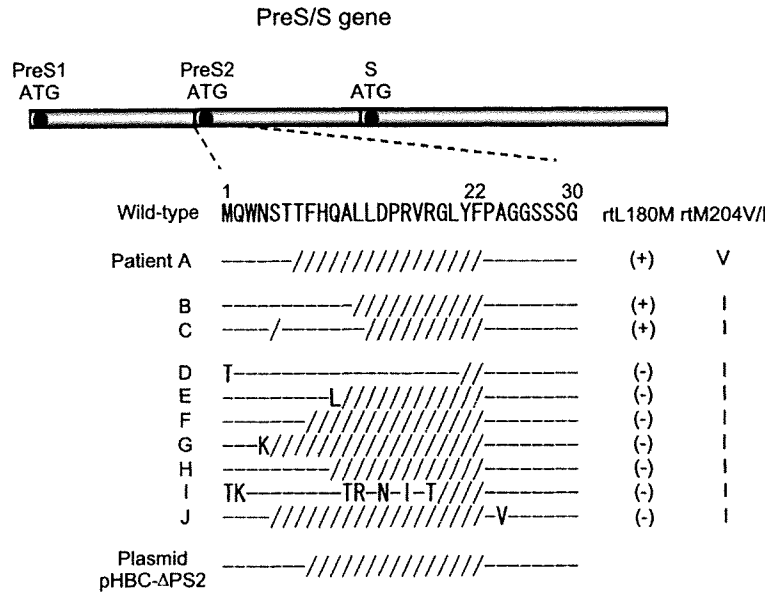

Figure 2. Patterns of the short deletion in the preS2 gene observed in lamivudine-resistant hepatitis B virus (HBV) strains. Ten of the 44 patients (patients A–J) had virus with the deletion in the preS2 gene of various patterns. The top sequence represents the amino acid sequence of the genotype C2 HBV DNA strain adr4 [25] as a representative strain. As for sequences derived from the patients, residues identical to the top sequence are indicated by dashes, whereas deletions of amino acid residues are shown by slashes. All deletions were found within the codon positions 5–22 of the preS2 gene. The bottom sequence represents the deletion pattern of the plasmid (pHBC-ΔPS2) used for in vitro transfection analysis (see figure 3), which expresses HBV DNA with the short deletion in the preS2 gene.

precore gene and results in the disability of HBeAg synthesis [26, 27], was found more frequently in viral strains without rtl80M than in those with it (64% vs. 17%; $P < .005$). Viral strains lacking rtL180M possessed the short deletion in the preS2 gene more frequently than those with rtL180M (50% vs. 10%; $P < .01$). The lengths of the deletion ranged from 12 to 54 bp, and all deletions were located within codon positions 5 to 22 of the preS2 gene (figure 2). Significant differences were also seen in the occurrences of 5 additional mutations—A373, T619, T2102, A/G2660, and A2860—between strains with and those without rtL180M. The detection rate of these 5 mutations was generally low among the lamivudine-resistant HBV strains obtained in this study. The G739 and T/C/A741 mutations are the causes of the rtM204V and rtM204I amino acid changes, and the occurrences of these mutations differed between viral strains with and those without rtL180M ($P < .001$), as described above.

Throughout the HBV genome, 5 mutations were significantly associated with the preference for the rtM204V or rtM204I mutation in the 44 lamivudine-resistant HBV strains (table 3). Of them, 3 mutations—C565, A853, and C1568—were found more frequently in strains with rtM204V than in those with rtM204I, but the frequencies of these mutations were considerably low in our lamivudine-resistant HBV strains. The occurrence of the A667 mutation, which accounts for rtL180M, was

Table 3. Differences in the viral genome between lamivudine-resistant hepatitis B virus (HBV) strains with the rtM204V and rtM204I mutations.

Mutation	Consensus nucleotide[a]	Amino acid substitution	No. (%) rtM204V (n = 16)	No. (%) rtM204I (n = 28)	P
C565	T	None	4 (25)	0 (0)	<.05
T/C646	A	Pol-V519L (rtV173L)	5 (31)	0 (0)	<.005
A667	T	Pol-L528M (rtL180M)	16 (100)	14 (50)	<.001
A853	C	None	3 (19)	0 (0)	<.05
C1568	T	Pol-L826P	3 (19)	0 (0)	<.05

[a] Consensus nucleotides are derived from the genotype C2 HBV strain adr4 (GenBank accession no. X01587) [25].

Table 4. Changing pattern of the precore defective A1896 mutation and short deletion in the preS2 gene from the pretreatment baseline to development of lamivudine resistance in relation to the presence or absence of the rtL180M mutation.

Type of mutation	Pattern of mutation		rtL180M, no.	
	Before therapy	After therapy[a]	Positive (n = 15)	Negative (n = 8)
Precore-defective A1896 mutation	−	−	8	2
	+	+	4	4
	−	+	1	2
	+	−	2	0
Short deletion in the preS2 gene	−	−	12	5
	+	+	0	1
	−	+	2	2
	+	−	1	0

[a] After development of lamivudine-resistant mutant virus.

higher in viral strains with rtM204V than in those with rtM204I ($P < .001$), as shown above. The T/C646 mutation, which causes the rtV173L change, was detected in 5 strains (31%) with rtM204V, compared with none of those with rtM204I ($P < .005$). It has been reported that the rtV173L mutation was detected together with the rtM204V and rtL180M mutations and was considered to be associated with lamivudine resistance [17, 28]. Our finding concerning the rtV173L mutation agreed with those of previous reports.

According to these observations, the relevance of the precore-defective A1896 mutation and the preS2 deletion to the absence of rtL180M was the most distinctive feature of the lamivudine-resistant HBV strains on screening of the whole genome. We therefore directed our attention to these precore and preS2 genomic changes and further investigated their role in the establishment of lamivudine-resistant virus.

Serial changes in the precore mutation and the preS2 deletion in lamivudine-resistant virus before and after lamivudine therapy. Serial changes in the precore-defective A1896 mutation, the short deletion in the *preS2* gene, and the drug resistance–associated rtM204V/I, rtL180M, and rtV173L mutations were investigated in the 23 (52%) of 44 patients with CH-B whose serum samples obtained before lamivudine therapy were available (table 4). Of the 11 patients with virus having the precore-defective mutation after the development of lamivudine resistance, 8 had virus that already possessed the mutation before therapy. Thus, the precore-defective mutation was generally a preexisting genomic change in most patients showing lamivudine resistance. On the other hand, of the 5 patients with virus that had the deletion in the preS2 gene after the development of drug resistance, 4 had virus that did not possess the deletion before therapy. The frequent detection of the preS2 deletion in lamivudine-resistant virus compared with virus before therapy indicates that this deletion may be coselected with drug resistance–associated mutations during the establishment of lamivudine-resistant mutant virus. As for the lamivudine-resistant rtM204V/I, rtL180M, and rtV173L mutations, they were not detected in any of the 23 viruses before lamivudine therapy, as expected.

Effect of the precore mutation and the preS2 deletion on the replicative competence of lamivudine-resistant HBV in vitro. We further conducted in vitro transfection analysis to explore the influence of the precore-defective mutation and the preS2 deletion on the replicative competence of lamivudine-resistant HBV. Three plasmids that expressed wild-type virus, precore-defective virus, and virus with the preS2 deletion were prepared. Next, plasmids with rtM204V plus L180M, rtM204I plus L180M, and rtM204I alone were synthesized in each of the 3 HBV-expressing backbone constructs. The level of intracellular HBV DNA was examined in cells transfected with these HBV-expressing plasmids. As shown in figure 3*A* and 3*B*, the introduction of lamivudine resistance–associated mutations into the virus with the wild-type backbone led to a decrease in viral replication (lanes 1–4). In addition, the replicative competence of the drug-resistant virus lacking rtL180M tended to be lower than that of the virus having rtL180M, although the difference was not statistically significant. As for the precore-defective virus, its replicative activity at baseline was higher than that of the wild-type virus (lanes 1 and 5). The decline in HBV replication due to the insertion of drug resistance–associated mutations was also observed for the virus with the precore-defective backbone. However, unlike for the virus with the wild-type backbone, the replicative activity of the precore-defective virus with lamivudine-resistant mutations was maintained at a considerable level (lanes 5–8). As for the virus with the preS2-deleted backbone, a reduction in viral replication due to the introduction of lamivudine resistance–associated mutations was also seen, but the degree of the reduction was not as great as that in the wild-type virus (lanes 9–12). Thus, both the precore-defective mutation and the preS2 deletion possessed activity supporting the viral replicative competence of lamivudine-resistant HBV, although the activity with the preS2 deletion was not as strong as that with the precore-defective mutation. The

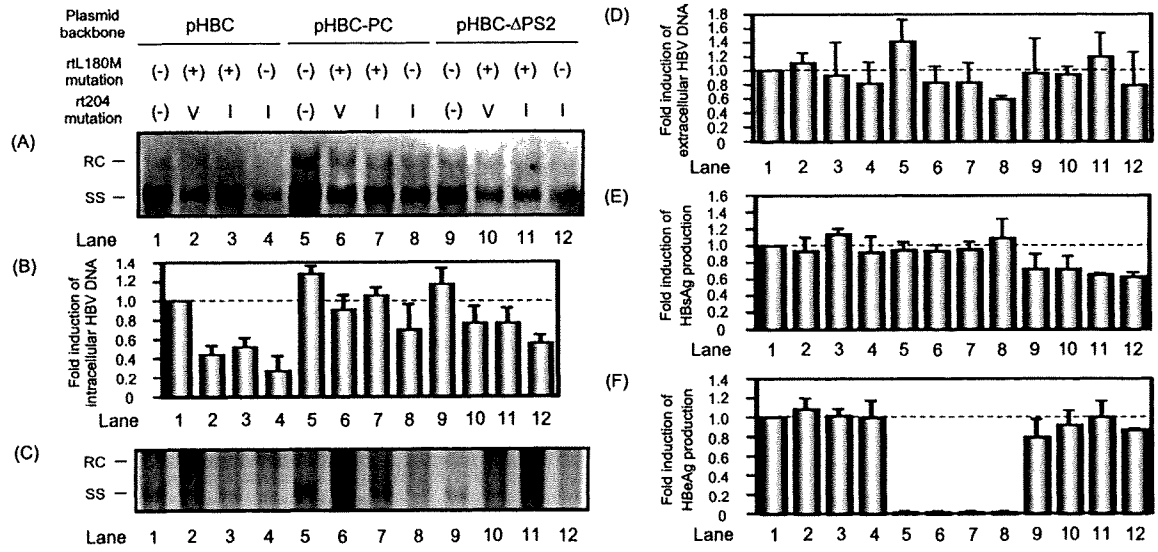

Figure 3. Levels of intracellular and extracellular progeny viral DNA and viral antigen production in cultured cells transfected with wild-type, precore-defective, or preS2-deleted hepatitis B virus (HBV)–expressing plasmids with or without lamivudine resistance-associated mutations. *A*, Representative result of Southern blot analysis to detect the intracellular progeny HBV DNA in cells transfected with various HBV-expressing plasmids. *B*, Quantitative analysis of the level of intracellular progeny HBV DNA. The progeny HBV DNA level for transfection with pHBC was considered to be 1, and the fold activities for transfection with the mutant HBV-expressing plasmids were calculated. The experiment was done 3 times, and results are shown as mean ± SD values. Statistically significant differences ($P < .05$) are as follows: lane 1 vs. 2–4, 1 vs. 5, 2 vs. 6 and 10, 3 vs. 7 and 11, 4 vs. 8 and 12, 5 vs. 6 and 8, and 9 vs. 10–12. *C*, Representative result of Southern blot analysis to detect extracellular progeny HBV DNA in cells transfected with various HBV-expressing plasmids. *D*, Quantitative analysis of the level of extracellular progeny HBV DNA. The progeny HBV DNA level for transfection with pHBC was considered to be 1, and the fold activities for transfection with the mutant HBV-expressing plasmids were calculated. The experiment was done 4 times, and results are shown as mean ± SD values. A statistically significant difference was not observed by 1-way analysis of variance. *E*, Levels of hepatitis B surface antigen (HBsAg) in culture medium of cells transfected with various HBV-expressing plasmids. The HBsAg level for transfection with pHBC was considered to be 1, and the fold activities for transfection with the mutant HBV-expressing plasmids were calculated. The experiment was done 3 times, and results are shown as mean ± SD values. Statistically significant differences ($P < .05$) are as follows: lanes 1 and 5 vs. 9, 3 and 7 vs. 11, and 4 and 8 vs. 12. *F*, Levels of hepatitis B e antigen (HBeAg) in culture medium of cells transfected with various HBV-expressing plasmids. The HBeAg level for transfection with pHBC was considered to be 1, and the fold activities for transfection with the mutant HBV-expressing plasmids were calculated. The experiment was done 3 times, and results are shown as mean ± SD values. Statistically significant differences ($P < .05$) are as follows: lanes 1 and 9 vs. 5, 2 and 10 vs. 6, 3 and 11 vs. 7, and 4 and 12 vs. 8. RC, relaxed circular HBV DNA; SS, single-stranded HBV DNA.

tendency appeared to be more evident in the drug-resistant virus without the rtL180M mutation. This may be a reason for the compensatory rtL180M mutation not being necessary during the establishment of lamivudine resistance in the HBV strain having the precore and preS2 genomic changes.

When the level of extracellular HBV DNA was examined in cells transfected with various HBV-expressing plasmids (figure 3*C* and 3*D*), no significant differences were observed among wild-type, precore-defective, and preS2-deleted viruses with respect to the reduction of viral secretion caused by the introduction of the lamivudine resistance–associated mutation. The discrepant results between the intracellular and extracellular viral DNA levels likely occurred because the extracellular viral DNA assay was less sensitive to minute changes in viral replication than the intracellular viral DNA assay.

As for the levels of production of HBsAg and HBeAg, the virus with the preS2-deleted backbone produced less HBsAg than did the viruses with the wild-type and precore-defective backbones (figure 3*E*). The wild-type and preS2-deleted viruses secreted HBeAg, whereas the precore-defective virus did not (figure 3*F*). The lamivudine resistance-associated mutations did not affect the production levels of HBV antigens.

DISCUSSION

HBV establishes lamivudine resistance via the resistance-causative rtM204V/I mutation and the replication-compensatory rtL180M mutation [15–20]. The present study aimed to investigate the genomewide peculiarity of lamivudine-resistant HBV. In particular, we elucidated the differences between viruses with and those without the compensatory rtL180M mutation. For this purpose, we conducted full-length sequencing analysis of lamivudine-resistant viruses derived from patients with CH-B by means of the PCR direct sequencing method. In some patients, the results were also confirmed by the PCR-subcloning method (data not shown). As a result, the precore-defective

A1896 mutation and the short deletion in the preS2 gene were identified as genomic changes significantly associated with the occurrence of the rtL180M mutation. These 2 viral genomic changes were found to be highly relevant to the observation that the rtL180M mutation was not needed for the establishment of the lamivudine-resistant mutant virus. This suggests that the precore-defective mutation and the preS2 deletion may function as surrogates for the compensatory rtL180M mutation and assist replication of lamivudine-resistant HBV. In the serial analysis of the mutations examined before and after lamivudine therapy, the preS2 deletion tended to be coselected with the drug resistance–associated mutation after therapy, although this tendency was not seen in the case of the precore-defective mutation. This also indicates that the preS2 deletion may have some advantage for establishment of lamivudine-resistant HBV.

We further conducted in vitro transfection analysis to verify the possible supportive role played by the precore and preS2 genomic changes in replication of lamivudine-resistant virus. The intracellular viral DNA was measured as a marker of viral replicative competence. In the wild-type virus, lamivudine resistance–associated mutations reduced viral replicative competence, and the rtL180M mutation compensated for viral replication to a certain degree. This agreed with previous findings of some other investigators [18–20]. On the other hand, the reduction in the viral replication level caused by the lamivudine-resistant mutations was lower in the precore-defective and preS2-deleted viruses than in the wild-type virus. Even the lamivudine-resistant virus without the rtL180M mutation maintained a substantial level of replicative activity in the viruses with precore and preS2 genomic changes. Thus, our results contribute evidence for a supportive role of both precore and preS2 genomic changes in the replicative competence of lamivudine-resistant HBV. This tendency was not evident in the case of the extracellular viral DNA assay, which may have been due to this assay's lower ability to detect slight changes in viral replicative activity.

As for the functional role played by the precore-defective A1896 mutation in the replication competence of lamivudine-resistant HBV, enhanced replicative activity of virus with lamivudine resistance caused by introduction of the precore-defective mutation has been reported for the recombinant HBV–expressing baculovirus system using the genotype D HBV strain [29]. Another previous in vitro transfection analysis using the genotype A HBV strain revealed that experimental insertion of the precore-defective mutation together with the T1858 mutation compensated for the replication competence of the virus possessing lamivudine-resistant mutations [30]. Our experimental result using the genotype C2 HBV strain is consistent with these previous findings. In addition, we showed in the present study that the preS2 deletion may also play a supportive role in the replication yield of lamivudine-resistant HBV, although the enhancement of viral replication caused by the preS2 deletion was not as strong as that caused by the precore-defective mutation.

It remains unclear why the precore-defective mutation leads to an increase in the viral replication of drug-resistant HBV. Previous in vitro transfection analyses have shown that the precore-defective mutation had no influence on viral replicative competence [29–31]. However, in our transfection analysis using the genotype C2 HBV strain, the replicative competence of the precore-defective virus tended to be higher than that of the wild-type virus, even when viruses without the lamivudine resistance–associated mutations were compared. It has recently been shown that the precore-defective mutation caused an elevation in viral replication in the particular HBV strain of genotype B1 [32]. According to this, the precore-defective mutation may in some way enhance HBV replication irrespective of the lamivudine resistance.

As for the involvement of the preS2 deletion in the replicative advantage of lamivudine-resistant HBV, the deletion results in truncation of the polymerase protein as well as the surface protein. Such truncation of the polymerase protein may increase the enzymatic activity and replication capacity of drug-resistant virus. As another possibility, the surface protein with the preS2 deletion may link to incomplete envelopment and subsequent intracellular accumulation of immature viral particles, resulting in an elevated intracellular HBV DNA level. However, this is improbable, because viral envelopment and secretion may be achieved efficiently in preS2-deleted virus as well as wild-type and precore-defective viruses, as was shown in the extracellular viral DNA assay.

In summary, our findings indicate that a precore-defective A1896 mutation and a short deletion in the preS2 gene may support viral replicative activity and substitute for the compensatory rtL180M mutation. Both the precore-defective mutation and the preS2 deletion have been shown to be frequently found during chronic HBV infection [26, 27, 33]. It is noteworthy that such naturally occurring frequent genomic changes in HBV significantly affect the establishment of drug-resistant viral strains. The lamivudine-resistant rtM204V/I mutation has also been reported to be completely or partially involved in resistance to other nucleos(t)ide analogues (emtricitabine, telbivudine, entecavir, and clevudine) [8, 9, 14, 34]. Our findings reveal novel aspects about the establishment of drug-resistant virus possessing the rtM204V/I and rtL180M mutations during the antiviral treatment of patients with CH-B.

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Decreased expressions of CD1d molecule on liver dendritic cells in subcutaneous tumor bearing mice☆

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Background/Aims: **α-Galactosylceramide (α-GalCer) has been attracting attention as a novel approach to treat metastatic liver cancer. However, the activation of liver innate immunity by α-GalCer should be examined because clinical trials of α-GalCer resulted in limited clinical responses.**

Methods: **We examined the activation of liver innate immunity by α-GalCer in subcutaneous Colon26 tumor bearing-mice (C26s.c.TB-mice).**

Results: **The expressions of CD1d molecule on liver dendritic cells (DCs) were significantly lower in C26s.c.TB-mice than those in tumor-unbearing normal mice. Although liver NK cells and NKT cells activated in normal mice after α-GalCer treatment, the activation of these cells were significantly inhibited in C26s.c.TB-mice. α-GalCer treatment resulted in significant antitumor effect against Colon26 metastatic liver tumor in normal mice, but not in C26s.c.TB-mice. The serum levels of TGF-β, known to suppress the CD1d expressions on DCs, in C26s.c.TB-mice were significantly higher than those in normal mice. Surgical subcutaneous tumor mass reduction resulted in the reduction of serum TGF-β, the recovery of CD1d expressions on liver DCs and the improvement of antitumor effect of α-GalCer against metastatic liver tumor.**

Conclusions: **These results suggested that tumor burden reduces CD1d expressions on liver DCs, thus impeding α-GalCer-mediated NK cell activation and antitumor activity in the liver.**

Keywords: **α-Galactosylceramide; CD1d; Liver dendritic cells; Antitumor immunity**

1. Introduction

The glycolipid antigen α-galactosylceramide (α-GalCer) induces activation of NKT cells in a CD1d-dependent manner [1]. α-GalCer presented by DCs efficiently stimulates NKT cells implicated in the innate immunity [2,3]. Recently α-GalCer has been attracting attention for novel anti-tumor therapy. *In vivo* animal studies have shown that systemic administration of α-GalCer can lead to anti-tumor effects against metastatic liver tumor [4,5], suggesting that α-GalCer treatment might be promising for clinical application against liver tumor. Metastatic liver tumors, one of the most common types of advanced malignancy, 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 carried

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Abbreviations: DC, dendritic cell; APC, antigen-presenting cells; CTL, cytotoxic T lymphocytes; α-GalCer, α-galactosylceramide; MNC, mononuclear cells; TB, tumor bearing.

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out in cancer immunotherapy using intravenous administration of α-GalCer, but with limited clinical responses [6,7]. Most clinical trials of cancer immunotherapy have been conducted with patients at advanced stages of cancer. Thus, for further development of α-GalCer treatment in such patients, the antitumor effect of α-GalCer should be examined in hosts with an advanced tumor burden.

In the current study, we evaluated the anti-tumor effect of administration of α-GalCer against liver tumor in subcutaneous tumor bearing animals. Both the antitumor effect of α-GalCer against liver tumor and liver NK cell and NKT cells activation were impaired in subcutaneous tumor bearing mice (s.c.TB-mice). The liver DCs were poorly activated by α-GalCer administration with lower expression of CD1d, NKT-activating molecules. However, the CD1d expression increased and the antitumor effect of α-GalCer against liver tumor was improved after surgical resection of the subcutaneous tumor mass. Our study has shed light toward understanding of the antitumor effect of α-GalCer in metasatic liver cancer patients.

2. Materials and methods

2.1. Mice

Six-to-eight week old female BALB/c mice were purchased from Shizuoka Experimental Animal Laboratory (Shizuoka, Japan), and maintained in micro-isolator cages. 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.

2.2. Cell lines

Colon26, a mouse colon adenocarcinoma cell line was kindly provided by Dr. Takashi Tsuruo (Institute of Molecular and Cellular Bioscience, The University of Tokyo, Tokyo, Japan). This cell line was maintained in complete medium (CM, RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 10 mM L-glutamine: all reagents from GIBCO/Life Technologies, Grand Island, New York) in a humidified incubator at 5% CO_2 and 37 °C.

2.3. α-GalCer

α-GalCer was kindly provided by Kirin Pharma Co. Ltd. (Gunma, Japan) and prepared as previously described [8].

2.4. Animal experiments

To establish Colon26 s.c.TB-mice (C26s.c.TB-mice), BALB/c mice were subcutaneously injected with 3×10^6 Colon26. On day 42, when the tumor size reached approximately 200 mm^2, bone marrow-derived DCs (BM-DCs) and liver DCs were prepared to evaluate the CD1d expression in C26s.c.TB-mice. BM-DC were generated as previously described [8]. Hepatic mononuclear cells (MNC) were prepared as previously described [8]. CD11c+ dendritic cells were isolated from hepatic MNC by magnetic cell sorting using MACS (Miltenyi Biotec, Gladbach, Germany) according to the manufacturer's protocol.

Hepatic metastasis of Colon26 cells was established as previously described [9]. To examine antitumor effect of α-GalCer in the liver of C26s.c.TB-mice, C26s.c.TB-mice or normal mice were injected with 5×10^5 Colon26 cells into the spleen 42 days after mice were subcutaneously injected with 3×10^6 Colon26 cells. Twenty-four hours later, α-GalCer (2 μg/100 μl) or 100 μl of the vehicle was administered intraperitoneally to each mouse. Ten days after tumor injection, the livers of the treated mice were removed, and the liver weight was measured to examine intrahepatic tumor growth.

2.5. Flow cytometry

For phenotypic analysis of BM-DCs and liver DCs, PE- or FITC-conjugated monoclonal antibodies (Ab) against mouse cell surface molecules [CD1d, CD80, CD86 CD11c (all from BD-Pharmingen, San Diego, CA), MHC class II (Miltenyi Biotec)], and appropriate isotype controls were used. We defined DCs with CD11c+ MHC class II+ cells by flow cytometry. To detect the NK cell and NKT cell population in liver MNCs, MNC were stained with PE-conjugated DX5 Ab and FITC-conjugated TCRβ (all from BD-Pharmingen). C26s.c.TB-mice and normal mice were injected intraperitoneally with α-GalCer (2 μg/100 μl) or 100 μl of vehicle. Hepatic MNC were prepared on day 0, 1, 3 and 7 after α-GalCer injection, and both NK cell and NKT cell populations in hepatic MNC were evaluated by flow cytometry. Flow cytometric analysis was performed using a FACscan (Becton Dickinson, San Jose, CA) flow cytometer. The results of flow cytometric analysis are reported in arbitrary mean fluorescence intensity (MFI) units.

2.6. TGF-β and IL-10 ELISA

Mice sera from C26s.c.TB-mice were harvested 42 days after intrahepatic tumor injection. Mice sera and the culture supernatants of Colon26 cells were subjected to mouse TGF-β ELISA (R&D systems, Minneapolis, MN) and mouse IL-10 ELISA (BD-Pharmingen), with lower levels of detection of 31.2 and 31.3 pg/ml, respectively.

2.7. Cytotoxic assay

To evaluate the activation of liver NK cells in C26s.c.TB-mice treated with α-GalCer, liver MNC were isolated 48 h after α-GalCer injection and subjected to ^{51}Cr release assay against NK-susceptible YAC-1 target as previously described [4]. Assays were performed in triplicate, with spontaneous release of all assays not exceeding 25% of the maximum release.

2.8. Surgical resection of subcutaneous tumor

To assess the impact of subcutaneous tumor on the CD1d expression of liver DCs, subcutaneous Colon26 tumors were surgically resected on day 42 after subcutaneous injection of Colon26 cells (C26s.c.TB-ope mice). Fourteen days after subcutaneous tumor resection, liver DCs were isolated and subjected to flow cytometry to evaluate the CD1d expression. To examine antitumor effect of α-GalCer in the liver of C26s.c.TB-ope mice, C26s.c.TB-mice or C26s.c.TB-ope mice were injected with 5×10^5 Colon26 cells into the spleen 10 days after subcutaneous tumor resection. Twenty-four hours later, α-GalCer (2 μg/100 μl) was administered intraperitoneally as above. Ten days later, the livers of the treated mice were removed, and the liver weights were measured to examine intrahepatic tumor growth.

2.9. Statistical analysis

The statistical significance of differences between the groups was determined by applying compared t test with Welch correction or Mann–Whitney U test. The statistical significance of the differences in more than three groups was determined by applying one-way ANOVA. We defined statistical significance as $p < 0.05$.

3. Results

3.1. Expressions of CD1d on DCs in C26s.c.TB-mice were lower than those in normal mice

Since α-GalCer induces activation of NKT cells in a CD1d-dependent manner [1], the expression of CD1d plays an important role in the activation of NKT cells. We examined the CD1d expressions on DCs in C26s.c.TB-mice. The expressions of CD1d on BM-DCs were similar in both normal and C26s.c.TB-mice (Fig. 1A and B). In contrast, those on liver DCs from C26s.c.TB-mice were significantly lower than those from normal mice (Fig. 1A and C). Spleen DCs from C26s.c.TB-mice were also significantly lower than those from normal mice (Fig. 1A and D). These results demonstrated that systemic decrease of CD1d expressions on DCs in each organ is observed in C26s.c.TB-mice, but the potential of differentiation of CD1d expressing DCs from precursor cells in bone marrow was similar between in C26s.c.TB-mice and normal mice.

3.2. The activation of liver NK cells, liver NKT cells and liver DCs was impaired in C26s.c.TB-mice

We next examined the activation of liver NK cells and liver NKT cells in C26s.c.TB-mice after administration of α-GalCer. The cytolytic activity of liver NK cells in α-GalCer-treated mice was stronger than that in vehicle-treated mice in normal mice. In marked contrast, the cytolytic activities in both α-GalCer and vehicle-treated mice were very weak in C26s.c.TB-mice (Fig. 2A). In normal mice, the liver NK cell proportions in whole liver MNCs increased with the peak at 1 day after α-

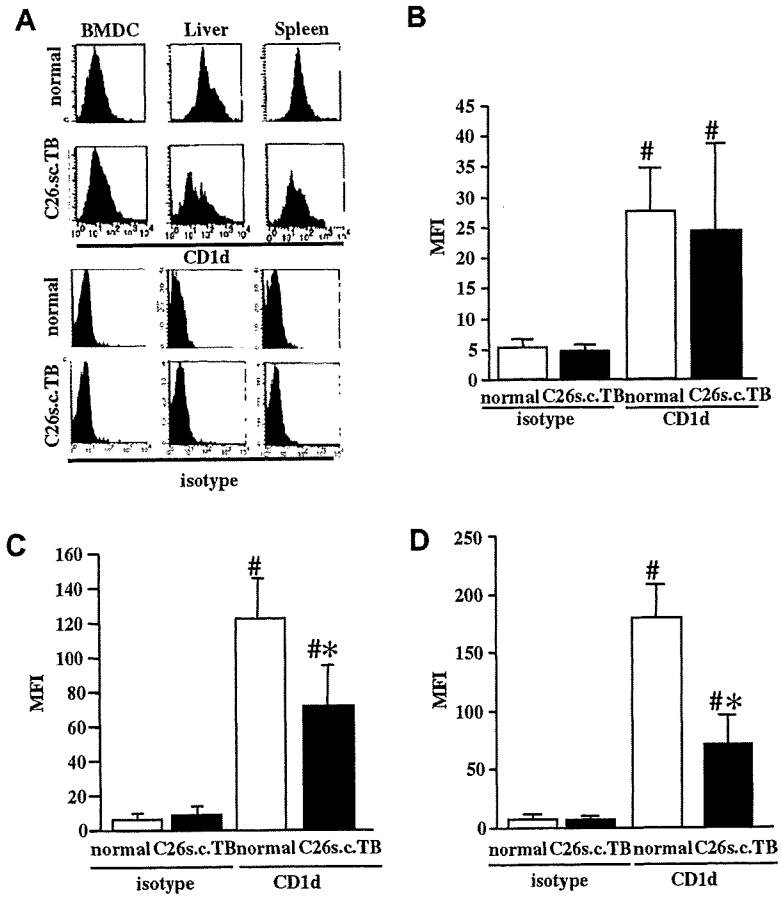

Fig. 1. CD1d expression on DCs in C26s.c.TB-mice. BM-DCs, liver and spleen DCs were prepared from C26s.c.TB-mice or normal mice ($N = 3$ in each group), and the expressions of CD1d molecules on DCs were evaluated by flow cytometry. The representative flow cytometry data of CD1d expressions on BM-DCs, liver DCs and spleen DCs were shown in Fig. 1A. The expression levels of CD1d molecules are reported in arbitrary MFI (mean ± SD). Normal: MFI of DCs from normal mice stained with anti-CD1d or isotype control antibody. C26s.c.TB: MFI of DCs from C26s.c.TB-mice stained with anti-CD1d or isotype control antibody. The CD1d expression on BM-DCs (B), on liver DCs (C), on spleen DCs (D). $^{\#}p < 0.05$ vs. respective isotype control $^{*}p < 0.05$ vs. CD1d expression in normal mice.

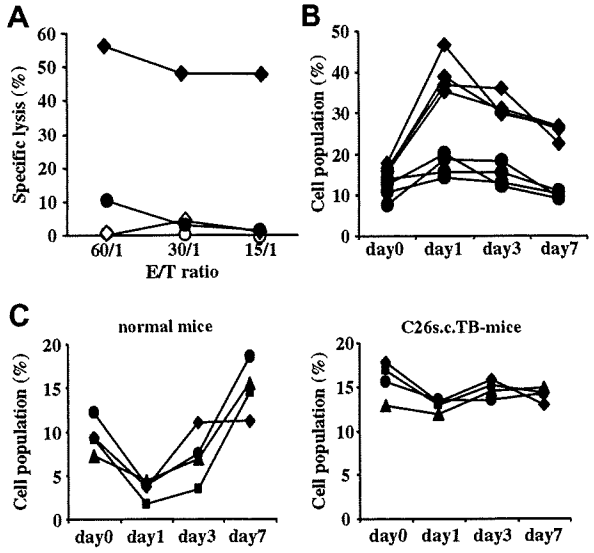

Fig. 2. Impaired activation of liver NK cells and NKT cells in C26s.c.TB-mice. (A) To evaluate the activation of liver NK cells in C26s.c.TB-mice treated by α-GalCer, liver MNC were isolated 48 h after α-GalCer injection and were subjected to ^{51}Cr release assay against NK-susceptible YAC-1 target. (♦) α-GalCer-treated normal mice, (◇) vehicle-treated normal mice, (•) α-GalCer-treated C26s.c.TB-mice, (○) vehicle-treated C26s.c.TB-mice. Representative data shown here is from three independent experiments. (B, C) BALB/c normal mice or C26s.c.TB-mice were injected intraperitoneally with α-GalCer. Hepatic MNC were prepared on day 0, 1, 3 and 7 days after α-GalCer injection. Liver NK cell and NKT cell populations in hepatic MNC were evaluated by flow cytometry. (B) Liver NK cell populations (DX5+/TCRβ− cells) in hepatic MNC after α-GalCer treatment. (♦) NK cell in each normal mice, (•) NK cell in each C26s.c.TB-mice ($N = 4$ in each group). (C) Liver NKT cell populations (DX5+/TCRβ+ cells) in hepatic MNC after α-GalCer treatment in normal mice and C26s.c.TB-mice ($N = 4$ in each group).

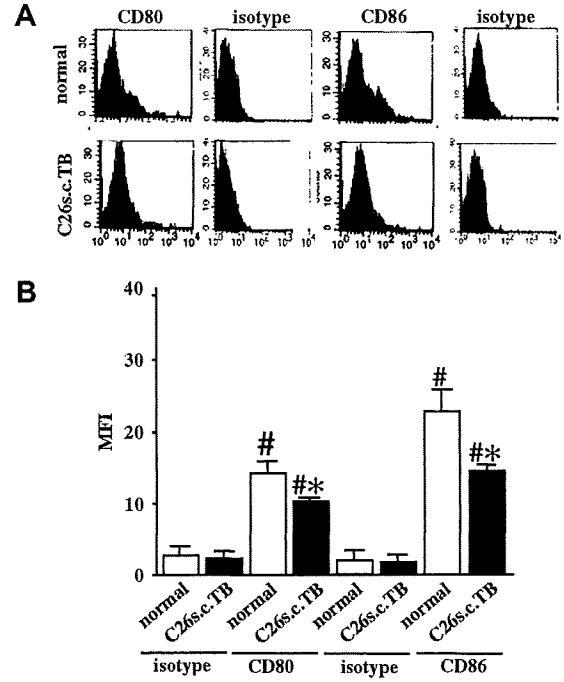

Fig. 3. The CD80 and CD86 expressions of liver DCs in C26s.c.TB-mice and normal mice. The expressions of CD80 and CD86 on liver DCs from both normal mice and C26s.c.TB-mice were evaluated by flow cytometry ($N = 3$ in each group). The representative flow cytometry data of CD80 and CD86 expressions on liver DC were shown in Fig. 3A. The expression levels of CD80 and CD86 molecules are reported as arbitrary MFI (mean ± SD of triplicate samples, Fig. 3B). $^{\#}p < 0.05$ vs. respective isotype control $^{*}p < 0.05$ vs. CD80 or CD86 expressions in normal mice.

GalCer administration, and the liver NK cell proportion at 7 days gradually decreased (Fig. 2B). C26s.c.TB-mice showed weaker increase of liver NK cell proportions in whole liver MNCs than normal mice (Fig. 2B). The liver NKT cell proportion decreased on day 1 and increased again on day 3 and day 7 after α-GalCer administration in normal mice. In marked contrast, those did not change on day 1, day 3 and day 7 after α-GalCer administration in C26s.c.TB-mice (Fig. 2C). The liver NK cell and NKT cell proportion in vehicle-treated mice exhibited no change in both mice groups (data not shown). These results demonstrated that the activation of liver NK cells and NKT cells by α-GalCer was impaired in C26s.c.TB-mice.

We also examined the CD80 and CD86 expressions of liver DCs in both C26s.c.TB-mice and normal mice, which are indicators of the antigen-presenting function of DCs. The expressions of CD80 and CD86 molecules on liver DCs from C26s.c.TB-mice were significantly lower than those from normal mice after α-GalCer administration (Fig. 3), suggesting that the antigen-presenting function of liver DC in C26s.c.TB-mice was also impaired compared with normal mice.

3.3. The antitumor effect of α-GalCer administration against metastatic liver tumor was impaired in C26s.c.TB-mice

We examined the antitumor effect of α-GalCer administration against metastatic liver tumor in both normal and C26s.c.TB-mice. With normal mice, no tumor formation was observed in the liver of any of the α-GalCer-treated mice although large Colon26 liver tumors had formed in all vehicle-treated mice. In contrast, with the C26s.c.TB-mice, large Colon26 liver tumors had formed in both α-GalCer-treated and vehicle-treated mice. The liver weights of the α-GalCer treatment group were significantly lighter than those of the vehicle treatment group for normal mice, while they were similar for both groups of the C26s.c.TB-mice (Fig. 4). These results demonstrated that the antitumor effect of α-GalCer against metastatic liver tumor was impaired in C26s.c.TB-mice.

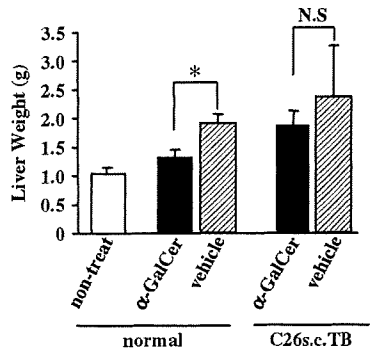

Fig. 4. Impaired antitumor effect of α-GalCer treatment against Colon26 liver tumor in C26s.c.TB-mice. To establish C26s.c.TB-mice, BALB/c mice were subcutaneously injected with 3×10^6 Colon26 cells 42 days before intrasplenic injection of tumor cells. BALB/c normal mice or C26s.c.TB-mice were injected into spleen with 5×10^5 Colon26 cells, and 24 h later either α-GalCer or vehicle was administered intraperitoneally ($N = 6$ in each treatment group). Ten days after treatment, the livers were removed from all treated mice and the liver weights of the groups were compared. As a control, the mean liver weights of untreated normal mice were 1.08 ± 0.09 g. $^*p < 0.05$. α-GalCer treatment group vs. vehicle treatment group in normal mice. N.S. α-GalCer treatment group vs. vehicle treatment group in C26s.c.TB-mice.

3.4. Serum TGF-β levels in C26s.c.TB-mice were increased compared with those in normal mice

Previous reports demonstrated that CD1d expressions on DCs decreased after co-culture with either TGF-β [10] or IL-10 [11]. The supernatants of 24 h cultures of Colon26 cells were subjected to TGF-β and IL-10 ELISA. The production of TGF-β in the supernatants of Colon26 was significantly higher than the control medium (Fig. 5A). No production of IL-10 was detected in the supernatants of Colon26 cells (data not shown). We next evaluate the serum TGF-β and IL-10 levels in C26s.c.TB-mice. The levels of TGF-β in C26s.c.TB-mice were significantly higher than that in normal mice (Fig. 5B). IL-10 was not detected in all mice sera from C26s.c.TB-mice and normal mice (data not shown).

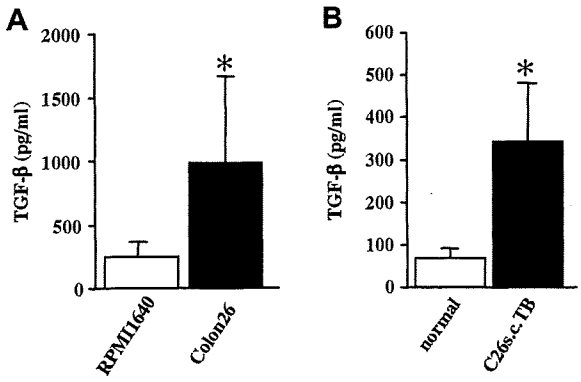

Fig. 5. The TGF-β production from Colon26 cells and the increase in serum TGF-β levels in C26s.c.TB-mice. (A) The culture supernatants of Colon26 cells or culture medium only (RPMI1640) were subjected to mouse TGF-β ELISA. (B) Mice sera from C26s.c.TB-mice were harvested 42 days after subcutaneous tumor injection and were subjected to mouse TGF-β ELISA. Mice sera from normal mice were used as controls. Cytokine levels are reported in pg/ml (mean ± SD of triplicate samples). Similar results were obtained in two independent experiments. $^*p < 0.05$.

3.5. Serum TGF-β levels decreased, the expression of CD1d molecules on liver DCs increased and the antitumor effect of α-GalCer was improved after tumor mass reduction

We next examined serum TGF-β levels and the CD1d expressions on liver DCs after surgical mass reduction in C26s.c.TB-mice. BALB/c mice were subcutaneously injected with 3×10^6 Colon26. On day 42, most Colon26 subcutaneous tumors were surgically excised (C26s.cTB-ope mice). Fourteen days later, serum TGF-β levels were evaluated, and liver DCs from C26s.c.TB-ope mice were prepared to evaluating the CD1d expression in comparison with those from C26s.c.TB-mice. The serum TGF-β levels in C26s.c.TB-ope mice were significantly lower than those in C26s.c.TB-mice (Fig. 6A). The expressions of CD1d on liver DCs from C26s.c.TB-ope mice were significantly higher than those from C26s.c.TB-mice and were similar to those from normal mice (Fig. 6B and C). These results demonstrated that surgical tumor mass reduction might lead to recovery of the impaired immune circumstances in the liver of C26s.c.TB-mice. We examined the antitumor effect of α-GalCer administration against metastatic liver tumor in both C26s.c.TB-mice and C26s.c.TB-ope mice. The liver weights of α-GalCer treated C26s.c.TB-ope mice were significantly lighter than those of α-GalCer treated C26s.c.TB-mice (Fig. 6D). These results demonstrated that the antitumor effect of α-GalCer against metastatic liver tumor was improved after subcutaneous tumor mass resection.

4. Discussion

A previous study showed that administration of α-GalCer resulted in complete rejection of Colon26 metastatic liver cancer in normal mice [5]. In the current study, we evaluated the antitumor effect of α-GalCer against the same Colon26 metastatic liver tumor model in C26s.c.TB-mice. α-GalCer treatment resulted in complete rejection of metasatic Colon26 liver tumor in normal mice, but the antitumor effect of α-GalCer against metastatic liver tumor was significantly impaired in C26s.c.TB-mice. These results were consistent with the clinical data of α-GalCer treatment in

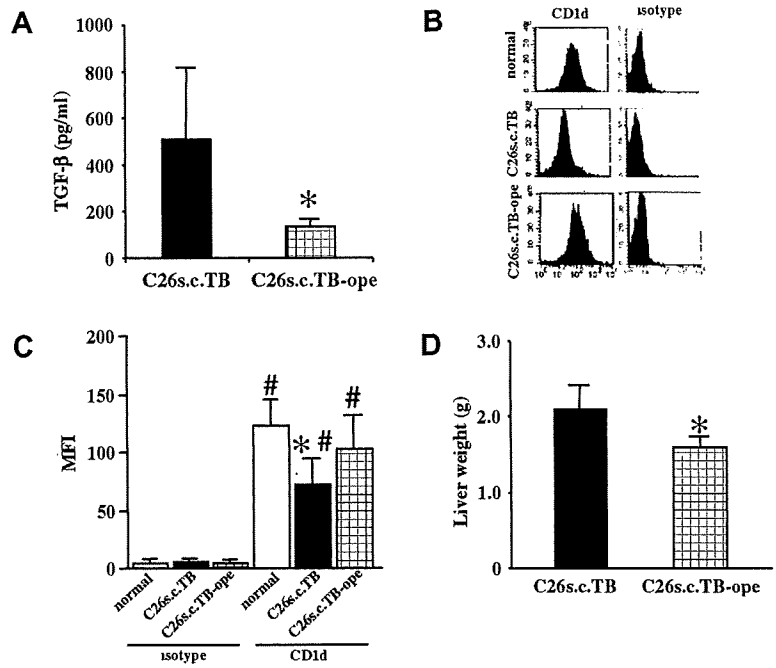

Fig. 6. Evaluation of serum TGF-β and CD1d expression on liver DCs and the antitumor effect of α-GalCer against metastatic liver tumor in surgical treated C26s.c.TB-mice. At 42 days, Colon26 subcutaneous tumors in C26s.c.TB-mice were surgically excised. Fourteen days later, liver DCs from surgically treated mice were prepared for comparison with liver DCs isolated from 42-day C26s.c.TB-mice. (A) Mice sera from C26s.c.TB-mice (C26s.c.TB) or surgically treated C26s.c.TB-mice (C26s.c.TB-ope) were harvested and were subjected to mouse TGF-β ELISA. Cytokine levels are reported in pg/ml (mean ± SD of triplicate samples). $^{*}p < 0.05$. (B, C) The expressions of CD1d on liver DCs from C26s.c.TB-mice (C26s.c.TB) or surgically treated C26s.c.TB-mice (C26s.c.TB-ope) were evaluated by flow cytometry. The representative flow cytometry data of CD1d expressions on liver DC were shown in Fig. 6B. The expression levels of CD1d molecules are reported as arbitrary MFI (mean ± SD of triplicate samples, Fig. 6C). $^{\#}p < 0.05$ vs. respective isotype control $^{*}p < 0.05$ vs. CD1d expression in normal mice. (D) C26s.c.TB-ope mice or C26s.c.TB-mice were injected into spleen with 5×10^5 Colon26 cells, and 24 h later α-GalCer was administered intraperitoneally ($N = 4$ in each group). Ten days after treatment, the livers were removed from treated mice and the liver weights of the groups were compared. $^{*}p < 0.05$. α-GalCer treated C26s.c.TB-ope mice vs α-GalCer treated C26s.c.TB-mice.

patients with advanced cancer, and encouraged us to investigate the detailed mechanism of the markedly reduced antitumor effect of α-GalCer in TB-mice to establish better α-GalCer treatment for cancer patients.

DCs have been implicated in the activation of NKT and NK cells in both mice and humans [1,6,12–17]. α-GalCer presented by CD1d molecules expressed on DCs activates NKT cells via recognition between CD1d molecules and Vα14–Jα281 invariant antigen receptor in mice [18]. Thus the expression of CD1d molecules on DCs is believed to be important for activation of NKT cells. Our study demonstrated that CD1d expressions on bone marrow-derived DCs were similar between normal and C26s.c.TB-mice, suggesting that the ability of differentiating DCs from precursor cells in bone marrow were same in both normal and C26s.c.TB-mice. In contrast, the CD1d expressions of liver DCs and spleen DCs in C26s.c.TB-mice were lower than those in normal mice. This is not unique to C26s.c.TB-mice, because decreased expression of CD1d molecules on liver DCs (not bone marrow-derived DCs) was also observed in CMS4 mouse sarcoma or BNL mouse hepatoma TB-mice (Tatsumi, unpublished data). These results suggested that some systemic immunosuppressive factors might modify the CD1d expression on DCs in TB-mice. Osman et al. demonstrated that α-GalCer administration resulted in activation of liver NKT cells with significant early disappearance of liver NKT cells in normal mice [19]. They also demonstrated that these phenomenon were not observed in CD1d(−/−) mice, suggesting that CD1d expressions play essential roles of liver NKT activation [19]. In our study, the early decreases of liver NKT cells were not observed after α-GalCer treatment in C26s.c.TB-mice. Based on these observations, the decreased expression of CD1d molecules on DCs might be associated with the impaired activation of liver innate immunity, thus resulting in an impaired antitumor effect of α-GalCer.

A normal mice liver contains lymphocytes that are usually enriched with NK and NKT cells; i.e., 25% NK cells and 30% NKT cells in contrast to peripheral blood that contains only 10% NK and 5% NKT cells

[20,21]. Efficient activation of abundant NKT cells and NK cells in the liver might be important in an antitumor effect against liver tumor. We and others have previously reported that sequential activation of both NKT cells and NK cells could be observed in the liver after α-GalCer administration. Although most NKT cells had disappeared from the liver within 12 h of α-GalCer administration [4,19], the antitumor effect against disseminated liver tumor depends on NK cells in the α-GalCer treatment, evidenced by that depletion of NK cells abolished the anti-metastatic tumor effect [4]. In the present study, we found the impairment of both the cytolytic activity of NK cells and an increase of the NK cell proportion in whole liver MNC in α-GalCer-treated C26s.c.TB-mice. These findings also offer the evidence that insufficient activation of liver NK cells might be associated with a poor antitumor effect of α-GalCer in TB-mice. The expressions of antigen-presenting related molecules, CD80 and CD86, on liver DCs in C26s.c.TB-mice were also lower than those in normal mice. Taken together, the presence of a tumor mass might modify the innate immune response in the liver and the maturation of liver DCs in TB-mice.

Several previous reports have demonstrated that TGF-β and IL-10 inhibit CD1d expression on DCs [10,11]. We hypothesize that the decreased expressions of CD1d might be associated with these immunosuppressive cytokines derived from the tumor mass. Our study demonstrated that Colon26 cells produce a large amount of TGF-β, but not IL-10, and that serum TGF-β level in C26s.c.TB-mice was significantly higher than that in normal mice, while the serum IL-10 level was not. Our results suggested that tumor-derived TGF-β might decrease CD1d expressions on liver DCs in C26s.c.TB-mice. Biswas et al. demonstrated that administration of anti-TGF-β neutralizing antibody inhibited metastatic cancer [22], suggesting that if the tumor-derived TGF-β had decreased in TB-mice, the liver immunological environment might be improved to develop antitumor immunity. Based on these results, we next examined serum TGF-β levels and the CD1d expression on liver DCs after surgical subcutaneous mass resection. Fourteen days after surgical resection, serum TGF-β in treated C26s.c.TB-mice had significantly decreased and the expressions of CD1d on liver DCs from treated C26s.c.TB-mice had significantly increased and recovered to the level of normal mice, suggesting that Colon26 tumor tissue derived TGF-β might modify the CD1d expression on liver DCs. More importantly, we demonstrated that the antitumor effect of α-GalCer against metastatic liver tumor was significantly improved in C26s.c.TB-ope mice. We believe that if complete resection of primary tumor could be achieved, the liver immune microenvironment might be expected to recover dramatically and cancer immunotherapy using α-GalCer might lead to better outcomes.

de Lalla et al. reported that the human invariant NKT cells are significantly enriched in chronically inflamed livers as compared with noninflamed ones although human liver harbors significantly less invariant NKT cells than the mouse one [23], suggesting that human invariant NKT cells might also play important roles in developing the chronic liver disease. Although the frequency of invariant Vα24 NKT cells is very low in humans, Vα24 NKT cells can be expanded by the stimulation of α-GalCer in cancer patients [7]. These suggested that the effector function of invariant NKT cells in human liver might be important for the establishing of new cancer treatments of α-GalCer.

The liver is the most common site of metastasis of gastrointestinal cancers (i.e., colorectal cancer, gastric cancer and pancreatic cancer). Thus, new therapeutic approaches of cancer immunotherapy for advanced liver tumor need to be developed. Our report is the first report demonstrating that the presence of a tumor mass might inhibit the activation of liver innate immune cells by α-GalCer due to decreased expression of CD1d on liver DCs. These findings indicate that α-GalCer treatment may represent a promising approach to preventing liver metastasis if the primary tumor can be completely controlled.

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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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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 I. 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 I.

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 (IU/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	47	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

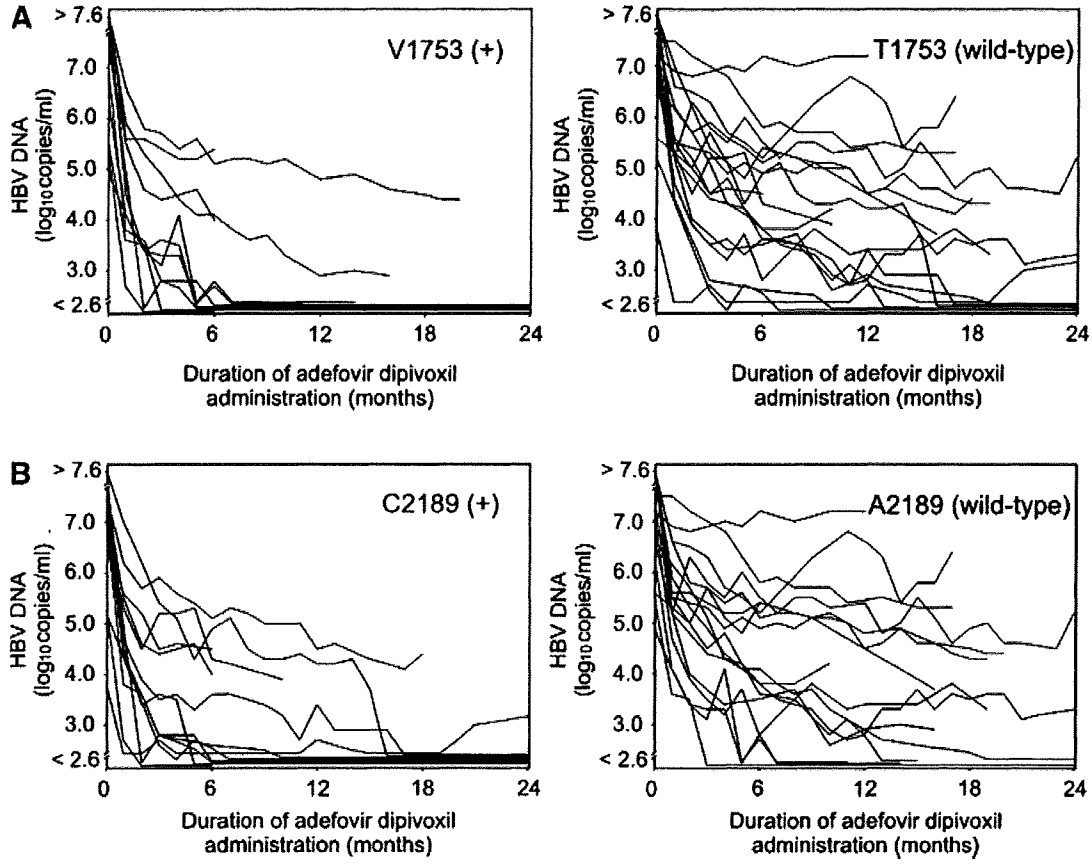

Fig. 1. Serial changes in the HBV DNA level up to 24 months of adefovir dipivoxil added to lamivudine treatment in lamivudine-resistant patients with type B chronic hepatitis in relation to the occurrences of (A) V1753 mutation and (B) C2189 mutation.

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.

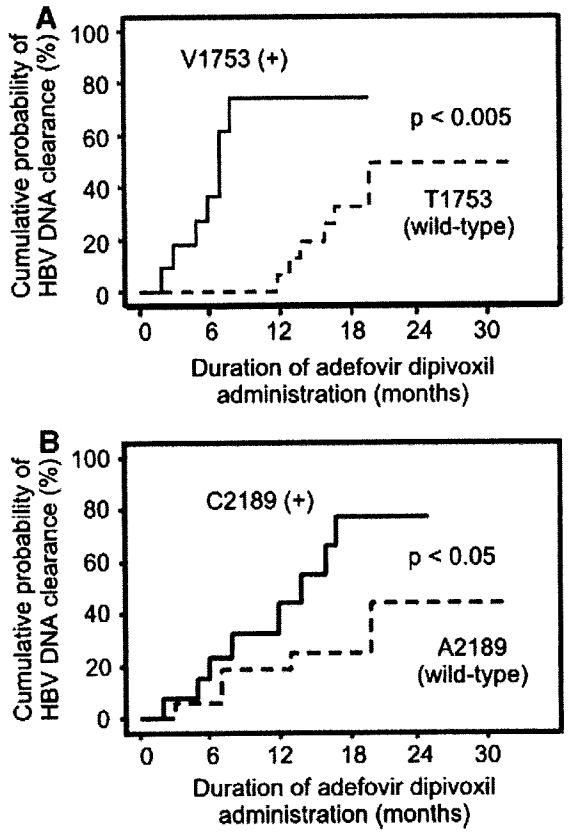

Fig. 2. Influence of the presence of the V1753 and C2189 mutant viruses on the efficacy of adefovir dipivoxil therapy added to lamivudine treatment in lamivudine-resistant patients with type B chronic hepatitis. The Kaplan–Meier estimates of the cumulative probability of sustained HBV DNA clearance correlated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

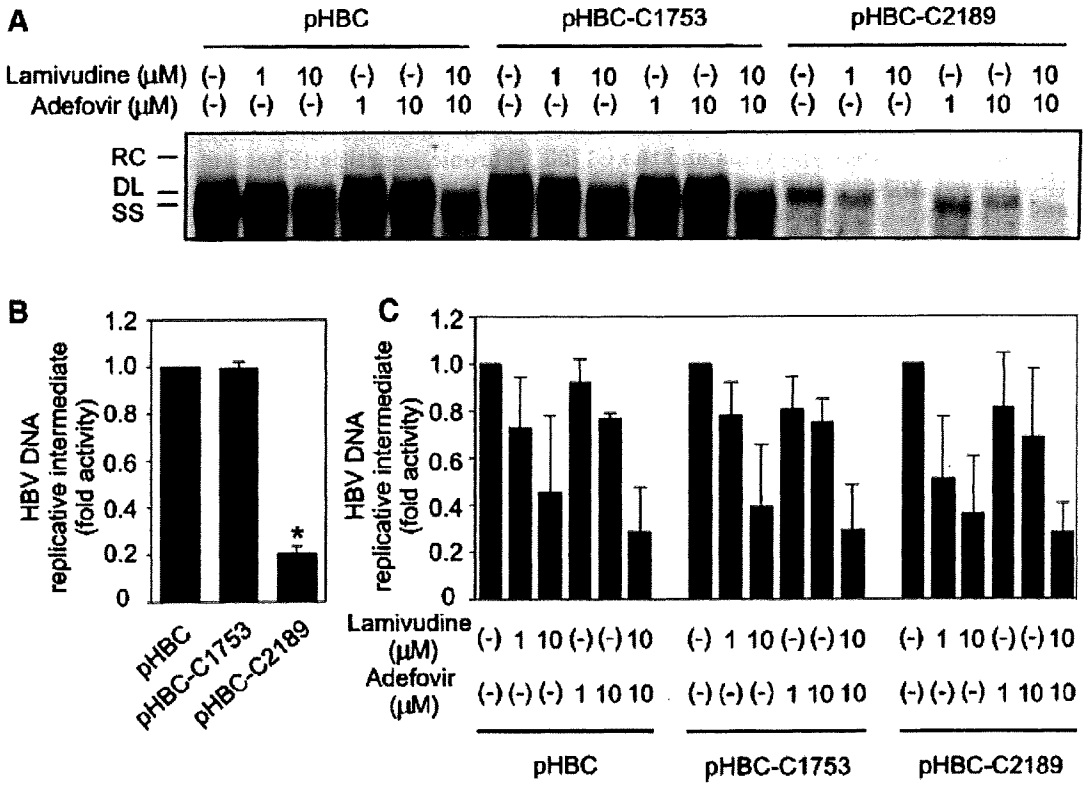

Fig. 3. In vitro transfection analysis to examine viral replicative competence and susceptibility to the treatment with lamivudine and/or adefovir. Huh-7 cells were transfected with pHBC, pHBC-C1753 and pHBC-C2189, and treated with lamivudine alone, adefovir alone, lamivudine plus adefovir, or left untreated. 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].