7)	yes (n = 12)	p value
rt180M/rt180wt	2/5	11/1	0.01
rt204I/rt204V/rt204V+I	5/2/0	5/4/3	NS
rt204V (yes/no)	2/5	7/5	NS
rt204I (yes/no)	5/2	8/4	NS

NS = Not significant.

83%. Patients with the rt180M mutation had a 27.5-fold higher probability of breakthrough hepatitis compared to those patients with rt180wt (logistic regression analysis, 95% CI 2.00–378.93, p = 0.013). In contrast, mutational patterns of rt204 were not associated with breakthrough hepatitis: only 8 of 13 patients with rt240V mutation and 5 of 10 patients with rt240I developed breakthrough hepatitis (p = 0.35, Fisher's exact test; table 2).

During viral breakthrough, patients with the rt180M mutation had a larger increase in HBV-DNA levels (3.7 ± 1.2 vs. 2.4 ± 1.2 LGE/ml, p = 0.044) and higher peak values of HBV-DNA (7.0 ± 1.3 vs. 5.5 ± 1.2 LGE/ml, p = 0.027) compared to those patients with rt180wt (fig. 3).

Discussion

In this study, we found that the rt180M mutation in domain B of the HBV polymerase gene was significantly associated with breakthrough hepatitis during long-term lamivudine therapy in patients with chronic hepatitis B from genotype C. The positive predictive value of this mutation for breakthrough hepatitis was 85%, and the negative predictive value was 83%. The rt180M mutation was always detected as a double mutation with an rt204 mutation. Moreover, it was linked to higher subsequent levels of HBV-DNA during viral breakthrough compared to similar episodes in patients with a single rt204 mutation. Conversely, the mutational patterns of rt204, namely rt204I or rt204V, were not significantly associated with breakthrough hepatitis. Thus, the development of an rt180M mutation in patients on long-term lamivudine therapy might be a useful predictor for breakthrough hepatitis. As such, the presence of this mutation may also be helpful in deciding whether or not to proceed with alternative or additive nucleoside analogues as salvage therapy.

Lamivudine plays an important role in the treatment of patients with chronic hepatitis B. Short-term treatment is insufficient for clearing the virus [8], while long-term treatment is associated with the development of drug-resistant HBV. These strains of drug-resistant HBV do not always precipitate a relapse of hepatitis [12, 15], but the mechanism and predictive factors for the differing clinical outcomes have not been identified.

Previous studies examined factors such as genotype [21], ALT levels [32–34], HBV-DNA levels prior to therapy [21, 33–35], the degree of decline in HBV-DNA levels during therapy [17, 36, 37], the presence of HBeAg [32, 35, 38], and the presence of core promoter mutations [39]. While all of these factors have been found to be related to the appearance of resistant virus strains, the data are inconsistent, and the ultimate role of these factors remains controversial [36]. Moreover, how these and other factors are related to different clinical outcomes after the reappearance of HBV has not been elucidated. This study attempts to identify those predictive factors for break-

through hepatitis during long-term lamivudine therapy that may have an important impact on clinical outcomes.

We found that pretreatment variables, such as mutations in the core promoter and precore genes, HBV DNA levels, ALT levels, and the presence of HBe antigen, were not associated with viral breakthrough or with breakthrough hepatitis. This finding leads us to believe that identifying high-risk patients prior to initiating lamivudine therapy may not be possible. All cases with breakthrough had mutation in the core promoter, but the difference did not reach statistical significance possibly due to the small number of patients with the wild-type sequence in the core promoter. Further study including a larger number of patients with the wild-type sequence in core promoter may be necessary to elucidate the significance of the mutation in the core promoter in breakthrough.

However, when patients were analyzed after 24 weeks of lamivudine therapy, we found that HBV-DNA levels were significantly associated with the development of viral breakthrough, a finding consistent with previous reports [17, 36, 37]. Additionally, higher rates of spontaneous mutations in the viral genome are likely to be associated with higher replication levels [40], leading to the emergence of resistant HBV. Given these findings, monitoring for HBV-DNA levels at 24 weeks may be useful in targeting patients at higher risk for viral breakthrough but not necessarily for breakthrough hepatitis.

When parameters at the time of viral breakthrough were analyzed, the rt180M mutation was significantly associated with the occurrence of breakthrough hepatitis. Among those we studied the rt180M was the sole maker that was associated with breakthrough hepatitis, therefore close monitoring and detection of this mutation may be useful clinically in the prediction of breakthrough hepatitis.

The mechanism underlying the association of rt180M mutation with breakthrough hepatitis is not well understood. Usually, the rt204V/I mutant virus is less efficient at replication than the rt204wt virus. However, in tissue culture, the mutants with both B and C domain mutations (rt180M and rt204I/V) have higher reverse transcriptase activity and replication capacity than those single C-domain mutants (rt180wt and rt204I/V). Domain C rt204 is located in the conserved YMDD motif of the RNA-dependent RNA polymerase and is involved in nucleotide binding [41], while domain B is involved in template positioning [42]. Since these two domains interact in the molecular model of HBV reverse transcriptase [43], it might be speculated that the rt180M mutation interacts with the rt204 mutations. In essence, in double mutants, the B-domain mutation rt180M may compensate for the replication defective C-domain mutants [44], thus accounting for the higher reverse transcriptase activity and replication capacity compared to single C-domain mutants. These in vitro findings are consistent with our findings that patients with both B and C domain mutants were associated with higher HBV-DNA levels during viral breakthrough than those with single C-domain mutants. It has also been suggested that cellular immune responsiveness to HBV may increase after suppression of viremia when using antiviral therapy such as lamivudine [45]. Thus, when viral breakthrough occurs in patients whose cellular immunity has been relatively restored, a relapse of hepatitis may occur. Additionally, the amino acid position 180 may be a more vulnerable target to the restored immune system, a possibility that needs to be examined by further study.

In conclusion, the results of this study offer potentially important clinical ramifications for patients infected with genotype C hepatitis B who are on long-term lamivudine therapy. The quantification of HBV viremia after 24 weeks of lamivudine therapy may predict patients who are at a higher risk for viral breakthrough. Closer monitoring may thus be warranted in those patients with HBV-DNA levels of >4 LGE/ml. When viral breakthrough does occur, genotypic assays for the rt180M mutation should be performed since this mutation may be predictive of hepatitis relapse.

It is our belief that using both a quantitative assay for HBV viremia at 24 weeks and genotypic assays for polymerase mutant detection after viral breakthrough may serve as an effective means of monitoring long-term lamivudine therapy in chronic hepatitis B genotype C patients. Together, the results from these assays can provide useful information that may influence the decision to initiate early induction of salvage therapy.

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Development of Hepatocellular Carcinoma after Interferon Therapy in Chronic Hepatitis C

Is It Possible to Reduce the Incidence by Ribanirin and IFN Combination Therapy?

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Key Words
Chronic hepatitis C · Hepatocellular carcinoma · Interferon · Ribavirin

Abstract
Objectives: Although the incidence of hepatocellular carcinoma (HCC) has been shown to be reduced after interferon (IFN) monotherapy in chronic hepatitis C, the risk factors for the development of HCC have not been fully understood. The aim of this study is to investigate the risk factors for the development of HCC after IFN in chronic hepatitis C as well as whether the incidence of HCC will be reduced by ribavirin and IFN combination therapy or not. ***Methods:*** 495 patients with chronic hepatitis C and which received IFN monotherapy were followed and the incidence and risk factors for the development of HCC were examined. On the other hand, in the patients which received ribavirin and IFN combination therapy, the sustained response rate was assessed and the reduction rate of HCC development was predicted. ***Results:*** Multivariate analysis by the Cox proportional hazard model revealed that the risk factors for HCC development were age, male gender, severe fibrosis and outcome of IFN therapy. On ribavirin and IFN combination therapy, the sustained response rate reached 17.3% in genotype 1b and 74% in genotypes 2a and 2b infection, thus reducing 20% of the estimated incidence of HCC. ***Conclusion:*** To reduce the incidence of HCC in chronic hepatitis C, improvement of the sustained response rate is an essential issue, and ribavirin and IFN combination therapy shows to be promising.

Introduction

Hepatitis C virus (HCV) infection is a major risk factor for the development of both liver cirrhosis and hepatocellular carcinoma (HCC) [1]. Recent epidemiological data highlight the fact that HCC associated with long-term HCV infection in a serious health care problem in regions such as Japan where HCV is widely endemic [2]. In Japan, HCV infection consists of 80% of the cause of hepatocellular carcinoma.

Interferon (IFN) monotherapy has been performed since 1992 in Japan for the treatment of hepatitis C which results in viral eradication in approximately 20–30% of the patients who received at least 6 months' treatment [3]. The viral eradication rate has been shown to be closely

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associated with genotype and viral load as well as viral mutation in genotype 1b infection [4]. An important question is whether IFN therapy is effective in reducing the incidence of HCC in the patients with chronic hepatitis C. Kasahara et al. [5] reported that the incidence of HCC was reduced by IFN in sustained responders; thus, improving the response rate is an essential issue to reduce the incidence of HCC.

Rivabirin and IFN combination treatment has been used in patients with chronic hepatitis C, which showed improvement of the sustained response rate from IFN monotherapy [6]. In Japan, this combination therapy is allowed for the treatment of patients with chronic hepatitis C at a limited duration of 24 weeks; however, the sustained response has been shown to improve especially in genotype 1b infection.

In the present study, the incidence and risk factors of the development of HCC after interferon therapy were examined. The reduction of occurrence in HCC was predicted after 24 weeks' treatment with ribavirin and IFN combination therapy.

Table 1. Clinical characteristics of the patients who received IFN monotherapy

Gender	
Male	282
Female	213
Age (mean ± SE)	52.3±0.57
Genotype	
1b	249
2a	63
2b	39
Unknown	141
HCVRNA level, kIU/ml (median 470)	1.1 to >850
Liver biopsy	
F1	132
F2	184
F3	123
F4	35
Total dose of IFN, MU (mean ± 53)	498±53
Outcome of IFN therapy	
SVR	155
NR	312
Development of HCC	
Yes	30
No	464

Patients and Methods

IFN Monotherapy Study

The first IFN monotherapy study included 495 consecutive patients with chronic hepatitis C in whom 24 weeks of IFN monotherapy was carried out from January 1994 to December 2001. The clinical characteristics of the patients are shown in table 1. The mean age is 52.3 years, and the HCV genotype was examined using the mixed-primer method [7]. Plasma level of HCVRNA was measured by amplicore monitor (version 2, Roche, Basel). The histological findings were classified according to established international criteria [8]. The median dosage of administered IFN was 640 MU, and sustained virological response (SVR) was defined as negative HCVRNA 6 months after interferon therapy and 155 patients achieved SVR. Otherwise, the patients were defined as non-responders. This study was in accordance with the Helsinki Declaration of 1975 (revised in 1983) and written informed consent was obtained from all the patients included in this study.

The diagnosis of HCC was established by CT scan during hepatic arteriography (CTHA) and arterio-portography via the superior mesenteric artery as well as needle biopsy of the nodule. Development of HCC was observed in 30 patients during the observation period.

Ribavirin and IFN Combination Study

In 227 patients with chronic hepatitis C from December 2001 to November 2002, ribavirin and IFN combination therapy were carried out. Ribavirin was administered 800 mg per day in the patients having body weight 60 kg or more, and 600 mg with less than 60 kg. IFNα-2b of 6 MU was administered everyday during the initial two weeks followed by 3 times per week for remaining 22 weeks. The clinical characteristics are shown in table 2. The therapy was discontinued in 12 patients because of anemia, appetite loss, depression, and skin rash. Dose reduction of ribavirin was necessary in 21 patients because of anemia. Thus, the outcome of the combination therapy was assessed in 215 patients.

Statistical significance was assessed by Student's t test, χ^2 analysis with Yates' correction, and Kaplan-Meier method using the log-rank test as indicated. Multivariate analysis was carried out by the Cox proportional hazard model.

Results

The development of HCC was observed in 31 patients after IFN monotherapy. The clinical characteristics of the patients which developed HCC was evaluated by univariate analysis. A statistically significant difference was noted in age, gender, genotype, fibrosis of the liver and outcome of interferon therapy. The serum HCVRNA level before treatment and the serum ALT level were not different between the two groups (table 3).

The incidence of HCC after interferon therapy was compared according to the fibrosis score of the liver. The incidence of HCC was 0.3% per year in the patients with F1 and 1.1% per year in F2; however, it was 3.8% in the F3 groups. The development of HCC was significantly higher in the patients in the F3 and F4 groups than those in the F1 and F2 groups (Kaplan-Meier method, log-rank test, $p < 0.01$; fig. 1).

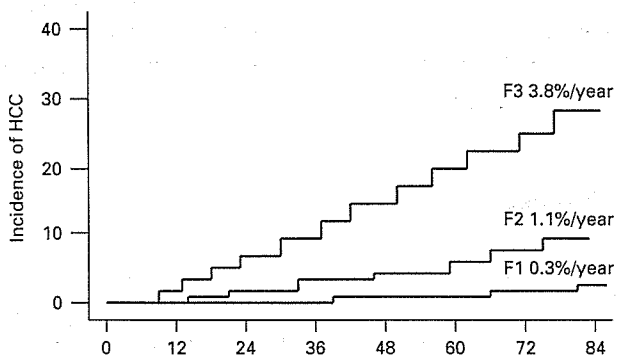

Fig. 1. Incidence of HCC was 0.1% in patients with fibrosis score F1, 1.1% in F2 and 3.8% in F3 (Kaplan-Meier method).

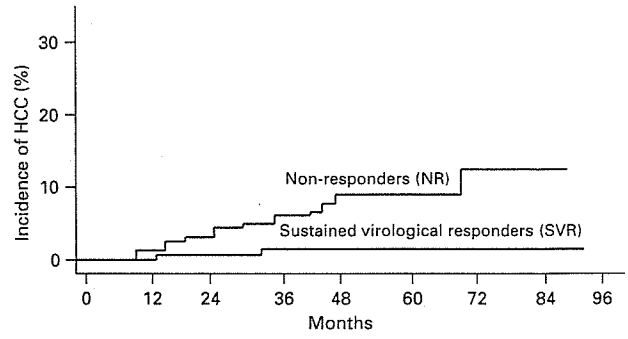

Fig. 2. Incidence of HCC was compared between sustained virological responder and non-responder patients. HCC development was significantly higher in the non-responders than in the sustained virological responders after IFN monotherapy.

Table 2. Clinical characteristics of the patients received ribavirin and IFN combination therapy

Gender	
Male	126
Female	101
Age (mean ± SE)	58.4 ± 1.2
Genotype	
1b	181
2a	30
2b	15
Mixed	1
HCVRNA level, kIU/ml (median 680)	67 to >850
Liver biopsy	
F1	86
F2	75
F3	64
F4	2
Outcome of IFN therapy	
SVR	61
NR	154
Withdrawal	12

Table 3. Comparison of the patients with or without development of HCC after IFN therapy (univariate analysis)

Development of HCC	Yes (n = 31)	No (n = 464)	p
Gender			
Male	22	260	<0.05
Female	9	204	
Age	60 ± 1.2	52 ± 0.6	<0.001
Genotype			
1b	22	228	<0.01
2a and 2b	1	102	
HCVRNA level, kIU/ml	512 ± 34	496 ± 18	n.s.
Liver biopsy			
F1 and F2	7	309	<0.001
F3 and F4	23	135	
ALT, IU/l	125 ± 8.3	118 ± 20	n.s.
Outcome of IFN therapy			
SVR	3	152	<0.01
NR	28	312	

The incidence of HCC was compared between sustained responders and non-responders. The incidence of HCC in sustained responders was 0.2% per year in the sustained responders; however, it was 3.9% per year in the non-responders. This difference was statistically significant (fig. 2).

Multivariate analysis using Cox hazard model was done. Age, gender, fibrosis of the liver and outcome of interferon were found to be independent risk factors (table 4). Among these risk factors, age, gender and fibrosis of the liver cannot be changed. Thus, to reduce the incidence of HCC, the improvement of sustained virological response is an important issue.

Since the end of 2001, ribavirin and IFN combination therapy for 24 weeks has been allowed in Japan, and 235 patients have been treated. The sustained virological response rate in genotype 1b dividing them according to their HCVRNA level before treatment. In the patient

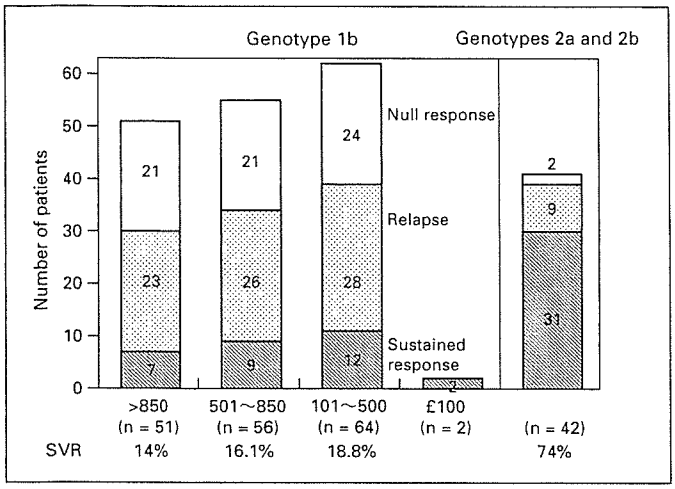

Fig. 3. Outcome of ribavirin and IFN combination therapy. ▨ = Sustained response; ▩ = relapse; □ = null response.

Table 4. Risk factors for the development of HCC after IFN monotherapy (Cox proportional hazard model)

Variable	Odds ratio	95% CI	p
Age (>56 vs. <55)	7.5	2.3–14.6	<0.005
Gender (male vs. female)	1.9	1.1–27.4	<0.05
Fibrosis (F3 and F4 vs. F1 and F2)	3.7	1.8–18.6	<0.01
Outcome of IFN (NR vs. SVR)	2.8	1.2–23.6	<0.05

group with a HCVRNA level higher than 850 kIU/ml, the sustained virological response rate was 0% by interferon monotherapy, while it was 14.0% by ribavirin and interferon combination therapy for 24 weeks. Similarly, it was 3.7% with a HCVRNA level from 500 to 850 kIU/ml on monotherapy, but it was 16.1% on combination therapy. The sustained virological response rate was 13.1% on monotherapy in those with a HCVRNA level from 100 to 500 kIU/ml, while it was 18.8% on combination therapy. However, a relapse rate, i.e. reappearance of HCVRNA after discontinuation of combination therapy, of 40–50% was observed in each group, and null response, i.e. no achievement of negative plasma HCVRNA during combination treatment, of around 30% was observed in each group. In the patients with genotype 2a and 2b infection, a sustained virological response was achieved in 74% (fig. 3).

Since the incidence of HCC reduced from 3.9% per year in non-responders to 0.2% per year in sustained virological responders, the incidence of HCC after treatment has been estimated to be reduced from 3.1 to 2.8% per year overall with 24 weeks' treatment with rivabirin and IFN combination therapy.

Discussion

HCC is the most life-threatening problem in the long-term course of chronic hepatitis C. The rising incidence of HCC has been pointed out not only in Japan but in the United States [9] and Europe [10]. Therefore, prevention of the development of HCC is an important issue in the clinical setting. In the present study, we analyzed the incidence and risk factors of HCC after IFN monotherapy in patients with chronic hepatitis C. The risk factors for the development of HCC were found to be age, male gender, fibrosis of the liver, and outcome of IFN therapy. Kasahara et al. [5] reported that the incidence of HCC was reduced by IFN in sustained responders, which is consistent with our data. They also reported that age, male gender and severe fibrosis of the liver were risk factors for the development of HCC. Imai et al. [11] reported similar risk factors for the development of HCC after IFN monotherapy in HCV-infected patients. Therefore, the liver fibrosis score is likely to be one of the most important risk factors for the subsequent development of HCC in HCV-infected patients, even following IFN therapy. Our data demonstrating that the degree of hepatic fibrosis is an independent risk factor for the development of HCC associated with HCV infection is certainly consistent with this supposition. Among these risk factors, age, male gender and fibrosis score of the liver cannot be changed before IFN therapy; thus, to reduce the incidence of HCC, improvement of the sustained response rate is an essential issue in patient care of HCV infection.

Recently, HCC-free survival could be obtained by IFN in patients with chronic hepatitis C, and the gain in HCC-free survival was greater when a patient was younger and fibrosis of the liver was more advanced [12]. The gain in HCC-free survival was calculated as difference between expected HCC-free survival with sustained virological response and that without. In this setting, improvement in achieving a sustained response is the central issue. Furthermore, the risk of death from liver-related disease was significantly reduced not only in sustained virological responders but also in biochemical responders in chronic hepatitis C [13].

Although the incidence of HCC has not been investigated after ribavirin and IFN combination therapy, HCC

development seems to be reduced by combination therapy by improving the sustained response rate, especially in genotype 1b infection. In the present study, the incidence of HCC is estimated to be reduced from 3.1 to 2.8% per year by combination therapy for 24 weeks. However, the sustained virological response rate has been shown to improve in genotype 1 infection by extended combination therapy for 48 weeks or by peginterferon-alfa-2b instead [14]. Thus, to reduce the incidence of HCC, extended treatment with ribavirin and IFN for 48 weeks is necessary in genotype 1b infection.

In the patients with HCV infection, the recurrence rate of HCC in the liver is as high as 20% per year, even after complete curative treatment was given to the primary HCC nodule [15]. The recurrence rate and prognosis was improved after elimination of hepatitis C virus RNA by IFN [16]. Furthermore, previous IFN therapy was shown to reduce the multicentric recurrence of HCC and improve the patients' survival in chronic HCV infection [17]. The rate of first recurrence of HCC was similar in patients treated with IFN and in untreated patients, but in the patients treated with IFN after curative treatment was given to the primary HCC nodule, the rate of second or third recurrence was lower than in the untreated group [15]. Moreover, IFN therapy enhanced patient survival after treatment of the HCC nodule.

From these results, it is concluded that IFN reduced the risk of the development of HCC when a sustained virological response was achieved in chronic hepatitis C. To reduce the risk of the development of HCC, it is an essential issue to improve the sustained response rate by prolonged ribavirin and IFN combination therapy.

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G to A Hypermutation of Hepatitis B Virus

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G to A hypermutation of the human immunodeficiency virus type 1 (HIV-1) is induced by a deaminase APOBEC3G and is related to host antiviral defense. APOBEC3G has also been found to reduce the replication of HIV-1 by an unknown mechanism. This enzyme also reduces the production of hepatitis B virus, although the mechanism for this action has not been clearly elucidated. The hypermutated hepatitis B virus (HBV) is rarely found in usual sequencing analyses. Using peptide nucleic acid mediated by polymerase chain reaction clamping, we detected the hypermutated HBV DNA in 1 of 8 patients with acute HBV infection and 4 of 10 with chronic HBV infection. In the latter group, hypermutated genomes were found only in eAb-positive patients. As much as 72.5% of G residues were mutated in the hypermutated clones. G to A substitutions were predominant in almost all clones sequenced compared with other substitutions. G to A mutated viral genomes also were found in HepG2–derived cell lines that continuously produced HBV into the supernatant. Both alpha and gamma interferon reduced virus production in these cell lines, but they did not alter the frequency of the hypermutation. Transcripts of APOBEC3G, as well as some other deaminases, were found in these cell lines. In conclusion, our results show that part of the minus strand DNA of HBV is hypermutated both *in vitro* (HepG2 cell lines) and *in vivo*. The role and mechanism of hypermutation in reducing HBV replication should be further investigated to understand the anti-HBV defense system. (HEPATOLOGY 2005;41:626-633.)

Hepatitis B virus (HBV) is a small enveloped DNA virus that replicates in hepatocytes in a noncytolytic manner. Chronic infection with the virus often leads to chronic hepatitis and liver cirrhosis. Hepatocellular carcinoma arises in chronic carriers at a higher frequency than noninfected individuals.[1-4]

The replication cycle of the HBV includes pregenome RNA synthesis and reverse transcription, resulting in the production of the minus strand DNA, which serves as a template of the plus strand DNA.[5] The life cycle of this virus resembles that of the human immunodeficiency virus 1 (HIV-1), which also replicates through reverse transcription.[6]

Recent reports showed that a cytosine deaminase APOBEC3G (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G), which is packaged in HIV-1 virions, induces G to A hypermutation to a nascent reverse transcript of HIV-1, which contributes in part to the innate antiviral activity.[7-10] The antiviral activity of APOBEC3G is species specific[11,12] and may represent the different actions of the protein.[13,14] The virion infectivity factor encoded by lentivirus genomes associates with APOBEC3G to prevent the enzyme from being packaged into virions and triggers its proteasomal degradation.[15-18] The negative strand DNA of the HBV might be a target of such antiviral deaminase activity. In fact, naturally occurring HBV genomes bearing the hallmarks of retroviral G to A hypermutation have been reported in clones obtained from 2 HBV carriers.[19] Both of these clones represented subgenomes arising from reverse transcrip-

Abbreviations: HBV, hepatitis B virus; HIV-1, human immunodeficiency virus type 1; APOBEC3G, apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B early antigen; PCR, polymerase chain reaction; PNA, peptide nucleic acid.

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tion of packaged spliced mRNA. However, such hypermutated genomes have otherwise never been reported, nor deposited in DNA databases. Moreover, whether such hypermutated sequences are generated in liver cells or in leukocytes is unknown.

Inhibition of HBV replication by APOBEC3G was observed recently in a transient transfection system.[20] However, no induction of hypermutations to the HBV genome was observed. Instead, prevention of pre-genome RNA packaging was observed.

The aims of the current study were to determine the frequency of viral genomes with G to A substitutions in HBV carriers and patients with acute HBV infection, and to determine whether the hypermutated sequences are generated in hepatic cell lines. We identified such hypermutated viral genomes in 5 of 18 HBV carriers and patients with acute HBV infection and the expression of known deaminases that are potentially responsible for the hypermutation in cultured hepatoma cell lines.

Materials and Methods

Serum Samples. Serum samples from 18 adult Japanese patients with HBV infection were studied. At the time of the study, 8 of these patients had acute HBV infection and tested positive for immunoglobulin M anti–hepatitis B core antibody. The remaining 10 patients were chronic carriers. All serum samples were stored at −80°C until examined. All patients were negative for serum markers of both hepatitis C virus and HIV-1 infection, and none was on antiviral treatment.

Serological Markers of HBV Infection. Hepatitis B surface antigen (HBsAg) was detected by enzyme immunoassay (Roche Diagnostics, Basel, Switzerland), and hepatitis B early antigen (HBeAg) as well as anti-HBe were detected by radioimmunoassay (Abbott Diagnostics, Abbott Park, IL). HBV DNA was determined by transcription-mediated amplification and hybridization-protection assay (Chugai Diagnostics, Tokyo, Japan), and the results were expressed as log genome equivalents per milliliter. The lower detection limit of this assay is 3.7 log genome equivalents/mL (equivalent to 5,000 copies/mL). The antibody against hepatitis C virus was tested for by the third-generation enzyme immunoassay (Roche Diagnostics).

Analysis of HBV DNA in Cell Lines That Stably Produce HBV. Two cell lines known to produce wild-type HBV and one cell line known to produce lamivudine-resistant HBV (with mutations of L528M and M552V) were created by transfecting 1.4 genome length sequences of HBV to HepG2 cell lines. These cell lines produced HBV that showed a similar sedimentation in sucrose density gradient centrifugation to HBV extracted from the serum of carriers (M. Tsuge et al., manuscript in preparation) and could infect human hepatocyte chimeric mice (manuscript in preparation). These cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal bovine serum at 37°C and 5% CO_2. Cells were seeded to semiconfluence in 6-well tissue culture plates and then treated with media containing interferon alpha or gamma. After 3 days of interferon treatment, the cells were harvested and lysed with 250 μL lysis buffer (10 mmol/L Tris-HCl [pH 7.4], 140 mmol/L NaCl, 0.5% [vol/vol] NP-40) followed by centrifugation for 2 minutes at 15,000*g*. Replicative intermediate of the HBV was immunoprecipitated and subjected to Southern blot analysis and quantitative analysis by light cycler. The effect of lamivudine was analyzed similarly, except that cells were harvested after 5 days of treatment.

Detection of Hypermutated Clones by Polymerase Chain Reaction With PNA Clamping, Cloning, and Sequencing. HBV DNA was extracted from 100 μL serum or culture supernatant by SMITEST (Genome Science Laboratories, Tokyo, Japan) and was dissolved in 20 μL H_2O. The first round of polymerase chain reaction (PCR) was performed with an outer primer set (PLF1 and BR112 [Table 1]) and a second-round PCR with an inner primer set (PLF2 and PLR2 [Table 1]). The peptic nucleic acid (PNA) oligonucleotide, initially designed to detect lamivudine-resistant variant genome,[21] was an 18-mer (PNA 552 [Table 1]) that exactly matched the 18-nucleotide sequence of the original YMDD sequence of DNA polymerase/reverse transcriptase, which contained GG and TG sequences (AGT TAT ATG GAT GAT GTG). The PCR with PNA clamping was performed in a total volume of 25 μL, consisting of a reaction buffer (100 mmol/L Tris-HCl [pH 8.3], 50 mmol/L KCl and 15 mmol/L $MgCl_2$), 0.2 mmol/L each of dNTPs, 1 μL of the DNA solution, 12.5 pmol each primer, 150 pmol PNA 552, and 1 unit of Taq DNA polymerase (Gene Taq, Wako Pure Chemicals, Tokyo, Japan) together with 0.2 μg anti-Taq high (Toyobo Co., Osaka, Japan). The amplification conditions included an initial denaturation at 95°C for 4 minutes and 25 cycles of amplification (denaturation at 95°C for 45 seconds, PNA annealing at 73°C for 2 minutes, annealing and extension of primer at 63°C for 50 seconds), followed by a final extension at 63°C for 7 minutes. Part of the X gene was amplified with an outer primer pair (HBV1 and HBV2) and an inner primer (PLF2 and HBV2) (Table 1) for the first- and second-round amplifications, respectively. The amplification for the first-round PCR included initial denaturation at 95°C for 4 minutes and 25 cycles of amplification (denatur-

Table 1. Oligonucleotides and PNAs Used in the Current Study

Primer	Sequence
	HBV amplification
PLF1	5′-GGT ATG TTG CCC GTT TGT CC-3′
BR112	5′-TTC CGT CGA CAT ATC CCA T-3′
PLF2	5′-CCT ATG GGA GTG GGC CTC AG-3′
PLR2	5′-CCA ATT ACA TAT CCC ATG AAG TTA AGG GA-3′
HBV1	5′-CCG GAA AGC TTG AGC TCT TCT TTT TCA CCT CTG CCT AAT CA-3′
HBV2	5′-CCG GAA AGC TTG AGC TCT TCA AAA AGT TGC ATG GTG CTG G-3′
BR109	5′-AAG GGA GTA GCC CCA ACG TT-3′
	PNA
PNA552	H2N-CAC ATC ATC CAT ATA ACT-CON2H
PNA552V	H2N-CAC ATC ATC CAC ATA ACT-CON2H
	Amplification of mRNAs of deaminases
APO1a	5′-CAG AGC ACC ATG ACT TCT-3′
APO1d	5′-ATT GTG GCC AGT GAG CTT CA-3′
APO2a	5′-AGA AGG AAG AGG CTG CTG TG-3′
APO2b	5′-AGA ACG GCT GCC TGC CAA CT-3′
APO2c	5′-GAA GGC TGG CAG GAT GGT GT-3′
APO2d	5′-CAG GTG ACA TTG TAC CGC AG-3′
APO3Aa	5′-TCT TAA CAC CAC GCC TTG AG-3′
APO3Ad	5′-GAA GAT GCG CAG TCT CAC GT-3′
APO3Ba	5′-AGA GCG GGA CAG GGA CAA GC-3′
APO3Bb	5′-GCG TAT CTA AGA GGC TGA AC-3′
APO3Bd	5′-CGA AGG ACC AAA GGG TCA TT-3′
APO3Be	5′-ACA AGT AGG TCT GGC GCC GT-3′
APO3Ca	5′-AGG ACG CTG TAA GCA GGA AG-3′
APO3Cb	5′-CCG ATG AAG GCA ATG TAT GG-3′
APO3Cc	5′-GTC GTC GCA GAA CCA AGA GA-3′
APO3Cd	5′-GAT GTG TAC CAG GTG ACC TG-3′
APO3Da	5′-CTG GGA CAA GCG TAT CTA AG-3′
APO3Dd	5′-AGT CTG AGA TGA AGA GGT GG-3′
APO3Fa	5′-GTT GGG TCC TGC CGC ACA GA-3′
APO3Fd	5′-TCA TCC TTG GCC GGC TAG TC-3′
APO3Ga	5′-GAC TAG CCG GCC AAG GAT GA-3′
APO3Gb	5′-CAC AGT GGA GCG AAT GTA TC-3′
APO3Gc	5′-GTT CGG AAT ACA CCT GGC CT-3′
APO3Gd	5′-ACT CCT GGT CAC GAT GCA GC-3′

ation at 95°C for 45 seconds, PNA annealing at 73°C for 2 minutes, primer annealing at 60°C for 1 minute, and extension of primer at 63°C for 4 minutes), followed by the final extension at 63°C for 7 minutes. The second-round amplification was performed under the same conditions without a primer extension for 3 minutes. The estimated error rate of the Taq DNA polymerase was 1.76×10^{-5} per site in amplifying approximately 10^2 copies of plasmid under the same conditions as described previously and cloning and sequencing.[21] Products (1 μL each) of the second-round of PNA PCR were subjected to PCR with primers PLF2 and BR109 for 35 cycles (94°C, 1 minute; 58°C, 1 minute; 72°C, 1.5 minutes) after initial denaturation at 94°C for 4 minutes and followed by the final extension at 72°C for 7 minutes. Amplicons were purified by electrophoresis on 2% (wt/vol) agarose gel and cloned into pGEM-T Easy Vector (Promega, Madison, WI) with the standard method, and then transformed into *Escherichia coli* JM 109 (Takara Shuzo Co., Otsu, Japan). Sequencing was performed in the ABI PLISMTM 310NT Genetic analyzer (Applied Biosystems, Tokyo, Japan) with Big Dye terminator version 3.0 Cycle Sequencing Ready Reaction kit (Applied Biosystems). Ten independent clones from each serum sample of patients or supernatant of cell cultures were sequenced for analysis and compared for nucleotide sequences obtained by direct sequencing of PCR products. Hypermutation was defined as clones with a statistically significant number of G to A substitutions.

Sequence Analysis. Nucleotide sequences were aligned and parameters of hypermutation were evaluated with Hypermut Program Package[22] (http://www.hiv.lanl.gov/HYPERMUT/hypermut.html). We used nucleotide sequences obtained by direct sequencing as reference sequences and tentatively labeled clones with a statistically significant ($P < .05$ by Fisher's exact test) number of G to A substitutions as "hypermutated."

Detection of mRNA of Known Deaminases by Reverse Transcription and PCR. Total RNA was extracted from HepG2 cell lines by using cell-to-cDNAII kit (Ambion, Austin, TX). The extracted RNA was reverse transcribed with random primer and M-MLV reverse transcriptase (ReverTra Ace, TOYOBO, Osaka, Japan) at 42°C for 60 minutes according to the instructions provided by the manufacturer. Synthesized cDNAs were used to detect mRNAs of known deaminases using primers listed in Table 1. Each of these primers was carefully designed to amplify only the target member of the APOBEC families. Amplification of specific deaminases was confirmed by amplifying each deaminase cDNA by using cDNAs obtained from organs reported to be positive for the expression of each deaminase. The amplicons were analyzed in 2% agarose gel, and the nucleotide sequences were confirmed by direct sequencing.

Results

Frequent Detection of G to A Substituted HBV Genomes by PCR With PNA Clamping in Patients With Acute or Chronic Hepatitis B Virus Infection. Using PCR with PNA clamping, clones with multiple G to A substitutions were found (Table 2). In contrast, only small numbers of other substitutions were identified in these clones. A hypermutated genome of HBV was found in 1 of 8 patients with acute HBV infection and 4 of 10 patients with chronic HBV infection (Table 2). We cloned and sequenced more than 20 clones without PNA and found no hypermutated clones. Among patients with chronic HBV infection, hypermutated clones were identified only in eAb-positive patients (Table 2). Figure 1

Table 2. Nucleotide Substitutions of Clones Amplified by PCR With PNA Clamping and Clinical Features of Patients With Acute and Chronic Hepatitis B Virus Infections

Patient	No. of Substitutions*		No. of Clones†	Pre-core‡	CP§	eAg	eAb	HBV DNA	ALT
	G to A	Other							
A-1	27	3	8 (1)	G	A/G	42	0	5.1	2,517
A-2	13	4	8	G	A/G	7.8	88	6.1	3,778
A-3	12	2	5	A/G	A/G	190	0	<3.7	1,417
A-4	11	0	4	G	A/G	58.3	0	4.5	2,550
A-5	11	3	9	G	A/G	170	0	8.3	175
A-6	7	7	9	A/G	Mixed	260	0	7.8	28
A-7	1	2	4	G	Mixed	0.1	99.4	4.1	2,295
A-8	1	1	3	A	T/A	0.7	91	7.1	6,183
C-1	152	2	10 (10)	A	T/A	0.3	100	5.5	394
C-2	44	12	9 (4)	A/G	T/A	18.2	73.4	6.2	340
C-3	30	4	10 (1)	A/G	T/A	0.3	97	7.3	53
C-4	23	1	3	G	A/G	140	0	5.9	2,770
C-5	22	1	8 (1)	A	T/A	0.4	95	6.5	105
C-6	19	9	9	A/G	Mixed	200	0	8.2	113
C-7	18	5	7	G	T/A	170	0	6.6	31
C-8	17	1	7	G	A/G	200	0	7.7	92
C-9	12	4	7	G	T/A	180	0	>8.8	56
C-10	6	4	7	A	A/G	2.5	95	8.3	267

*Total number of nucleotide substitutions in 10 clones compared with sequences obtained by direct sequencing.

†Number of different clones of 10 clones sequenced. Figures in parentheses represent the number of clones with hypermutation (those with a statistically significant number of G to A substitutions).

‡Nucleotide sequence of codon 28 of pre-core protein (nucleotide 1896).

§Nucleotide sequence of basic core promoter (nucleotides 1762 and 1764). Mixed represents mixture of A/G and T/A.

illustrates hypermutations found in an eAb-positive patient with chronic HBV infection (C-1 in Table 2). As much as 72.5% (29 of 40) of G residues were mutated in such hypermutated clones. Hypermutation was found in both the envelope/polymerase region (Fig. 1A) and x region (Fig. 1B) of HBV genome obtained from this patient. Preference of G to A mutation was similar with those reported in HIV-1; that is, G residues in GA sequences were the most frequently hypermutated (Fig. 2). In contrast, the G residues in CxG context were less frequently substituted (Fig. 2). Numerous G to A nucleotide substitutions were identified in clones lacking a statistically significant number of G to A hypermutations (Table 2). The number of such substitutions was apparently greater than "other substitutions" (Table 2). There was no relationship between the degree of hypermutation and serum alanine aminotransferase concentration or HBV DNA level (Table 2).

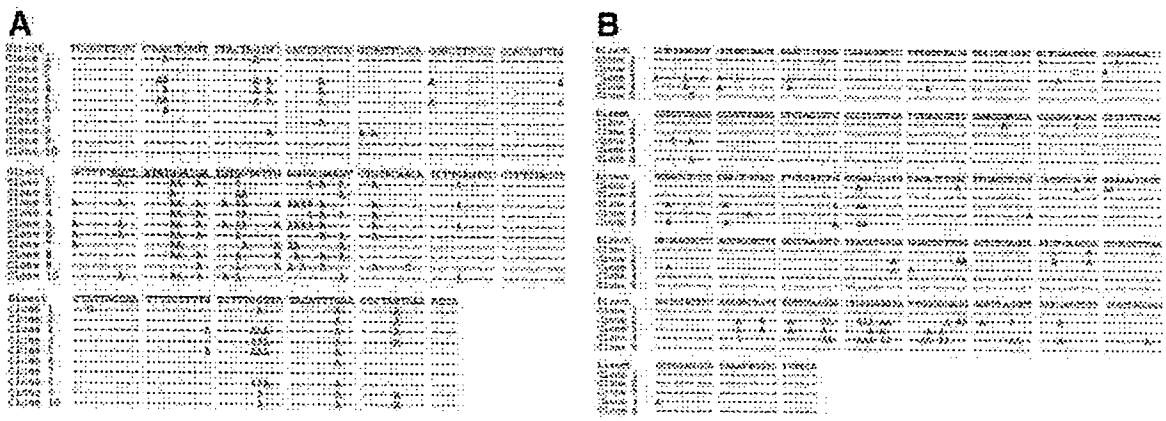

Fig. 1. G to A hypermutations detected in sequences of HBV DNA in sera extracted from an HBe antibody-positive HBV carrier (Patient C-1, Table 2) by PCR with PNA clamping. (A) DNA sequence alignment in the HBs antigen/polymerase region of the HBV. The nucleotide sequences that were obtained by direct sequencing were used as a reference sequence (**top line**). The target sequence of PNA annealing is **underlined**. (B) DNA sequence alignment in the x region of the HBV.

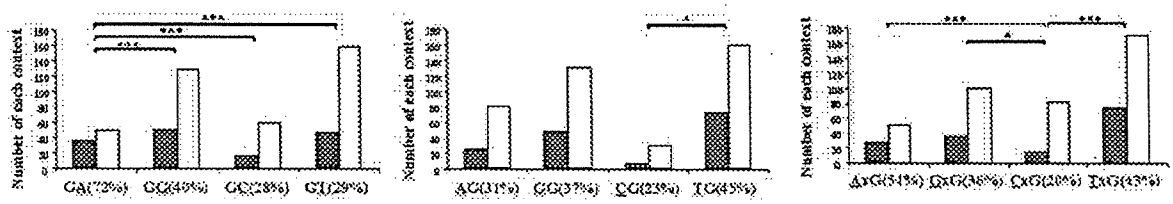

Fig. 2. Preferred nucleotide contexts of G to A hypermutation in 10 clones from patient C-1 (Table 2). The preferred nucleotide letter one letter after (left), one letter before (middle), and two places before (right, x = any) the target G residue. **Open bars**: number of occurrences of each context in the sequence analyzed. **Gray bars**: number of G residues mutated to A. The percentage in parentheses represents the rate of mutated G residues.*$P < .05$, *** $< .001$ (Fisher's exact test or chi-square test).

G to A Hypermutation in HBV-Producing Cell Lines. We established HepG2 cell lines that continuously produced HBV into the medium and examined the frequency of hypermutation. Hypermutated clones were identified in one of these cell lines (Table 3 and Fig. 3). The preference of G to A mutation was similar to that found in serum samples obtained from patients (data not shown). Various levels of HBsAg, HBeAg, and HBV DNA were released into the medium from these cells (Table 3). No relationship was found between the frequency of the hypermutated genome and intracellular intermediates of HBV DNA and HBsAg and HBeAg levels (Table 3). Figure 4 shows replicative intermediates of the HBV produced in these cell lines detected by Southern blot analysis (Fig. 4). No noticeable difference was observed between a cell line with hypermutated genomes and those without hypermutated genomes (lanes 1 and 2 in Fig. 4).

G to A Hypermutation During Antiviral Treatment. We treated the cell lines with alpha and gamma interferon and lamivudine. Both interferons reduced HBV DNA production from these cells in a dose-dependent manner (Fig. 5). The frequency of G to A hypermutation did not increase in those treated cell lines (Fig. 6), suggesting that G to A hypermutation is not responsible for antiviral defense through these interferons. Treatment of a cell line with lamivudine resulted in marked reductions in the production of HBV in the supernatant as well as intracellular viral intermediates (Fig. 7) and completely abolished identification of G to A substitution (Fig. 6). A similar reduction of detection of hypermutated clones was observed in serum samples obtained from patients who were treated with lamivudine (data not shown).

Expression of Deaminases in HepG2 Cell Lines. We examined the expression of known deaminases to see whether any such enzymes are active in HepG2 cells. As shown in Fig. 8, mRNA expression of 5 of 8 of these deaminases was detected, although the expression level of some deaminases was very low. mRNA of Apobec3G, a key enzyme for the hypermutation of HIV-1, was expressed in HepG2 cells, but the cDNA of this enzyme was only found by nested PCR. The expression level of the mRNA was similar in HBV-producing cells with various levels of hypermutations of HBV as well as parent HepG2 cells (detected by only nested PCR).

Table 3. Nucleotide Substitutions of Clones Amplified by PCR With PNA Clamping in Three Cell Lines That Produce the Hepatitis B Virus

Cell Line	No. of Substitutions* G to A	No. of Substitutions* Other	No. of Clones†	eAg	HBs Ag	HBV DNA
Cell line 1	102	0	10 (7)	17	4.7	5.2
Cell line 2	19	0	7	10	4.9	4.6
Cell line 3	21	1	6	14	2.8	4.6

*Total number of nucleotide substitutions in ten clones compared with sequences of the transfected clone.

†Number of different clones of 10 clones sequenced. The figure in parentheses represents the number of clones with hypermutation (those with a statistically significant number of G to A substitutions). Codon 28 of the pre-core gene of the transfected clone was wild (Trp), and nucleotides 1762/1764 were T/A.

Discussion

In this study, we detected the mutated HBV genome in some patients by using PCR with PNA clamping. PNA is a DNA analog in which the ribose-phosphodiester backbone of DNA has been replaced by *N*-(2-aminoethyl) glycine linkages.[23] The PNA anneals strongly to DNA like a complementary DNA, but with higher affinity.[23] The annealing of the PNA to the target sequence thus prevents amplification of the target DNA in the PCR. In our previous study,[21] we attempted to block the amplification of lamivudine-sensitive wild-type YMDD motif strain and detected a very small amount (1/10,000) of YMDD motif mutant. Because the target sequence of this system contained many Gs with GA and GG (AGT TAT ATG GAT GAT GTG), we assumed that we could detect very rare hypermutated genomes.

Because we did not detect any hypermutated sequence without PNA, we assumed that the rate of the hypermutated genome is very low. This low frequency of hyper-

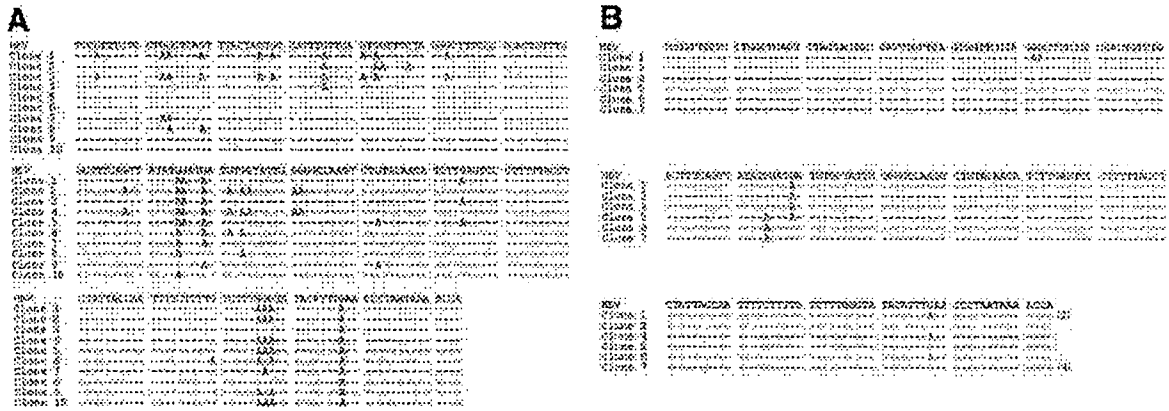

Fig. 3. G to A hypermutations detected in sequences of the HBV DNA (produced by HBV DNA-transfected cell lines to the supernatants). The nucleotide sequences of the transfected clone were used as a reference sequence (**top line**). DNA sequence alignments in the HBs antigen/polymerase region of cell line 1 (A) and cell line 2 (B) of the HBV. Numbers in parentheses are numbers of clones.

mutated genomes accounts for the lack of reports of such sequences with only one exception until recently,[19] in which the presence of two clones of hypermutated sequences in spliced genomes was reported. One may assume that the rare hypermutated genome might be produced in peripheral blood mononuclear cells because the HBV genome was previously found in such cells.[24-28] However, we showed that these genomes are found in HBV-transfected cell lines. Our results clearly demonstrate that hypermutation actually occurs in hepatocytes. The reason(s) for such a low frequency of hypermutation is not clear. The low expression level of deaminases in hepatocytes might account for the low frequency. In fact, we observed a very low expression level of APOVEC3G (transcripts was only detected by nested PCR [Fig. 8]) in HepG2 cell lines.

Recently, Turelli et al.[20,29] suggested that overexpression of APOBEC3G inhibits the replication of HBV by preventing encapsidation of the virus. However, they did not observe an increase in G to A hypermutation. In contrast, Rosler et al.[30] reported that G to A substitutions significantly increased in HepG2 cells when co-transfected with APOBEC3G cDNA. They found only 50 G to A substitutions by cloning 223 clones,[30] suggesting that the frequency of G to A substitutions is rare despite overexpression of APOBEC3G. Our preliminary data suggest that overexpression of APOBEC3G does not produce a

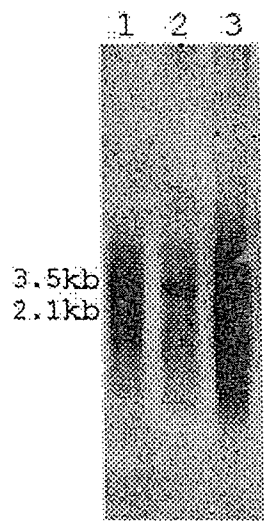

Fig. 4. Southern blot analysis of the HBV DNA extracted from cell lines that stably produce HBV into the supernatant. Two YMDD wild-type virus sequences (lanes 1 and 2) and one YVDD mutant virus sequence (lane 3) were transfected into the HepG2 cell line.

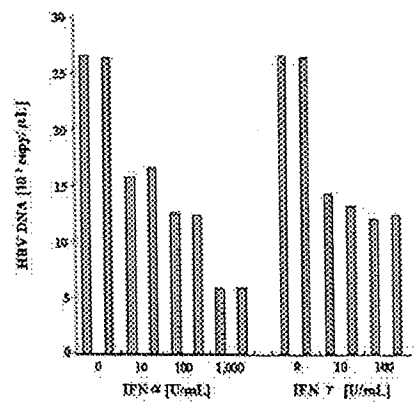

Fig. 5. Effects of interferon alpha and gamma on production of HBV DNA by cell line 1. Experiments were performed in duplicate with increasing amounts of each interferon.

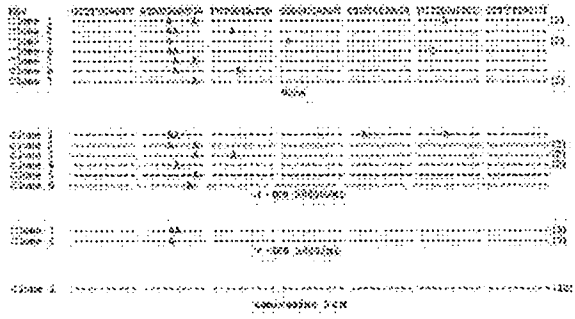

Fig. 6. Nucleotide sequence substitutions around YMDD motif of reverse transcriptase detected by PCR with PNA clamping after treating a HepG2 cell line (cell line 2 in Table 3). The nucleotide sequence of the transfected clone was used as a reference sequence (**top line**). Cells were treated with interferons and lamivudine as shown in Figs. 5 and 7, respectively.

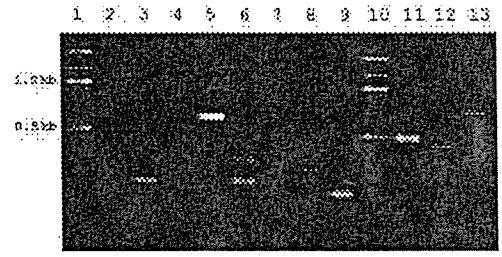

Fig. 8. Agarose gel electrophoresis of mRNAs of known deaminases amplified by reverse transcription-polymerase chain reaction. Lane 1: molecular weight size marker; lane 2: APOBEC1; lane 3: APOBEC2; lane 4: APOBEC3A; lane 5: APOBEC3B; lane 6: APOBEC3C; lane 7: APOBEC3D; lane 8: APOBEC3F; lane 9: APOBEC3G; lane 10: molecular weight size marker. Only mRNA of APOBEC3F was detected by one-stage PCR. To confirm the predictability of the assay, 3 negative mRNAs in Hep3G (APOBEC1, 3A and 3D) were amplified by using mRNAs from tissues known to express it. Lanes 11 and 12: APOBEC1 and APOBEC3A from the ileum; lane 13: APOBEC3D from the duodenum. All detected cDNAs were cloned, and nucleotide sequences were confirmed.

noticeable increase in HepG2 cells by our detection method (C. Noguchi and K. Chayama, unpublished data). However, the method employed to detect hypermutation is not quantitative. Moreover, no antibody to detect APOBEC3G is available. Measurement of activity of this enzyme might be necessary to address this issue.

Because the patterns of hypermutations found in patients as well as cell lines are in agreement with strong dinucleotide preferences of a retroviral genome[31-35] edited by APOBEC3G,[7-9] we assume that hypermutations might also be induced by a similar enzyme. As pointed out by Turelli et al.,[20-29] another deaminase including APOBEC3F might be responsible for the generation of hypermutation. We actually detected the expression of deaminases in HepG2 cell lines. The expression levels of these deaminases are very low because they were detected by only two-stage PCR with one exception (only APOBEC3F was detected by a single-stage PCR).

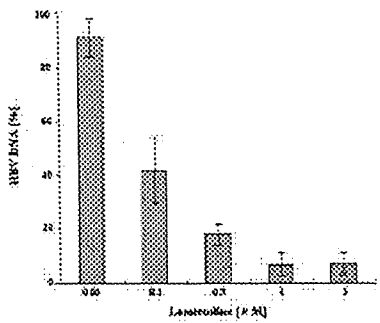

Fig. 7. Effects of lamivudine on production of HBV DNA by cell line 1. After 5 days of lamivudine treatment, the HBV DNA in core particles was immunoprecipitated and quantitated by real-time PCR. Data are mean ± SD of 4 independent experiments.

However, other possibilities should not be ignored. For example, some viral proteins might prevent such editing activity of deaminase by associating with this enzyme, as virion infectivity factor does in HIV-1–infected cells. Possibly the edited HBV genomes are degraded in liver cells rapidly by removal of the U residues by uracil DNA glycocylase followed by cellular nucleases.[36]

We found hypermutated genomes only in patients positive for eAb. The G to A nucleotide substitution of codon 28 of pre-core protein, which induces premature stop of this protein and basal core promoter mutations (A1762T/G1764A), might be related to the clearance of eAg.[28] Further studies should be conducted to investigate the relationship between G to A substitutions in these regions by deaminase(s), production of eAg, and replication efficacy of the virus.

A recent study showed that the amount of HBV DNA reduction occurs noncytopathologically through the action of cytokines, especially interferon alpha/beta and gamma.[37,38] We thus examined whether interferon can alter the occurrence of hypermutation. However, the results showed no increase in the number of hypermutation in HepG2–derived cell lines treated by interferon alpha and gamma (Fig. 6). Thus, the antiviral action of the mechanism responsible for G to A substitution in liver cells is likely to be independent of the action of interferon.

In conclusion, numerous innate intracellular defense systems exist, and the precise pathways of such systems are not fully understood. The role of editing of the HBV genome in such defense systems should be further investigated to understand the natural antiviral mechanisms and to develop an antiviral strategy against HBV.

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Suppression of Macrophage Infiltration Inhibits Activation of Hepatic Stellate Cells and Liver Fibrogenesis in Rats

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Background & Aims: Monocytes/macrophages infiltrate into injured livers. We tried to clarify their roles in inflammation and subsequent fibrogenesis by inhibiting their infiltration with a mutated form (7ND; 7 amino acids at the N-terminal were deleted) of monocyte chemoattractant protein 1, which may function as a dominant-negative mutant. *Methods:* Rats were injected via the tail vein with an adenovirus expressing either human 7ND (Ad7ND), a truncated type II transforming growth factor β receptor (AdTβ-TR), which works as a dominant-negative receptor, bacterial β-galactosidase (AdLacZ), or saline. Seven days later, the rats were treated with dimethylnitrosamine for 1–21 days. *Results:* Within 24 hours after a single dimethylnitrosamine injection, macrophages were observed in livers. With a 3-day dimethylnitrosamine treatment, activated hepatic stellate cells were detectable in livers in AdLacZ-, AdTβ-TR–, and saline-injected rats. In contrast, in the Ad7ND-treated rats, infiltration of macrophages was markedly reduced, and activated hepatic stellate cells were not detectable. After a 3-week dimethylnitrosamine treatment, fibrogenesis was almost completely inhibited, and activated hepatic stellate cells were hardly seen in livers in both Ad7ND- and AdTβ-TR–treated rats. *Conclusions:* Our results show that blockade of macrophage infiltration inhibits activation of hepatic stellate cells and leads to suppression of liver fibrogenesis. The presence of activated hepatic stellate cells in the initial phase after injury and its absence at a later phase in the AdTβ-TR–treated livers indicate that transforming growth factor β is not an activating factor for hepatic stellate cells, and this suggests that transforming growth factor β is required for the survival of activated hepatic stellate cells. Our study suggests that infiltrated macrophages may themselves produce an activating factor for hepatic stellate cells.

Inflammation is always accompanied by an infiltration by leukocytes,[1] a process that is thought to be regulated by chemotactic cytokines called *chemokines*.[1,2] Monocyte chemoattractant protein (MCP)-1, one of these chemokines, induces infiltration by monocytes/macrophages and lymphocytes[3] by binding to a specific receptor, CCR2.[1,2] In animal models of liver injury[4,5] and in patients with chronic hepatitis,[6,7] MCP-1 is detectable in both livers and serum. Injury-induced inflammation results in tissue remodeling or liver fibrosis. However, the actual roles performed by infiltrated monocytes/macrophages and MCP-1 in liver fibrogenesis are largely unknown.

During liver fibrogenesis, hepatic stellate cells (HSC) are activated to myofibroblast-like cells expressing α-actin. These activated HSC and myofibroblasts already existing in the portal field and around central veins may play a central role in fibrogenesis,[8] after which they produce extracellular matrix through the generation of various cytokines, including transforming growth factor (TGF)-β.[9] For fibrogenesis, HSC are considered to be the responsible cells, and TGF-β is one of the critical factors for fibrogenesis. In fact, when we inhibited the action of TGF-β by using a dominant-negative mutated receptor for TGF-β,[10] the activated HSC were markedly reduced in number, and fibrogenesis, as well as the progression of already-established fibrosis, was almost completely suppressed.[11–13] This shows the essential roles played by TGF-β and HSC in fibrotic remodeling after liver injury. However, the mechanism underlying the activation of HSC is not fully understood, although TGF-β has been believed to be an activating factor.[14]

In this study, to try to answer these questions, we introduced a mutated form of MCP-1 (7ND), which is

Abbreviations used in this paper: DMN, dimethylnitrosamine; ELISA, enzyme-linked immunosorbent assay; HSC, hepatic stellate cells; MCP, monocyte chemoattractant protein; MOI, multiplicity of infection; TGF, transforming growth factor; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling.

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considered to inhibit the action of MCP-1 as a dominant-negative mutant,[15,16] into dimethylnitrosamine (DMN)-treated rats, an established model of liver fibrosis with a pathology closely resembling that of human cirrhosis.[17,18] Some rats were given a dominant-negative TGF-β receptor to eliminate signaling by TGF-β.[11,12] We compared these rats in terms of (1) infiltration by monocytes/macrophages and activation of HSC, both of which occur in the acute phase after injury, and (2) fibrotic changes in the chronic phase after injury. Although inhibition of MCP-1 and blockade of TGF-β each led to a marked suppression of liver fibrogenesis, we were interested to find that some responses in the initial phase after injury were quite different between these 2 groups. Our study indicates that TGF-β is not an activating factor for HSC and suggests that infiltrated monocytes/macrophages may produce the activating factor(s).

Materials and Methods

Preparation of Adenoviruses

Replication-defective $E1^-$ and $E3^-$ adenoviral vectors expressing an amino-terminal deletion mutant of human MCP-1 (Ad7ND) with a FLAG epitope tag in its carboxyl-terminal (complementary DNA, a generous gift from Dr. B. Rollins, Harvard University),[15,16] a truncated human TGF-β type II receptor (AdTβ-TR),[10–12] or bacterial β-galactosidase (AdLacZ)[19] under a CA promoter comprising a cytomegalovirus enhancer and a chicken β-actin promoter[20] were prepared as previously described.[21]

Detection of Mutated Human Monocyte Chemoattractant Protein 1 (7ND) and Rat Wild-Type Monocyte Chemoattractant Protein 1

COS cells were infected with either Ad7ND (multiplicity of infection [MOI] of 1, 10, and 100) or AdLacZ (MOI of 10), as previously described.[10] One day after infection, the medium was replaced with serum-free medium, and cells were incubated for a further 24 hours. A mutant MCP-1 (7ND) secreted into culture media was analyzed by Western blotting by using monoclonal antibodies against either FLAG (Abcam, Cambridge, UK) or human MCP-1 (Sanbio, 5400 AM Uden, The Netherlands), as previously described.[13]

7ND and rat MCP-1 were also detectable by enzyme-linked immunosorbent assay (ELISA). Livers were homogenized in phosphate-buffered saline with 1% Triton X-100, 0.1% sodium dodecyl sulfate, and 0.5% sodium deoxycholate. The homogenates were centrifuged at 20,000*g* for 30 minutes. 7ND and rat MCP-1 were measured in the supernatant of liver homogenates and in sera from rats by using a human MCP-1 ELISA kit (Biosource, Camarillo, CA) and a rat kit (Biosource), respectively, according to the manufacturer's instructions. These ELISA kits are species specific, and cross-reaction between human and rat MCP-1 is less than 5%. In fact, no human MCP-1 protein was detectable in samples from either intact or AdLacZ-infected rats (data not shown).

Animal Models

All animals were treated under protocols approved by the institutional animal care committees, and the experiment was performed under both the institutional guidelines for animal experiments and by the Law (No. 105) and Notification (No. 6) of the Japanese government. Male Sprague–Dawley rats, 10 weeks old and weighing approximately 350 g, were given a single infusion of 0.5 mL of Ad7ND, AdTβ-TR, AdLacZ (2×10^9 plaque-forming units per milliliter), or saline via the tail vein, as previously reported.[12] By this method, virtually all cells in the liver were infected and expressed the introduced molecule.[11,12] Seven days later, rats were given an intraperitoneal injection of DMN (10 μg/g body weight; Wako, Osaka, Japan) either once or at the indicated times (3 consecutive daily injections or 3 consecutive daily injections and 4 days off per week for 3 weeks), as previously reported.[11–13] After DMN treatment, blood was collected, and the rats were killed. Biochemical parameters were measured by using standard methods. The liver was either fixed with 4% buffered paraformaldehyde for histological examination or frozen immediately in liquid nitrogen for the extraction of hydroxyproline, the content of which was measured as described elsewhere.[22]

Histological Examination

Liver sections were stained with hematoxylin or Masson trichrome or subjected to immunohistostaining by using antibodies against either CD68 (ED-1; Serotec, Raleigh, NC) or α-actin (Dako, Tokyo, Japan). Immunoreactive materials were visualized by using a streptavidin-biotin staining kit (Histofine SAB-PO kit; Nichirei, Tokyo, Japan) and diaminobenzidine. Macrophages (CD68-positive cells) and lymphocytes were counted by a technician blinded to the treatment regimen. Four random high-power (200×) fields from each section were examined. As negative controls, immunohistostaining was performed without the first antibodies.

Determination of Hepatic Stellate Cells in Apoptosis

Fragmented DNA in apoptotic cells in liver sections was stained with diaminobenzidine (dark brown) by the terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) technique by using a commercially available kit (Roche Diagnostics, Mannheim, Germany). Then, the sections were double-stained against α-actin and visualized with the aid of 3-amino-9-ethyl carbazole liquid substrate chromogen (red; Dako). As negative controls, the TUNEL reaction mixture was used without terminal transferase.