Table 2. Area Under the ROC Curves, Sensitivity, Specificity, and Negative and Positive Predictive Values of Non-Virologic Responses

Variables	Az	95% CI	Cut-off	Sensitivity	Specificity	NPV[a]	PPV[b]
RIG-I	0.89	0.78–0.95	0.68	0.80	0.87	0.92	0.70
MDA5	0.92	0.86–0.98	0.84	0.82	0.89	0.93	0.74
LGP2	0.76	0.63–0.90	1.03	0.65	0.72	0.85	0.46
RIG-I/Cardif	0.91	0.84–0.99	0.88	0.75	0.91	0.91	0.75
RIG-I/RNF125	0.81	0.69–0.93	1.05	0.82	0.62	0.91	0.43
ISG15	0.91	0.85–0.97	0.36	0.90	0.81	0.96	0.64
USP18	0.90	0.84–0.96	0.67	0.90	0.83	0.96	0.67

[a]NPV, negative predictive value.
[b]PPV, positive predictive value.

we determined the basal protein expression levels of Cardif in the liver in NVR and SVR patients. Western blot analysis demonstrated a single Cardif product in all samples (Figure 4*A*). Similar to Cardif mRNA expression, mean Cardif expression in NVR patients was significantly lower than that in SVR (Figure 4*B*, $P = .01$). The cleavage product of Cardif, which has been reported by Loo et al,[23] was not detected in our analyses.

Transcriptional Responses to PEG-IFN-α-2b and Ribavirin Therapy in PBMC

Sequential analysis in response to PEG-IFN-α-2b and ribavirin demonstrated a rapid and strong induction of RIG-I, ISG15, and USP18 mRNA expression, which peaked 8 hours after PEG-IFN-α-2b administration (Figure 5). A greater fold change of these peak inductions was observed in SVR patients compared with NVR patients, although statistical significance was not achieved. In marked contrast, RNF125 expression profile in response to PEG-IFN-α-2b was triphasic, and consisted of (1) rapid and strong suppression peaked at 8 hours after administration, (2) increased 1.5- to 2-fold above baseline level during 24–48 hours after the administration, and (3) gradually decreased to baseline level (Figure 5). The rapid suppression and subsequent increase following PEG-IFN-α-2b administration tended to have a greater fold change in NVR patients compared with those in SVR patients. In contrast from RIG-I, ISG15, USP18, and RNF125, Cardif expression profile was relatively constitutive, and transcriptional response to PEG-IFN was weak (Figure 5).

Discussion

In the present study, we found that baseline expression levels of intrahepatic viral sensors and related regulatory molecules were significantly associated with the final virologic outcome in patients with chronic hepatitis C who were treated with PEG-IFN-α-2b and ribavirin combination therapy: up-regulation of RIG-I, MDA5, LGP2, ISG15, and USP18 and lower expression of Cardif and RNF125 could predict nonresponse to subsequent treatment with PEG-IFN-α-2b and ribavirin. The positive predictive value of a high ratio of expression of RIG-I to Cardif (>0.88) for NVR was the highest at a value of 0.75, and the negative predictive values of high expression of ISG15 (>0.36/internal control) and USP18 (>0.67/internal control) were the highest at values of both 0.96. These data may be of use in predicting clinical responses to the PEG-IFN-α and ribavirin combination before initiating therapy.

Table 3. Multivariate Analysis for the Factors Associated With Non-Virologic Response

Variable	Odds ratio	95% CI	*P* value
RIG-I/Cardif Ratio (by 0.1)	1.5	1.1–2.1	.008
RIG-I/RNF125 Ratio (by 0.1)	1.2	1.0–2.5	.1
ISG15 (by 0.1/internal control)	1.5	1.1–2.0	.01
Age (by 1 y)	1.0	0.9–1.1	.6
Platelet count (by $1 \times 10^4/\mu L$)	1.2	0.9–1.5	.07

Previously, large randomized controlled trials identified several pretreatment factors associated with the final virologic outcome, such as genotype, HCV RNA level, degree of fibrosis, age, body weight, ethnicity, and steatosis.[24] However, these findings lead us to believe that predicting the final virologic response before initiating PEG-IFN-α and ribavirin is difficult. Indeed, only age and platelet count were associated with the outcome in our patients with genotype 1b and a high viral load. Currently, the final response can be gauged only after treatment has been initiated. Although an early viral response at 12 weeks suggests the eventual outcome with 60%–90% accuracy,[25] a 12-week regimen is associated with adverse effects and is expensive. Therefore, this study investigated the baseline expression of genes involving innate immunity that may have significant effects on clinical outcomes.

In the present study, we demonstrated that RIG-I and MDA5 were inducible upon HCV infection and that expression of these intrahepatic positive viral sensors was up-regulated in NVR. In vitro studies have suggested that RIG-I and MDA5 play a pivotal role in the regulation of IFN production and augment the production of IFN via an amplification circuit. These results suggest that expression of RIG-I and MDA5 and related amplification system may be up-regulated by endogenous IFN at a higher baseline level in NVR patients. However, HCV elimination by subsequent exogenous IFN is insufficient

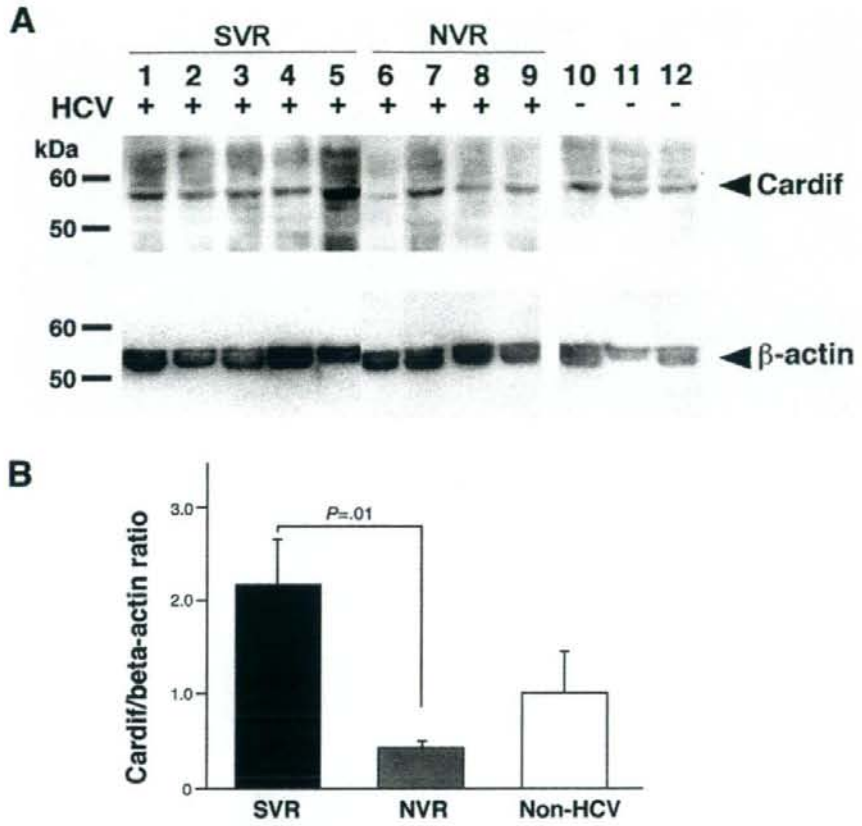

Figure 4. (*A*) Western blot analysis. Five lanes were SVR (*lanes 1–5*), 4 lanes were NVR (*lanes 6–9*), and 3 lanes were non-HCV control (*lanes 10–12*). Specific bands for Cardif and β-actin are indicated by *arrows*. (*B*) Expression level of Cardif protein normalized to β-actin in the liver biopsy specimens according to ultimate treatment response. *Error bars* indicate the standard error.

in these patients, suggesting that NVR patients may have adopted a different equilibrium in their immune response to the virus. In contrast to the expression of RIG-I and MDA5, Cardif mRNA, which was expressed in a relatively constitutive fashion, was significantly lower in NVR. Our ROC analysis highlights that lower expression of Cardif relative to that of RIG-I was one of the strongest predictors for NVR. Moreover, Western blot analysis further confirmed the down-regulation of Cardif in NVR patients, as demonstrated by its protein level. Because Cardif is one of the substantial target molecules of HCV evasion,[11,20] it is likely that Cardif expression is suppressed by HCV with resistant phenotype or is inadequate in NVR patients. Loo et al have demonstrated a Cardif cleavage product in 2 of 4 liver tissue samples of chronic HCV infection.[23] In our study, however, the Cardif cleavage product was not detected, presumably because the product could be unstable in vivo, resulting in rapid degradation. Although further studies are necessary to elucidate mechanisms of Cardif down-regulation, our findings of lower expression of Cardif in NVR suggested that the status of Cardif expression in the liver might have a significant effect on the ultimate outcome of antiviral treatment.

The antiviral effect brought by RIG-I/Cardif signaling is regulated by the coordination of negative and positive regulators. It has been shown that RNF125 functions as a negative regulator of RIG-I/Cardif signaling. RNF125 is an ubiquitin E3-ligase with activity against protein containing CARD domains, such as RIG-I, MDA5, and Cardif, and these ubiquitinated molecules undergo proteasomal degradation. In contrast, RNF125 do not have negative function against LGP2, a negative regulator of RIG-I signaling, because LGP2 lacks CARD domain. In contrast to RIG-I, RNF125 expression was rapidly suppressed by exogenous IFN; therefore, observed lower basal hepatic level of RNF125 in NVR could be explained by the suppressive effect of endogenous IFN, which may be up-regulated in NVR patients. Hence, RNF125 may constitute a negative regulatory circuit for IFN production and is responsible for responsiveness to PEG-IFN and ribavirin therapy.

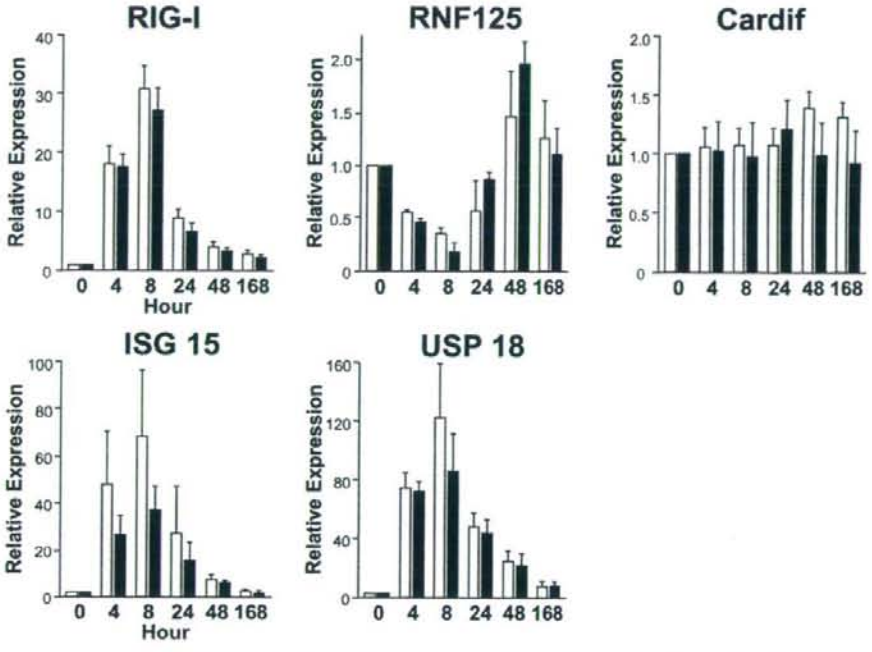

Figure 5. Transcriptional responses during PEG-IFN-α-2b and ribavirin therapy in PBMC (n = 14). *Open columns* indicate SVR (n = 7), and *solid columns* indicate NVR (n = 7). *Error bars* indicate the standard error. The *P* values determined by Mann–Whitney *U* test between 2 groups at 8 hours were as follows: RIG-I, *P* .3; RNF125, *P* .3; Cardif, *P* .7; ISG15, *P* .3; USP18, *P* .2.

It has been shown that RIG-I function is modified by ISG15 via ISGylation.[17] Consistent with our data, Chen et al identified 18 genes, including ISG15 and USP18, whose expression differed between responders and nonresponders.[26] Interestingly, a recent study has shown that USP18 negatively regulates IFN signaling independently of its isopeptidase activity toward ISG15 by binding to the IFNAR2 receptor subunit and blocking the interaction between Janus kinase and the IFN receptor.[27] Moreover, the siRNA knockdown of USP18 in human cells has consistently been shown to potentiate the ability of IFN to inhibit HCV RNA replication.[28] Therefore, USP18 is suggested as a novel in vivo inhibitor of signal transduction pathways that are specifically triggered by type I IFN. Consistent with a role for USP18 in down-regulating the antiviral IFN response, we confirmed that up-regulation of USP18 was one of the factors predicting a lack of response to treatment with IFN.

The mechanism underlying the association of gene expression involving innate immunity with resistance to therapy is not well understood. Our human study with HCV patients treated by PEG-IFN and ribavirin highlights RIG-I/Cardif, RIG-I/RNF125, and ISG15/USP18, which is partly responsible for the clinical responsiveness to antiviral therapy. RIG-I signaling by viral pathogens may affect a wide variety of responses in not only innate but also acquired immunity. Our study is the first to demonstrate the potential relevance between molecules involving innate immunity and the clinical response to antiviral therapy.

In addition, sequential analysis of expression profile during PEG-IFN-α-2b and ribavirin treatment was also performed in this study. Lanford et al demonstrated transcriptional response to IFN-α in chimpanzee by genome microarray analysis, which included RIG-I, ISG15, and USP18.[29] An association of transcriptional response with early phase of virologic response has been also reported in PBMC or liver biopsy specimen.[30–32] We recently reported that the transcriptional double-stranded RNA-activated protein kinase response during treatment with PEG-IFN-α-2b and ribavirin was associated with the ultimate clinical response.[30] Similarly, the present study demonstrated a strong and rapid increase of RIG-I, ISG15, and USP18 mRNA in response to clinical PEG-IFN treatment especially in SVR patients, although few patients were available to achieve statistical significance between SVR and NVR. In marked contrast, transcriptional response of RNF125 exhibited a triphasic pattern. Rapid suppression seen in the first phase was presumably because of a negative regulatory effect of IFN. However, increase of RNF125 mRNA in the second phase, which tended to be grater in NVR, may be responsible for inhibiting RIG-I expression seen 8–48 hours after PEG-IFN-α-2b administration. Although limitations includ-

ing the use of PBMC and small sample size still deserve mention, the sequential expression profile during treatment may provide further valuable information regarding the prediction of the clinical response to the therapy and the mechanism of action of antiviral treatment.

In the present study, we have included patients with genotype 1b because it is imperative to designate a virologically homogeneous patient group to associate individual treatment responses with different gene expression profiles that direct innate immune responses. We have preliminarily studied genotype 2 patients and found that Cardif and RNF125 gene expression levels in NVR patients were significantly lower than those with SVR patients ($P = .03$ and $P = .04$, respectively) and that RIG-I/Cardif and RIG-I/RNF125 ratios were significantly higher in NVR patients ($P = .02$ and $P = .009$, respectively, see Supplementary Figure 2 online at www.gastrojournal.org). These findings suggest that the differences in gene expression profiles between SVR and NVR were almost identical to those demonstrated in patients with genotype 1b. However, the correlation between treatment responses in all the genotypes and the different status of innate immune responses needs to be explored. Further studies may be necessary to clarify this issue.

In conclusion, the results of the present study offer potentially important clinical implications for patients with chronic hepatitis C who are treated with PEG-IFN-α and ribavirin. Quantifying hepatic gene expression of the RIG-I/Cardif system, including its regulators before treatment, is useful in identifying patients who are at a higher risk for NVR. The data from these assays can provide valuable information that may influence the decision about the treatment strategy in each individual patient. Finally, this clinical human study demonstrates the potential relevance of the molecules involving innate immunity to the clinical response to therapy. Our data will help understand the pathogenesis of HCV resistance and development of new antiviral therapy targeted toward the innate immune system.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2008.02.019.

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Received August 30, 2007. Accepted January 31, 2008.

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Supported by grants from the Miyakawa Memorial Research Foundation; the Japanese Ministry of Education, Culture, Sports, Science and Technology; and the Japanese Ministry of Welfare, Health and Labor.

Financial disclosures: The authors who participated in this study have had no affiliation with the manufacturers of the drugs involved either in the past or in present and have not received funding from the manufacturers to conduct this research.

Journal of Medical Virology 79:1811–1817 (2007)

Successful Treatment of an Entecavir-Resistant Hepatitis B Virus Variant

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Emergence of a lamivudine (LAM)-resistant hepatitis B virus (HBV) with amino acid substitutions in the YMDD motif is a well-documented problem during long-term LAM therapy. Entecavir (ETV) is a new drug approved for treatment of HBV infection with or without LAM-resistant mutants. This report describes an ETV-resistant strain of HBV, which emerged after prolonged ETV therapy in a patient who did not respond to LAM therapy. Direct sequence analysis of the ETV-resistant strain showed appearance of amino acid substitution rtS202G in the reverse transcriptase (RT) domain, together with rtL180M + M204V substitution that had developed at the emergence of LAM-resistant mutant. In vitro analysis demonstrated that the rtL180M + M204V + S202G mutant strain displayed a 200-fold and a 5-fold reduction in susceptibility to ETV compared with the wild-type and the rtL180M + M204V mutant strain, respectively. Adefovir was effective against the ETV-resistant strain both in vitro and during the clinical course. In conclusion, this study showed that virological and biochemical breakthrough due to ETV could occur in patients infected with LAM-resistant HBV and confirmed that the addition of rtS202G substitution to the rtL180M + M204V mutant strain is responsible for ETV resistance and we could treat the resistant mutant successfully. ***J. Med. Virol. 79:1811–1817, 2007.***

KEY WORDS: HBV; rtS202G; lamivudine; adefovir; in vitro

INTRODUCTION

Hepatitis B virus (HBV) is a small enveloped DNA virus known to cause chronic hepatitis and often leads to liver cirrhosis and hepatocellular carcinoma [Bruix and Llovet, 2003; Ganem and Prince, 2004]. To date, interferon and three nucleoside and nucleotide analogs (lamivudine [LAM], adefovir dipivoxil [ADV], and entecavir [ETV]) have been approved for the treatment of chronic HBV infection. Nucleoside and nucleotide analogues suppress HBV replication in most patients and improve transaminase levels and liver histology [Nevens et al., 1997; Lai et al., 1998; Suzuki et al., 1999]. However, prolonged therapy results in the emergence of drug-resistant mutants.

LAM is associated with a higher rate of emergence of drug-resistant mutants than ADV or ETV, which is 24% and 70% after 1 and 4 years of therapy, respectively, followed by increases in viral load and re-elevation of transaminase levels [Lai et al., 2003]. Most LAM-resistant

Abbreviations used: HBeAg,hepatitis B e antigenHBsAg,hepatitis B surface antigenHBV,hepatitis B virusORF,open reading framePCR,polymerase chain reactionRT,reverse transcriptase

Grant sponsor: Ministry of Education, Sports, Culture, and Technology; Grant sponsor: Ministry of Health, Labor and Welfare.

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Accepted 28 June 2007
DOI 10.1002/jmv.20981
Published online in Wiley InterScience (www.interscience.wiley.com)

strains show amino acid substitutions in the YMDD (tyrosine–methionine–aspartate–aspartate) motif in the C domain of HBV polymerase. In addition to the emergence of the YMDD mutation, rtL180M and rtV173L mutations in the B domain of HBV polymerase are frequently observed [Allen et al., 1998; Delaney et al., 2003].

Both in vitro and clinical studies have shown recently that ADV and ETV could suppress both wild-type and LAM-resistant strains and were confirmed as salvage therapy for LAM-refractory patients [Levine et al., 2003; Sherman et al., 2006; Rapti et al., 2007]. However, a few studies have already reported the emergence of resistant mutants to these drugs.

ADV-resistant mutations are infrequent and their appearance is delayed in treatment-naïve patients; mutation occurs at 0% after 1 year and 28% after 5 years and the selection of rtA181V/T or rtN236T mutant was associated with resistance to ADV [Maecellin and Asselah, 2005]. On the other hand, the emergence rate of ADV-resistant mutations in LAM-resistant patients was 18% after 48 weeks of ADV monotherapy [Lee et al., 2006]. A recent study reported patients treated with combination therapy of ADV with LAM did not develop resistance to ADV for 3 years [Rapti et al., 2007].

ETV is the most novel nucleotide analogue of the three drugs and displays greater in vitro potency than LAM or ADV against wild-type HBV. ETV-resistance is reported to be rare in treatment-naïve patients [Colonno et al., 2006]. However, ETV-resistant mutants appeared at 6–9% per year in LAM-refractory patients [Tenney et al., 2004, 2007; Sherman et al., 2006].

In the present study, an ETV-resistant strain of HBV was identified after prolonged ETV therapy in a patient who did not respond to LAM therapy. To our knowledge, this is the first report that breakthrough hepatitis was induced by emergence of an ETV-resistant strain and was successfully treated with ADV. This study checked the importance of amino acid substitutions in the HBV polymerase for resistance to ETV in vitro. Furthermore, the susceptibility of the mutant strain to ADV was analyzed.

MATERIALS AND METHODS

Antiviral Compounds

LAM [(−)-β-L-2′, 3′-dideoxy-3′-thiacytidine] was provided by GlaxoSmithKline (Stevenage, Herts, UK). Adefovir {9-[2-(phosphonomethoxy)ethyl]-adenine} was provided by Gilead Sciences (Foster City, CA), and ETV {2-amino-1,9-dihydro-9-[(1S,3R,4S)-4-hydroxy-3-(hydroxymethyl)-2-methylenecyclopentyl]-6H-purin-6-one, monohydrate} was provided by Bristol-Myers Squibb Pharmaceutical Research Institute (Wallingfold, CT).

Analysis of Virological Markers

Hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg), and antibody against HBeAg (anti-HBe) were determined by enzyme immunoassay kits (Abbot Diagnostics, Chicago, IL). HBV-DNA was measured by real-time PCR using the Light Cycler (Roche, Mannheim, Germany) by the polymerase chain reaction (PCR). The primers used for amplification were 5′-TTTGGGCATGGACATTGAC-3′ and 5′-GGTGAA-CAATGTTCCGGAGAC-3′. The amplification condition included initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 sec, annealing at 58°C for 5 sec and extension at 72°C for 6 sec. The lower detection limit of this assay was 300 copies.

Cloning of HBV-DNA and Plasmid Construction

HBV-DNA was extracted from 100 μl of serum samples by SMITEST (Genome Science Laboratories, Tokyo, Japan) and was dissolved in 20 μl H_2O. The full-length HBV-DNA was amplified using the above HBV-DNA samples by the method of Gunther et al. [1998]. Nucleotide sequence positions were numbered from the unique *Eco*RI site. The 1.4 genome lengths HBV-DNA amplified from the serum of a patient who showed ETV resistance was cloned into a plasmid vector pcDNA3 (Invitrogen, San Diego, CA). In brief, the PCR product amplified using serum from the patient was cleaved with *Bam*HI and *Apa*I (HBV positions 1,400–2,600) and cloned into pcDNA3, which was named pcDNA3-1. Similarly, the PCR product was cleaved with *Apa*I and *Bam*HI (HBV positions 2,600–3,215, 1–1,400) and cloned into pBluerscript SK+ (Stratagene, La Jolla, CA), which was named pB-1. The *Kpn*I-*Bam*HI fragment from pB-1 and *Kpn*I-*Apa*I fragment from pcDNA3-1 were cloned into pcDNA3-1. To introduce the nucleotide substitutions into the rtL180M, M204V, and S202G, site-directed mutagenesis was performed using the QuickChange Site-Directed Mutagenesis kit (Stratagene). Four plasmids with/without amino acid substitutions were created and are listed in Table IV.

Cell Culture, Transfection, and Determination of IC_{50}

HepG2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (FBS) at 37°C under 5% CO_2. Cells were seeded to semi-confluence in 6-well tissue culture plates. Transient transfection of the plasmids into HepG2 cell lines was performed using TransIT-LT1 (Mirus, Madison, WI) according to the instructions provided by the supplier. To determine 50% inhibitory concentrations (IC_{50}s) for each anti-viral drug, various concentrations of LAM, ADV, and ETV were added after 24 hr to the culture plate containing the cells, and harvested after 5 days. The medium containing the drugs was changed at days 1, 3, and 4. All experiments were performed in triplicate. GraphPad prism (GraphPad Prism Software, Inc., San Diego, CA) was used to determine the best-fit values for individual dose–response equations.

Analysis of Replicative Intermediate of HBV by Quantitation

The cells were harvested at 5 days after transfection and lysed with 250 μl of lysis buffer (10 mM Tris-HCl [pH

7.4], 140 mM NaCl, and 0.5% (v/v) NP-40) followed by centrifugation for 2 min at 15,000*g*. The core-associated HBV genome was immunoprecipitated by mouse anti-core monoclonal antibody 2A21 (Institute of Immunology, Tokyo) and subjected to Southern blot analysis after SDS/proteinase K digestion followed by phenol extraction and ethanol precipitation. Quantitative analysis was performed by real-time PCR with cyber green using Light Cycler. The HBV-specific primers used for amplification were 5′-TTTGGGCATGGACATTGAC-3′ and 5′-GGTGAACAATGTTCCGGAGAC-3′. The amplification conditions included initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 sec, annealing at 58°C for 5 sec, and extension at 72°C for 6 sec. The lower detection limit of this assay was 300 copies.

Statistical Analysis

Data are expressed as mean ± SD. Group comparisons were performed using the Student's *t*-test. A *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Patient's Profile

An ETV-resistant strain of HBV was isolated from a 44-year-old Japanese woman with hepatitis B e antigen-positive chronic HBV infection (Fig. 1A). In this patient, LAM successfully reduced the HBV at the initial stage of treatment. However, viral breakthrough was observed at 11 months after the beginning of LAM therapy and the HBV viral load reached up to 7.5 log copies/ml. After 17 months of LAM, interferon was added to LAM therapy for 6 months. However, after withdrawal of IFN, the viral load and ALT rebounded. Thus, the patient was switched to 0.5 mg of ETV. This resulted in reduction of HBV-DNA and normalization of ALT. After 12 months of ETV therapy, the viral load rebounded, and following 12 more months of ETV, breakthrough hepatitis was observed. After stopping ETV, because of the inadequate effect of IFN monotherapy for one month, the patient was switched to 10 mg of ADV. This treatment reduced both the viral load and ALT level to acceptable levels (Fig. 1).

treatment		month	ALT (IU/L)	HBV-DNA (log copies/ml)
		-3	246	7.2
LAM		0	46	5.2
		5	28	3.7
		11	33	4.1
	IFN	17	72	7.5
		18	1184	5.6
		20	39	3.9
		23	34	3.4
		27	117	7.1
ETV		31	112	7.2
		39	40	2.9
		43	28	4.2
	IFN	56	140	6.8
ADV		57	313	6.8
		60	38	4
	LAM	71	24	3.3
		75	19	3.1

Fig. 1. Clinical course of a patient who developed entecavir resistant mutant.

Isolation of a Multiple Drug-Resistant Hepatitis Strain

Isolates from this patient were analyzed for substitutions in HBV reverse transcriptase (RT). Comparison of the nucleotide sequences by the direct sequence method obtained throughout the clinical course showed three amino acid substitutions in the RT domain of the polymerase (Table I). At the baseline of LAM, all three substitutions were of the wild-type by direct sequence analysis and clonal analysis (Table II). After breakthrough hepatitis induced by LAM, direct sequence analysis showed mixed type (YIDD and YVDD) mutant strain. The rtM204V mutant was detected in 65% of HBV clones and the rest were all the YIDD type. Importantly, at this point, there was no amino acid substitution at rt202. After 12 months of ETV therapy when the viral load was slightly increased, the rtL180M + M204V + S202G mutant was detected in 45% of the HBV clones, followed by decrease of the YIDD and YVDD mutants without substitution at rtS202G. Finally, after 24 months of ETV therapy, when the breakthrough hepatitis occurred, the rtL180M + M204V + S202G mutant was detected in 92% of the HBV clones and the rest were rtL180M + M204V mutants without substitution at rtS202G. Interestingly, the rtM204I + S202G strain never appeared during nucleotide therapy.

Susceptibility of Mutants to Entecavir In Vitro

To analyze the role of the rtL180M, rtG202S, and rtM204V substitutions in ETV resistance, four patient-specific strains were transfected into HepG2 cells (Table III). ETV was added after 24 hr to the culture plate containing the cells, and harvested after 5 days. The core-associated HBV genome was extracted from cells and quantified by real-time PCR. The double amino acid substitutions rtL180M + M204V, which is related to LAM resistance, displayed a 38-fold decrease in susceptibility to ETV compared with the wild-type. Moreover, triple amino acid substitutions rtL180M + M204V + S202G, isolated from the patient

TABLE I. Direct Sequence Analysis of Samples From Our Patient With Entecavir (ETV) Resistance

	rt L180	rt S202	rt M204
(1) At the beginning of LMV	—	—	—
(2) At the beginning of ETV	L/M	—	I/V
(3) One year after ETV	M	G/S	V
(4) Two years after ETV	M	G	V

LMV, lamivudine.

who developed breakthrough hepatitis during ETV therapy, induced 198 times greater resistance than the wild-type. In agreement with the above data, the appearance of the rtS202G substitution in the rtL180M + M204V mutant strain resulted in a fivefold decrease in ETV susceptibility. On the other hand, only a single amino acid substitution rtS202G, which was artificial and did not truly exist, had little effect on the susceptibility to ETV (Table III, Fig. 3).

Susceptibility of Mutants to Lamivudine and Adefovir In Vitro

The susceptibility of the rtL180M + M204V and rtL180M + M204V + S202G mutants to LAM was also analyzed using transient transfection assay with HepG2 cells. Both strains displayed strong resistance to LAM (>1,000-fold). We also examined whether ADV was as effective against the rtL180M + M204V + S202G mutant strain as the wild-type. The IC_{50} values of the mutant strain and wild-type for adefovir were almost the same, which displayed the same result in vivo (Fig. 2, Table IV).

DISCUSSION

The present study describes the identification of an ETV-resistant strain of HBV after prolonged ETV therapy in a patient who was resistant to LAM therapy. Using direct sequencing and clonal analysis, the results demonstrated that the addition of rtS202G mutation to the LAM-resistant mutant strain correlated with the ETV-resistance. To our knowledge, this is the first report of a patient who developed not only virologic breakthrough but also biochemical breakthrough, followed by successful treatment with ADV (Fig. 1).

Clonal analysis showed mixed type of LAM-resistant strains at the commencement of ETV treatment. All of the rtM204V mutant strains were accompanied by rtL180M mutation, but none of the rtM204I mutant did. After 1 year of ETV therapy, the rtL180M + M204V + S202G mutant emerged in 45% of the HBV clones. Furthermore, almost all clones became the rtL180M + M204V + S202G variant 2 years after ETV therapy. These results suggest two important things. Firstly, the addition of the rtS202G mutant to the rtM204V mutant induced the ETV resistance. Secondly, the S202G was induced only in the mutant strains with rtM204V not in the rtM204I.

The in vitro study described in this article demonstrated that the rtL180M + M204V mutation reduced the susceptibility to ETV by 38-fold compared with wild-type (Table III). Furthermore, the addition of the rtS202G substitution to the rtL180M + M204V mutant strain resulted in a fivefold decrease in ETV susceptibility. Interestingly, the single S202G substitution did not induce ETV resistance in vitro. Thus, it appears that the rtS202G substitution never reduced the susceptibility to ETV in the absence of rtM204V substitution. The amino acid substitutions rtS202G have been reported to emerge with resistance against ETV [Yim et al., 2006; Tenney et al., 2007; Villet et al., 2007]. In all previous studies, the rtS202G mutation was accompanied by rtM204V substitution and our results are similar to those of the reported in vitro studies. It is known that other amino acid substitutions, rtT184 and rtM250 in the RT domain are associated with ETV resistance and they also need the substitution at rt204 to achieve such resistance. Tenney et al. [2004] reported that the rates of T184, S202, and M250 mutations in LAM-resistant patients before ETV treatment were 5.2%, 1.2%, and 1.8%, respectively. Moreover, these ETV-resistance-related residues emerged in 6% more patients by 1-year ETV therapy and 8% more patients by 2-year therapy.

TABLE II. Clonal Analysis of Samples From the Patient With Entecavir (ETV) Resistance

	Relative rate (%) of clones (no. of clones/total)			
	Wild	M204I	L180M + M204V	L180M + M204V + S202G
(1) At the beginning of LMV	100 (6/6)	0	0	0
(2) At the beginning of ETV	0	35 (7/20)	65 (13/20)	0
(3) 12 months after ETV	0	14 (3/22)	41 (9/22)	45 (10/22)
(4) 24 months after ETV	0	0	8 (1/13)	92 (12/13)

LMV, lamivudine.

TABLE III. In Vitro Susceptibility of rtL180/rtM204/rtS202 Mutants to Entecavir

				ETV	
	rt L180	rt M204	rt S202	IC_{50} (μM)	Resistance (fold)
Wild	—	—	—	0.00081	1
S202G	—	—	G	0.00054	0.67[a]
L180M + M204V	M	V	—	0.031	38**
L180M + M204V + S202G	M	V	G	0.16	198**

Experiments were performed in triplicates.
[a]NS, not significant.
**$P < 0.001$ compared with the wild-type.

In the present study, clonal analysis showed the rtS202G substitution was induced only in the mutant strains with rtM204V but not in the rtM204I, as described recently [Yim et al., 2006; Tenney et al., 2007; Villet et al., 2007]. A recent study demonstrated similar results; all 16 patients with virologic rebounds with ETV resistance had the rtM204V substitution, either alone or in combination with rtM204I substitution [Tenney et al., 2007]. Ono et al. [2001] reported that the clinical frequency of LAM-resistant mutants was 18.6% for the rtM204I, 1.4% for the rtM204V, 11.4% for the rtL180M + M204I, and 64.3% for the rtL180M + M204V. In other words, most of the YVDD mutants were accompanied with rtL180M mutation. On the other hand, only about one-third of YIDD mutants were accompanied with rtL180M. Previous in vitro studies demonstrated that both the rtM204I and rtL180M + rtM204V substitutions had incomplete cross-resistance to ETV, and reported that the rtL180M + rtM204V mutant was more susceptible than the rtM204I mutant. The replication capacity of the rtL180M + rtM204V was four-times larger than the rtM204I mutant [Ono et al., 2001]. Thus, it was considered that the addition of rtS202G substitution to the rtL180M + rtM204V mutant could strengthen the replication ability, or could reduce susceptibility to ETV more strongly than the rtM204I mutant. Further studies are needed to confirm the above hypothesis.

There is no consensus regarding the management of patients with ETV resistance. There are few reports of successful treatment of ETV resistant viruses in vivo. Villet et al. [2007] reported that ADV was clinically effective for virological breakthrough caused by ETV-resistant HBV variant. However, different from the previous report, the present study demonstrated the emergence of biochemical breakthrough after viral rebound caused by ETV resistance. Moreover, it was confirmed that ADV was effective in not only viral breakthrough but also biochemical breakthrough. Our in vitro study also indicated that the rtL180M + M204V + S202G mutant had no resistance against ADV. This result is compatible with the response in vivo. In this regard, recent studies demonstrated that ADV and tenofovir are effective for ETV-resistance in vitro and that ADV was definitely effective against other ETV-related amino acid substitutions S184 and M250 in vitro [Tenney et al., 2007; Villet et al., 2007]. However, the clinical effect has never been reported.

In conclusion, the present study showed that virological and biochemical breakthrough due to ETV could occur in patients infected with LAM-resistant HBV. It was confirmed that the addition of rtS202G substitution to the rtM204V mutant strain is responsible for ETV resistance and the resistant mutant could be treated successfully. While ETV resistance is rare in treatment-naïve patients, the amino acid substitution associated with ETV resistance is similar to the substitution seen in patients with LAM-resistance. Thus, it is considered that the successful salvage therapy described in this study could be a potentially helpful for similar events during ETV therapy. The possibility of emergence of novel mutants resistant to

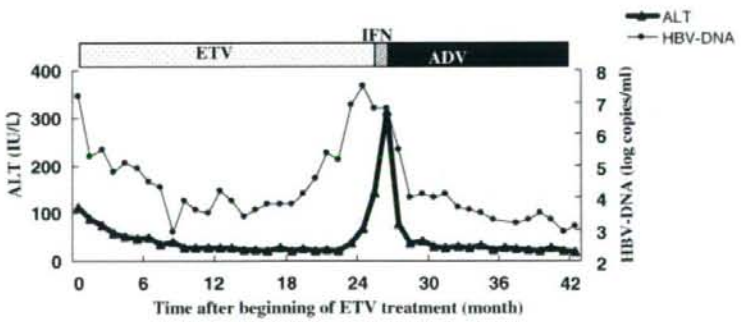

Fig. 2. Clinical course of a patient who developed breakthrough during entecavir therapy.

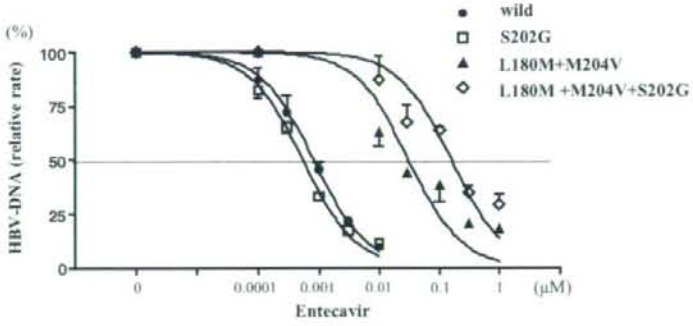

Fig. 3. In vitro analyses of susceptibilities of wild-type HBV and three mutants (rtS202G, rtL180M + M204V, rtL180M + M204V + S202G) to entecavir (ETV) after transient transfection into HepG2 cells. Cells were transiently transfected with plasmids containing 1.4 genome lengths HBV and treated with the indicated amount of entecavir. Data are the dose–response curves of the four HBV strains against entecavir. The strains were used to estimate the entecavir IC_{50} values for each HBV strains. Values are relative to no entecavir treatment controls for each strain. Experiments were performed in triplicates.

TABLE IV. In Vitro Susceptibility of rtS202/rtM204 Mutant to Lamivudine (LAM) and Adefovir (ADV)

	LAM		ADV	
	IC_{50} (μM)	Fold resistance	IC_{50} (μM)	Fold resistance
Wild	0.1	1	0.39	1
L180M + M204V	>100	>1,000**	—	—
L180M + M204V + S202G	>100	>1,000**	0.32	0.82[a]

Experiments were performed in triplicates.
[a]NS, not significant.
**$P < 0.001$ compared with the wild-type.

multiple anti-HBV drugs is real. Therefore, further studies are necessary to develop safes and more useful treatment strategies.

ACKNOWLEDGMENTS

This work was carried out at the Research Center for Molecular Medicine, Faculty of Medicine, Hiroshima University. The authors thank Kana Kunihiro, Rie Akiyama, Yoshiko Seo, Yoshiko Nakata, and Eiko Miyoshi for their excellent technical assistance. This work was supported in part by Grants-in-Aid for scientific research and development from the Ministry of Education, Sports, Culture, and Technology and the Ministry of Health, Labor and Welfare.

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«paper no. vir83658 charlesworth ref: vir83658»

Animal RNA

Journal of General Virology (2008), **89**, 000–000 DOI 10.1099/vir.0.83658-0

Establishment of an infectious genotype 1b hepatitis C virus clone in human hepatocyte chimeric mice

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The establishment of clonal infection of hepatitis C virus (HCV) in a small-animal model is important for the analysis of HCV virology. A previous study developed models of molecularly cloned genotype 1a and 2a HCV infection using human hepatocyte-transplanted chimeric mice. This study developed a new model of molecularly cloned genotype 1b HCV infection. A full-length genotype 1b HCV genome, HCV-KT9, was cloned from a serum sample from a patient with severe acute hepatitis. The chimeric mice were inoculated intrahepatically with *in vitro*-transcribed HCV-KT9 RNA. Inoculated mice developed viraemia at 2 weeks post-infection, and this persisted for more than 6 weeks. Passage experiments indicated that the sera of these mice contained infectious HCV. Interestingly, a similar clone, HCV-KT1, in which the poly(U/UC) tract was 29 nt shorter than in HCV-KT9, showed poorer *in vivo* infectivity and replication ability. An *in vitro* study showed that no virus was produced in the culture medium from HCV-KT9-transfected cells. In conclusion, this study developed a genetically engineered genotype 1b HCV-infected mouse. This mouse model will be useful for the study of HCV virology, particularly the mechanism underlying the variable resistance of HCV genotypes to interferon therapy.

Received 13 December 2007
Accepted 14 May 2008

INTRODUCTION

Hepatitis C virus (HCV), a positive-sense, single-stranded RNA virus, infects and replicates efficiently only in the hepatocytes of humans and chimpanzees. There are many genotypes of HCV distributed worldwide (Simmonds *et al.*, 1993); among them genotype 1b is the major genotype in Asia, including Japan, and is known to be one of the most resistant genotypes to interferon (IFN) therapy (Fried *et al.*, 2002). Until recently, studies of HCV replication have long been hampered by the lack of a virus culture system. The development of HCV replicon systems has allowed the

The GenBank/EMBL/DDBJ accession numbers for the sequences of HCV-KT9 and HCV-KT1 determined in this work are AB435162 and AB426117, respectively.

study of the mechanisms of replication HCV (Lohmann *et al.*, 1999). However, these replicons lack structural proteins, do not replicate efficiently without adaptive mutations and do not produce infectious virions. Recently, it was reported that the genotype 2a full-length JFH-1 genome replicated efficiently in Huh7 cells without adaptive mutations and produced virions that were infectious for both naïve cells and chimpanzees, as well as for a human hepatocyte-transplanted chimeric mouse (Wakita *et al.*, 2005; Zhong *et al.*, 2005; Lindenbach *et al.*, 2006). To date, five full-length genotype 1b clones, HCV-N (Beard *et al.*, 1999), Con-1 (Bukh *et al.*, 2002), HCV-J4 (Okamoto *et al.*, 1992), HCV-CG1b (Thomson *et al.*, 2001) and HCV-BK (Takamizawa *et al.*, 1991), have been demonstrated to be infectious by intrahepatic inoculation of transcribed HCV RNA into the liver of chimpanzees. Among these, only the HCV-CG1b genome is reported to produce HCV particles when transfected into Huh7 cells (Heller *et al.*, 2005).

Although the chimpanzee is a useful animal model for the study of HCV infection, there are ethical restrictions on the use of this animal. Instead, Mercer *et al.* (200) developed a useful small-animal model for the study of HCV infection using chimeric urokinase-type plasminogen activator (uPA)/severe combined immunodeficiency (SCID) mice (which are immunodeficient and undergo liver failure) with engrafted human hepatocytes (Mercer *et al.*, 2001). This HCV-infected mouse model is reported to be useful for evaluating anti-HCV drugs such as IFN-α and anti-NS3 protease (Kneteman *et al.*, 2006). We have previously described methods to improve the replacement levels of human hepatocytes in this mouse model (Tateno *et al.*, 2004) and we have developed a reverse genetics systems for hepatitis B virus (Tsuge *et al.*, 2005) and HCV (Hiraga *et al.*, 2007). In the present study, we report the establishment of an infectious genotype 1b HCV clone that infects and replicates efficiently in human hepatocyte chimeric mice.

METHODS

Cloning of infectious genotype 1b HCV isolate. Serum samples were obtained from a 43-year-old physician who developed severe acute hepatitis after needle stick exposure from a patient with chronic hepatitis C. On admission, the serum total bilirubin concentration was 10.0 mg dl^{-1} and the prothrombin time was 40 %. The patient tested positive for HCV antibodies by a third-generation radioimmunoassay (Ortho-Clinical Diagnostics) and for HCV RNA by RT-PCR. Serum HCV RNA was quantified using an Amplicor Monitor HCV test (Roche Diagnostics). The HCV RNA titre was 2.5×10^6 copies ml^{-1} on admission and then decreased gradually. Fig. 1 shows the serial changes in alanine aminotransferase (ALT) as a measure of liver function and HCV RNA levels in this patient. Serum samples obtained in the early phase of infection were used for cloning the full-length genome.

RNA extraction, cDNA synthesis, plasmid construction and RNA transcription. Total RNA was extracted from 100 µl serum samples using SepaGene RV-R (Sanko Junyaku) and reverse transcribed with random hexamers and ReverTra Ace reverse transcriptase (Toyobo) according to the manufacturer's instructions. PCR primers were designed based on the sequence of HCV-Con1 (GenBank accession no. AJ238799; Bukh *et al.*, 2002). Five overlapping cDNA segments (nt 1–2292, 2269–6715, 6696–9094, 7564–9404 and 9361–9605; nucleotide numbers are those of HCV-Con1) were amplified by PCR with TaKaRa LA *Taq* polymerase (Takara Biochemicals) using the above cDNA. Amplified products were separated by agarose gel electrophoresis. Nucleotide sequences were determined using a Big Dye Terminator Mix Cycle Sequencing kit (Applied Biosystems Japan) with an automated DNA sequencer (model 310; PE Biosystems). We corrected the nucleotide sequences of the obtained clones by site-directed mutagenesis and made them identical to the nucleotide sequences obtained by direct sequencing. Naturally occurring restriction enzyme cutting sites were utilized to clone each segment. We utilized the vector pBR322 and created a multiple-cloning site under the control of the T7 promoter by ligating a linker at restriction enzyme cutting sites as they appeared in order from 5′ to 3′ in the HCV sequences (Fig. 2a). Each segment of HCV was cloned into this vector to generate the full-length clones. The HCV-KT9 clone was established using the 3′-terminal fragment with the longest poly(U/UC) tract length (115 nt), which should have a high replication ability (Friebe & Bartenschlager, 2002; Yi & Lemon, 2003; You & Rice, 2008). A clone with a shorter poly(U/UC) tract length (86 nt), HCV-KT1, was also generated. A polymerase-deficient mutant with an amino acid substitution in the GDD motif (GDD→GND; HCV-KT9-GND) was generated using a Quick Change Site-Directed Mutagenesis kit (Stratagene). After digesting the plasmid with *Xba*I (New England BioLabs) at the 3′ end of the HCV cDNA, HCV RNA was transcribed using T7 RNA polymerase (MEGAscript; Ambion) at 37 °C for 3 h in a 100 µl reaction mixture, according to the manufacturer's instructions. The RNA was analysed using denaturing agarose gel electrophoresis and kept at −80 °C until use.

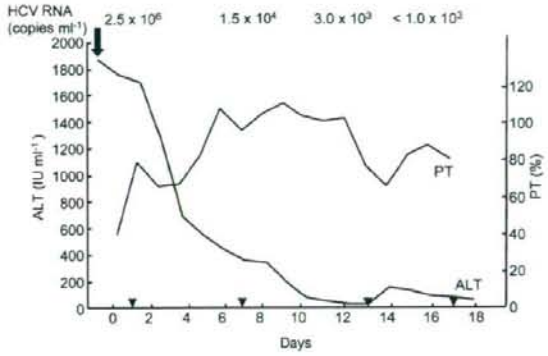

Fig. 1. Clinical course of a patient with severe acute hepatitis C. Alanine aminotransferase (ALT) and prothrombin time (PT) are shown from the day of admission (day 1). The patient was treated daily with 10^6 U IFN-β intravenously for 5 days, followed by 106 U IFN-α intramuscularly three times a week for 6 months. HCV RNA was measured on days 1, 7, 13 and 17 (arrowheads). A serum sample was taken on day 1 (arrow) and used to clone the full-length HCV genome.

Construction of a phylogenetic tree. A phylogenetic tree was constructed based on the entire nucleotide sequences of 26 full-length genotype 1b clones plus HCV-KT9. The total number of synonymous and non-synonymous substitutions among the nucleotide sequences

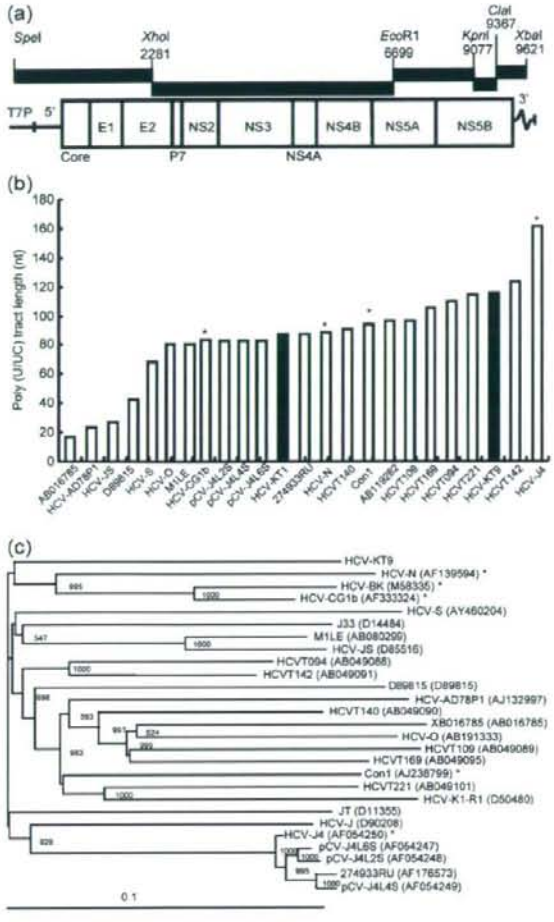

Fig. 2. (a) Schematic diagram of the organization of the cDNA clone HCV-TK9. The T7 RNA promoter (T7P) is located immediately upstream of the HCV genome. Restriction enzyme sites that were used to create clone HCV-KT9 are labelled according to their nucleotide position within the HCV sequence. Amino acid sequences unique to HCV-KT9 compared with 26 other HCV genotype 1b isolates are indicated at the bottom of the figure, with the position of the repaired amino acid residues noted within the polyprotein. (b) Length of the poly(U/UC) tracts of HCV-KT1, HCV-KT9 and 22 other HCV genotype 1b clones reported previously. Asterisks indicate clones confirmed to be infectious by experiments using chimpanzees. (c) Phylogenetic tree constructed with HCV-KT9 and 26 genotype 1b HCV whole-genome sequences. Bar, number of nucleotide substitutions per site. Asterisks indicate clones confirmed to be infectious in experiments using chimpanzees.

was estimated using the method of Gojobori *et al.* (1982) and a phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987).

Intrahepatic injection experiments in human hepatocyte chimeric mice. We used methods described previously (Tateno *et al.*, 2004) to generate $uPA^{+/+}/SCID^{+/+}$ mice and transplant human hepatocytes. All mice used in this study were transplanted with frozen human hepatocytes obtained from the same donor. Mouse serum concentrations of human serum albumin (HSA) correlate with the repopulation index and were measured as described previously (Tateno *et al.*, 2004). Intrahepatic injection of RNA, extraction of serum samples and euthanasia were performed under ether anaesthesia. Briefly, 500 µl RNA solution containing 30 µg transcribed HCV RNA was injected into the liver of anaesthetized chimeric mice through a small abdominal incision. RNA extraction from mouse serum samples, quantification of HCV RNA and nested PCR were performed as described previously (Hiraga *et al.*, 2007). All animal protocols described in this study were performed in accordance with the guidelines of the local committee for animal experiments and under the approval of the Ethics Review Committee for Animal Experimentation of the Graduate School of Biomedical Sciences, Hiroshima University.

Cell culture, RNA transfection and measurement of HCV core antigen. The human hepatoma cell line Huh7 was maintained in Dulbecco's modified Eagle's medium (Sigma) containing 10 % fetal calf serum. RNA transfection and measurement of HCV core antigen in the culture medium were performed as described previously (Wakita *et al.*, 2005).

Statistical analysis. The infectious ratio of chimeric mice was compared and the differences assessed using a χ^2 test. Differences in HCV RNA replication ability *in vitro* were analysed statistically by one-way analysis of variance followed by Scheffe's test. A P value of less than 0.05 was considered statistically significant.

RESULTS

Characteristics of genotype 1b clones HCV-KT9 and HCV-KT1

The entire genome of HCV cDNA was assembled from five DNA fragments (Fig. 2a). We obtained 24 3′-extremity clones with different poly(U/UC) tract lengths. We selected the clone with the longest (U/UC) tract because a previous study indicated that the length of poly(U/UC) tract correlates with HCV replication in an HCV replicon system (Friebe & Bartenschlager, 2002; Yi & Lemon, 2003; You & Rice, 2008). The length of the poly(U/UC) tract in the longest 3′ clone was 115 nt. The entire genome length of the HCV-KT9 clone using this longest 3′ clone was 9621 nt. We also generated the clone HCV-KT1 with a shorter (86 nt) poly (U/UC) tract to compare the replication abilities of these clones. The lengths of the poly (U/UC) tracts of 22 clones deposited in GenBank are shown in Fig. 2(b). All infectious clones had a poly (U/UC) tract longer than 80 nt. Fig. 2(c) shows a phylogenetic tree constructed using the nucleotide sequences of the 26 full-length genotype 1b clones published to date. Interestingly, the sequence of HCV-KT9 was closest to that of HCV-CG1b (GenBank accession no. AF333324), which has been reported to be infectious, and formed a cluster with two other infectious clones, HCV-N (Beard *et al.*, 1999) and HCV-BK (Takamizawa *et al.*, 1991). We compared the amino acid

sequences of HCV-KT9 with an alignment of the sequences of the 26 other genotype 1b strains. All HCV full-length clones reported from Japan were included in these 26 strains. Based on these comparisons, we identified 25 aa unique to HCV-KT9 (Fig. 2a). We found that the amino acid sequence of the IFN sensitivity-determining region in the NS5A region, which has been suggested to mediate IFN resistance via interaction with the cellular protein kinase R (Enomoto *et al.*, 1996; Gale *et al.*, 1997), was that of the wild-type.

Intrahepatic injection of HCV-KT1 and HCV-KT9 RNAs into human hepatocyte chimeric mice

In the next experiments, 30 μg *in vitro*-transcribed RNA of HCV-KT1, HCV-KT9 or HCV-KT9-GND was injected into the livers of chimeric mice. Eight out of 10 (80 %) HCV-KT9-injected mice developed measurable viraemia at 2 weeks post-inoculation (Table 1 and Fig. 3), with the HCV RNA titre reaching 1.1×10^6 to 8.8×10^6 copies ml^{-1} at 6 weeks post-inoculation (Fig. 3). To check for the presence of infectious HCV in the serum of HCV-KT9-infected mice, each of five naïve mice was injected with 10 μl serum sample (containing 3.5×10^5 copies of HCV) obtained from an HCV-KT9-infected mouse 6 weeks after inoculation. All five naïve mice became positive for HCV RNA, as confirmed by nested PCR, at 2 weeks post-inoculation and two mice developed persistent viraemia (Fig. 4). These results indicated that the serum of HCV-KT9-injected mice contained infectious HCV. In contrast to HCV-KT9, none of the three mice injected with HCV-KT9-GND RNA developed viraemia (Table 1). These results indicated that HCV-KT9 replicates efficiently in mice livers and produces infectious virus continuously. On the other hand, only one out of seven HCV-KT1-injected mice (14 %) developed measurable viraemia (Table 1 and Fig. 3). The level of viraemia was low in this HCV-KT1-infected mouse, HCV RNA was negative by nested PCR at 2 weeks after inoculation and the titre was only 2.2×10^4 copies ml^{-1} at 4 weeks post-inoculation (Fig. 3). These results confirmed the importance of the poly(U/UC) tract length in experimentally induced viraemia.

The nucleotide and amino acid sequences of the viral genome isolated from an HCV-KT9-injected mouse (Fig. 3) at 6 weeks after RNA injection were identical to the injected HCV-KT9 (data not shown). We tried to reclone the poly(U/UC) tract in the HCV-KT1-infected mouse, but it was impossible to reamplify the HCV cDNA using the remaining small amount of serum.

Table 1. Correlation between length of the poly(U/UC) tract and HCV infection

Clone	Length of poly(U/UC) tract	Number of mice			Infection ratio
		Infected	Not infected	Total	
HCV-KT1	86	1	6	7	14 %
HCV-KT9	115	8	2	10	80 %*
HCV-KT9-GND	115	0	3	3	0 %

*P=0.015, compared with HCV-KT1.

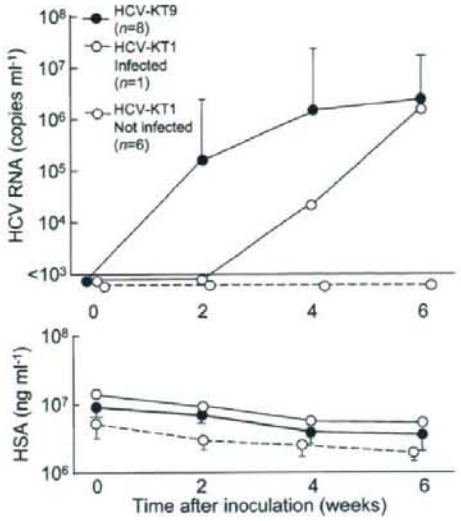

Fig. 3. Changes in HCV RNA levels and HSA concentrations in the sera of mice infected with clonal HCV. Mice were inoculated intrahepatically with 30 μg *in vitro*-transcribed HCV RNA. Eight of the ten HCV-KT9-infected mice (80 %), one of the seven HCV-KT1-infected mice (14 %) and none of the three HCV-KT9-GND-infected mice became positive for HCV RNA. The results for six HCV-KT1-uninfected mice are also shown. Mice serum samples were obtained every 2 weeks post-infection for analysis of HCV RNA titres. Data are shown as mean ± SD.

Analysis of virus production from HCV-KT9-transfected cells

Next, we evaluated the ability of the HCV-KT9 clone to replicate in transfected Huh7 cells. In these experiments, we used JFH-1 RNA, which is known to replicate efficiently in cell cultures, as control (Wakita *et al.*, 2005). Core protein was secreted efficiently from JFH-1 RNA-transfected Huh7 cells. In contrast, we did not observe any measurable levels of core protein in the supernatant of HCV-KT9-transfected cells (Fig. 5), suggesting a minimal replication ability of HCV-KT9 to produce and release virus into the supernatant.

DISCUSSION

In this study, we described the establishment of a genotype 1b clone, HCV-KT9, that replicated efficiently following injection of the transcribed RNA into chimeric mouse liver.

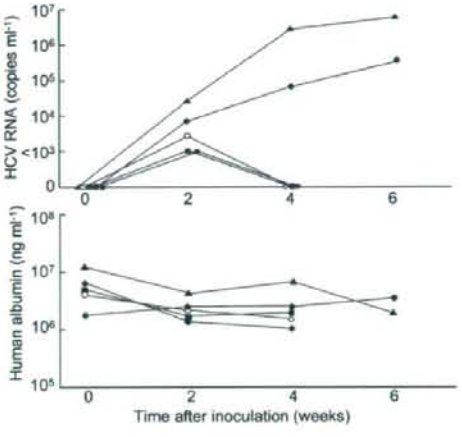

Fig. 4. Passage experiments of HCV in naïve chimeric mice. Five naïve chimeric mice were inoculated intravenously with 10 µl serum sample (containing 3.5×10^5 copies HCV) obtained from an HCV-KT9-infected mouse at week 6 post-inoculation. Serum samples were obtained at the indicated time intervals for the measurement of HCV RNA levels and HSA concentrations. Data represent the changes in five individual mice.

The key factor that determines the infectivity of HCV clones has not yet been established. We previously established a clone from HCV that replicated in a chimeric mouse after injection of serum from a chronically HCV-infected patient. However, we did not observe viraemia after intrahepatic injection of the transcribed RNA from this clone (unpublished results). In contrast, injection of HCV-KT9 RNA in the present study resulted in viraemia in eight out of ten mice (80 %). The fact that the nucleotide and amino acid sequences of the virus recovered from the infected mice were identical to those of the HCV-KT9 clone indicated that no adaptive mutation was necessary for this clone to replicate in the chimeric mouse.

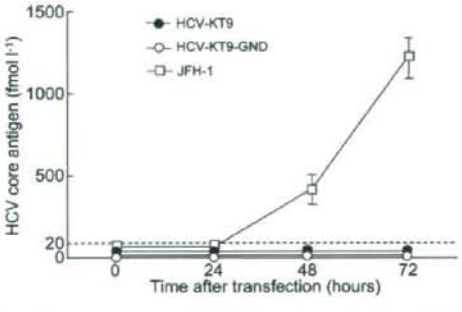

Fig. 5. Time-course studies of HCV core protein secretion into the culture medium of HCV RNA-transfected cells. Huh7 cells were transfected with 10 µg HCV-KT9, HCV-KT9-GND or JFH-1 RNA. HCV core antigen in the culture medium was measured at 24, 48 and 72 h after transfection. Data are shown as mean ± SD of HCV core protein levels obtained from three independent transfection experiments.

Interestingly, the clone was obtained from a patient with severe acute hepatitis. This is similar to JFH-1, an HCV clone with a strong replication ability in cultured cell lines, chimpanzees and chimeric mice, which was cloned from serum samples of a patient who developed acute fulminant hepatitis with a high virus titre (Wakita *et al.*, 2005). A virus that replicates in the early stage of infection may have strong replication ability, which may be lost in the chronic phase of infection.

A key amino acid substitution may be present in one (or some) of the amino acids unique to this clone (Fig. 2a). We also showed that clone HCV-KT1, which differs from HCV-KT9 only in the length of the poly(U/UC) tract, had a poorer replication ability in mice (Table 1 and Fig. 3). However, there is a possibility that a shorter poly(U/UC) tract only slows down the rate of infection, as the HCV RNA titre in the HCV-KT1-infected mouse at 6 weeks after inoculation was similar to that in HCV-KT9-infected mice (Fig. 3). It has been reported that the length and composition of the poly(U/UC) tract is important for the replication of HCV replicons (Friebe & Bartenschlager, 2002; Yi & Lemon, 2003; You & Rice, 2008). However, no replication advantage of a poly(U/UC) tract longer than 86 bp was revealed in this study. This may be due to differences *in vitro* and *in vivo*, where the innate immune response against the virus may be more robust than in cell culture.

As shown in the present study, reverse genetics of HCV has become available for studies of HCV replication. The important factors for virus replication suggested above can be analysed further using this system.

We also examined the response of HCV-KT9-infected mice to IFN treatment. Three HCV-KT9-infected mice were treated with daily intramuscular injections of 1000 IU IFN-α (g body weight)$^{-1}$ for 2 weeks. This regimen resulted in a reduction in HCV RNA levels of only 1.0 log copies ml^{-1} (data not shown). These results are consistent with our previous study, which showed a similar low-level reduction in HCV RNA in mice infected with a genotype 1a clone, and differ from our previous results in mice infected with HCV genotype 2a, which became negative for HCV RNA following daily treatment with 1000 IU IFN-α (g body weight)$^{-1}$ for 2 weeks (Hiraga *et al.*, 2007). These results are in agreement with our clinical experience that genotype 1 is more resistant to IFN therapy than genotype 2. As shown in the present study and previously (Hiraga *et al.*, 2007), reverse genetics of HCV with three genotypes, 1a, 1b and 2a, is now available. By recombination of these clones or the establishment of mutants with nucleotide and amino acid sequences similar to each other, it may be possible to clarify the mechanism underlying the variability in susceptibility of HCV genotypes to IFN.

In this study, HCV-KT9 showed no virus production ability *in vitro*. Recently, Kato *et al.* (2007) reported that the genotype 1b HCV clone CG1b replicated in Huh7.5.1 cells and produced infectious HCV. It will be of interest to create chimeric viruses of HCV-KT9 and HCV-CG1b, and to determine the mutations that are important for virus production *in vitro*.

In summary, we established an infection model of a genotype 1b HCV clone using human hepatocyte chimeric mice. This model will be useful for studies of HCV replication, particularly the mechanism underlying the variable resistance of HCV genotypes to IFN therapy.

ACKNOWLEDGEMENTS

The authors thank Rie Akiyama and Kana Kunihiro for their technical help and Dr Francis V. Chisari for providing the Huh7.5.1 cells. This work was supported in part by Grants-in-Aid for scientific research and development from the Ministry of Education, Sports, Culture and Technology and the Ministry of Health, Labor and Welfare, Japan.

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Tumor cell apoptosis induces tumor-specific immunity in a CC chemokine receptor 1- and 5-dependent manner in mice

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Abstract: The first step in the generation of tumor immunity is the migration of dendritic cells (DCs) to the apoptotic tumor, which is presumed to be mediated by various chemokines. To clarify the roles of chemokines, we induced apoptosis using suicide gene therapy and investigated the immune responses following tumor apoptosis. We injected mice with a murine hepatoma cell line, BNL 1ME A.7R.1 (BNL), transfected with HSV-thymidine kinase (tk) gene and then treated the animals with ganciclovir (GCV). GCV treatment induced massive tumor cell apoptosis accompanied with intratumoral DC infiltration. Tumor-infiltrating DCs expressed chemokine receptors CCR1 and CCR5, and T cells and macrophages expressed CCL3, a ligand for CCR1 and CCR5. Moreover, tumor apoptosis increased the numbers of DCs migrating into the draining lymph nodes and eventually generated a specific cytotoxic cell population against BNL cells. Although GCV completely eradicated HSV-tk-transfected BNL cells in CCR1-, CCR5-, or CCL3-deficient mice, intratumoral and intranodal DC infiltration and the subsequent cytotoxicity generation were attenuated in these mice. When parental cells were injected again after complete eradication of primary tumors by GCV treatment, the wild-type mice completely rejected the rechallenged cells, but the deficient mice exhibited impairment in rejection. Thus, we provide definitive evidence indicating that CCR1 and CCR5 and their ligand CCL3 play a crucial role in the regulation of intratumoral DC accumulation and the subsequent establishment of tumor immunity following induction of tumor apoptosis by suicide genes. *J. Leukoc. Biol.* 84: 1001–1010; 2008.

Key Words: *dendritic cells · gene therapy*

INTRODUCTION

Hepatocellular carcinoma (HCC) occurs in individuals with chronic liver disease related to hepatitis B or C virus infections [1–3]. Even after the curative treatments for HCC, such as surgical resection and radiofrequency ablation, tumor recurrence often occurs because of the multicentric development of HCC in the cirrhotic liver [4]. Immune-based therapies, particularly those based on dendritic cells (DCs), may be theoretically effective in preventing the recurrence because of their potential capacity to search for and eradicate tumor cells irrespective of site [5]. However, DC-based therapy is still considered to be in its infancy, probably as a result of the lack of effective techniques for enhancing the immune response to human cancer cells including HCC, which are generally poor in immunogenicity.

Apoptotic tumor cells are generally less immunogenic than necrotic cells, but they can sometimes induce efficient antitumor immune responses depending on the type of apoptosis inducer. Indeed, some anticancer drugs can induce apoptosis of tumor cells and simultaneously enhance the immunogenicity of apoptotic cancer cells [6–8]. Ganciclovir (GCV) can activate the protease family of caspases and induce apoptosis selectively in the cells transfected with the HSV-thymidine kinase (tk) gene [9, 10]. Thus, when GCV is administered systemically to tumor-bearing individuals, it induces apoptosis of HSV-tk-transfected tumor cells but not normal cells. This treatment strategy, designated as suicide gene therapy, can induce immunogenic apoptosis of the tumor cells [11], as evidenced by a massive intratumoral infiltration of macrophages and T cells [12]. Moreover, the expression of various proinflammatory cytokines is augmented at the tumor sites following GCV treatment [12, 13]. Furthermore, to enhance the suicide gene therapy-induced immune responses, the simultaneous use of cytokines such as GM-CSF, IL-2, and MCP-1/CCL2 has been used with some success [14–16]. To design more effective methods of preventing tumor recurrences, it is necessary to fully understand the immune responses after tumor apoptosis induced by HSV-tk/GCV suicide gene therapy.

DCs are potent APC that play a crucial role in the establishment of adoptive immune response. Immature DCs capture and process antigens at the inflammatory sites and thereafter migrate to the draining lymph node, where they

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Received November 26, 2007; revised June 12, 2008; accepted June 30, 2008.

doi: 10.1189/jlb.1107791

undergo phenotypical and functional maturation. At the draining lymph node, the mature DCs interact with naïve T cells and present the captured and processed antigen to T cells [17, 18].

Chemokines are presumed to play an essential role in the regulation of DC trafficking and DC–T cell interaction in general [19–22]. Circulating immature DCs express inflammatory chemokine receptors such as CCR1, CCR2, CCR5, and CCR6, and these DCs can reach the source of the inflammatory stimulus under the guidance of the ligand gradient for the expressed receptors such as CCL2, CCL3, CCL4, CCL5, CCL7, and CCL20. After capturing antigens, DCs undergo maturation, resulting in a decrease in inflammatory chemokine receptor expression and a reciprocal increase in CCR7 expression. Mature DCs expressing CCR7 migrate to T cell-rich areas of the draining lymph nodes, where the ligands for CCR7, CCL19, and/or CCL21 are expressed abundantly. However, it still remains elusive whether similar mechanisms operate in the DC migration process following massive tumor apoptosis induced by treatments such as gene therapy, chemotherapy, and radiation therapy.

Here, we demonstrate the induction of specific tumor immunity by tumor apoptosis after HSV-tk/GCV suicide gene therapy and essential roles of DCs in this process. Moreover, we provide definitive evidence to indicate that CCR1 and CCR5 and their ligand CCL3 play a key role in the regulation of intratumoral DC accumulation and the subsequent establishment of tumor immunity following induction of tumor apoptosis by HSV-tk/GCV suicide gene therapy. These observations might lay the foundation for devising novel measures to enhance antitumor immune responses to prevent tumor recurrence.

MATERIALS AND METHODS

Mice

Specific pathogen-free, 7- to 9-week-old male BALB/c mice were purchased from Charles River Japan (Yokohama, Japan) and were designated as wild-type (WT) mice. CCL3-deficient [CCL3 knockout (CCL3KO)] mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA). CCR1KO mice were a gift from Dr. Philip M. Murphy [National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIAID, NIH), Bethesda, MD, USA]. CCR5KO mice were generated as described previously [23]. All mice were backcrossed to BALB/c mice for eight to 10 generations. All animal experiments were performed under specific pathogen-free conditions in accordance with the Guideline for the Care and Use of Laboratory Animals of Kanazawa University (Japan).

Tumor cell lines

A murine HCC cell line, BNL 1ME A.7R.1 (BNL), was cultured in DMEM (Sigma Chemical Co., St. Louis, MO, USA) containing 10% FBS (Gibco, Long Island, NY, USA). BNL cells were infected with the retroviral vector pG1Sv.Na harboring HSV-tk cDNA. The infected BNL cells were cultured in 10% FBS-containing DMEM in the presence of 400 μg/ml G418 (Gibco). The surviving cells were tested for sensitivity to GCV in vitro as described previously [24]. GCV-sensitive cells were designated as BNL-tk and were used in the experiments.

Apoptosis detection assay

After culturing for 1 day with 5 μg/ml GCV, BNL-tk cells were harvested, and phosphatidyl serine levels were determined by staining the cells with propidium iodide (PI) and the Annexin V-FITC apoptosis detection kit (Calbiochem, Darmstadt, Germany) according to the manufacturer's instructions. At least 50,000 stained cells were analyzed on a FACSCalibur system (BD Biosciences, San Diego, CA, USA) for each determination.

Tumor injection

Seven- to 9-week old male WT, CCR1KO, CCR5KO, and CCL3KO mice were inoculated s.c. into the left flank with 2×10^5 BNL-tk cells on Day 0. From Days 14 to 18 (5 consecutive days), 75 mg/kg GCV (i.p.) was administered daily (see **Fig. 1C**). Tumors were removed at the indicated time intervals for immunohistochemical analysis and quantitative real-time RT-PCR. In another series of experiments, WT, CCR1KO, CCR5KO, or CCL3KO mice were inoculated with 1.5×10^5 BNL-tk on Day 0. The mice were i.p.-injected with 75 mg/kg GCV from Days 2 to 5. The animals were then rechallenged s.c. with 1.0×10^5 BNL in their right flank on Day 18, after confirming that the primary tumors were eradicated completely (see Fig. 5A). Tumor sizes were evaluated twice each week using calipers, and tumor volume was calculated by the following formula: Tumor volume (mm^3) = (the longest diameter) × (the shortest diameter)^2/2.

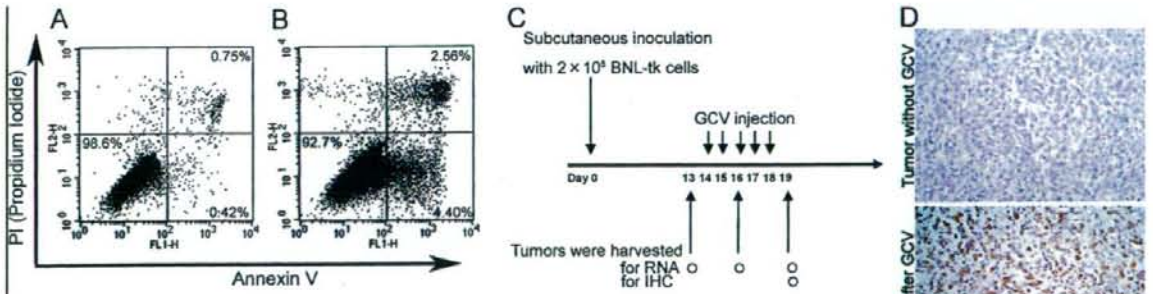

Fig. 1. Apoptosis of the murine hepatoma cell line induced by GCV treatment. After 1 day of culture without (A) or with (B) 5 μg/ml GCV, BNL-tk cells were harvested and stained with the Annexin V-FITC apoptosis detection kit and PI. Then, the cells were analyzed by flow cytometry. Representative results from three individual experiments are shown here. FL1- and -2-H, Fluorescence 1- and 2-height. (C) Schematic representation of GCV treatment in vivo. Mice were s.c.-injected with 2×10^5 BNL-tk cells on Day 0. Then, GCV was i.p.-injected into mice from Days 14 to 18. Tumors were harvested on the day before GCV injection (Day 13), on Day 3 or 6 after GCV injection (Day 16 or 19) for real-time RT-PCR analysis, and on Day 19 for immunohistochemistry (IHC). (D) Apoptotic cells detected in tumor tissues with or without GCV treatment using anti-ssDNA antibody. Original magnification, ×400. Original bar, 50 μm.