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Ⅳ. 研究成果の刊行物・別刷

Journal of Medical Virology 81:798–806 (2009)

Mutations Associated With the Therapeutic Efficacy of Adefovir Dipivoxil Added to Lamivudine in Patients Resistant to Lamivudine With Type B Chronic Hepatitis

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Factors influencing the therapeutic efficacy of adefovir dipivoxil added to continuing lamivudine have not been elucidated in lamivudine-resistant patients with type B chronic hepatitis. The viral mutations influencing the efficacy of treatment with adefovir dipivoxil were investigated by sequencing analysis of the whole virus genome. Thirty patients resistant to lamivudine receiving adefovir dipivoxil therapy added to lamivudine were studied. From serum samples obtained before the administration of adefovir dipivoxil, full-length viral DNA sequences were determined by PCR-direct sequencing. Susceptibility of the virus to adefovir was examined further using in vitro transfection analysis. By screening the whole viral genome, the presence of two mutations, a T-to-C/G/A mutation at nt1753 (V1753) and an A-to-C mutation at nt2189 (C2189), correlated with the higher incidence of sustained viral DNA clearance during therapy ($P < 0.005$ and $P < 0.05$). In multivariate analysis, the V1753 ($P = 0.001$) and the C2189 ($P = 0.007$) mutations, and elevated transaminase ($P = 0.011$) and low viral load ($P = 0.008$) at the baseline were selected as significant independent factors associated with improved antiviral efficacy. In vitro transfection analysis showed no differences in susceptibility to adefovir among wild-type virus and C1753 and C2189 mutant viruses, suggesting that the virus possessing these mutations may be eradicated more efficiently than the wild-type virus by treatment regardless of a direct antiviral effect of adefovir. ***J. Med. Virol. 81:798–806, 2009.***

KEY WORDS: antiviral therapy; hepatitis B virus; mutation

INTRODUCTION

Treatment of patients with chronic hepatitis B virus (HBV) infection is aimed at suppressing continuously viral replication thereby preventing progression of liver disease. Lamivudine has been shown to achieve reduction of HBV DNA and histological improvement in the liver in most patients with type B chronic hepatitis [Lai et al., 1998; Dienstag et al., 1999]. However, its long-term administration often causes the emergence of drug-resistant virus, resulting in loss of antiviral activity. The frequency of lamivudine resistance has been reported to be 24% after 1 year and 70% after 4 years of therapy [Lai et al., 2003]. This resistance is conferred by an rtM204V/I mutation occurring within the reverse transcription domain of the HBV polymerase gene [Liaw et al., 2000; Leung et al., 2001; Lai et al., 2003]. The rtL180M mutation is also combined frequently with rtM204V/I [Lai et al., 2003].

Adefovir dipivoxil has been shown to result in significant virological and histological improvement in both nucleoside-naïve and lamivudine-resistant patients with type B chronic hepatitis [Hadziyannis et al., 2003; Marcellin et al., 2003; Perrillo et al., 2004; Peters et al., 2004]. The incidence of adefovir dipivoxil resistance in nucleoside-naïve patients has been reported to be 6% after 3 years and 29% after 5 years of therapy [Hadziyannis et al., 2005, 2006], which is less frequent than that occurring with lamivudine therapy

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Accepted 21 August 2009
DOI 10.1002/jmv.21348
Published online in Wiley InterScience (www.interscience.wiley.com)

alone. As for lamivudine-resistant patients, adefovir dipivoxil resistance has been observed in 18% of patients after 1 year after the change from lamivudine to adefovir dipivoxil [Lee et al., 2006]. Two mutations, rtA181V/T and rtN236T, have been shown to confer resistance to adefovir dipivoxil [Hadziyannis et al., 2005, 2006; Lee et al., 2006]. In the case of adefovir dipivoxil administration added to lamivudine therapy, mutant strains resistant to both drugs develop rarely, although there have been reports of a few patients with resistance to both [Villet et al., 2006; Karatayli et al., 2007].

Thus far, factors affecting the efficacy of adefovir dipivoxil added to lamivudine treatment have not been clarified fully in lamivudine-resistant patients with type B chronic hepatitis. In the present study, sequencing analysis of full-length HBV DNA was undertaken in lamivudine-resistant patients with type B chronic hepatitis, who received adefovir dipivoxil added to the continuing lamivudine treatment, and attempts were made to identify the viral mutations associated significantly with therapeutic efficacy.

PATIENTS AND METHODS

Patients and Treatment

Thirty consecutive patients with type B chronic hepatitis at Osaka University Hospital or the National Hospital Organization Osaka National Hospital participated in this study. These patients were also subjects in a previous study that investigated the correlation of viral genomic changes with occurrences of lamivudine-resistant rtM204V/I and rtL180M mutations [Ohkawa et al., 2008]. All 30 patients had been treated with 100 mg/day of lamivudine and had shown lamivudine resistance with the detection of lamivudine-resistant mutant virus. The total duration of the preceding lamivudine therapy ranged from 20 to 60 (median 35) months. All the patients received 10 mg/day of adefovir dipivoxil continuously in addition to lamivudine. Liver function tests and HBV markers were measured every month for the initial 6 months and every 2 months thereafter. The follow-up period of adefovir dipivoxil therapy ranged from 6 to 40 (median 19) months.

Clinical Features of the Patients

The clinical and virological features of the patients at the commencement of adefovir dipivoxil administration are shown in Table 1. The 26 males and 4 females, aged 25–71 (median 49) years, all had hepatitis B surface antigen (HBsAg). Hepatitis B e antigen (HBeAg) was found in 21 (70%) patients. HBV DNA ranged from 4.9 to >7.6 (median 7.2) $\log_{10}$ copies/ml, and alanine aminotransferase (ALT) ranged from 16 to 455 (median 108) IU/L. With respect to liver diseases, 23 (77%) were diagnosed with chronic hepatitis, 3 (10%) with cirrhosis and 4 (13%) with hepatocellular carcinoma (HCC) according to a liver biopsy and/or imaging procedures. None of the patients had evidences of hepatitis C virus- or human immunodeficiency virus-related diseases, alcoholic liver disease, autoimmune hepatitis and drug-induced liver injury. Serum samples for sequencing analysis were collected within 2 months before the commencement of adefovir dipivoxil administration and stored at −80°C. Pairwise serum samples obtained before lamivudine therapy were also collected and used for analysis in 14 of the 30 patients with type B chronic hepatitis. Informed consent was obtained from all patients.

Measurement of HBV Markers

HBsAg, HBeAg and antibody to HBeAg (anti-HBe) were measured by enzyme immunoassay. Serum HBV DNA was quantitated by a PCR-based assay (Amplicor HB Monitor, Roche Diagnostics Co. Ltd, Tokyo, Japan) having a lower detection limit of 2.6 $\log_{10}$ copies/ml.

Sequencing of Full-Length HBV DNA

The full-length HBV DNA was amplified by PCR and subjected to direct sequencing analysis as described elsewhere [Kanada et al., 2007]. In 25 of the 30 patients with type B chronic hepatitis, sequence data of full-length HBV DNA, that had been determined in a previous study [Ohkawa et al., 2008], were also used in this study. In the remaining five patients, HBV DNA sequences were updated using serum samples obtained just before the commencement of adefovir dipivoxil administration. The Genbank accession numbers of the nucleotide sequences in HBV strains determined in this study are shown in Table 1.

Plasmid and Transfection

The HBV-expressing plasmid pHBC carried approximately 1.2 times the genomic length of HBV adr4 strain of genotype C (Genbank accession no. X01587) [Fujiyama et al., 1983]. pHBC-C1753 and pHBC-C2189, which had the C1753 and C2189 mutations in their inserted HBV sequences, were generated by site-directed mutagenesis. pCMV-SEAP was the expression plasmid of a secreted alkaline phosphatase.

Huh7 cells (3×10^5 cells) were seeded on a 35-mm-diameter culture dish and transfected with 1 μg of HBV-expressing plasmid and 0.06 μg of pCMV-SEAP using the FuGENE6 reagent (Roche Diagnostics Co. Ltd). After overnight culture, the cells were treated with 1 or 10 μM of lamivudine (GlaxoSmithKline Co. Ltd, Tokyo, Japan), 1 or 10 μM of adefovir (Toronto Research Chemicals, Inc., North York, Canada), 10 μM of lamivudine plus 10 μM of adefovir, or left untreated. The cells were harvested 3 days after transfection. The culture supernatant was used for measurement of alkaline phosphatase activity to evaluate the efficiency of transfection.

Detection of HBV DNA Replicative Intermediate

For detection of the HBV DNA replicative intermediate, the cells were lysed with buffer containing

TABLE I. Clinical and Virological Features in 30 Patients With Type B Chronic Hepatitis Treated With Adefovir Dipivoxil Added to Continuing Lamivudine

Patient no.	Age (years)	Gender (M/F)	Liver disease	Duration of preceding lamivudine therapy (months)	ALT (μ/l)	HBeAg/ anti-HBe	HBV DNA (log_{10} copies μl)	rtM204V/I mutation (V/I)	rtL180M mutation	V1753 mutation	C2189 mutation	Follow-up period of lamivudine plus adefovir-dipivoxil therapy (months)	Sustained HBV DNA clearance	Genbank accession no.
1	51	M	Chronic hepatitis	33	64	+/−	7.2	I	+	−	+	40	+	AB367415
2	54	M	Chronic hepatitis	20	87	+/−	>7.6	V	+	−	+	33	+	AB367393
3	38	M	Chronic hepatitis	30	429	+/−	>7.6	I	+	+ (C)	+	32	+	AB367414
4	33	M	Chronic hepatitis	33	331	+/−	7.5	I	+	+ (C)	+	31	+	AB367413
5	71	F	Cirrhosis	21	148	−/+	7.1	I	−	−	+	31	+	AB367430
6	51	M	Cirrhosis	38	272	−/+	6.9	I	−	+ (G)	+	29	+	AB367804
7	51	M	Chronic hepatitis	36	327	−/+	5.5	V	+	+ (C)	−	28	+	AB367406
8	25	M	Chronic hepatitis	41	455	+/−	6.6	V	+	−	−	27	+	AB367394
9	55	M	HCC	35	96	+/+	5.2	V	+	−	+	26	+	AB367401
10	62	M	Chronic hepatitis	49	401	+/+	7.4	I	−	−	−	15	+	AB367428
11	38	M	Chronic hepatitis	41	122	+/+	7.1	V	+	+ (G)	−	14	+	AB367407
12	27	M	Chronic hepatitis	21	28	+/−	4.9	I	−	+ (A)	−	11	+	AB367422
13	37	F	Chronic hepatitis	54	16	−/+	5.3	I	+	+ (C)	+	6	+	AB367803
14	47	M	Chronic hepatitis	59	59	+/−	>7.6	I	+	−	−	32	−	AB367408
15	36	M	Chronic hepatitis	28	340	+/−	7.1	I	−	−	−	28	−	AB367425
16	39	M	HCC	28	47	+/−	5.0	I	−	−	+	25	−	AB367802
17	64	M	Cirrhosis	45	32	+/−	>7.6	V	+	+ (C)	−	20	−	AB367403
18	60	F	Chronic hepatitis	25	41	−/+	6.6	I	−	−	−	19	−	AB367427
19	57	F	Chronic hepatitis	55	140	+/−	>7.6	I	+	−	−	19	−	AB367410
20	38	M	Chronic hepatitis	28	112	+/−	7.5	V	+	−	+	18	−	AB367398
21	39	M	Chronic hepatitis	35	193	+/−	>7.6	I	+	−	−	17	−	AB367418
22	36	M	Chronic hepatitis	46	115	−/−	7.5	V	+	−	−	17	−	AB367404
23	56	M	HCC	26	35	+/−	>7.6	I	−	−	−	17	−	AB367435
24	46	M	Chronic hepatitis	48	78	−/+	>7.6	V	+	+ (G)	−	16	−	AB367800
25	54	M	Chronic hepatitis	40	104	−/+	7.2	V	+	−	−	13	−	AB367405
26	71	M	Chronic hepatitis	56	184	+/−	7.1	I	−	−	−	11	−	AB367434
27	53	M	HCC	27	120	−/+	6.7	I	−	−	+	10	−	AB367433
28	40	M	Chronic hepatitis	27	56	+/−	7.4	I	−	+ (C)	+	6	−	AB367423
29	63	M	Chronic hepatitis	26	20	+/−	>7.6	V	+	+ (C)	−	6	−	AB367399
30	36	M	Chronic hepatitis	60	66	−/−	6.8	I	−	−	+	6	−	AB367801

50 mM Tris–Cl (pH 7.5), 1 mM EDTA and 1% NP40, followed by the 15-min incubation on ice. Then, the sample was centrifuged to remove the nuclei pellet at 15,000 rpm at 4°C, and treated with 30 μg of DNase I at 37°C for 30 min. The sample was subjected to overnight incubation at 37°C in lysis buffer containing 1% SDS and 200 μg of proteinase K. After phenol/chloroform extraction and ethanol precipitation, the DNA sample was electrophoresed, transferred onto a nylon membrane and hybridized with an alkaline phosphatase-labeled HBV DNA probe. The signals were detected with the chemiluminescent substrate CDP-star (GE Healthcare Bio-Sciences Co. Ltd, Tokyo, Japan) and quantitated using an image analyzing software (ImageJ 1.38, supplied online by the National Institutes of Health, Bethesda, MD).

Statistical Analysis

Group comparisons of continuous and categorical variables were done using Fisher's exact probability test, χ^2 test with Yate's correction and Mann–Whitney's non-parametric *U*-test as appropriate. The group comparison of Kaplan–Meier curves for the cumulative probability of sustained HBV DNA clearance was performed by the log-rank test. The correlation of various clinical and virological factors with the cumulative probability of sustained HBV DNA clearance was evaluated by a Cox proportional-hazards model using univariate and stepwise multivariate procedures. The one-way analysis of variance and the Fisher's PLSD test were used for the in vitro transfection analysis.

RESULTS

Overall Therapeutic Efficacy of Adefovir Dipivoxil Added to Lamivudine in Lamivudine-Resistant Patients With Type B Chronic Hepatitis

Among the lamivudine-resistant patients with type B chronic hepatitis examined in this study, HBV DNA decreased to an undetectable level (<2.6 $\log_{10}$ copies/ml) in 6 (20%) of 30 patients at 6 months, 6 (26%) of 23 patients at 12 months and 9 (56%) of 16 patients at 18 months after the beginning of adefovir dipivoxil administration. Thirteen (43%) of the 30 patients achieved sustained HBV DNA clearance during follow-up. ALT normalization was observed in 21 (70%) of 30 patients at 6 months, 14 (61%) of 23 patients at 12 months and 11 (69%) of 16 patients at 18 months of therapy.

Viral Mutations Associated With Efficacy of Adefovir Dipivoxil Added to Lamivudine Treatment

The lengths of the 30 HBV DNA sequences obtained from the lamivudine-resistant patients with type B chronic hepatitis ranged from 3,161 to 3,230 nucleotides. All 30 patients were infected with HBV of genotype C as determined by phylogenetic tree analysis of the HBV isolates obtained in this study and the representative HBV isolates of major genotypes (data not shown). Viral mutations were sought that showed a relationship with the therapeutic efficacy of adefovir dipivoxil added to lamivudine over the whole HBV genome. As a result, there were only two mutations; one was a T-to-C/G/A mutation at nt1753 (V1753 mutation) located in the basic core promoter (BCP) [Yuh et al., 1992], and the other was an A-to-C mutation at nt2189 (C2189 mutation) in the core gene.

The V1753 mutation was detected in 11 (37%) of the 30 patients studied; the C1753 mutation was found in 7 patients, G1753 in three patients, and A1753 in 1 patient. Figure 1A shows the serial change in HBV DNA before and after the commencement of adefovir dipivoxil administration added to lamivudine treatment in patients with type B chronic hepatitis with and without the V1753 mutation. In patients with V1753, HBV DNA clearance was observed in 5 (45%) of 11 patients at 6 months, 5 (71%) of 7 patients at 12 months and 4 (80%) of 5 patients at 18 months of therapy. By contrast, in patients having T1753, HBV DNA clearance was seen in only 1 (5%) of 19 patients at 6 months, 1 (6%) of 16 patients at 12 months and 5 (45%) of 11 patients at 18 months. Significant differences in the frequency of HBV DNA clearance were observed at 6 and 12 months of therapy between patients with and without V1753 ($P < 0.02$ and $P < 0.005$). Thus, patients with V1753 tended to achieve HBV DNA clearance more frequently by adefovir dipivoxil added to lamivudine treatment than those with T1753.

The C2189 mutation was found in 13 (43%) of the 30 lamivudine-resistant patients with type B chronic hepatitis. Figure 1B shows the serial change in HBV DNA during adefovir dipivoxil administration added to lamivudine treatment in patients with type B chronic hepatitis with and without the C2189 mutation. In patients with C2189, HBV DNA was cleared in 5 (38%) of 13 patients at 6 months, 4 (44%) of 9 patients at 12 months and 8 (89%) of 9 patients at 18 months of therapy. On the other hand, in patients having A2189, HBV DNA was undetectable in 1 (6%) of 17 patients at 6 months, 2 (14%) of 14 patients at 12 months and 1 (14%) of 7 patients at 18 months of therapy. There was a significant difference in the frequency of HBV DNA clearance after 18 months of therapy between patients with and without C2189 ($P < 0.01$). HBV DNA tended to be cleared more frequently by adefovir dipivoxil administration added to lamivudine treatment in patients with C2189 than in those with A2189.

A group comparison of the Kaplan–Meier curves was undertaken for the cumulative probability of sustained HBV DNA clearance with respect to the occurrence of these two viral mutations. Patients with V1753 had a significantly higher cumulative probability of sustained HBV DNA clearance than those with T1753 ($P < 0.005$) (Fig. 2A). The cumulative probability of sustained HBV DNA clearance was also higher in patients with C2189 than in those with A2189 ($P < 0.05$) (Fig. 2B). The presence of the V1753 and C2189 mutations showed no

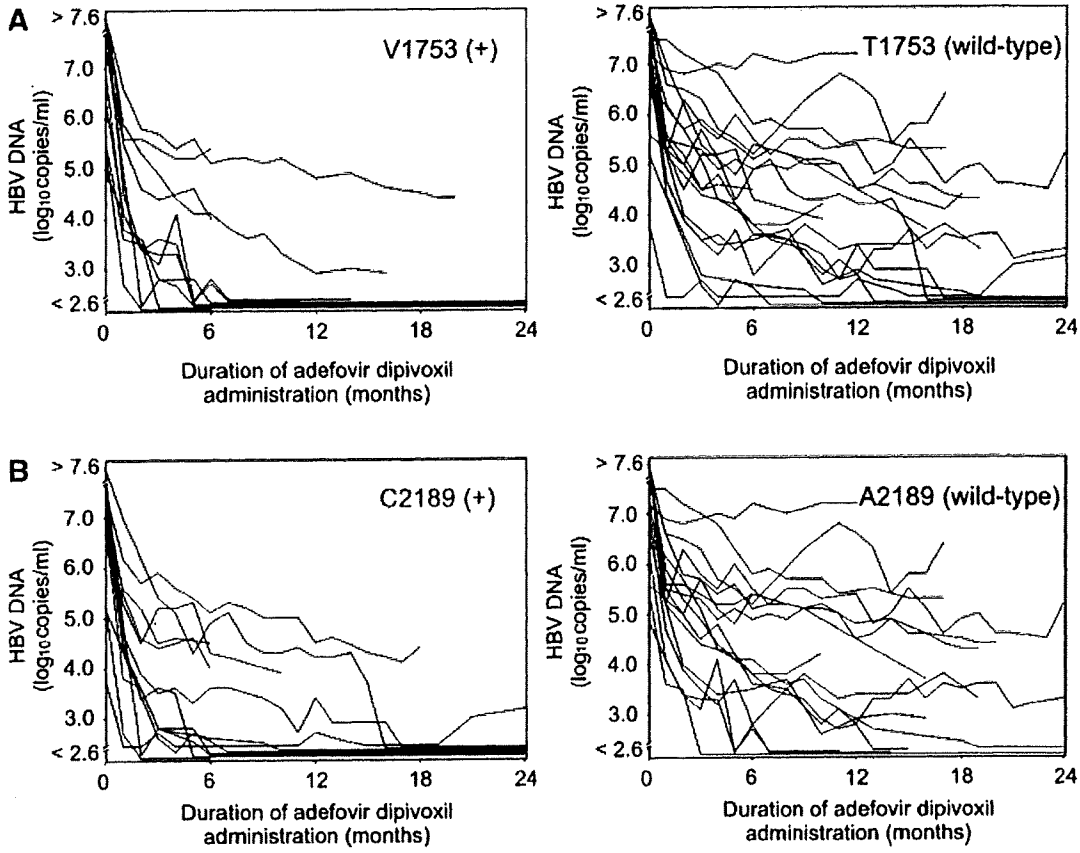

Fig. 1. Serial changes in the HBV DNA level up to 24 months of adefovir dipivoxil added to lamivudine treatment in lamivudine-resistant patients with type B chronic hepatitis in relation to the occurrences of (A) V1753 mutation and (B) C2189 mutation.

relationship with ALT normalization during adefovir dipivoxil added to lamivudine treatment.

Factors Associated With the Efficacy of Adefovir Dipivoxil Added to Lamivudine Treatment Determined by Univariate and Multivariate Analyses

Next, the clinical and virological factors affecting the therapeutic effect of adefovir dipivoxil added to lamivudine were investigated in the 30 patients with type B chronic hepatitis. Six clinical factors (age, gender, liver disease, ALT, HBeAg positivity, and HBV DNA), two lamivudine resistance-associated viral mutations (rtM204V/I and rtL180M) [3], two major naturally occurring viral mutations (A1896 and T1762/A1764) [Carman et al., 1989; Okamoto et al., 1994], and V1753 and C2189 mutations were investigated. As shown in Table II, only the V1753 and C2189 mutations were significant factors contributing to sustained clearance of HBV DNA ($P = 0.006$ and $P = 0.047$) by univariate analysis. High ALT and low HBV DNA (<7.5 $\log_{10}$ copies/ml) at baseline were selected as significant independent factors contributing to sustained clearance of HBV DNA ($P = 0.011$ and $P = 0.008$) in addition to the V1753 and C2189 mutations ($P = 0.001$ and $P = 0.007$) in multivariate analysis.

Serial Changes in the V1753 and C2189 Mutations During Antiviral Therapy

The V1753 and C2189 mutations were also examined using serum samples obtained before lamivudine therapy, which were available in 14 of the 30 type B chronic hepatitis patients. These mutations were assayed by PCR-direct sequencing. Of the 14 patients, the V1753 mutation was found in only 1 patient before lamivudine therapy and detected in additional 4 patients before adefovir dipivoxil administration. The C2189 mutation was found in three patients before lamivudine therapy, one of whom lost the mutation before adefovir dipivoxil administration. The additional three patients acquired this mutation before adefovir dipivoxil administration. Thus, both the V1753 and C2189 mutations tended to appear during lamivudine therapy accompanied by the lamivudine resistance in these patients.

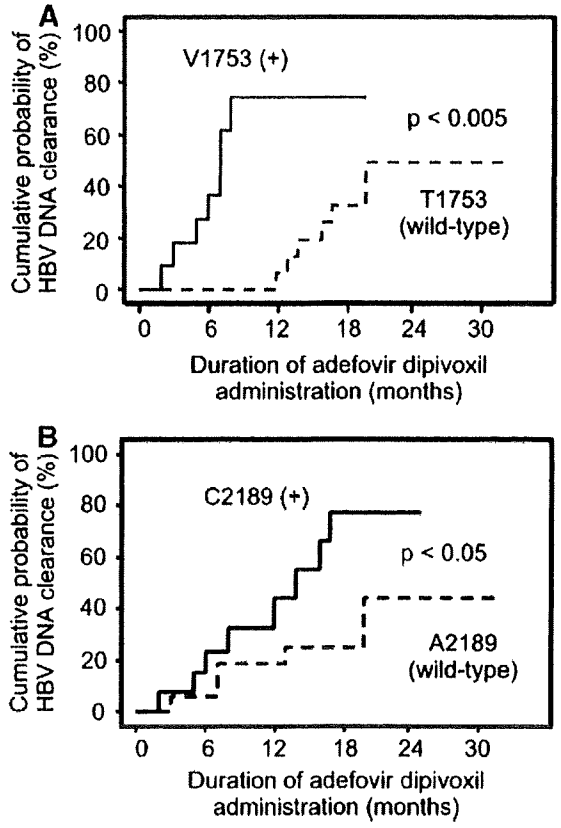

Fig. 2. Influence of the presence of the V1753 and C2189 mutant viruses on the efficacy of adefovir dipivoxil therapy added to lamivudine treatment in lamivudine-resistant patients with type B chronic hepatitis. The Kaplan–Meier estimates of the cumulative probability of sustained HBV DNA clearance correlated with the presence or absence of (A) V1753 mutation and (B) C2189 mutation.

Susceptibility to Lamivudine and/or Adefovir of Wild-Type and Mutant Viruses In Vitro

Finally, susceptibility to lamivudine and/or adefovir of the wild-type and C1753 and C2189 mutant viruses was examined in vitro. Cultured cells with forced expression of the wild-type or mutant virus were treated with lamivudine alone, adefovir alone, lamivudine plus adefovir, or left untreated, and the cellular HBV DNA replicative intermediate was examined. In the HBV-expressing cells without treatment (Fig. 3A,B), the replicative competence of the C1753 mutant virus was the same as that of the wild-type virus, whereas the C2189 mutant virus showed an approximately fivefold lower replicative competence than the wild-type virus. As for susceptibility to nucleos(t)ide analogs, the degree of reduction in viral replication by treatment with lamivudine alone, adefovir alone or lamivudine plus adefovir did not differ significantly among the wild-type virus and the C1753 and C2189 mutant viruses (Fig. 3A,C).

DISCUSSION

Adefovir dipivoxil added to ongoing lamivudine treatment has been accepted as a reliable therapeutic

TABLE II. Univariate and Multivariate Analyses to Investigate Factors Associated With Sustained HBV DNA Clearance in Patients With Type B Chronic Hepatitis Treated With Adefovir Dipivoxil Added to Continuing Lamivudine

Factors	Univariate analysis				Multivariate analysis			
	Hazard ratio	95% confidence interval	χ^2-value	*P*-value	Hazard ratio	95% confidence interval	χ^2-value	*P*-value
Clinical factors								
Age (/1 year increment)	0.987	0.843–1.033	0.302	0.583	—	—	—	—
Gender (female)	1.315	0.287–6.020	0.124	0.725	—	—	—	—
Liver disease (cirrhosis and HCC)	1.199	0.328–4.384	0.076	0.783	—	—	—	—
ALT (/1 IU/l increment)	1.003	1.000–1.007	3.405	0.065	1.005	1.001–1.010	6.443	0.011
HBeAg (negative)	1.919	0.566–6.503	1.096	0.295	—	—	—	—
HBV DNA (<7.5 $\log_{10}$ copies/ml)	3.175	0.862–11.765	3.017	0.082	14.706	1.996–111.111	6.956	0.008
Virological factors								
rtM204V/I mutation (rtM204I)	1.060	0.346–3.254	0.011	0.918	—	—	—	—
rtL180M mutation (+)	0.766	0.234–2.505	0.195	0.659	—	—	—	—
A1896 mutation (+)	1.138	0.371–3.493	0.051	0.821	—	—	—	—
T1762/A1764 mutation (+)	0.500	0.062–4.013	0.426	0.514	—	—	—	—
V1753 mutation (+)	4.986	1.603–15.506	7.705	0.006	58.322	5.054–673.002	10.619	0.001
C2189 mutation (+)	3.155	1.017–9.804	3.957	0.047	7.042	1.704–29.412	7.270	0.007

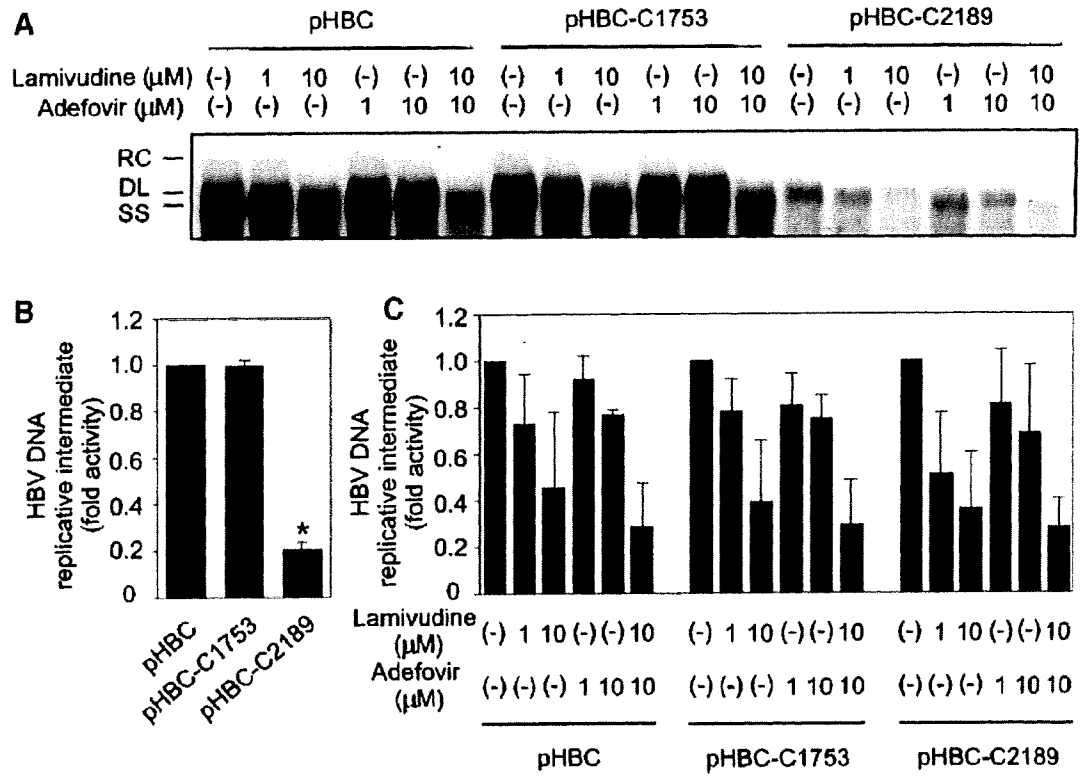

Fig. 3. In vitro transfection analysis to examine viral replicative competence and susceptibility to the treatment with lamivudine and/or adefovir. Huh-7 cells were transfected with pHBC, pHBC-C1753 and pHBC-C2189, and treated with lamivudine alone, adefovir alone, lamivudine plus adefovir, or left untreated. The HBV DNA replicative intermediate in the cytoplasmic fraction of the cells was detected by Southern blot analysis. A: Representative result of Southern blot analysis to detect the HBV DNA replicative intermediate. SS, single-stranded HBV DNA. DL, double-stranded linear HBV DNA. RC, relaxed circular HBV DNA. B: Quantitative analysis of the HBV DNA replicative intermediate in cells transfected with pHBC, pHBC-C1753 and pHBC-C2189 without nucleos(t)ide analog treatment. The level of the HBV DNA replicative intermediate in the case of transfection with pHBC was considered as 1, and its fold activity in the case of transfection with the mutant HBV-expressing plasmid was calculated. The experiment was done three times, and the results are presented as the mean ± SD. *$P < 0.001$ versus pHBC and pHBC-C1753 groups. C: Degree of reduction in the HBV DNA replicative intermediate after treatment with lamivudine and/or adefovir in cells transfected with pHBC, pHBC-C1753 and pHBC-C2189. The level of the HBV DNA replicative intermediate in untreated cells was considered as 1, and its fold activity in cells treated with lamivudine and/or adefovir was calculated. The experiment was done three times, and the results are presented as the mean ± SD.

regimen for lamivudine-resistant patients with type B chronic hepatitis. In the present study, the viral mutations associated with the effect of this regimen were investigated by screening the whole HBV genome via sequencing analysis of full-length viral DNA. Two mutations, V1753 and C2189, were identified as significant determinants of the therapeutic efficacy. Using adefovir dipivoxil added to lamivudine treatment, HBV DNA tended to decline to the undetectable level more frequently in patients with the V1753 or C2189 mutation than in those without it. In univariate analysis, only the presence of the V1753 or C2189 mutation was shown to be a factor contributing to sustained clearance of HBV DNA during adefovir dipivoxil therapy. Multivariate analysis also revealed that the V1753 and C2189 mutations, as well as high ALT and low HBV DNA at baseline, were independent factors associated with a better antiviral effect. Reports from the United States and European countries have revealed that female gender, high ALT, low viral load, absence of HBeAg and genotype D rather than genotype A were related to a better outcome of adefovir dipivoxil therapy in nucleoside-naïve and lamivudine-resistant patients with type B chronic hepatitis [Lampertico et al., 2005; Fung et al., 2006; Buti et al., 2007]. The findings of the present study from Japan, a genotype C HBV-endemic area, agreed in part with these reports. Of particular interest is the finding that the therapeutic efficacy of adefovir dipivoxil added to lamivudine may be affected not only by clinical factors but also the genomic background of HBV such as the presence of the V1753 or C2189 mutation in lamivudine-resistant patients with type B chronic hepatitis. In addition, serial sequencing analysis revealed that both the V1753 and C2189 mutations tended to be selected during lamivudine therapy associated with the establishment of lamivudine resistance, although they have been shown to be mutations which occur naturally during the course of HBV infection [Ehata et al., 1991; Bozkaya et al., 1996; Takahashi et al., 1999; Imamura et al., 2003; Ozasa et al., 2006; Tanaka et al., 2006].

The findings of the present study suggest higher sensitivity to adefovir dipivoxil therapy of the V1753 and C2189 mutant viruses compared to the wild-type virus in vivo. However, in vitro transfection analysis showed no differences in susceptibility to adefovir, as well as to lamivudine, among the wild-type virus and the C1753 and C2189 mutant viruses. This indicates that the V1753 and C2189 mutant viruses may be eradicated more efficiently by adefovir dipivoxil therapy than the wild-type virus regardless of a direct antiviral effect of adefovir dipivoxil. The V1753 and C2189 mutant viruses may induce stronger immune responses against the viral pathogens than the wild-type virus, which might result in more frequent viral eradication under adefovir dipivoxil therapy in patients having the V1753 or C2189 mutant virus compared to those with the wild-type virus.

Of the 1421 HBV strains, whose nucleotide sequences of the BCP, precore and core regions had been identified and registered in the Hepatitis Virus Database (http://s2as02.genes.nig.ac.jp), there were 259 (18%) strains with the V1753 mutation and 127 (9%) strains with the C2189 mutation. The V1753 mutation was found in strains of all HBV genotypes, whereas the C2189 mutation was found in strains of genotypes A, B, C, and E. Thus, the V1753 and C2189 mutations were not specific for genotype C but common in other HBV genotypes.

The V1753 mutation occurring in the BCP not only influences the core promoter activity but also causes the I127T/N/S amino acid change of the overlapping X gene. This mutation has been detected in a considerable proportion of chronic HBV carriers, especially coupled with the adjacent T1762/A1764 mutation [Kidd-Ljunggren et al., 1997; Takahashi et al., 1999]. Indeed, all 11 patients with the V1753 mutation possessed the T1762/A1764 mutation in the current study. It has also been shown that, among patients with type B chronic hepatitis of genotype C, the V1753 mutation was found more frequently in patients with HCC than in those without it [Tanaka et al., 2006]. In acute HBV infection, the frequency of mutation has been reported to be higher in patients with fulminant hepatitis than in those with non-fulminant hepatitis [Imamura et al., 2003; Ozasa et al., 2006]. In vitro transfection assay revealed that the C1753 mutant virus possessed similar replicative competence to the wild-type virus, though viruses having the G1753 and A1753 mutation were not examined. Also, the in vitro replicative competence did not differ between the wild-type and C1753 mutant viruses when the T1762/A1764 mutation was introduced into the backbone HBV structure (data not shown). According to these observations, the serious disease course and better response to adefovir dipivoxil therapy caused by the V1753 mutation, as suggested by the present study and other previous investigations [Imamura et al., 2003; Ozasa et al., 2006; Tanaka et al., 2006], may not be due to the modification of the viral replicative competence. Further studies should be done to clarify why the V1753 mutation is involved in the active liver disease and the better outcome of adefovir dipivoxil therapy in patients with HBV infection.

The C2189 mutation, which leads to the I97L amino acid change in the core gene, has also been shown to be detected frequently in patients with type B chronic hepatitis [Ehata et al., 1991; Bozkaya et al., 1996], although the relevance of the mutation to a particular disease course has not been elucidated fully. Previous in vitro transfection studies have suggested that the virus with the C2189 mutation resulted in excessive secretion of the immature virion and enhanced viral replication [Yuan et al., 1999; Suk et al., 2002]. This does not agree with the present result showing lower replicative competence of the C2189 mutant virus than the wild-type virus. This discrepancy may be due to the usage of HBV-expressing plasmids of different viral strains. The virological and clinical significance of the C2189 mutant virus should be assessed by further detailed investigation.

In summary, the results of the present study indicate that the presence of the two viral mutations, V1753 and C2189, may be associated with a better therapeutic effect of adefovir dipivoxil added to lamivudine based on the results of screening of the full-length HBV genome obtained from lamivudine-resistant patients with type B chronic hepatitis. As the present study examined a limited number of patients with HBV of genotype C, further studies with a larger number of patients with different genotypes should lead to a better understanding of how identifying these mutations can be useful in a clinical setting.

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Journal of Hepatology 50 (2009) 1155–1162

Journal of Hepatology

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Activated liver dendritic cells generate strong acquired immunity in α-galactosylceramide treatment☆

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***Background/Aims*: α-Galactosylceramide (α-GalCer) presented by dendritic cells (DCs) activates NKT cells that in turn drive DC maturation. However, the potential of generating acquired immunity of liver DCs in α-GalCer treatment remains unclear.**

***Methods*: We examined the activation of acquired immunity in the α-GalCer treatment against liver or spleen tumor and the ability of liver and spleen DCs in the generation of acquired immunity.**

***Results*: Administration of α-GalCer resulted in generation of p53 peptide-specific cytotoxic T lymphocytes (CTLs) in mice bearing liver CMS4 tumor, aberrantly expressing p53, but not in mice bearing spleen CMS4 tumor. The growth of rechallenged CMS4 subcutaneous tumor was inhibited in α-GalCer-treated mice against liver CMS4 tumor, but not in α-GalCer-treated mice against CMS4 spleen tumor. The antigen presenting related functions of liver DCs were significantly higher than those of spleen DCs in α-GalCer-treated mice. Vaccination of normal mice with p53 peptide pulsed liver DCs isolated from α-GalCer treated mice resulted in generation of p53 peptide-specific CTLs, but that with p53 peptide pulsed spleen DCs did not.**

***Conclusions*: These results demonstrated that α-GalCer treatment induced unique immunologic activation of liver DCs in comparison with spleen DCs, which might be favorable to generate liver acquired immunity.**

***Keywords*: α-Galactosylceramide; Liver dendritic cells; Acquired antitumor immunity**

1. Introduction

α-Galactosylceramide (α-GalCer) presented by CD1d molecules expressing on dendritic cells (DCs) efficiently stimulates NKT cells implicated in innate immunity [1,2]. Recently, *in vivo* animal studies have shown that systemic administration of α-GalCer can lead to anti-tumor effects against metastatic liver tumor [3,4], suggesting that α-GalCer treatment might be promising for clinical application against liver tumor. Metastatic liver tumors resist conventional chemotherapy and radiotherapy, and present with a poor prognosis. Thus novel and more effective immunotherapy is needed, especially for metastatic liver cancer. Several phase I clinical studies have been done in cancer immunotherapy using intravenous administration of α-GalCer, but with limited clinical responses [5,6]. For further development of α-GalCer treatment in liver cancer patients, the antitumor effect of α-GalCer should be more precisely examined in the liver.

DCs effectively elicit immune responses to self and foreign antigens [7,8]. These specialized antigen-presenting cells (APCs) can induce the generation of both

Received 9 September 2008; received in revised form 9 December 2008; accepted 13 December 2008; available online 26 February 2009
Associate Editor: V. Barnaba

☆ The authors declare that they do not have anything to disclose regarding funding from industries or conflict of interest with respect to this manuscript.

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Abbreviations: DC, dendritic cell; APC, antigen-presenting cells; CTLs, cytotoxic T lymphocytes; α-GalCer, α-galactosylceramide; M-NC, mononuclear cells.

0168-8278/$36.00 © 2009 Published by Elsevier B.V. on behalf of the European Association for the Study of the Liver.
doi:10.1016/j.jhep.2008.12.027

antigen-specific cytotoxic T lymphocytes (CTLs) and T helper cells. α-GalCer administration resulted in maturation of spleen DCs and activation of the CD8+ T cell immune response via costimulatory molecules expressed on the spleen DCs [9,10]. However, in contrast to well-characterized spleen DCs, the details of activation of liver DCs by α-GalCer treatment remains to be clarified because of the difficulty of procuring adequate numbers of isolated liver DCs for functional analysis [11]. Although most previous studies reported that α-GalCer treatment induces early activation of liver NKT and NK cells [3,4,12], which were the main effector cells to eradicate metastatic tumor cells, little is known regarding the induction of liver acquired immunity after early rejection of liver tumor. Nakagawa et al. reported that CD122+CD8+ memory T cells play critical roles in metastatic liver tumor rejection by α-GalCer treatment [13]. However, the ability of α-GalCer to activate liver DCs and generate acquired immunity remains to be clarified.

In the current study, we evaluated the induction of acquired immunity by α-GalCer activated liver DCs in comparison with spleen DCs. We demonstrated that α-GalCer treatment resulted in generating strong acquired immunity after liver tumor treatment, but not after spleen tumor treatment. We also show that α-GalCer treatment activated liver DCs more strongly with respect to the antigen-presenting function and antigen-specific CTL induction than spleen DCs. Thus, α-GalCer treatment resulted in unique immunologic activation of liver DCs, which might contribute to induction of acquired immunity in the liver.

2. Materials and methods

2.1. Mice and cell lines

Six-to-ten-week-old female BALB/c mice and C57BL/6 mice were purchased from Shizuoka Experimental Animal Laboratory (Shizuoka, Japan). The animals were handled under aseptic conditions. Procedures were performed according to approved protocols and in accordance with recommendations for the proper care and use of laboratory animals. CMS4 sarcomas (H-2^d) express mutated p53 and present the wild-type $p53_{232\text{-}240}$ epitope recognized by H-$2K^d$-restricted CTLs [14,15], and MC38 colon cancer cell lines were maintained as previously described [16]. α-Galactosylceramide (α-GalCer) was kindly provided by Kirin Pharma (Gunma, Japan) and prepared as previously described [15].

2.2. IFN-γ ELISPOT assays for p53 peptide-reactive CD8+ T cells responses after α-GalCer treatment for CMS4 tumor and animal experiments

To examine the induction of the acquired antitumor immunity, BALB/c mice were injected intrahepatically or intrasplenically with 5×10^5 CMS4 cells on day 0 and treated intraperitoneally (i.p.) with α-GalCer (2 μg/100 μl) or 100 μl of vehicle on day 1. Fourteen days after α-GalCer treatment, CD8+ T cells were isolated from the spleen of immunized mice by using magnetic beads (MACS, Miltenyi Biotec, Gladbach, Germany). Next, CD8+ T cells (1×10^5 cells/well) and syngeneic bone marrow derived DCs (BMDCs) generated from normal BALB/c mice (2×10^4 cells/well) were cocultured with $p53_{232\text{-}240}$ peptide in ELISPOT culture plate. We used mouse IFN-γ ELISPOT kit (R & D Systems, Minneapolis, MN) to detect the $p53_{232\text{-}240}$ peptide-specific CD8+ T cell responses, as previously described [16]. To assess the systemic acquired immunity due to α-GalCer treatment, mice were injected in the liver or the spleen with 5×10^5 CMS4 cells or MC38 cells on day 0 and were injected i.p. with α-GalCer on day 1. On day 14 after α-GalCer treatment, 1×10^6 CMS4 cells or MC38 cells were injected as a rechallenge into the right flank of treated mice, respectively. Tumor size was assessed every 7 days.

2.3. Preparation of liver and spleen DCs and flow cytometry

Twenty-four hours after i.p. treatment with α-GalCer or vehicle, hepatic mononuclear cells (MNC) and splenic MNC were prepared as previously described [15]. CD11c+ dendritic cells were isolated from liver MNC and spleen MNC by magnetic cell sorting using MACS (Miltenyi Biotec) according to the manufacturer's protocol. For phenotypic analysis of liver and spleen DCs, PE- or FITC- or APC-conjugated monoclonal antibodies against mouse cell surface molecules [CD11c (Miltenyi Biotec), CD40, CD80, CD86, MHC class II, CD8α and CD11b (all from BD-Pharmingen, San Diego, CA)] were used, and flow cytometric analysis was performed using a FACS Calibur (Becton Dickinson, San Jose, CA) flow cytometer. We defined DCs with CD11c+ MHC class II+ cells by flow cytometry and evaluated the expressions of these antigen presenting related molecules. Data were analyzed using FlowJo software (Tree Star, Ashland, OR) and reported as the mean fluorescence intensity (MFI).

2.4. Cytokine measurement

Twenty-four hours after i.p. treatment with α-GalCer or vehicle, liver and spleen DCs were prepared as above. To assess cytokine production, we cultured 2×10^5 DCs in 1 ml of complete medium with LPS (R & D Systems Inc., 10 μg). After 48 h, cell culture supernatants were harvested and tested using a species-specific enzyme linked immunosorbent assay (ELISA) kit for IL-12, IFN-γ and TNF-α (BD-Pharmingen) according to the manufacturer's protocols.

2.5. T cell proliferation assay

Twenty-four hours after i.p. treatment with α-GalCer or vehicle, liver and spleen DCs were prepared as above. The DCs were added in various numbers to 5×10^5 allogeneic T lymphocytes (purified using Thy-1.2 immunomagnetic microbeads from C57BL/6 mice) in 96-well U-bottom plates and then pulsed with [^{3}H] thymidine (1 μ Ci/well) on day 3 for an additional 20 h as previously described [17].

2.6. Immunization of p53 peptide-pulsed liver or spleen DCs from α-GalCer-treated mice

Twenty-four hours after i.p. treatment with α-GalCer or vehicle, liver and spleen DCs were prepared as above. Isolated DCs were incubated with $p53_{232\text{-}240}$ peptide at concentration of 10 μg/mL per 10^6 DCs/mL for 2 h as previously described [14]. 1×10^6 $p53_{232\text{-}240}$ peptide pulsed liver or spleen DCs were injected i.p. into normal BALB/c mice. Five days after i.p. immunization, CD8+ T cells were isolated from the spleen of immunized mice by using magnetic beads (MACS) and were subjected to mouse IFN-γ ELISPOT assay as above described.

2.7. Statistical analyses

The statistical significance of differences between the groups was determined by applying Student's *t*-test with Welch correction after each group had been tested with equal variance and Fisher exact probability test. The statistical significance of the differences in more than three groups was determined by applying one-way ANOVA. Statistical significance was defined as $p < 0.05$.