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Possible Molecular Mechanism of the Relationship Between NS5B Polymorphisms and Early Clearance of Hepatitis C Virus During Interferon Plus Ribavirin Treatment

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We previously reported the relationship between viral polymerase polymorphisms and the initial decline in viral load induced by interferon-α and ribavirin therapy in genotype 1b-related chronic hepatitis C patients. The presence of E124K and I85V of NS5B was closely associated with viral clearance at 8 weeks of treatment. The aim of this study was to investigate the mechanisms by which this polymorphism of NS5B protein affects early viral clearance. We used a replicon system derived from strain O, genotype 1b virus. Three mutants (V85I), (K124E), and (V85I/K124E) were introduced to the replicon. OR6c, a derivative of HuH7 cells, was transfected with the replicon including a luciferase reporter gene. Luciferase activities were measured 72 hr post-transfection. All three mutants showed higher luciferase activity than that of the wild type, and the V85I mutant showed the highest activity. This result was also confirmed by neomycin gene-containing replicons with same mutations. All replicons were down-regulated by ribavirin, but the level of reduction in the V85I mutant was the lowest. Our results suggested that this mutation at least partly contributes to resistance to early viral clearance during interferon and ribavirin combination therapy. ***J. Med. Virol. 80:632–639, 2008.*** © 2008 Wiley-Liss, Inc.

KEY WORDS: hepatitis C virus; NS5B polymorphism; replicon; interferon and ribavirin combination therapy; viral proliferation

INTRODUCTION

With an estimated 170 million infected individuals, hepatitis C virus (HCV) has a major impact on public health. An estimated 65–80% of the individuals infected with HCV develop persistent infection while 20–50% develop cirrhosis and 5% develop hepatocellular carcinoma (HCC) [Liang et al., 2000; Gao et al., 2004]. Until recently, interferon (IFN)-α and IFN-β were the only available treatments for HCV infection, although only 10–15% of treated subjects achieved sustained viral eradication with IFN monotherapy, and early viral clearance after initiation of IFN monotherapy was correlated with sustained viral clearance [Saito et al., 2000].

The current approved treatment for HCV infection is pegylated IFN-α (peg-IFN) in combination with ribavirin (RBV). This combination therapy leads to viral clearance in 50–80% of cases, depending on the infecting HCV genotype, and 50% of patients with HCV genotype 1b and high baseline levels of viral RNA do not achieve a sustained virological response with the combination therapy after 48 weeks [Manns et al., 2001; Fried et al., 2002; Feld and Hoofnagle, 2005]. Several prior studies have attempted to predict the efficacy of IFN plus RBV combination therapy. A quantitative measurement of HCV viremia or the initial decline in viral load is a reliable marker for early prediction of the therapeutic response to IFN and RBV combination therapy [Zeuzem et al., 1998; Bouvier-Alias et al., 2002; Takahashi et al., 2005; Lukasiewicz et al., 2007].

RBV is a broad-spectrum nucleoside analogue antiviral drug which is especially noted for its actions

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against RNA viruses and exhibits in vitro activity against some DNA and RNA viruses, including certain members of *Flaviviridae* [Sidwell et al., 1972]. It has recently been demonstrated that the antiviral activity of RBV can result from the ability of a viral RNA-dependent RNA polymerase (RdRP) to utilize RBV triphosphate and to incorporate this nucleotide into the viral genome with reduced specificity, thereby mutagenizing the genome and decreasing the yield of infectious virus [Crotty et al., 2000; Lanford et al., 2003]. Moreover, RBV exhibits an antiviral effect through a mechanism of error-prone replication in the HCV subgenomic replication system [Contreras et al., 2002]. Although RBV by itself cannot decrease serum HCV RNA levels in patients, it has been demonstrated that combination therapy with RBV and either IFN-α or peg-IFN yields a higher sustained response rate than is achieved with IFN-α monotherapy [Pol et al., 2000; Poynard et al., 2000; Saracco et al., 2001].

We previously reported the relationship between viral RdRP polymorphisms and the initial decline in viral load induced by IFN-α and RBV therapy in genotype 1b-related chronic hepatitis C patients [Kumagai et al., 2004]. Substitution of glutamic acid to lysine at the 124th position (E124K) and of isoleucine to valine at the 85th position (I85V) of NS5B was closely associated with viral clearance at 8 weeks of treatment.

In this study, we used the genotype 1b HCV replicon system [Ikeda et al., 2005] to generate NS5B mutants (E124K, I85V, and both) and we compared the replication activity with that of the wild-type replicon and to analyze how this polymorphism of NS5B protein affects early viral clearance during combination therapy with IFN and RBV. We also examined the significance of NS5B polymorphisms in the RBV-induced decrease in viral replication. We concluded that the identified polymorphism of NS5B partly affects viral replication.

MATERIALS AND METHODS

Cell Culture System

OR6 cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Invitrogen Life Technology, Carlsbad, CA) supplemented with 10% fetal calf serum, penicillin, and streptomycin (complete DMEM), in addition to G418 (300 μg/ml; Geneticin, Invitrogen), and were then passaged twice a week at a 5:1 split ratio. OR6c cells are cured OR6 cells from which genome-length HCV RNA was eliminated by IFN-α treatment (500 IU/ml for 2 weeks) without G418, as previously described [Ikeda et al., 2005].

Plasmids

The plasmids pON/C-5B/KE (Fig. 1a) and pHCV-O were described previously [Ikeda et al., 2005]. This plasmid includes the adaptive mutation of K1609E of NS3 to enhance the efficiency of replication, this adaptive mutation was reported by Lohman et al. (22) The plasmid pON/C-5B/KE contains neomycin phosphotransferase (Neo) downstream of HCV IRES and the full length HCV-O polyprotein coding sequence downstream of encephalomyocarditis virus (EMCV) IRES. To introduce a pON/C-5B/KE/(V85I), pON/C-5B/KE/(K124E), pON/C-5B/KE/(V85I&K124E), we first made PCR fragments including the partial NS5B region with the primers 5′-ggatcccgatctcagcgacgg-3′ and 5′-tctagaggtcc-cattcgccattac-3′. This 2.4-kb fragment was subcloned into pSTBlue1 Blunt vector (Novagen, Madison, WI) to generate pSTBlue-1MN002. Each vector expressing the V85I mutant, K124E mutant, and V85I&K124E double mutant of HCV-O was generated by Quick Change mutagenesis (Stratagene, La Jolla, CA) to generate pSTBlueMN002(V85I), pSTBlueMN002(K124E) pSTBlueMN002(V85I&K124E). Next, pON/C-5B was

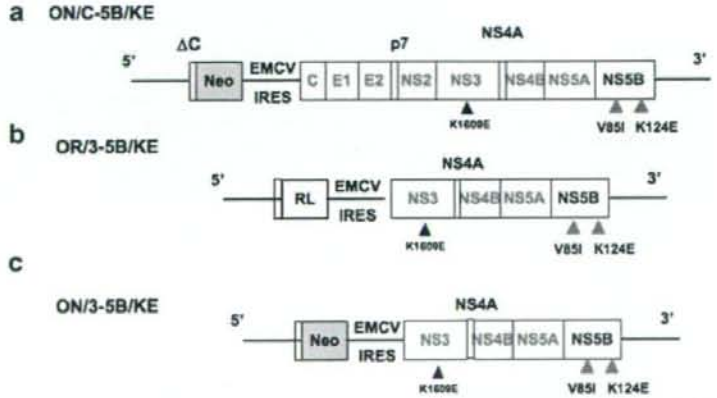

Fig. 1. **a**: Organization of genome-length HCV RNA derived from HCV-O. Open reading frames, untranslated regions, EMCV IRES, and Neo genes are depicted as shaded boxes, thin lines, thick lines, and open boxes, respectively. ΔC indicates the 12 N-terminal amino acid residues of the core as a part of IRES. This construct also contains adaptive mutation K1609E which is indicated by a black triangle. We use this construct as a wild type. Grey triangle is the position of 85 and 124 in NS5B which we generated mutation to the replicon for this experiments. **b**: The construct of the reporter subgenomic HCV replicon carries the renilla luciferase gene (RL). **c**: The construct of the reporter subgenomic HCV replicon carries the Neo gene.

digested with *Sna*b1 and *Xba*1 and subcloned into pSTBlue-1 to create pSTBlueMN001. All of the pSTBlue-MN002mutants were digested with *Bam*H1 and *Xba*1, which were subcloned into pSTBlueMN001 to create pSTBlueMN001mutant. The pSTBlueMN001 mutants were digested with *Sna*b1 and *Xba*1 and re-ligated in pON/C-5B/KE to introduce pON/C-5B/KE/(V85I), pON/C-5B/KE/(K124E), pON/C-5B/(V85I&K124E). The plasmids pOR/3-5B/KE/(V85I), pOR/3-5B/KE/(K124E) and pOR/3-5B/KE/(V85I&K124E), were constructed from pOR/3-5B/KE (Fig. 1b) by swapping for fragments of pSTBlueMN001 mutants digested with *Sna*b1 and *Xba*1. DNA sequencing of the manipulated regions of the plasmids verified all mutations.

RNA Transfection and Selection of G418-Resistant Cells

For electroporation, OR6c cells were washed twice with ice-cold phosphate buffered saline (PBS) and resuspended at 10^7 cells/ml in PBS. Twenty microgram of ON/C-5B/KE or its mutant derived RNA was mixed with 500 μl of the cell suspension in a cuvette with a gap width of 0.2 cm (Bio-Rad, Hercules, CA). The mixture was immediately subjected to two pulses of current at 1.2 kV, 25 μF, and maximum resistance. Following 10 min of incubation at room temperature, cells were seeded into 10-cm dishes. Cells were selected in complete DMEM with 300 μg/ml G418. About 3 weeks after transfection and G418 selection, cells were fixed and stained with Coomassie brilliant blue (0.6 g/l in 50% methanol–10% acetic acid) and the number of colonies was counted.

Transient-Replication Assays With Luciferase Replicons

OR6c cells were transfected by electroporation as the same protocol described above using 20 μg of OR/3-5B/KE or its mutants derived RNAs carrying the renilla luciferase (RL) gene. After addition of 2 ml of complete DMEM, 2×10^4 of aliquot OR6c cells were plated in 24-well plates at least in triplicate for each assay and harvested at various time points with renilla lysis reagent (Promega KK, Tokyo, Japan) and subjected to the RL assay according to the manufacturer's protocol (Promega). Values obtained with cells harvested 6 hr after electroporation were used to correct for the transfection efficiency.

IFN and Ribavirin Treatment

To monitor the anti-HCV effect of IFN and RBV on replication, OR6c cells were transfected by electroporation using 10 μg of OR/3-5B/KE derived RNAs as described elsewhere [Crotty et al., 2000]. OR6c cells (2×10^4/well) were plated onto 24-well plates at least in triplicate for each assay and cultured for 4 hr. Then the cells were treated with IFN at a final concentration of 1, 2, 4, 10, and 20 units/ml or RBV at a final concentration of 50,100, and 200 μM for 72 hr, harvested with renilla lysis reagent (Promega), and assayed for luciferase activity according to the manufacturer's protocol. We also studied about the additional effect of RBV (100 μM) on IFN (1 u/ml).

Cell Viability

We checked toxic effect of IFN and RBV. Effect of IFN (1 and 4 units/ml), and RBV (50 and 100 μM) on cell viability was investigated. To examine the cytotoxic effect of IFN and RBV on OR6c cells with OR/3-5B/KE replicon RNA, the cells were seeded at a density of 2×10^5 cells per dish onto 6-well plates. After 24-hr culture, the cells were treated with IFN or RBV at final concentrations of 2 and 4 units/ml or 50 and 100 μM, respectively, in the absence of G418. After incubation for 72 hr, the number of viable cells was counted in an improved Neubauer-type hemocytometer after trypan blue dye (Invitrogen) treatment.

Indirect Immunofluorescence

Cells were grown on four-well chamber slides until 70–80% confluent, washed three times with PBS, and fixed in methanol–acetone (1:1, v/v) for 10 min at room temperature. Dilutions of primary murine monoclonal antibody to residues 21–40 of the core protein (2Zcp11; Tokushu Men-eki Institute, Tokyo) (1:1,000), were prepared in PBS containing 3% bovine serum albumin and incubated with fixed cells for 2 hr at room temperature. After additional washes with PBS, specific antibody binding was detected with a goat anti-mouse immunoglobulin G-fluorescein isothiocyanate-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) diluted 1:500. Cells were washed with PBS, and mounted in DAKO Fluorescent mounting medium (DAKO Japan, Tokyo, Japan) prior to examination using a Zeiss AxioPlan2 fluorescence microscope.

Statistical Analysis

Difference in relative luciferase activity among mutant replicons and differences in anti-HCV activity of RBV among mutant replicons were tested using Student's *t*-test and Mann–Whitney *U*-test as appropriate. *P*-values <0.05 were considered statistically significant.

RESULTS

Mutation in NS5B Enhances Levels of Replication on Transient Assay

To investigate whether the mutations in NS5B of the HCV genome affect replication, we used subgenomic HCV replicons with the renilla luciferase gene for transient assay [Ikeda et al., 2005]. In a previous study [Kumagai et al., 2004], substitution of glutamic acid at the 124th position with lysine and substitution of isoleucine at the 85th position with valine in NS5B yielded a complete match with the population of good

responders (5 out of 5 patients). We introduced mutations to two different types of replicon to obtain ON/C-5B/KE(V85I), ON/C-5B/KE/(K124E), ON/C-5B/KE (V85I&K124E), OR/3-5B/KE/(V85I), OR/3-5B/KE(K124E), and OR/3-5B/KE(V85I&K124E) as described in Materials and Methods Section. The subgenomic replicons with V85I showed higher replication activity than the wild-type replicon in OR6c cells (Fig. 2). Also the replicon with K124E and the replicon with V85I&K124E showed slightly higher replication activity than the wild type, but the replicon with K124E single amino acid mutation did not show statistically higher replication activity than the wild type (Fig. 2). We initially expected that double mutations (V85I&K124E) would lead to better replication than either of the single mutations (V85I or K124E), but interestingly the V85I mutation on NS5B replicated best. This result indicated that the level of replication was affected by amino acid substitution at the 85th position.

Mutation in NS5B Enhances the Efficiency of Colony Formation in Cured Cells

In colony formation assay, we used cured subgenomic replicon cells (OR6c), since cured cells enhanced colony formation of the replicon more efficiently than did parental HuH-7 cells. We examined the effect of these mutations in full-length replicon, ON/C-5B/KE by a colony formation assay. In the initial experiment, we introduced each 20 μg of RNA derived from the ON/C-5B/KE, ON/C-5B/KE/(V85I), ON/C-5B/KE/(K124E), and ON/C-5B/KE/(V85I&K124E) into OR6c cells. After 3 weeks of G418 selection at a concentration of 300 μg/ml, only one colony was obtained and the same result was obtained with ON/C-5B/KE(K124E) and ON/C-5B/KE/(V85I&K124E) transcripts. In repeated experiments, the number of G418-resistant colonies was reproducibly one or zero, but when ON/C-5B/KE/(V85I) transcripts was electroporated, G418 resistant 4–6 colonies was obtained in repeated experiments. These results also confirm that the replication level of ON/C-5B//KE/(V85I) is higher than that of ON/C-5B/KE, ON/C-5B/KE/(K124E) and ON/C-5B/KE/(V85I&K124E).

As the efficiency of colony formation with full-length replicon (ON/C-5B/KE; Fig. 1a) was quite low, we investigated colony formation with subgenomic replicon, ON/3-5B/KE (Fig. 1c). Figure 3 shows the representative result of three independent colony formation assays. The efficiency of colony formation of ON/3-5B/KE was better than that of full-length replicon and the colony formation of in vitro transcript of ON/3-5B/KE, ON/3-5B//KE/(V85I), ON/3-5B/KE/(K124E) and ON/3-5B/KE/(V85I&K124E) was 39 (61), 157 (132), 44 (54), and 134 (107), respectively (the numbers in parentheses show another set of result). The efficiency of colony formation of ON/3-5B//KE/(V85I) was greater than that of ON/C-5B/KE and it showed a similar result with that obtained from genome-length replicon.

Inhibition of HCV RNA Replication by IFN and RBV

We examined the inhibitory effect of IFN and RBV on the replication of OR/3-5B/KE. In this experiment, the subgenomic replicon system was used. OR6c cells were treated with IFN at concentrations of 1–20 μM (Fig. 4) and RBV at concentrations of 50, 100, and 200 μM (Fig. 5) after transfection of OR/3-5B/KE derived RNA. Since it is important to know how IFN and RBV treatment is toxic to the cells, we examined cell viability after treatment with 50 and 100 μM of RBV or 2 and 4 units/ml of IFN. The cell viability of OR6c was not

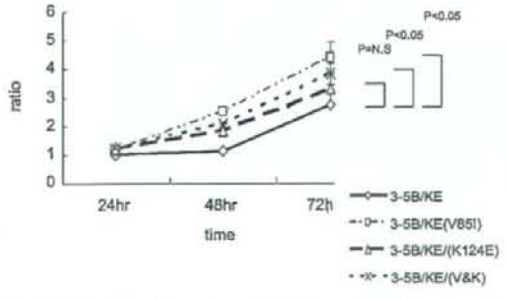

Fig. 2. Effect of amino acid substitutions in NS5B on transient replication activity of replicon. The replication activity of reporter subgenomic HCV replicon with mutation of V85I, K124E, or V85I and K124E (V&K) was compared with that of wild-type in OR6c cells (transient transfection). After 72 hr of transfection, the Renilla luciferase (RL) assay was performed as described in the Materials and Methods section. The relative RL activity (ratio) of mutants was calculated in comparison to that of subgenomic replicon of wild-type (assigned as 1). The data indicate means ± SD of triplicates from three independent experiments. 3-5B/KE: OR/3-5B/KE, 3-5B/KE(V85I): OR/3-5B(K124E), 3-5B/KE/(K124E): OR3-5B/KE/(K124E), 3-5B/KE/(V&K): OR/3-5B/KE/(V85I&K124E).

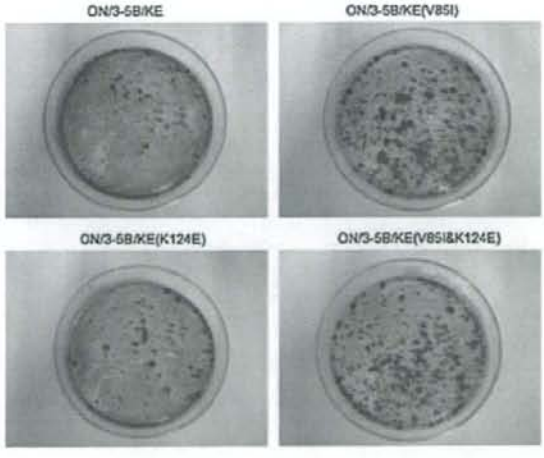

Fig. 3. Colony formation assay of OR6c cells transfected with wild-type and three different mutant replicons. A representative result of colony formation assay using subgenomic replicon RNA (ON/3-5B/KE) system. The efficacy of colony formation was much higher than that of full-length replicon RNA (ON/C-5B/KE). In this series of photographs, colony forming unit of ON/3-5B/KE, ON/3-5B//KE/(V85I), ON/3-5B/KE/(K124E) and ON/3-5B/KE/(V85I&K124E) was 2.7/μg, 6.7/μg, 3.0/μg and 5.4/μg, respectively.

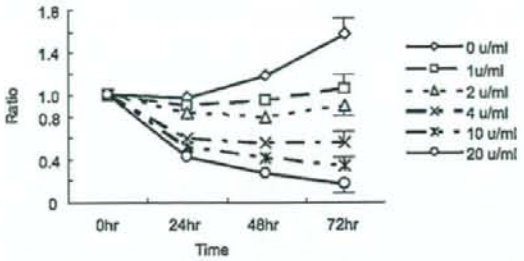

Fig. 4. Dose-dependent inhibition of replication by interferon-α (IFN). OR6 cells were transfected with wild-type replicon (OR/3-5B/KE). Inhibition of HCV RNA replication in the OR6c cell treated with IFN-α was shown at the indicated time (24, 48, and 72 hr) from the start of treatment. The cells were treated with IFN-α (0, 1, 2, 4, 10, and 20 u/ml), and the Renilla luciferase assay was performed as described in Materials and Methods Section. The relative luciferase activity (%) calculated at each point, where the luciferase activity of non-treated cells at 0 hr was assigned to be 100%, is presented. The data indicate means ± SD of triplicates from three independent experiments.

changed by these treatments (Fig. 6), indicating that both IFN and RBV were not toxic to the cells at the indicated concentrations. As shown in Figures 4 and 5, the inhibition of HCV RNA replication occurred in a dose-dependent manner with IFN or RBV treatments. RBV at a concentration of 100 μM inhibited replication of RNA (Fig. 5), but was not toxic to OR6c cells (Fig. 6).

The inhibitory effect of 100 μM RBV on RNA replication in each mutant was also examined. Various biological effect of IFN has been investigated and its effect on cell cycle or cell-differentiation is strong, and we focused on the effect of mutants on RBV treatment. To see this effect, we compared between IFN alone and IFN + RBV. As shown in Figure 7, no difference between three mutants was seen in the treatment with 1 unit/ml of IFN. The proliferation of each mutant RNA was similarly reduced to around a ratio of 0.6. On the other hand, addition of 100 μM of RBV was differently affected by each mutation pattern (Fig. 8). The single mutant with V85I and double mutants with V85I and K124E

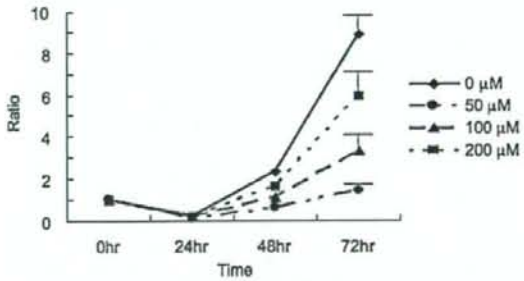

Fig. 5. Dose-dependent inhibition of HCV RNA replication by ribavirin. OR6 cells were transfected with wild-type replicon (OR/3-5B/KE) and treated with ribavirin at concentrations of 50, 100, and 200 μM for 72 hr. Inhibition of HCV RNA replication in the OR6c cell treated with ribavirin (RBV) was shown at the indicated time (24, 48, and 72 hr) from the start of treatment. The relative luciferase activity (%) calculated at each point, where the luciferase activity of non-treated cells at 0 hr was assigned to be 100%, is presented. The data indicate means ± SD of triplicates from three independent experiments.

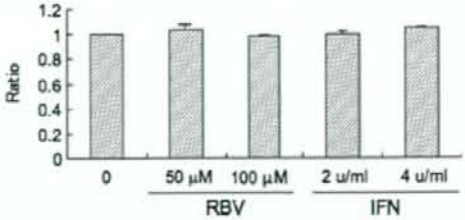

Fig. 6. Cytotoxicity of ribavirin (RBV) or interferon-α (IFN) on replicon RNA in OR6c cells. OR6c cells with OR/3-5B/KE RNA were cultured in the absence or presence of RBV or IFN (50 and 100 μM or 2 and 4 u/ml) for 72 hr, and then the cell viability was determined as described in Materials and Methods Section. The relative cell viability (%) calculated at each point, when viability of non-treated cells was assigned to be 100%, is presented. The data indicate means ± SD of triplicates from three independent experiments.

were significantly increased in RNA proliferation. The degree of inhibition by RBV in OR/3-5B/KE(V85I) and OR/3-5B/KE(V85I&K124E) was significantly lower than that in OR/3-5B/KE, although the difference of OR/3-5B/KE(K124E) was not significant.

Indirect Immunofluorescence

To confirm the presence of replicating full-length RNAs in cells selected for G418 resistance following transfection with ON/C-5B/KE(V85I), one G418-resistant cell colony was selected at random and clonally cultured. We confirmed HCV protein expression by indirect immunofluorescence imaging and observed core protein in the replicon cells (OR6) (Fig. 9c), HCV core protein was demonstrated in the clonally isolated cell line selected after transfection with ON/C-5B/KE(V85I)

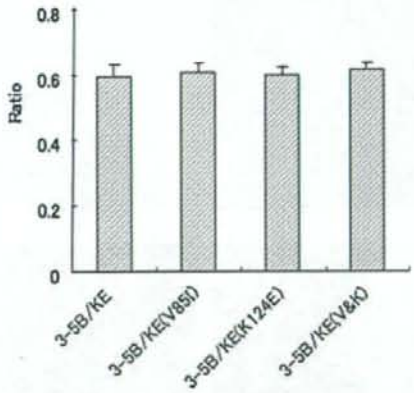

Fig. 7. Effect of interferon-α (IFN) on the subgenomic HCV replicon possessing the Renilla luciferase reporter. The replication levels of the subgenomic HCV replicons were monitored by luciferase reporter assay with IFN 0 u/ml or IFN 1 u/ml for 72 hr. Renilla luciferase assay was performed as described in Materials and Methods Section. The relative luciferase unit with IFN (1 u/ml) treatment were calculated, where the luciferase unit without IFN treatment was assigned to be 1, and compared between wild type (OR/3-5B/KE) and other three mutants (OR/3-5B/KE(V85I), OR/3-5B/KE(K124E), OR/3-5B/KE(V&K)). The data indicate means ± SD of triplicates from two independent experiments.

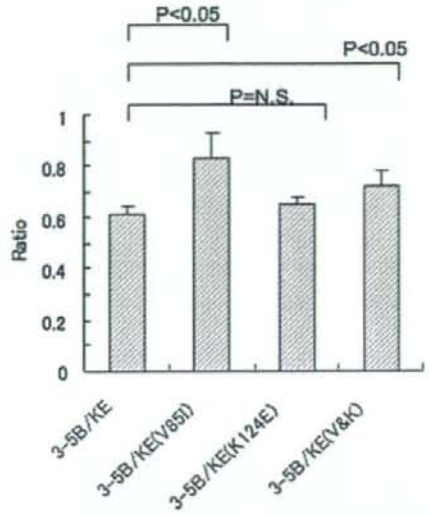

Fig. 8. Effect of interferon-α (IFN) and ribavirin (RBV) combination treatment on the replication levels of the subgenomic HCV replicon possessing the Renilla luciferase reporter. The replication levels of the subgenomic HCV replicons were monitored by luciferase reporter assay with IFN 1 u/ml or IFN 1 u/ml and ribavirin 100 μM for 72 hr. The relative luciferase unit of IFN 1 u/ml and ribavirin 100 mM treatment were calculated, where the luciferase unit of IFN 1 u/ml treatment was assigned to be 1, and compared in wild type (OR/3-5B/KE) and other three mutants (OR/3-5B/KE(V85I), OR/3-5B/KE(K124E), OR/3-5B/KE(V&K)). The data indicate means ± SD of triplicates from three independent experiments.

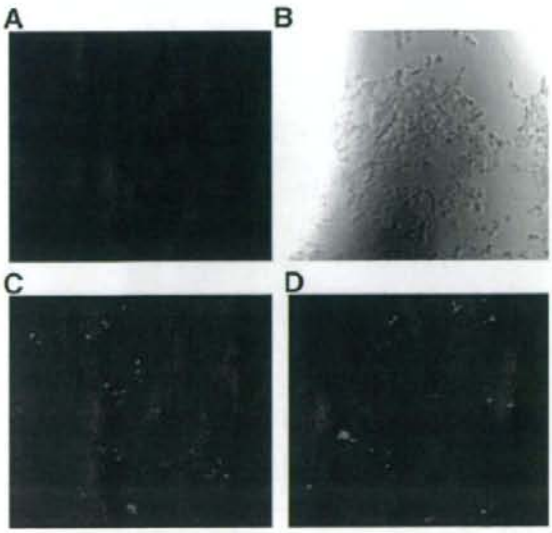

Fig. 9. Indirect immunofluorescence detection of HCV core antigen in normal OR6c cells (cured cell) (**a**), OR6 cells (wild-type HCV replicon) (**c**), and a clonally isolated cell line selected following transfection of OR6c cells with ON/3-5B/V85I (cell line 1) (**d**) and the correspondent phase-contrast microscopic photograph of OR6c cells (**b**).

(Fig. 9d), while it was not observed in the cured cell line (OR6C) (Fig. 9a,b).

DISCUSSION

Predictive factors for a sustained viral response (SVR) in IFN monotherapy or combination therapy have been vigorously investigated in prior studies. In addition to several host and viral factors, such as HCV genotypes, baseline viral load, stage of fibrosis, gender, age, and obesity [Saito et al., 2000, 2006], disappearance of serum HCV RNA during the early phase of therapy or a rapid decrease in HCV RNA levels are significant factors for achieving a SVR [Ferenci et al., 2001]. In our previous study, two distinct amino acid substitutions in the NS5B region of the HCV genome correlated with early viral responses in combination therapy [Kumagai et al., 2004]. NS5B of the HCV genome codes for RdRP, which regulates viral replication. Thus, the detected mutations might increase replication efficacy of HCV or induce resistance to the anti-viral effect of RBV, which could lead to resistance to therapy in the early phase. It was thought that the HCV replicon system would be a good tool for examining the correlation between viral mutation and replication capability. One of the mutation-introduced replicons (V85I) showed a higher replication activity than that of the wild type, and, consistent with our previous clinical study, this mutant was resistant to in vitro RBV treatment. The present study is the first to examine the precise relationship between such mutations and clinical data on the early clearance of HCV during IFN and RBV combination therapy. The mutations of V85I and K124E in NS5B have never been reported in the replicon system.

We investigated the effect of both IFN and RBV on the wild type and three mutants in NS5B at non-toxic concentration to the host cell (Fig. 6). One unit of IFN did not affect the replication of mutants (Fig. 7) but RBV significantly affected the replication of three mutants in the presence of IFN (Fig. 8). These results indicated that the polymorphism of NS5B affect sensitivity to RBV treatment. Although it has been known in the clinical setting that HCV RNA levels are not changed in patients with chronic hepatitis C during RBV monotherapy, our in vitro results showed the reduction of HCV RNA replication with RBV treatment. It was reported that serum levels of RBV in patients with chronic hepatitis C under IFN + RBV combination therapy was very low such as 10^{-14} mM [Naka et al., 2005], however, we can examine the anti-viral effect of much higher levels of RBV on the replicon system without a direct toxic effect of RBV in HuH7 cells. The possibility of a difference between circulating HCV particles and the replicon system in terms of RBV sensitivity may still exist, but this question will be further investigated using a recently developed cell culture system.

We used a dicistronic genome length and subgenome HCV RNA replication systems, which were established previously using HCV RNA from HCV-O infected in non-neoplastic human hepatocyte PH5CH8 cells. For the

cells into which genome-length and subgenomic HCV RNA were introduced, we chose the cloned cell line OR6c, prepared by IFN treatment from subgenomic HCV replicon-supporting cells, since OR6c had a higher efficiency of colony formation (ECF) than its parental HuH-7 cell line in a study of subgenomic HCV replicons [Blight et al., 2002]. It is known that the efficiency of colony formation is unstable, so that the luciferase activity and the colony-forming unit are always discrepant. The impact of ON/C-5B/KE(V85I) on colony formation was about 4 times that of the wild-type replicon in genome length and subgenomic RNAs, and the V85I mutation in NS5B showed 1.5 times higher replication activity in luciferase assay than the wild type in the subgenomic replicon system. Young et al. reported an RBV-resistant NS5B mutation during RBV monotherapy [Young et al., 2003], but this phenylalanine to tyrosine amino acid substitution located at the 415th position in NS5B differed from our amino acid substitution. Replicon cells were selected after G418 exposure, and the replication may be amplified by this selection culture. We sequenced the NS5B region, which includes the 85th and 124th nucleotide portions, from some clones 2 months after G418 selection culture, and we did not found significant mutations. From the present in vitro study and previous clinical study, it may be concluded that at least V85I mutation in NS5B increases viral replication that may cause resistance to RBV treatment.

Two of the patients in the clinical study [Kumagai et al., 2004] had previously been treated with IFN-α monotherapy in our previous study: one patient (Pt 3) has V85 and K124 in the HCV RdRP and the other (Pt 7) had I85 and E124. The former was a good responder to IFN-α and RBV combination therapy, but the latter was not. This result indicated that I85V and E124K substitutions did not affect the response to IFN-α monotherapy, because both types had failed to respond previously to IFN monotherapy. Therefore, we surmised that this amino acid substitution influenced the response to RBV anti-viral activity, which prompted us to examine the effect of RBV on viral replication. Several mechanisms of anti-viral activity of RBV have been proposed [Tam et al., 1999; Maag et al., 2001; Lau et al., 2002], but it is unclear why only the V85I single amino acid substitution induced replication better than the wild type. As shown previously [Kumagai et al., 2004], the 85th amino acid of HCV RdRP is distant from the active site of polymerase but is located near the RNA primer binding site, and this substitution may influence nucleotide misincorporation during polymerization. This 85th position is more important than the 124th position for replication of HCV-O.

This study is the first to examine whether NS5B polymorphism affects the replication efficiency and anti-HCV effect of RBV in an HCV RNA replicon system. It will be interesting to know whether these mutations in other genotypes (genotypes 2 and 3) replicate more efficiently and are more resistant than genotype 1b to RBV alone. Our data suggested that during clinical use of RBV, several mutations in the HCV genome might occur, such as in the isoleucine residue at the 85th position of HCV NS5B, which then affect viral replication and RBV resistance. This viral mutation may be one of the reasons for the failure in early viral clearance by IFN and RBV. There are, however, many factors that influence the success of IFN and RBV combination therapy. The resistance or sensitivity to IFN or peg-IFN, not to RBV, might also affect the early viral response, and many factors in both viral and host sides are known to affect IFN responsiveness, such as NS5A mutations [Enomoto et al., 1996], immunological status [Saito et al., 2000], or irf-1 gene promoter polymorphisms [Saito et al., 2002, 2005]. Together, these factors might determine the efficacy of anti-viral therapy in vivo, and the present in vitro data provides evidence partially supporting our clinical observations that NS5B polymorphisms are associated with early viral clearance during IFN and RBV therapy. However, it is unclear whether this single mutation occurs with peg-IFN plus RBV combination therapy and further studies are necessary. Nevertheless, our report is useful for modeling targets for antiviral compounds for the treatment of HCV.

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BASIC STUDIES

Mitochondrial electron transport inhibition in full genomic hepatitis C virus replicon cells is restored by reducing viral replication

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fluvastatin – interferon – oxidative stress – reactive oxygen species

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Abstract

Background/Aim: Hepatitis C virus (HCV) core protein has been shown to inhibit mitochondrial electron transport and to increase reactive oxygen species (ROS) *in vitro* and *in vivo*. The aim of this study was to investigate whether inhibiting HCV replication could restore the mitochondrial redox state and electron transport activity. *Methods:* We measured ROS, mitochondrial reduced glutathione content, and mitochondrial complex I, II, III and IV activities and protein expression in full genomic HCV replicon cells and cured cells that had been prepared by eliminating HCV RNA from replicon cells by interferon (IFN)-α treatment. *Results:* Cured cells had significantly lower ROS production and greater mitochondrial glutathione content than replicon cells. Complete inhibition of HCV replication by IFN-α restored complex I and IV activities by 20–30% ($P < 0.01$) and complex I expression ($P < 0.05$). Treatment with fluvastatin, one of the 3-hydroxy-3-methylglutaryl co-enzyme A reductase inhibitors, which is known to have anti-HCV activity, partially inhibited core protein expression and restored complex I activity in full genomic HCV replicon cells to a lesser degree ($P < 0.05$). *Conclusions:* Our results show that the mitochondrial redox state and electron transport activity can be restored by reducing HCV replication.

Hepatitis C virus (HCV) causes acute and chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC) (1). Because current antiviral treatment can only eliminate the virus in about 50% of patients (2, 3), therapies to reduce disease progression in chronically infected individuals would be of great benefit. In this respect, it is still a matter of debate whether reduction of HCV replication, even if not eliminating HCV, is beneficial to the outcome of disease. Although the mechanisms of its pathogenesis are incompletely understood, there have been several lines of evidence suggesting that oxidative stress is present in chronic hepatitis C to a greater degree than in other inflammatory liver diseases and is closely related to disease progression (4, 5). We and others have shown that HCV core protein induces the production of reactive oxygen species (ROS) (6–8) and that mitochondrial electron transport inhibition by HCV is associated with ROS production (9). Therefore, whether reduction of HCV replication restores mitochondrial electron transport activity is of interest in exploring treatments to reduce disease progression in HCV-associated chronic liver disease.

Establishment of the HCV subgenomic replicon has made it possible to assess the antiviral activities of interferon (IFN) and other reagents *in vitro* (10). We also developed a genome-length HCV RNA replication reporter system (11) and found that different statins, which are 3-hydroxy-3-methylglutaryl co-enzyme A reductase inhibitors, have different anti-HCV profiles while using this reporter system (12). In the present study, we chose to use fluvastatin, which exhibited the strongest inhibition of HCV replication among the statins (12), to reduce HCV replication in full genomic HCV replicon cells without complete inhibition. The aim of this study was to examine whether mitochondrial electron transport activity was restored by reduction of HCV replication.

Materials and methods

Cell cultures

Full genomic HCV replicon cells were described in detail by Ikeda *et al.* (11). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin, streptomycin and G418 (300 μg/ml; Calbiochem, Darmstadt, Germany) and passaged twice a week at a 5:1 split ratio. Cured cells were established by eliminating genome-length HCV RNA from replicon cells by IFN-α treatment (500 IU/ml for 2 weeks; Sigma-Aldrich, St Louis, MO, USA) without G418, as described (11). In some experiments, full genomic HCV replicon cells were incubated in the presence of 10 μmol/L fluvastatin (Novartis Pharmaceutical, Tokyo, Japan) for 96 h.

Measurement of reactive oxygen species

The cellular ROS level was measured by oxidation of the cell-permeable, oxidation-sensitive fluorogenic precursor dihydrodichlorocarboxyfluorescein diacetate (DCFDA; Molecular Probes Inc., Eugene, OR, USA). Cells in six-well plates were treated with tertiary butyl hydroperoxide (t-BOOH) for 5 h or not, followed by a 30-min incubation with DCFDA (500 nmol/L final concentration) at 37 °C. Fluorescence was measured with a CytoFluorII fluorescence plate reader (PerSeptive Biosystems, Framingham, MA, USA) at an excitation wavelength of 486 nm and an emission wavelength of 530 nm as described (7).

Localization of ROS production on the subcellular level was observed with a Zeiss (Oberkochen, Germany) LSM5 Pascal inverted laser scanning confocal microscope. Cells were pre-incubated with 5 μmol of hydroxyphenyl florescein (HPF, Alexis Corporation, Lausen, Switzerland) (13) for 5 min at 37 °C. They were then imaged at 30-s intervals after treatment with 10 nmol/L t-BOOH. The green fluorescence of HPF (excitation, 488 nm; emission, 505–530 nm) was observed after excitation with an argon–krypton laser.

Isolation of mitochondria

Mitochondrial pellets were obtained as described previously with some modification (7, 9). Briefly, harvested cells were centrifuged at 500*g* for 5 min. The pellets were homogenized with 25 strokes using a Dounce homogenizer (Wheaton Science Products, Millville, NJ, USA) and a tight-fitting pestle with isolation buffer [70 mM sucrose, 1 mM KH_2PO_4, 5 mM HEPES, 220 mM mannitol, 5 mM sodium succinate and 0.1% bovine serum albumin (BSA), pH 7.4]. The homogenate was centrifuged at 1330*g* for 5 min at 4 °C. The supernatant fraction was retained, whereas the pellet was washed with isolation buffer and centrifuged again. The combined supernatant fractions were centrifuged at 1000*g* for 15 min at 4 °C to obtain a crude mitochondrial pellet. Purified mitochondria were prepared by sucrose gradient (1.5 M sucrose and 1 M sucrose) centrifugation as described (14) with some modification. An aliquot was removed for determination of the protein concentration with the Bio-Rad protein DC assay kit (Bio-Rad, Hercules, CA, USA), using BSA as the standard.

Measurement of reduced glutathione content

Crude mitochondrial samples (3–4 mg of mitochondrial protein) were sonicated using a Sonifier cell disruptor 200 (VWR Scientific, Danbury, CT, USA) for 15 s at power setting 3 in ice-cold 5% metaphosphoric acid and centrifuged at 3000*g* at 4 °C for 10 min. The concentration of reduced glutathione was measured by the thioester method using a GSH-400 kit (Oxis International Inc., Portland, OR, USA).

Immunoblotting

Crude mitochondrial pellets were suspended in lysis buffer (T-PER Tissue Extraction Reagent; Pierce, Rockford, IL, USA) and centrifuged at 10 000*g* for 15 min at 4 °C. The supernatant (20 μg of protein) was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis on 16% gel. The proteins were electrophoretically blotted onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA), blocked overnight at 4 °C with 5% skim milk and 0.1% Tween 20 in Tris-buffered saline, and subsequently incubated for 1 h at room temperature with an anti-hepatitis C core protein antibody (1:1000, Affinity Bio Reagents, Golden, CO, USA), anti-OxPhos complex I antibody (1:1000), anti-OxPhos complex II antibody (1:2000), anti-OxPhos complex III antibody (1:2500) or anti-OxPhos complex IV antibody (1:1000, Molecular Probes Inc). The membranes were washed, incubated with appropriate secondary antibodies and detected with ECL™ Western blot detection reagents (Amersham Biosciences, Piscataway, NJ, USA). The degree of protein expression was expressed as the normalized quotient, which was derived by dividing the intensity of the blot density of each protein by that of β-actin protein.

Measurement of complex I, II, III and IV activities

Submitochondrial particles were prepared from mitochondria by incubation for 3 min at 37 °C followed by sonication in a microcentrifuge tube immersed in ice water. Forty micrograms of submitochondrial

particles was pelleted at 15 000*g* for 10 min. Enzyme activity assays were performed at 25 °C by a previously established method (15). Complex I [nicotinamide adenine dinucleotide (NADH)-decylubiquinone oxidoreductase] activity was measured as the initial (5 min) rate of decrease of A_{340} using the acceptor 2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzoquinone (DB 80 μM) and 200 μM NADH as the donor in 10 mM Tris (pH 8.0) buffer containing 1 mg/ml BSA, 0.24 mM KCN and 0.4 μM antimycin A. Complex II (succinate decylubiquinone 2,6-dichlorophenolindophenol reductase) activity was measured at 600 nm using 80 μM DCPIP as the acceptor and 10 mM succinate as the donor in 10 mM KH_2PO_4 (pH 7.8), 1 mg/ml BSA, 2 mM EDTA, in the presence of 0.24 mM KCN, 4 μM rotenone, 0.2 mM ATP and 0.4 μM antimycin A. Complex III (ubiquinol cytochrome *c* reductase) activity was measured at 550 nm using 40 μM oxidized cytochrome *c* as the acceptor and 80 μM decylubiquinol as the donor in 10 mM KH_2PO_4 (pH 7.8), 1 mg/ml BSA, 2 mM EDTA, in the presence of 0.24 mM KCN, 4 μM rotenone and 0.2 mM ATP for 2 min. Complex IV (cytochrome *c* oxidase) activity was measured using a cytochrome *c* oxidase assay kit (Sigma-Aldrich), following the manufacturer's instructions.

Statistical analysis

Quantitative values are expressed as mean ± standard deviation. Two groups were compared by the Student *t*-test. A P value of < 0.05 was considered to be significant. Two groups among multiple groups were compared by the rank-based, Kruskal–Wallis analysis of variance test followed by Scheffe's test.

Results

Increased reactive oxygen species production and mitochondrial oxidant status in full genomic hepatitis C virus replicon cells

To assess the effect of HCV replication on ROS production, we used the ROS-sensitive fluorescent probe DCFDA. As compared with cured cells, HCV replication increased ROS 1.4-fold (Fig. 1A). Because HCV infection results in an inflammatory response and an increase in the basal oxidative stress, we next determined the effect of an exogenous oxidant, 500 nmol/L t-BOOH, on ROS production. This treatment had no effect on cured cells, but increased ROS production in full genomic HCV replicon cells to a level 2.5-fold greater than that of cured cells (Fig. 1A; $P < 0.01$). Cells were then imaged by confocal microscopy at 30-s intervals after exposure to HPF, which is more sensitive to ROS production than DCFDA. As shown in Figure 1B, treatment with t-BOOH significantly increased the oxidized fluorescent product as time passed in full genomic HCV replicon cells, but not in cured cells ($P < 0.0005$). Thus, a small volume of exogenous oxidant (10 nmol/L) that did not induce ROS production in cured cells significantly increased ROS production in full genomic HCV replicon cells.

We previously demonstrated, by confocal microscopy, that the mitochondria are the primary site of initial ROS production in cells expressing HCV core protein and cytochrome P450 2E1 (7). Because confocal microscopic images of the oxidized fluorescent product in replicon cells were almost the same as those in our previous study, we measured mitochondrial reduced glutathione content to assess mitochondrial antioxidant capacity. The level of mitochondrial reduced glutathione was significantly lower in full genomic HCV replicon cells than in cured cells (Fig. 1C; $P < 0.05$), suggesting that HCV replication was responsible for the mitochondrial oxidant status and sensitized to exogenous oxidative stress.

Restoration of mitochondrial electron transport activity by complete inhibition of hepatitis C virus replication

Our previous study has demonstrated that core protein causes oxidation of the glutathione pool, increases ROS production and inhibits complex I activity (9). Because increased ROS production and mitochondrial oxidant status were found in full genomic HCV replicon cells as well, we next measured complex I, II, III and IV activities in submitochondrial particles to determine whether inhibiting HCV replication restored mitochondrial electron transport activity. Complete inhibition of HCV replication by IFN-α restored complex I and IV activities by 20–30% ($P < 0.01$) (Fig. 2). However, complex II and III activities were not changed after treatment with IFN-α in these cells (Fig. 2).

We further assessed the expression levels of complexes I, II, III and IV in full genomic HCV replicon cells and cured cells. As shown in Figure 3, immunoblotting revealed that complete inhibition of HCV replication by IFN-α restored the complex I expression as well ($P < 0.05$). Although the complex IV activity was restored by IFN-α, the expression of complex IV did not change after complete inhibition of HCV replication. Thus, it should be noted that both activity and expression of complex I were restored by completely inhibiting HCV replication in full genomic HCV

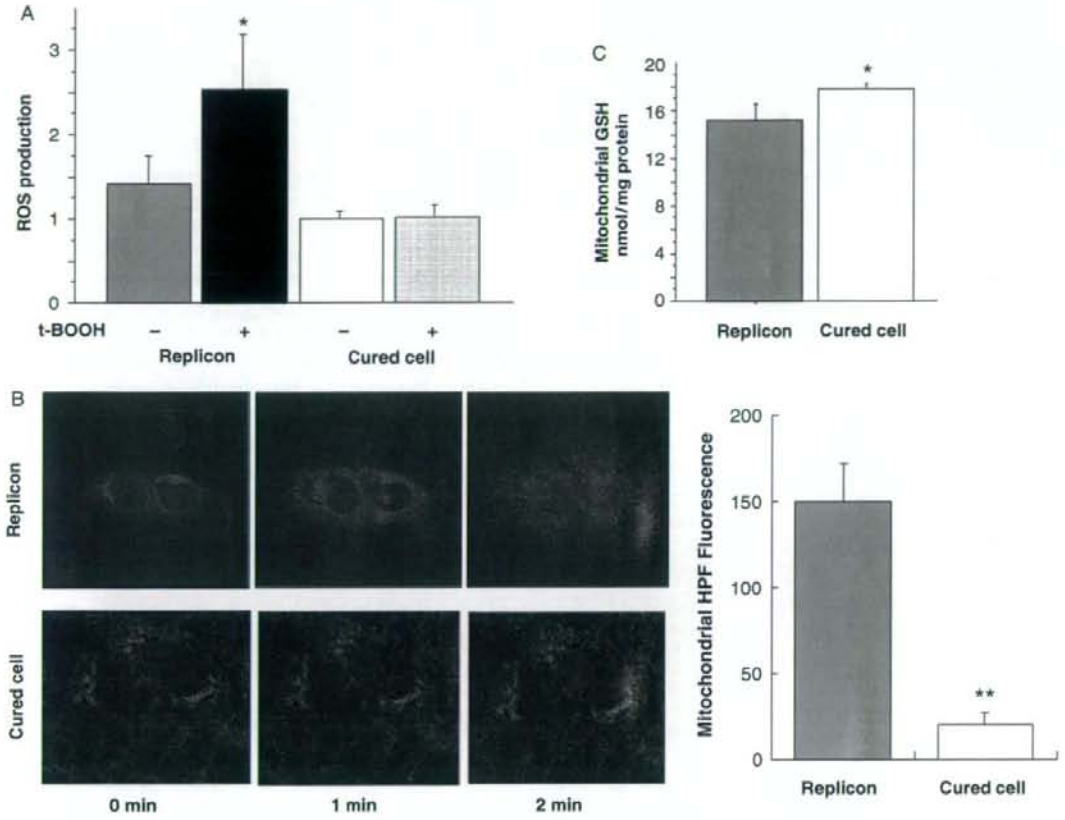

Fig. 1. Effects of HCV replication on ROS production and mitochondrial reduced glutathione level. (A) ROS production was measured by oxidation of DCFDA in HCV replicon cells and cured cells under control conditions or after 5-h incubation with t-BOOH (500 nmol/L). $^{*}P < 0.01$ compared with untreated cured cells. (B) Confocal images of ROS generation. HCV replicon cells and cured cells were pre-incubated with HPF, subsequently treated with t-BOOH (10 nmol/L) and imaged at 30-s intervals. The increase in HPF fluorescence intensity 2 min after treatment with t-BOOH was compared between HCV replicon cells and cured cells. $^{**}P < 0.0005$ compared with HCV replicon cells. (C) Reduced glutathione content was measured in crude mitochondrial fractions prepared from HCV replicon cells and cured cells. $^{*}P < 0.05$ compared with HCV replicon cells. DCFDA, dihydrodichlorocarboxyfluorescein diacetate; HCV, hepatitis C virus; HPF, hydroxyphenyl florescein; ROS, reactive oxygen species.

replicon cells, even though the significance of reduced complex IV activity remains elusive.

Incompletely inhibited hepatitis C virus replication partially restores mitochondrial electron transport activity

Fluvastatin, a 3-hydroxy-3-methylglutaryl co-enzyme A reductase inhibitor, has been shown to have an inhibitory effect on HCV replication in the present full genomic HCV replicon cells (12). We used fluvastatin for partially inhibiting HCV replication in full genomic HCV replicon cells, because it has a lesser inhibitory effect on HCV replication than IFN-α (12). In fact, expression of core protein was present in mitochondria, but was significantly lowered by treatment with fluvastatin in full genomic HCV replicon cells ($P < 0.05$; Fig. 4A). Partial inhibition of HCV replication restored complex I activity by ~13% ($P < 0.05$; Fig. 4B). Although statins including fluvastatin are known to have an anti-oxidative effect (16, 17), treatment with fluvastatin did not improve complex I activity in cured cells (Fig. 4B), suggesting that this activity was restored by its inhibitory effect on HCV replication rather than its anti-oxidative property. However, partial inhibition of HCV replication did

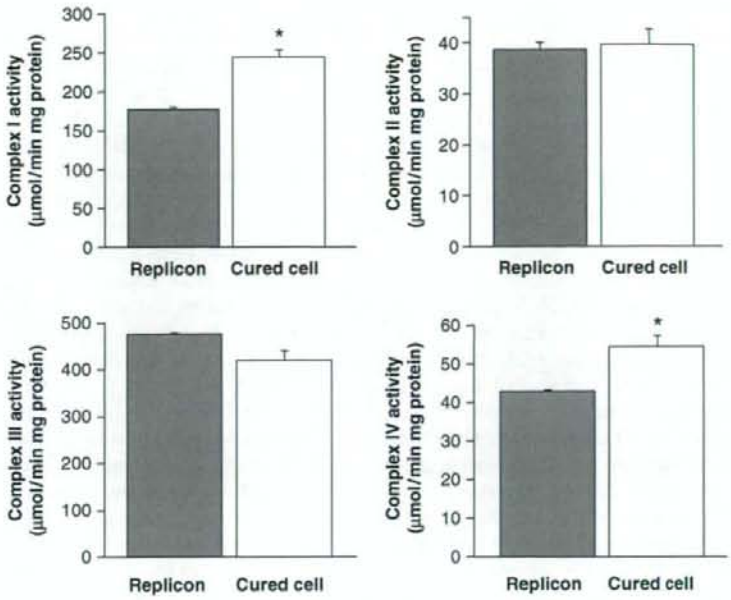

Fig. 2. Mitochondrial complex I, II, III and IV activities. Complex I (NADH-decylubiquinone oxidoreductase) activity, complex II (succinate decylubiquinone 2,6-dichlorophenolindophenol reductase) activity, complex III (ubiquinol cytochrome *c* reductase) activity and complex IV (cytochrome *c* oxidase) activity were measured in submitochondrial fractions prepared from HCV replicon cells and cured cells. $^*P < 0.01$ compared with HCV replicon cells. HCV, Hepatitis C virus; NADH, nicotinamide adenine dinucleotide.

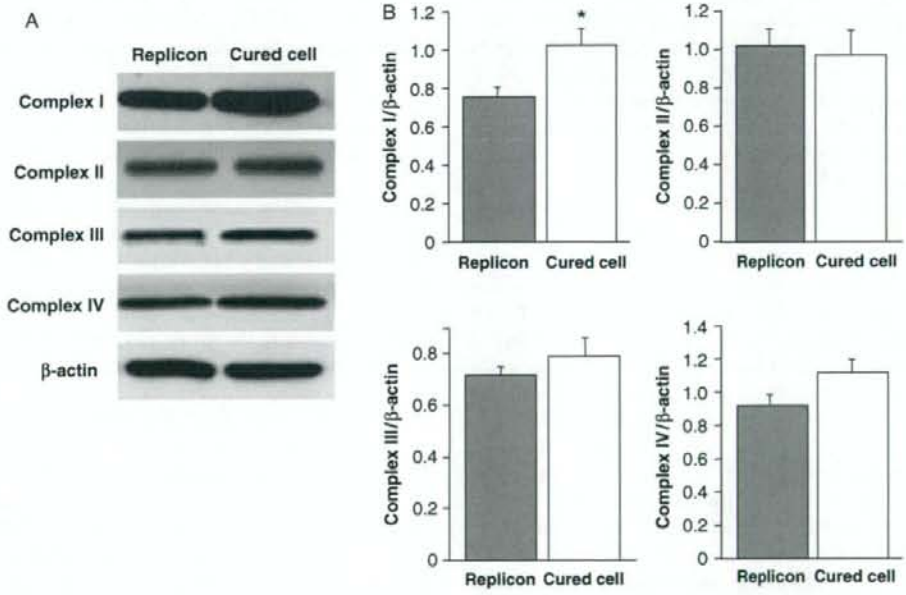

Fig. 3. Expression of mitochondrial complex I, II, III and IV. (A) Immunoblots for complex I, II, III and IV were performed using crude mitochondrial fractions prepared from HCV replicon cells and cured cells. (B) The degree of protein expression was normalized with β-actin protein. $^*P < 0.05$ compared with HCV replicon cells. HCV, hepatitis C virus.

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not lead to a significant reduction of ROS production (Fig. 4C), a significantly increased level of mitochondrial reduced glutathione (Fig. 4D) or complex I expression (Fig. 4A). Thus, incomplete inhibition of HCV replication restored mitochondrial electron transport activity in full genomic HCV replicon cells, even though it was not sufficient to reduce mitochondrial oxidative status.

Discussion

We have previously reported that HCV core protein inhibits mitochondrial electron transport and increases ROS production in the transgenic mouse liver (9). Our present results have shown that these phenomena can be reproduced in the presence of HCV replication. As replication of a full-length HCV genome rather than mere core protein expression is closer to the disease condition occurring in patients with chronic hepatitis C, the present results have strengthened the possibility that mitochondrial oxidation, ROS production and inhibition of mitochondrial electron transport are actually caused in chronic hepatitis C. Thanks to the establishment of HCV replicon cells, we could investigate the effect of inhibiting HCV replication on mitochondrial electron transport activity that was closely related to ROS production. In the present study, we focused on restoration of mitochondrial electron transport activity by inhibiting HCV replication, regarding complete inhibition of HCV replication *in vitro* as that of HCV eradicated with IFN therapy and partial inhibition of HCV replication *in vitro* as that in cells undergoing IFN therapy without HCV eradication.

Consistent with a previous observation, complex I activity, but not complex III activity, was reduced in full genomic HCV replicon cells. Complex I appeared to be the source of HCV-induced ROS, because mitochondrial ROS generation can occur at either complex I or complex III (18–20). We also found decreased expression of complex I in full genomic HCV replicon cells as compared with cured cells. Complex I is the site most sensitive to oxidative damage of the electron transport carriers, and inhibition of complex I occurs during the early stages of mitochondrial damage (21). Increased mitochondrial ROS production due to reduction of complex I activity amplifies mitochondrial oxidation, which in turn may inhibit the expression of complex I. Complex IV activity, i.e. that of cytochrome *c* oxidase, was reduced in full genomic HCV replicon cells as well. Complex IV localizes at the end of mitochondrial electron transport, accepts one electron at a time from cytochrome *c* and passes them four at a time to oxygen. Therefore, decreased activity of complex IV may amplify mitochondrial ROS production, possibly by inhibiting electron flow in the respiratory chain.

Thus, it is likely that HCV replication increases mitochondrial ROS production through inhibition of electron transport, causing oxidative stress within the liver in patients with chronic hepatitis C. Several different experimental models of HCV protein expression reproduced this finding (6–8). However, whether reduction of HCV replication restores mitochondrial function remains unknown. In the present study full genomic HCV replicon cells had ~30% reduction of complex I activity ($P = 0.0001$) and ~20% reduction in complex IV activity ($P < 0.01$) as compared with cured cells (Fig. 2). In other words, complete inhibition of HCV replication by IFN-α restored the activities of complex I and complex IV, leading to reduced ROS production in the presence of an exogenous oxidant and to an increase of mitochondrial reduced glutathione content (Fig. 1). There have been several lines of clinical evidence suggesting that HCV elimination by IFN treatment reverses the progression of liver fibrosis and significantly suppresses the development of HCC afterwards (22, 23). Restoration of mitochondrial electron transport activity by complete inhibition of HCV replication may well account for this clinical evidence, because the progression of liver fibrosis and development of HCC in chronic hepatitis C have been shown to be closely related to excess oxidative stress within the liver (24, 25).

In the clinical setting, however, HCV eradication by combination therapy with peginterferon-α and ribavirin has been successful in 50–60% of patients with refractory chronic hepatitis C at most (2, 3). Therefore, it is a critical issue for patients for whom this is unsuccessful if needed, whether prolonged reduction of HCV replication, not elimination of HCV, reduces or delays the progression of liver fibrosis and development of HCC. Although there have been several studies suggesting the inhibitory effect of IFN therapy on HCC development in patients with HCV-associated chronic liver diseases (26, 27), it is still controversial (28). Cured cells did not show core protein expression at all (data not shown), whereas fluvastatin-treated replicon cells had core protein expression that was significantly lower than in replicon cells without treatment. Thus, incomplete inhibition of HCV replication by fluvastatin was useful as a model for assessing if reduction of HCV replication, not elimination of HCV, could restore mitochondrial function. We found that incomplete inhibition of HCV replication restored complex I activity, but did

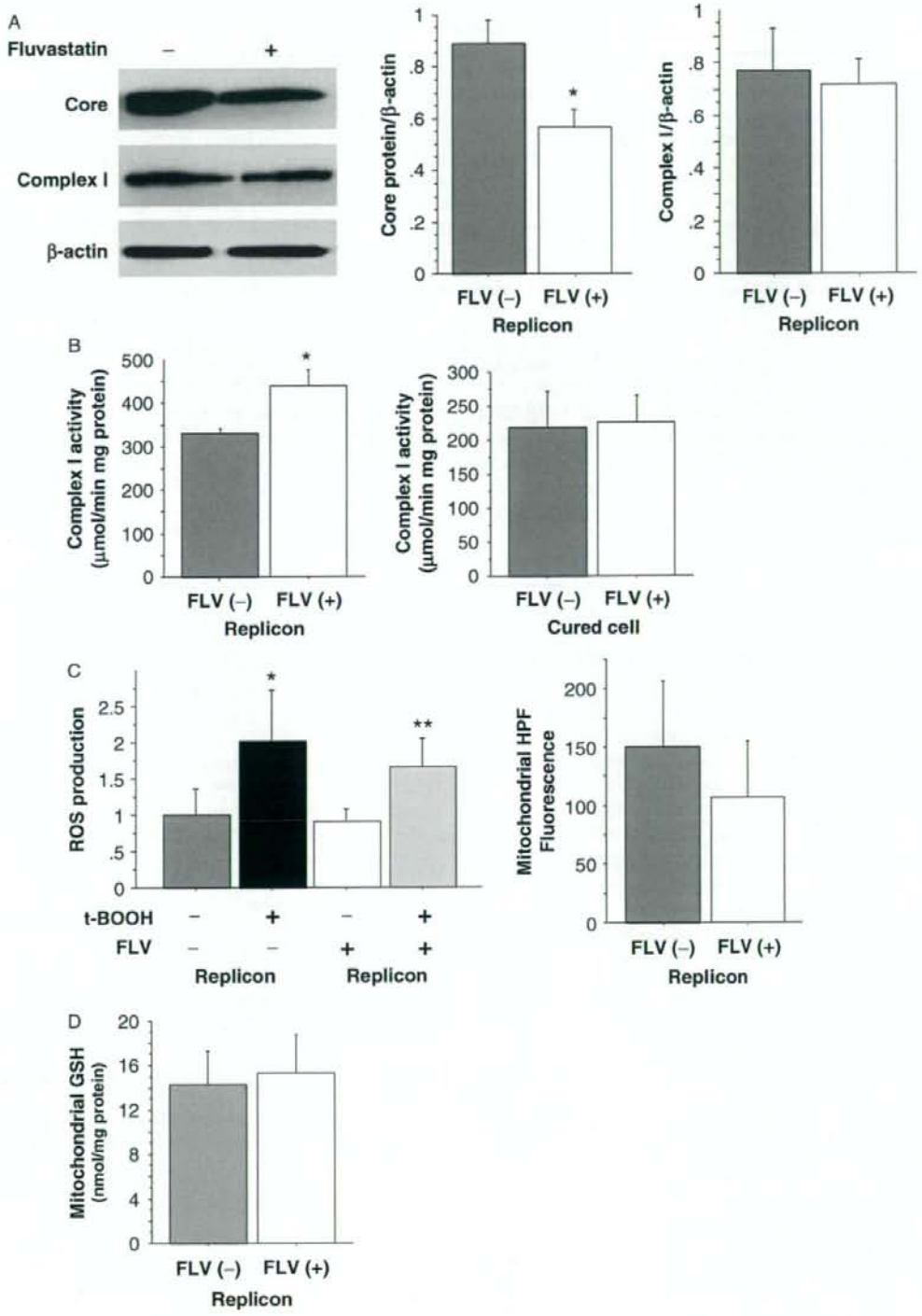

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Fig. 4. Effect of fluvastatin on core protein expression, mitochondrial complex I activity and expression, ROS production and mitochondrial reduced glutathione level. (A) Immunoblots for core protein and complex I were performed using crude mitochondrial fractions prepared from HCV replicon cells treated with/without fluvastatin. The degree of protein expression was normalized with β-actin protein. (B) Complex I (NADH-decylubiquinone oxidoreductase) activity was measured in submitochondrial fractions prepared from HCV replicon cells and cured cells, both of which were treated with/without fluvastatin. (C) ROS production was measured by oxidation of DCFDA in HCV replicon cells treated with/without fluvastatin under control conditions or after 5-h incubation with t-BOOH (500 nmol/L). HCV replicon cells treated with/without fluvastatin also were pre-incubated with HPF and subsequently treated with t-BOOH (10 nmol/L). The increase in HPF fluorescence intensity 2 min after treatment with t-BOOH was compared between HCV replicon cells with fluvastatin and those without. $^{*}P < 0.05$ as compared with HCV replicon cells without both t-BOOH and fluvastatin treatment. $^{**}P < 0.005$ as compared with fluvastatin-treated HCV replicon cells without t-BOOH. (D) Reduced glutathione content was measured in crude mitochondrial fractions prepared from HCV replicon cells treated with/without fluvastatin. DCFDA, dihydrodichlorocarboxyfluorescein diacetate; FLV, fluvastatin; HCV, hepatitis C virus; HPF, hydroxyphenyl florescein; NADH, nicotinamide adenine dinucleotide; ROS, reactive oxygen species; t-BOOH, tertiary butyl hydroperoxide.

not lead to the reduction of mitochondrial oxidative status. Thus it should be noted that restoration of complex I activity resulted from the inhibitory effect of HCV replication by fluvastatin rather than its antioxidant property. Even if incomplete inhibition of HCV replication fails to reduce mitochondrial oxidative status *in vitro*, restoration of complex I activity for a certain period *in vivo* may lead to a reduction of mitochondrial oxidative status. However, we need to recognize that inhibition of HCV replication *in vitro* by statins does not necessarily imply the same effect *in vivo*, because the absence of a clinical anti-HCV effect of statins has been reported (29). The present results showing that even incomplete inhibition of HCV replication can restore mitochondrial function to a lesser degree than completely inhibited HCV replication provides us with a rationale for suppressing HCV replication by anti-HCV agents in nonsustained responders to the current combination therapy.

In conclusion, our study shows that HCV replication causes oxidation of the mitochondrial glutathione pool, increases ROS production and inhibits mitochondrial electron transport activity, and that these changes in the mitochondrial redox state can be reversed by reducing HCV replication.

Acknowledgements

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Case Report

Early emergence of entecavir-resistant hepatitis B virus in a patient with hepatitis B virus/human immunodeficiency virus coinfection

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The efficacy of entecavir for patients with hepatitis B virus/human immunodeficiency virus coinfection has not been fully elucidated. Here we examined a patient coinfected with both viruses in whom entecavir-resistant hepatitis B virus appeared. The 60-year-old Japanese male with the coinfection received antiretroviral therapy including lamivudine. The therapy initially suppressed replication of both viruses, followed by reactivation of the hepatitis B virus alone by 2 years of therapy. He subsequently received entecavir therapy in addition to the antiretroviral regimen. After entecavir administration, the hepatitis B virus DNA level was slightly reduced, but then increased after 6 months of entecavir therapy. In the sequencing analysis of hepatitis B virus, no drug resistance-associated amino acid substitutions were observed in the reverse transcriptase (rt) domain before antiretroviral therapy. The lamivudine-resistant amino acid substitutions at rt173, rt180 and rt204 were detected before entecavir administration, and further the entecavir-resistant rt202 substitution was observed after 6 months of entecavir therapy. The full-length hepatitis B sequences showed that the viral strain derived from the patient belonged to genotype H. In summary, this report describes a patient with hepatitis B virus/human immunodeficiency virus coinfection who received entecavir therapy in addition to an antiretroviral regimen and showed the early emergence of entecavir-resistance hepatitis B virus. In entecavir therapy for patients infected with both viruses, great care should be taken with respect to the emergence of entecavir-resistant hepatitis B virus, especially in patients with pre-existing lamivudine-resistant virus.

Key words: coinfection, drug-resistant hepatitis B virus, entecavir, hepatitis B virus, human immunodeficiency virus, lamivudine

INTRODUCTION

CHRONIC CARRIERS OF hepatitis B virus (HBV) number more than 350 million worldwide.[1] Chronic HBV infection is seen in approximately 10% of human immunodeficiency virus (HIV)-infected patients,[2] and coinfection with HBV and HIV is a serious health problem due to the shared mode of transmission. Since the prognosis of HIV-infected patients can be dramatically improved by highly active antiretroviral therapy (HAART), one of the major causes of mortality in HIV-infected patients is chronic liver disease due to HBV infection.[3]

Lamivudine (LAM, also abbreviated to 3TC), one of the antiretroviral drugs, has also been used for the reduction of HBV replication and improvement of HBV-related liver diseases.[4,5] However, the anti-HBV effect of LAM is hampered by the emergence of LAM-resistant mutant virus in cases of HBV monoinfection and HBV/

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HIV coinfection.[6,7] The LAM-resistant HBV strain is based on point mutation occurring within the reverse transcriptase (rt) domain of the polymerase gene. A methionine-to-valine/isoleucine amino acid substitution at rt204 (rtM204V/I) is known to confer LAM resistance.[8,9] A leucine-to-methionine substitution at rt180 (rtL180M) and a valine-to-leucine substitution at rt173 (rtV173L) have also been shown to appear in association with LAM resistance.[8,10,11] The emergence rate of LAM-resistant virus in patients coinfected with HBV and HIV has been reported to be approximately 50% after 2 years of therapy.[9]

Recently, entecavir (ETV) has been reported to be superior to LAM for the suppression of viral replication and disease activity in patients with HBV monoinfection who had not received previous treatment with other anti-HBV drugs (naïve patients).[12,13] ETV has also been shown to be effective in HBV-infected patients who had been treated with LAM and showed LAM resistance.[14] It has been demonstrated that ETV resistance occurs based with amino acid substitution(s) at rt184, rt202 and/or rt250, together with the LAM-resistant rtM204V/I and rtL180M substitutions.[15] The emergence rate of ETV-resistant virus after 3 years of therapy has been reported to be less than 1% in naïve patients and 15% in LAM-resistant patients with chronic HBV monoinfection.[16] However, the anti-HBV efficacy of ETV for HBV/HIV coinfection has not been fully clarified.

In this study, we examined a patient with concomitant HBV/HIV infection who underwent HAART including LAM, and showed the appearance of LAM-resistant HBV. Subsequent ETV administration did not lead to an adequate reduction of the HBV replicative level, followed by the early emergence of the ETV-resistant virus. We investigated the serial change in the drug resistance-associated mutation status within the rt domain of the HBV polymerase gene, as well as full-length nucleotide sequences of the ETV-resistant HBV strain derived from the patient.

CASE REPORT

Patient and serum sampling

A 60-YEAR-OLD JAPANESE heterosexual male first visited to the National Hospital Organization Osaka National Hospital in December 2001 due to a positive result from an HIV antibody (anti-HIV) test in voluntary HIV screening. From his anamnestic record, he had been admitted with type B acute hepatitis to another hospital 3 years earlier. Anti-HIV had been negative at that time. On his first visit, the anti-HIV positivity was confirmed by Western blot analysis. Antibodies to HIV-1 proteins, gp160, gp110/120, p68, p52, gp41, p40 and p34 were positive. As for antibodies to HIV-2 proteins, only an antibody to p68 was positive. According to these, he was judged to be infected with HIV-1. The HIV-RNA level was $10^{4.3}$ copies/mL, and the CD4+ T cell counts were 275/mm^3 (normal range, >300/mm^3). He tested positive for hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg), and negative for antibody to HBsAg (anti-HBs) and antibody to HBeAg (anti-HBe). The HBV-DNA level was $>10^{7.6}$ copies/mL, and the alanine aminotransferase (ALT) level was 106 IU/L. The patient was free of HIV-related symptoms and had no opportunistic infectious diseases. HAART with LAM (300 mg/day), zidovudine (AZT) (600 mg/day) and efavirenz (EFV) (600 mg/day) was started in April 2002. AZT and EFV were then substituted for sanilvudine (d4T) (60 mg/day) and avacavir (ABC) (600 mg/day) in July 2002 because of anemia and dizziness. By July 2002, HIV-RNA decreased to below the detection limit ($<10^{1.7}$ copies/mL), whereas the CD4+ T cell counts tended to rise up to >500/mm^3. In August 2006, fosamprenavir (FPV) (2400 mg/day) was commenced in place of d4T due to peripheral nerve palsy. Suppression of HIV-RNA below the detection limit continued at the end of follow-up, irrespective of repeated alterations in the therapeutic regimen of HAART. As for HBV status, HBV-DNA declined to $10^{3.9}$ copies/mL in April 2003 but increased again to $>10^{7.6}$ copies/mL in May 2005. To control HBV replication, ETV (0.5 mg/day) was added in October 2006. After the ETV administration, HBV-DNA slightly decreased from $>10^{7.6}$ to $10^{6.2}$ copies/mL in January 2007 but rose to $10^{7.2}$ copies/mL 3 months later. ALT remained abnormal and HBeAg continued to be positive throughout the follow-up period. The clinical course of the patient is summarized in Figure 1a.

For the nucleotide sequencing of HBV-DNA, the serum samples were obtained in December 2001 (before HAART), August 2006 (before ETV administration), and April 2007 (after 6 months of ETV therapy). These serum sampling points were designated as P1, P2 and P3 (see Fig. 1a). Serum samples were stored at −80°C until use. Informed consent was obtained from the patient.

Virus markers and nucleotide sequencing

HBsAg, anti-HBs, HBeAg, anti-HBe and anti-HIV were tested by chemiluminescent immunoassay. A