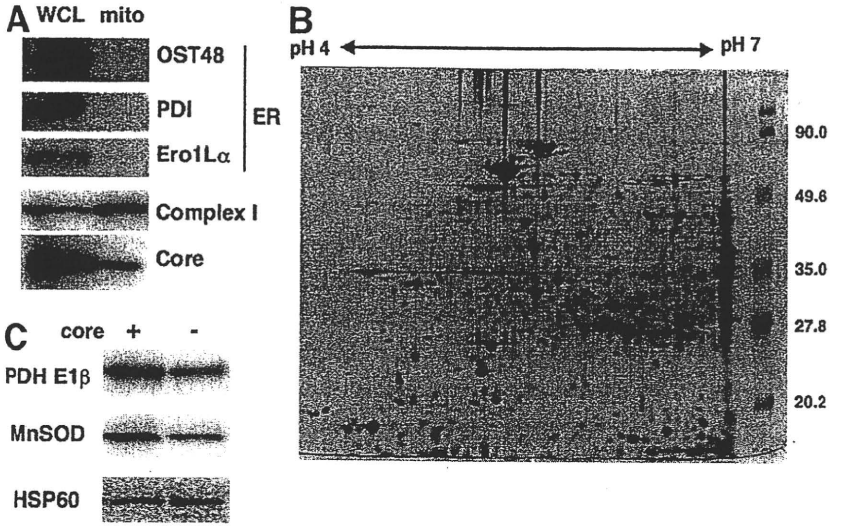

Fig. 1. 2D-PAGE of mitochondria purified from core-expressing cells. (A) Whole-cell lysates (WCL) and purified mitochondria (mito) derived from core-expressing cells were subjected to SDS-PAGE and immunoblotted with anti-core, anti-subunit of complex I (mitochondrial protein), or anti-OST48, PDI, Ero1La (ER proteins) antibodies. (B) Purified mitochondria of core-expressing cells were subjected to 2D-PAGE and the gel was stained with silver. The numbers shown on the right are molecular weights. (C) Purified mitochondria of core-expressing and control cells were subjected to SDS-PAGE and blotted with an anti-E1β subunit of PDH (PDH E1β), anti-MnSOD, or anti-HSP60 antibody.

kyo, Japan). The membrane was subsequently incubated with specific primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies and visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce). Antibodies against the core protein (Anogen, Mississauga, Canada), manganese superoxide dismutase (MnSOD) (BD Biosciences, San Jose, CA), prohibitin (Neomarkers, Fremont, CA), oligosaccharyltransferase-48 (OST48), heat shock protein (HSP) 60 (Santa-Cruz Biotechnology, Santa Cruz, CA), pyruvate dehydrogenase (PDH), ubiquinol-cytochrome c oxidoreductase, COX (Molecular Probes, Eugene, OR), protein disulfide isomerase (PDI), ER protein endoplasmic oxidoreduction-1 (Ero1)-Lα, and IκBα (Cell Signaling Technology, Danvers, MA), were used as primary antibodies. For immunoprecipitation experiments, cells were lysed in NET-N buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) and the lysates were incubated with anti-prohibitin overnight followed by the addition of protein Sepharose 4B (GE Healthcare), then washed with the same buffer five times. Immunoprecipitates were subjected to SDS-PAGE followed by immunoblotting with specific antibodies.

Determination of COX Activity. COX activity was determined with a MitoProfile Rapid Microplate Assay Kit (MitoSciences, Eugene, OR) using 10 μg of purified mitochondria. The assay was performed three times independently.

Statistical Analysis. Results are expressed as means $\pm$ SE. The significance of the difference in means was determined by Student's t test or Mann-Whitney's U test.

Results

Presence of HCV Core Protein in Purified Mitochondria. Increasing evidence suggests that the HCV core protein is localized to mitochondria as well as to ER and the nucleus. Therefore, we first investigated whether the core protein is expressed in the mitochondria of core-expressing (Hep39) cells used in this study. We used Nycodenz discontinuous gradients to extract mitochondria as described.[17] In the mitochondria derived from core-expressing HepG2 cells, the core protein was detected by immunoblotting, whereas ER resident proteins such as an ER-specific type I transmembrane protein OST48, ER-resident molecular chaperon PDI, and ER membrane-associated N-glycoprotein Ero1-Lα, were not (Fig. 1A). In this fraction, reduced nicotinamide adenine dinucleotide (NADH)-ubiquinone oxidoreductase, complex I of mitochondrial oxidative phosphorylation system, was more strongly expressed than that in the whole cell. These results indicate that the purified mitochondria fraction was free of ER, and that a portion of the core protein was localized to the mitochondria in core-expressing cells.

Proteomics Analysis of Mitochondria by 2D-PAGE. For proteomics analysis, purified mitochondrial proteins derived from core-expressing cells were subjected to 2D-PAGE followed by silver-staining of the gel. In this study we analyzed only acidic proteins using IPG strips covering pH 4 to pH 7 because the analysis of acidic proteins by 2D-PAGE is relatively easy. The mitochondrial fraction was also extracted from Hepswx, a control cell line resistant to G418 but does not express the core protein, then similarly subjected to 2D-PAGE and used for comparing the expression pattern. We repeated the above procedure (purification of mitochondria, 2D-PAGE, and silver-staining) five times, and confirmed a similar expression pattern in core-expressing cells. The representative gel image is shown in Fig. 1B. ImageMaster 2D Elite software detected about 1100 spots on the silver-stained acidic gel, i.e., at pH 4-7 and Mrs of 20-100 kDa. The number of

Table 1. Proteins of Differential Expression in Mitochondria of Core-Expressing Cells

Protein Name	Fold Change (Mean ± SD)
Increased	
Succinyl-CoA:ketoacid CoA transferase	10.43 ± 1.29
NADH-specific isocitrate dehydrogenase a subunit precursor	9.64 ± 4.66
Unknown	8.65 ± 2.40
GrpE-like protein co-chaperon	5.71 ± 0.49
Leucine aminopeptidase	4.26 ± 1.14
Pyruvate dehydrogenase E1 component b subunit	3.79 ± 1.34
CGO15alt2	3.18 ± 0.80
HSP70	3.11 ± 1.39
Prohibitin	2.60 ± 0.24
3-Hydroxyisobutyrate dehydrogenase	2.47 ± 0.77
HSPC108	2.46 ± 0.69
MnSOD	2.35 ± 0.65
Ubiquinol-cytochrome c oxidoreductase core I protein	2.00 ± 0.23
Decreased	
Aldehyde dehydrogenase 2	0.12 ± 0.02
Aldehyde dehydrogenase 5 precursor	0.25 ± 0.03
ATP synthase a subunit isoform 1	0.50 ± 0.09
Reference protein	
HSP60	1.02 ± 0.02

protein spots was smaller than those reported in a recent study investigating the human placental mitochondrial proteome.[15]

We then compared the intensity of the spots between core-expressing and control cells. Analysis of repeated experiments by Student's *t* test revealed 13 increased and three decreased spots in intensity in core-expressing cells. These spots were excised and digested with trypsin, then proteins were identified by mass spectrometry. The names of the identified proteins are listed in Table 1. Among them were proteins related to mitochondrial respiratory chain, protein chaperons, and lipid metabolism. Because antibodies to some of these proteins are commercially available, expression levels of the proteins were examined by immunoblotting. The expression levels of the PDH-E1β subunit and MnSOD, which were identified as increased proteins, were higher in core-expressing cells than in control cells (Fig. 1C), whereas that of HSP60, which was identified as having a similar expression, was unchanged.

Up-regulation of Prohibitin by the Core Protein. Among the identified proteins, we focused on prohibitin, an up-regulated protein in mitochondria of core-expressing cells (Fig. 2A). Prohibitin is a mitochondrial protein associated with cell proliferation.[20] It also works as a chaperon of mitochondrial proteins.[21,22] We confirmed an increased prohibitin expression level in core-expressing cells

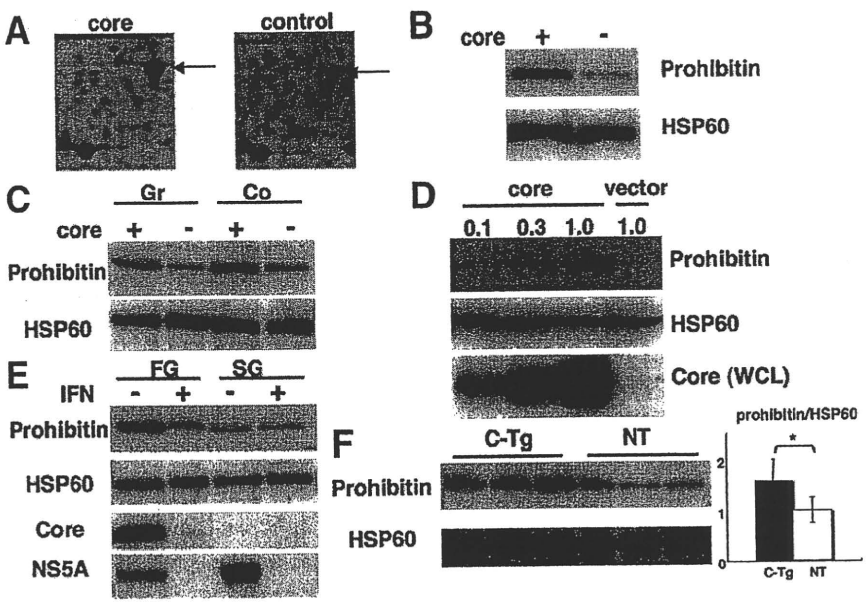

Fig. 2. Up-regulation of prohibitin in core-expressing cells. (A) Protein spot corresponding to prohibitin (arrow) in 2D-PAGE. (B) Purified mitochondria from core-expressing or control cells were subjected to SDS-PAGE and immunoblotted with anti-prohibitin or anti-HSP60 antibody. (C) Mitochondria were purified from growing (Gr) or confluent (Co) cells in 100-mm dishes and subjected to SDS-PAGE, then immunoblotted with an anti-prohibitin or anti-HSP60 antibody. (D) HepG2 cells in six-well plates were transfected with different amounts (μg) of core-expressing plasmid and mitochondrial proteins were analyzed by immunoblotting with anti-prohibitin or anti-HSP60 antibody. The expression levels of the core protein in whole-cell lysates (WCL) were also determined. (E) Cells harboring HCV replicon were untreated or treated with IFN and expression levels of prohibitin in mitochondria were determined. Expression of HCV core and NS5A proteins was also examined. FG, full-genomic replicon cells; SG, subgenomic replicon cells. (F) Expression levels of prohibitin in mitochondria were determined in liver tissues HCV core-gene transgenic and nontransgenic mice. Prohibitin/HSP60 expression levels were determined by densitometry. C-Tg, core-gene transgenic mouse; NT, nontransgenic littermate (n = 3) *$P < 0.05$.

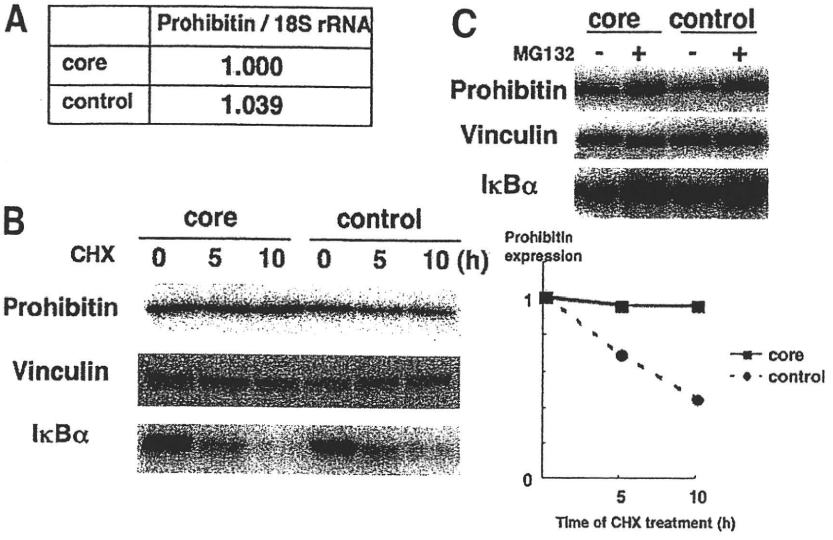

Fig. 3. Increased protein stability of prohibitin in core-expressing cells. (A) RNA was extracted from core-expressing and control cells, and the amount of specific mRNA was determined by real-time PCR with specific primers/probe against prohibitin. The amount of prohibitin mRNA was standardized by that of 18S ribosomal RNA (18S rRNA). (B) Cells were incubated with 100 ng/mL cycloheximide and harvested at the timepoints indicated above the lanes (numbers are hours of cycloheximide treatment). Whole-cell lysates were subjected to SDS-PAGE and immunoblotted with anti-prohibitin, anti-IκBα, or anti-vinculin (as an internal standard) antibody. The intensity of each band was measured by densitometry, and expression levels (prohibitin/vinculin) are shown in the right panel. (C) Cells were harvested after incubation with 20 μM MG132 for 8 hours and subjected to immunoblotting with anti-prohibitin, anti-IκBα, or anti-vinculin antibody.

by immunoblotting (Fig. 2B). Because prohibitin is associated with cell proliferation, it is possible that prohibitin expression changed according to the cell proliferative status. As shown in Fig. 2C, core-expressing cells had high prohibitin expression levels in the cells in both confluent growth and growing statuses compared with control cells. We also determined the expression levels in cells synchronized with aphidicolin followed by l-mimosine treatment and found an increased expression level in core-expressing cells (data not shown). To exclude the possibility that the increased prohibitin expression level is due to the expansion of limited cell clones, not specific to the core protein expression, we examined prohibitin expression in cells transiently expressing the core protein and found that prohibitin expression level increased dose-dependently in core-expressing cells (Fig. 2D). We also examined the prohibitin expression levels in Huh7 cells harboring full- or subgenomic HCV replicon. For this purpose, we used interferon (IFN)-treated replicon cells (cured cells) as a control. Core and nonstructural (NS)5A proteins were not detected after treatment of full-genomic replicon cells with IFN, suggesting a successful elimination of replicon. Prohibitin expression levels in cells with full-genomic replicon were increased compared with those in IFN-treated cured cells, whereas levels of prohibitin expression were low in subgenomic replicon cells regardless of IFN-treatment (Fig. 2E). In addition, prohibitin expression levels were also increased in livers of 3-month-old transgenic mice expressing the core protein compared with those in nontransgenic littermates (Fig. 2F).

We next sought to determine the mechanism of the increased steady-state level of prohibitin in core-expressing cells. To determine prohibitin messenger RNA (mRNA) expression, we performed a real-time polymerase chain reaction (PCR) using specific primers/probe. No difference in prohibitin mRNA was observed between core-expressing and control cells (Fig. 3A). We next determined the stability of prohibitin in these cells. By treating the cells with cycloheximide, the expression levels of prohibitin gradually decreased in control cells (Fig. 3B). On the other hand, in core-expressing cells prohibitin was hardly degraded by cycloheximide treatment for 10 hours, whereas IκBα was equally degraded in both cells. This result suggests that prohibitin was stabilized in the presence of the core protein. Because prohibitin has been shown to be degraded by proteasome,[23] we examined expression levels of prohibitin in the presence of proteasome inhibitor MG132. By treatment with MG132, prohibitin expression was increased to the similar level in core-expressing and control cells. These results suggest that the core protein may inhibit proteasomal degradation of prohibitin by some mechanism, including the prevention of degradation by interaction with the core protein. Then, core-expressing cells were lysed and subjected to immunoprecipitation with an anti-prohibitin antibody. As shown in Fig. 4, the core protein was coimmunoprecipitated with an anti-prohibitin antibody. To exclude a nonspecific interaction with the antibody or Sepharose beads, cells expressing a small amount of prohibitin by transfection with small interfering RNA (siRNA) against prohibitin were also examined. In these cells the amount of the coimmunoprecipitated core protein decreased. In addition, the core protein was not coimmunoprecipitated by control immunoglobulin G (IgG), indicating a specific interaction of prohibitin with the core protein. These results suggest that prohibitin expression increased in core-expressing cells owing to the increased stability presumably by interaction with the core protein.

Impaired Chaperon Function of Prohibitin in Core-Expressing Cells. We next examined the effect of

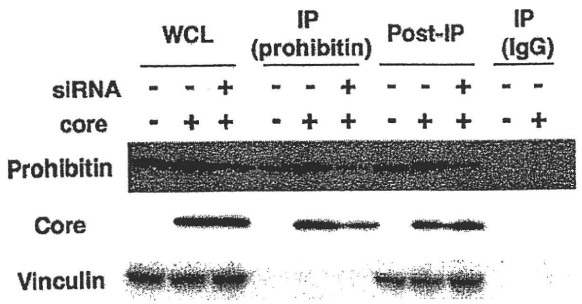

Fig. 4. Interaction of the core protein with prohibitin. Core-expressing and control cells were transfected with or without siRNA against the prohibitin gene, then harvested and lysed in NET-N buffer 3 days after transfection. Whole-cell lysates (WCL) were immunoprecipitated (IP) with an anti-prohibitin antibody or control IgG and immunoblotted with anti-prohibitin or anti-core antibody. Supernatants after the immunoprecipitation were harvested and similarly immunoblotted (Post-IP).

the interaction of prohibitin with the core protein on the function of prohibitin. Prohibitin works as a chaperon of mitochondrial proteins. Nijtmans et al.[21] demonstrated that prohibitin exerts a chaperon function particularly for the stabilization of mitochondrial DNA-encoded proteins. COX is a mitochondrial respiratory complex IV formed by 14 subunits, 10 of which are encoded by nuclear DNA and the rest by mitochondrial DNA.[24] We examined the interaction of prohibitin with subunit II of COX encoded by mitochondrial DNA. As shown in Fig. 5A, the level of COX II coimmunoprecipitated with an anti-prohibitin antibody was decreased in core-expressing cells, although the amount of immunoprecipitated prohibitin was higher than that in control cells. On the other hand, the subunit IV of COX encoded by nuclear DNA was similarly coimmunoprecipitated between core-expressing and control cells. When prohibitin expression was decreased by siRNA transfection, coimmunoprecipitation of COX subunits was similarly decreased with the amount of immunoprecipitation of prohibitin itself being low. We next determined expression levels of COX subunits in the mitochondria in these cells. Expression levels of mitochondrial DNA-encoded subunits I and II in core-expressing cells were decreased, whereas the levels of nuclear DNA-encoded subunits IV and VIb were similar to those in control cells. When transfected with prohibitin-siRNA, expression levels of all of the COX subunits examined were decreased in both core-expressing and control cells, suggesting that protein levels of these subunits are dependent on prohibitin (Fig. 5B, see Supporting Fig. 1 for densitometry). Similar data were observed when blots for COX II and IV were developed together in the same membrane (Supporting Fig. 2). We also determined COX activity in these cells and found that core-expressing cells had a significantly decreased COX activity (about 70% of that in control cells, Fig. 5C). These results suggest that interaction of prohibitin with the core protein is associated with an impaired function of prohibitin as a mitochondrial chaperon, which may trigger disordered assembly and function of mitochondrial respiratory complexes.

Discussion

In the present study we analyzed expression levels of mitochondrial proteins in HepG2 cells expressing the HCV core protein and identified a set of proteins with different expressions. Some of those proteins were related to the mitochondrial respiratory chain (Table 1). Because the core protein was shown to be associated with the induction of oxidative stress,[7-9] the core protein may modulate the expression and function of proteins forming mitochondrial respiratory complexes, which naturally

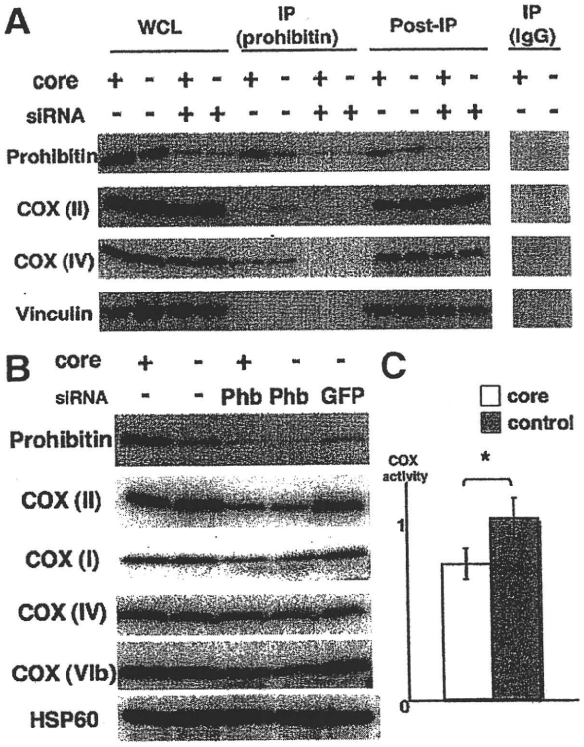

Fig. 5. Effects of core-prohibitin interaction on interaction/expression of COX subunit proteins and COX activity. (A) Whole-cell lysates (WCL) of core-expressing and control cells were subjected to immunoprecipitation with an anti-prohibitin antibody or control IgG, and the interaction of prohibitin with COX subunits was determined by immunoblotting of immunoprecipitated proteins (IP). Supernatants after the immunoprecipitation were harvested and similarly immunoblotted (Post-IP). (B) Cells were transfected with or without siRNA against the prohibitin (Phb) or GFP gene and harvested 3 days after transfection for purification of mitochondria. Purified mitochondria were subjected to SDS-PAGE and immunoblotted with several anti-COX subunits antibodies. The expression levels of HSP60 were also examined as an internal control. (C) COX activity was determined by measuring cytochrome c oxidation. The activity was normalized by taking the average rate of control cells as 1. Data shown are means ± SE (n = 5). $*P < 0.05$.

leads to ROS accumulation. In addition, MnSOD, which plays a key role in protecting cells from oxidative damage, was up-regulated in core-expressing cells, reflecting ROS increase in the cells. Several protein chaperons such as HSP70 and GrpE-like protein co-chaperon were also identified as up-regulated proteins. Because these proteins are known to be important in the mitochondrial protein-import mechanisms, the modulated expression of these proteins may be associated with the different expressions of the identified mitochondrial proteins.

Prohibitin, a mitochondrial protein chaperon, was identified as an up-regulated protein in core-expressing cells. Prohibitin is a ubiquitously expressed and highly conserved protein that was originally determined to play a predominant role in inhibiting cell-cycle progression and cellular proliferation by attenuating DNA synthesis.[20,25] Prohibitin is present in the nucleus and interacts with transcription factors that are important in cell cycle progression. In core-expressing cells used in this study, prohibitin was also detected in the nucleus and its expression level was also higher than that in control Hepswx cells or HepG2 cells (data not shown). The growth rate of core-expressing cells, however, was similar to that of control cells (data not shown). The physiological significance of the high expression level of prohibitin in the nucleus remains to be determined, but it may be related to enhanced apoptosis by Fas ligand, as shown by Ruggieri et al.,[16] because prohibitin interacts with E2F, Rb, and p53 and modulates the transcription activity of these factors and induces apoptosis.[26,27]

Mitochondrial prohibitin acts as a protein chaperon by stabilizing newly synthesized mitochondrial translation products through direct interaction.[21] We examined the interaction between prohibitin and mitochondrially encoded subunit II of COX and found a suppressed interaction between these proteins in core-expressing cells. In addition, there are several studies that showed the association of prohibitin with the assembly of mitochondrial respiratory complex I as well as complex IV (COX).[21,28] Complex I also consists of both nuclear- and mitochondrial-DNA-encoded subunits; therefore, it is probable that the assembly and function of complex I are impaired by the core protein. We attempted to examine the interaction of prohibitin with the mitochondrial DNA-encoded subunit of complex I, but commercially available antibodies against this subunit could not detect the protein itself by immunoblotting (data not shown). With respect to the complex I function, we found a decreased complex I activity in core-expressing cells (H. Miyoshi et al., manuscript in preparation). Other groups have also shown that complex I activity is decreased in the liver of transgenic mice harboring HCV core and envelope genes[9] as well as in cultured cells.[29] From these findings, the interaction between prohibitin and the core protein may impair the function of complex I as well as complex IV, leading to an increase in ROS production. In fact, the suppression of the prohibitin function is shown to result in an increased production of ROS,[30] a phenomenon observed in core-expressing cells used in this study (Miyoshi et al., in prep.) as well as in the liver of core-gene transgenic mice.[7,8] Interestingly, Berger and Yaffe[31] showed that loss of function of prohibitin leads to an altered mitochondrial morphology, that is, the loss of the normal reticular morphology and organized mitochondrial distribution. In hepatocytes from the core-gene transgenic mice, we observed a change in morphology of mitochondria, a disappearance of the double structure of mitochondrial membranes.[2] These changes in mitochondrial morphology are somewhat different, but the dysfunction of prohibitin may be responsible for the morphological abnormality of mitochondria observed in the core-gene transgenic mice.

We concluded that prohibitin overexpression is due to increased stability induced by the interaction with the core protein. In this study we showed that prohibitin might be degraded by proteasome, although we could not detect ubiquitinated forms of prohibitin. If the degradation is mediated by ubiquitin as reported,[23] it is possible that the interaction with the core protein interferes with ubiquitin-binding and protects prohibitin from degradation by proteasome. Some posttranslational protein modifications such as phosphorylation are other possible factors for the stabilization, because prohibitin can be serine-phosphorylated[32]; however, in our examination no serine/threonine/tyrosine phosphorylation of prohibitin was detected in core-expressing cells (data not shown). Thus far, there are no studies showing that prohibitin stabilization leads to a suppressed function as a mitochondrial chaperon. Therefore, this finding is novel and noteworthy because the prohibitin expression level has been considered to be proportional to the chaperon function. Prohibitin is highly expressed in several human tumors.[33,34] In addition, a 2D-PAGE of the hepatoma cell line HCC-M identified prohibitin as a positively regulated protein.[35] In these studies, the mechanism of prohibitin overexpression was not elucidated, but considering that prohibitin is associated with the inhibition of cell proliferation, the function of prohibitin is suppressed by stabilization by some molecules in the tumor, similar to the mechanism we suggest in the current study.

In addition to HepG2 cells constitutively expressing the core protein, increased prohibitin expression levels were also found in livers of core-gene transgenic mice.

The difference in expression levels between the transgenic mice and nontransgenic littermates, however, was a little bit smaller than that in the studies of HepG2 cells. This may be due to the low expression level of the core protein in the transgenic mice compared with that in core-expressing HepG2 cells because the expression level of prohibitin was proportionally increased to that of the core protein as shown in this study (Fig. 2D). Otherwise, there might be some *in vivo* mechanism for suppressing prohibitin expression in mice.

In this study, COX subunit IV as well as II were found to interact with prohibitin (Fig. 5A). Although there are no studies demonstrating that prohibitin also works as chaperon for nuclear DNA-encoded mitochondrial proteins as far as we investigated, knockdown of prohibitin expression by siRNA led to decreases in expression levels of both nuclear (COX IV, VIb) and mitochondrial (COX I, II) DNA-encoded subunits in mitochondria (Fig. 5B and Supporting Figs. 1 and 2). We showed that COX IV interacts with prohibitin (Fig. 4), suggesting that prohibitin also works for stable expression of nuclear DNA-encoded COX IV. Degrees of decrease in COX IV and VIb expression, however, were smaller than those in I and II. Prohibitin might contribute to stabilization of COX IV and VIb by mechanism(s) other than chaperon function. Steglich et al.[36] showed that prohibitin regulates protein degradation by the m-AAA protease in mitochondria. Recently, Da Cruz et al.[37] showed that SLP-2, a member of the stomatin gene family, interacts with prohibitin and regulates the expression of mitochondrial proteins such as COX IV and ND6 of complex I encoded by nuclear DNA by AAA proteases. In view of these findings, COX IV and VIb expression in mitochondria is dependent on prohibitin but other factors may also be involved in the attainment of stable expression of these subunits. The expression levels of COX II and IV in the whole-cell lysates were not so drastic among cell samples (Fig. 5A) compared to those in the mitochondria (Fig. 5B). The reason is not clear, but it is possible that redundant proteins such as improperly folded proteins by lack of chaperons were included in the whole-cell lysates.

In summary, we analyzed mitochondrial proteins in core-expressing HepG2 cells by proteomics analysis and identified prohibitin as an up-regulated protein. The dysfunction of prohibitin induced by the core protein may lead to ROS overproduction in the mitochondrion, which plays a key role in the pathogenesis of chronic hepatitis C. The restoration of prohibitin function might be a therapeutic option for correcting the dysregulated assembly and dysfunction of mitochondrial respiratory chain complexes.

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Evaluation of Hepatitis C Virus Core Antigen Assays in Detecting Recombinant Viral Antigens of Various Genotypes▽

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A single substitution within the hepatitis C virus core antigen sequence, A48T, which is observed in ~30% of individuals infected with genotype 2a virus, reduces the sensitivity of a commonly used chemiluminescence enzyme immunoassay. Quantitation of the antigen is improved by using a distinct anticore antibody with a different epitope.

Hepatitis C virus (HCV) is a major cause of chronic liver disease throughout the world. Accurate diagnosis of HCV infection is important due to the morbidity associated with the virus, and determining the level of viral replication is important in predicting and monitoring the effect of antiviral treatment. Although quantifying viral RNA represents the standard method for identifying active infection (5, 8, 13), several sensitive immunoassays that detect the viral core antigen (Ag) have now been developed as an alternative to HCV RNA testing (3, 4, 6, 9, 10, 12, 16). The amino acid sequence of the core Ag is largely conserved among different viral isolates (14); however, genetic variability of the virus constitutes one of the major challenges to using core Ag assays for diagnosis. In this study, we examined the effects of sequence heterogeneity on the sensitivity of diagnostic kits for detection of the core Ag by using recombinant Ag derived from each of the major HCV genotypes. Expression plasmids for epitope-tagged core Ag were generated by inserting cDNA for the full-length core region of genotype 1a (17; GenBank accession no. AF011751), 1b (1; D89815), 2a (7; AB047639), 2b (AB030907), or 3a virus, with a FLAG tag sequence attached at its 5′ end, into the EcoRI site of the pCAG mammalian expression vector (11). HEK293T cells transiently transfected with the expression plasmids were harvested 48 h after transfection using a passive lysis buffer (Promega, Madison, WI). Centrifugation was performed to remove the debris after ultrasonication. Total protein was quantified in aliquots of cell lysate by using the bicinchoninic acid method (Pierce, Rockford, IL) and then used for determining the concentrations of HCV core Ag.

Figure 1A shows comparable levels of core Ag in each sample of cell lysate, as determined by immunoblotting with anti-FLAG antibody (Ab). The ability of HCV core Ag assays to detect five different HCV genotypes were compared using a commercially available chemiluminescence enzyme immunoassay (CLEIA) (Lumipulse II HCV core assay [assay detection range, approximately 50 to 50,000 fmol/liter]; Fujirebio, Japan) (15) and enzyme-linked immunosorbent assay (ELISA) (Ortho HCV Ag ELISA test [assay detection range, approximately 44.4 to 3,600 fmol/liter]; Ortho-Clinical Diagnostics, Japan) (2) to detect HCV core Ag in cell lysate. As shown in Fig. 1B,

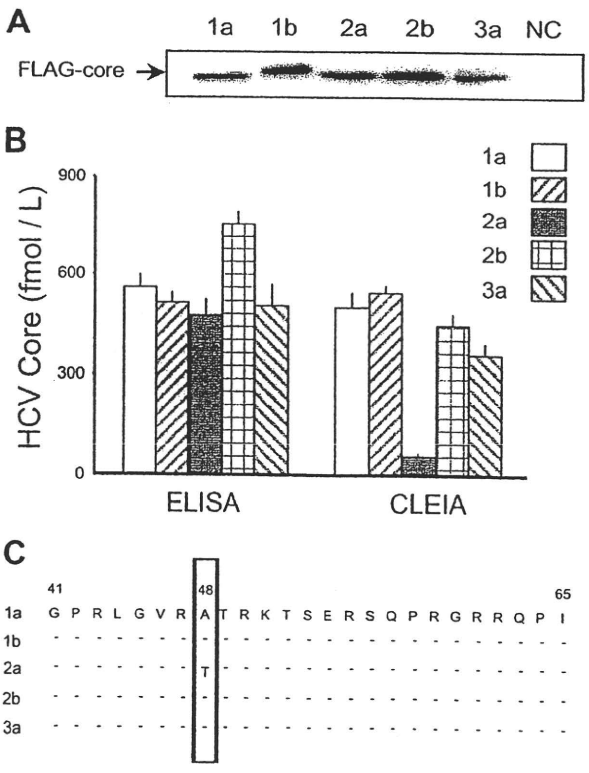

FIG. 1. Detection of recombinant HCV core Ag derived from genotype 1a, 1b, 2a, 2b, and 3a isolates by immunoblotting using an anti-FLAG Ab (A) as well as ELISA and CLEIA (B). The data shown in panel B represent the mean values and standard deviations (n = 3). NC, negative control. (C) The amino acid sequence from amino acids 41 to 65 of the core Ag used in this study. Key residues at the 48th position are boxed. Hyphens indicate conservation.

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TABLE 1. Comparison of the 48th residues of HCV core Ags of genotypes 1a, 1b, 2a, 2b, and 3a

Genotype	No. of isolates	No. (%) of isolates with residue at 48th position		
		T	A	Other
1a	263	9 (3.5)	254 (96.5)	0 (0)
1b	298	2 (0.7)	294 (98.6)	2 (0.7)
2a	17	5 (29.5)	12 (70.5)	0 (0)
2b	17	0 (0)	17 (100)	0 (0)
3a	23	0 (0)	23 (100)	0 (0)
Total	618	16 (2.6)	600 (97.1)	2 (0.3)

although the ELISA measured similar concentrations of core Ag in all samples, apparent low levels of the genotype 2a core Ag, originally from an isolate known as the JFH-1 isolate (7), were detected using the CLEIA method, suggesting that some differences in the amino acid sequences corresponding to particular HCV genotypes or isolates may influence the sensitivity of core Ag detection. A comparison of the core Ag sequences, including the monoclonal Ab epitopes used in the development of CLEIA, revealed conservation of alanine at the 48th position in four clones, of genotypes 1a, 1b, 2b, and 3a, but not genotype 2a, for which there is a threonine at this position (Fig. 1C). Based on our analysis of sequences available from the HCV database (http://hcv.lanl.gov/content/sequence/NEWALIGN/align.html), alanine is highly conserved at the 48th residue of the core Ag for HCV isolates of genotypes 1a, 1b, 2b, and 3a (Table 1). In contrast, alanine and threonine occur in this position in 70.5% and 29.5%, respectively, of genotype 2a isolates. To examine whether the low sensitivity of the CLEIA method might be due to this particular amino acid change, we next replaced threonine with alanine at the 48th position of the JFH-1 core Ag (for

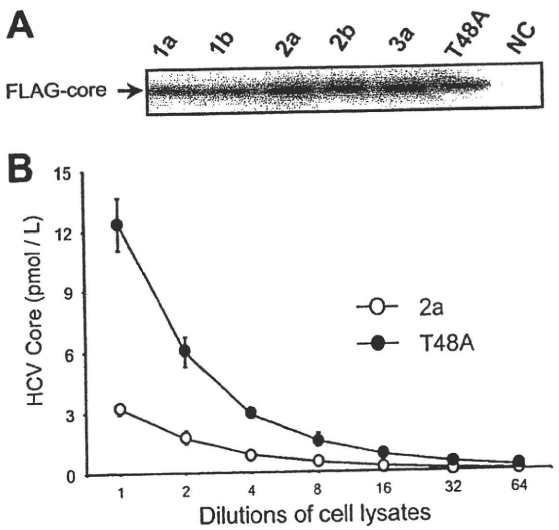

FIG. 2. Effect of T48A substitution in the core Ag of the JFH-1 isolate with regard to sensitivity of the CLEIA method. Samples of wild-type or mutated core Ag cell lysate were analyzed by immunoblotting (A) and CLEIA (B). The data shown in panel B represent the mean values and standard deviations (n = 3). NC, negative control.

TABLE 2. Comparison of the modified CLEIA with the original version for detection of the core Ags of genotypes 1a, 1b, 2a, 2b, and 3a[a]

Genotype	CLEIA	HCV core antigen concn (fmol/liter) in serially diluted cell lysates at indicated fold dilution							
		1	2	4	8	16	32	64	128
1a	Original	11,147	5,527	2,611	1,484	691	403	195	101
	Modified	10,511	5,700	2,676	1,420	716	444	200	111
1b	Original	11,612	5,618	3,081	1,551	779	409	223	113
	Modified	11,192	6,028	2,824	1,522	804	431	197	101
2a	Original	3,216	1,710	844	480	232	104	48	36
	Modified	12,101	6,255	3,153	1,676	805	422	212	106
2b	Original	10,559	5,635	2,811	1,286	762	387	194	94
	Modified	10,977	6,179	3,381	1,624	842	437	219	129
3a	Original	11,478	5,891	2,922	1,414	756	422	212	112
	Modified	11,208	6,225	3,126	1,555	791	445	215	100

[a] Data represent the mean values in triplicate measurements.

the mutant JFH-1coreT48A) and measured the HCV core Ag concentration in cells expressing both mutated and wild-type JFH-1 core Ag. After confirming comparable levels of FLAG-tagged core Ag in the cell lysate samples by immunoblotting (Fig. 2A), HCV core Ag was quantified in the samples by serial dilution via the CLEIA method. As shown in Fig. 2B, the core Ag concentrations of JFH-1coreT48A were assessed to be 3.2- to 3.8-fold higher than those of the wild-type core Ag, suggesting that the sensitivity of HCV core Ag detection may have been affected by the 48th residue in the core Ag. Data for samples derived from genotypes 1a, 1b, 2b, and 3a were analogous to data for JFH-1coreT48A (data not shown). Although HCV isolates with threonine at the 48th position of the core Ag sequence comprise a relatively small proportion of the major genotype population, only 2.6% of the genotype 1a, 1b, 2a, 2b, and 3a isolates here (16 of 618 isolates; Table 1), attempts to overcome this problem would improve the overall sensitivity and usefulness of the assay. To achieve this aim, another monoclonal anticore Ab, whose epitope is comprised of amino acids 50 to 65, which are completely conserved among all the genotypes examined (Fig. 1C), was therefore used as a second Ab in a modified version of the CLEIA. We compared this modified assay with the original version by measurement of core Ag concentrations of the various genotypes (Fig. 2A) as illustrated in Table 2. The modified assay was able to quantify core Ag from genotypes 1a, 1b, 2a, 2b, and 3a with no significant differences observed between Ag levels in samples from different genotypes at each dilution.

It has been demonstrated that the HCV core Ag assay is a useful alternative to HCV RNA quantification for the diagnosis of hepatitis C and for monitoring the antiviral effects of treatment. Compared to various reverse transcription-PCR methods, HCV core assays are less expensive and easier to perform, without the requirement of sophisticated laboratory equipment and specially trained laboratory personnel. In addition, the core Ag assay can be used to measure a more diverse set of blood samples, such as sera stored for a long period of time, because the viral Ag is generally more stable than the RNA in sera or plasma. Despite the adequate performance of core Ag assays, we have shown that a single amino acid substitution at the 48th position of the core Ag changes the detection sensitivity. It is also noted that, although the original CLEIA should be improved, the ELISA used in this study may be substituted for it.

In conclusion, we have identified a distinct anticore Ab with a different epitope that might enable improved detection across all of the major HCV isolates. The findings of this study would provide useful information for the development of an improved assay with greater accuracy.

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Identification of hepatitis C virus genotype 2a replicon variants with reduced susceptibility to ribavirin

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ABSTRACT

Ribavirin (RBV), a nucleoside analogue, is used in the treatment of hepatitis C virus (HCV) infection in combination with interferons. However, potential mechanisms of RBV resistance during HCV replication remain poorly understood. Serial passage of cells harboring HCV genotype 2a replicon in the presence of RBV resulted in the reduced susceptibility of the replicon to RBV. Transfection of fresh cells with RNA from RBV-resistant replicon cells demonstrated that the RBV resistance observed is largely replicon-derived. Four major amino acid substitutions: T1134S in NS3, P1969S in NS4B, V2405A in NS5A, and Y2471H in NS5B region, were identified. Site-directed mutagenesis of these mutations into the replicon indicated that Y2471H plays a role in the reduced susceptibility to RBV and leads to decrease in replication fitness. The results, in addition to analysis of sequence database, suggest that HCV variants with reduced susceptibility to RBV identified are preferential to genotype 2a.

1. Introduction

Hepatitis C virus (HCV) is a leading cause of chronic liver diseases, such as chronic hepatitis, cirrhosis and hepatocellular carcinoma, affecting approximately 170 million people worldwide (WHO, 2000). HCV belongs to the genus Hepacivirus of the family Flaviviridae, and its genome is a single-stranded, positive-sense RNA of 9.6 kb. HCV displays marked genetic heterogeneity and is currently classified into 6 major genotypes and more than 50 subtypes. HCV genotypes have regional distribution and, of those, genotypes 1 and 2 are detected worldwide (Simmonds et al., 2000). Current standard therapy for chronic hepatitis C consists of the combination of pegylated interferon alpha (IFN-α) in combination with ribavirin (RBV). However, approximately 50% of treated patients infected with genotype 1 do not respond or show only a partial or transient response and treatment is limited by the adverse effects of both agents (Manns et al., 2001; Fried et al., 2002).

HCV replication is associated with a high rate of mutation that gives rise to a mixed and changing population of mutants, known as quasispecies (Martell et al., 1992; Domingo, 1996). The characteristic of HCV may have important implications concerning viral persistence, pathogenicity and resistance to antiviral agents (Domingo, 1996; Forns et al., 1999; Farci and Purcell, 2000). Most previous studies on the possible relationship between HCV quasispecies and response to chemotherapy have been carried out in HCV genotype 1 patients. In addition, several studies have successfully demonstrated that the HCV subgenomic replicon is derived from genotype 1, which typically contains HCV nonstructural genes placed downstream of the neomycin phosphotransferase gene, in selecting variants resistant to antiviral inhibitors. Two studies have demonstrated the identification of HCV genotype 1 mutants responsible for decreased sensitivity to RBV (Young et al., 2003; Pfeiffer and Kirkegaard, 2005). However, little is known about the generation of genotype 2 isolates resistant to antivirals including RBV, or the molecular mechanisms that confer resistance.

In this study, we report the generation and characterization of HCV genotype 2a replicon variants with reduced susceptibility to RBV. The impacts of major amino acid substitutions observed on RBV susceptibility and viral replication capacity were also examined.

2. Materials and methods

2.1. Compounds

RBV and IFN-α were purchased from MP Biomedicals (Eschwege, Germany) and Dainippon Sumitomo Pharma (Osaka, Japan), respectively.

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Table 1
Primers used for PCR and nucleotide sequencing.

Region	Primer name	Nucleotide sequence	Position[a]	Polarity
NS3–4A–4B region	PCR primers			
	JF1S	GAAAAACACGATGATACCATG	1756–1776	Sense
	JF1AS	AACCCAGTCCCACACGTC	4650–4633	Antisense
	Sequencing primers			
	JF5S	CACTTTCAGTGACAACAGCA	2322–2341	Sense
	JF6S	CGCCACCGACGCCCTCATGA	3003–3022	Sense
	JF4AS	CTGGTCGACAACGGACTGGT	4109–4090	Antisense
NS5A–NS5B region	PCR primers			
	JF2S	TGCTCCGGATCCTGGCTC	4612–4629	Sense
	JF2AS	TACCTAGTGTGTGCCGCTCTA	7786–7806	Antisense
	Sequencing primers			
	JF3S	TGAGGTCCATGCTAACAGA	5209–5228	Sense
	JF4S	TCGAGGGGGAGCCTGGAGAT	5870–5889	Sense
	JF3AS	GAGTGTCTAACTGTTTCCCAG	7220–7200	Antisense

[a] Reference strain: Gene Bank accession no. AB114136.

2.2. Cell culture

The human hepatoma cell line Huh-7 was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with MEM non-essential amino acids (Invitrogen) 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (FBS) at 37 °C in a 5% CO_2 incubator. HCV replicon cells JFH-1/4-1 (Miyamoto et al., 2006), which are Huh-7-derived cells carrying a subgenomic replicon of JFH-1 (Kato et al., 2003) were maintained in the Huh-7 medium as above, supplemented with 1 mg/ml G418 (Nacalai Tesque, Kyoto, Japan).

2.3. Quantification of HCV RNA

Total RNA was isolated from harvested cells using Trizol (Invitrogen). Copy numbers of the viral RNA were determined by real-time RT-PCR involving single-tube reactions and performed using TaqMan EZ RT-PCR Core Reagents (PE Applied Biosystems, Foster City, CA, USA), as described previously (Aizaki et al., 2003; Takeuchi et al., 1999).

2.4. Cell viability assay

Cells were seeded at density of 5×10^4 cells/well in 24-well plates and RBV at various concentrations was added on the next day. Cultures were further incubated for 3 days at 37 °C under a humidified 5% CO_2 atmosphere. Cytotoxicity assay was performed by Cell Titer-GLO™ Luminescent Cell Viability Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Luciferase activities were quantified with LUMAT LB 9501 (Berthold Technologies, Bad Wilbad, Germany).

2.5. Isolation and nucleotide sequencing of HCV nonstructural regions from replicon-containing cells

Total cellular RNA was isolated from replicon cells with or without RBV treatment as described above. cDNA synthesis was carried out by using Super ScriptTM III First-Strand Synthesis System for RT-PCR (Invitrogen) with primer JF1AS for NS34AB region and JF2AS for NS5AB region. Two cDNA fragments, corresponding to NS3–NS4B and NS5A–NS5B regions, were amplified by PCR using Takara EX Taq DNA polymerase (Takara BIO, Kyoto, Japan) and specific primers (Table 1; Date et al., 2004). PCR products were subcloned into pGEM-T vector (Promega) and inserts were sequenced using QIA prep^R Spin Mini Prep kit (QIAGEN, Tokyo, Japan). Nucleotide sequences were analyzed with the 3100 Avant Genetic Analyzer (PE Applied Biosystems).

2.6. Plasmid constructions

pSGR-JFH1/luc, a subgenomic replicon construct with luciferase reporter derived from HCV genotype 2a JFH-1 isolate was reported previously (Miyamoto et al., 2006). Mutant replicons carrying T1134S, P1969S, V2405A, and Y2471H were created by PCR-based site-directed mutagenesis and cDNA fragments containing the above mutations were inserted into the corresponding sites of pSGR-JFH/luc. All plasmids were confirmed by sequencing the entire PCR-generated inserts. Each mutant is referred to by the original amino acid (one letter code) followed by the residue positions within the complete open reading frame of full-length JFH-1 and the substituted amino acid (one letter code).

2.7. RNA synthesis and transient replication assay

The transient replication assay method was described previously (Kato et al., 2005). Briefly, purified plasmids of pSGR-JFH1/Luc, -JFH1/Luc-T1134S, -JFH/Luc-P1969S, -JFH/Luc-V2405A and -JFH/Luc-Y2471H were linearized with XbaI and were treated with proteinase K and SDS, followed by phenol–chloroform extraction. RNA was synthesized with Ampliscribe™ T7 Transcription Kits (Epicentre BIO Technologies, Madison, WI, USA). Each transcribed RNA (5 μg) was electroporated into 2.5×10^6 of Huh7 cells pulsed at 290 mV, 975uFD with Gene pulser II apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Transfected cells were resuspended in growth medium without selection antibiotics and were plated in 24-well plates at 6×10^4 cells per well. Cells were harvested at different time points post-transfection and were lysed in Passive Lysis Buffer (Promega). Luciferase activity in cells was determined using the Luciferase Assay System (Promega).

3. Results

3.1. Selection of replicon variants derived from genotype 2a with reduced susceptibility to RBV

It has been reported that RBV inhibits HCV RNA replication in Huh-7 cells bearing the viral subgenomic replicon RNAs with the EC_{50} (50% effective concentration) values of 15–225 μM (Zhou et al., 2003; Tanaka et al., 2004; Kato et al., 2005; aus dem Siepen et al., 2007). To select for RBV-associated replicon variants, cells bearing a genotype 2a HCV replicon were serially passed in the presence of 200 μM RBV as well as 1 mg/ml G418. After 20-week treatment, variant cells were then tested for RBV resistance. HCV RNA levels were determined after a 72-h incubation with various concentrations of RBV in the absence of G418, and about 5-fold-reduced susceptibility to RBV was observed in the variant replicon

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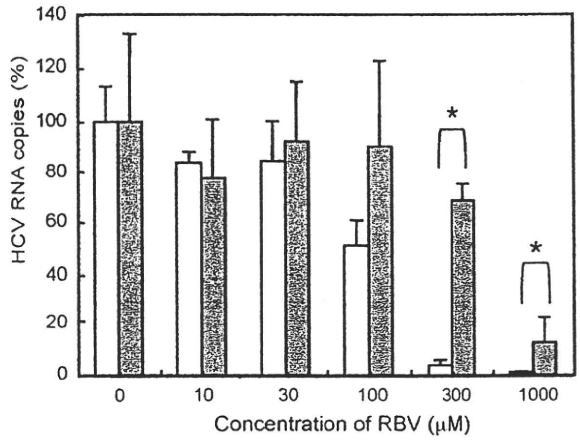

Fig. 1. Inhibitory effect of RBV on HCV RNA levels in genotype 2a replicon cells after long-term treatments with RBV. The replicon cells were serially passaged in 0 or 200 μM RBV for 20 weeks. The cells were then split and incubated with fresh RBV at various concentrations in the absence of G418 for 3 days, followed by the determination of HCV RNA. Clear bars, passage in the absence of RBV: gray bars, passage in the presence of RBV. HCV RNA copies per microgram of total RNA were normalized as percentages of those of untreated (RBV 0 μM). Each data point is presented as the mean of three independent determinations with standard deviation. $^*p < 0.05$.

cells; the EC_{50} values for the variant and wild-type replicon cells were 470 and 102 μM, respectively (Fig. 1). Comparable cytotoxic effects of RBV were observed against wild-type and variant replicon cells, with the CC_{50} (50% cytotoxicity concentration) values of 151 and 156 μM, respectively (data not shown).

3.2. Mapping RBV resistance to cell line or replicon RNA

To test whether reduced susceptibility to RBV in the variant cells observed as above was due to the appearance of mutations within the viral RNA or was cell-derived, total RNAs from the variant and wild-type replicon cells were extracted and used for retransfection of naïve Huh7 cells. Retransfected cells resistant to G418 were established after 4 weeks of cultures in the presence of 1 mg/ml G418 and were assessed for HCV RNA replication sensitivity to RBV (Fig. 2A). HCV RNA levels in the cells obtained from the wild-type replicon were inhibited by 56, 89 and 97% with 100, 300 and 1000 μM RBV, respectively. By contrast, the culture retransfected with RNA derived from the variant replicon cells exhibited inhibition levels of 13, 29 and 89% with the corresponding concentrations of RBV. EC_{50} values were calculated to be 93 and 449 μM, respectively. We confirmed the presence of replicon mutations, as described below, in the cells retransfected with RNA derived from the variant replicon cells.

In order to explore the possibility for cell-derived resistance, both wild-type and variant replicon cells were cured of viral RNAs by IFN treatment; cells were passaged with media containing 100 IU/mL IFN-α in the absence of G418 for 2 months. To compare RBV sensitivity, cured cells were transiently transfected with the wild-type JFH-1 subgenomic replicon RNA and were treated with various concentrations of RBV for 72 h. Similar anti-HCV effects of RBV were observed in the cured cells derived from wild-type and variant replicons, with the EC_{50} values of 147 and 118 μM, respectively (Fig. 2B). Thus, the results suggest that the RBV resistance observed may arise by mutations in the replicon rather than by changes in the cells.

3.3. HCV mutations in replicon variant with reduced susceptibility to RBV

It has been reported that mutations in RNA virus genomes responsible for RBV resistance are mostly present in the coding region for the viral RNA-dependent RNA polymerase (RdRp). On the other hand, it is known that RBV works as an RNA mutagen to generate rapidly mutating viral RNA and that NS5B RdRp and other nonstructural proteins in HCV are involved in the viral replication complex, playing key roles in genome replication. Therefore, we sequenced the coding regions for NS3 through NS5B proteins of the replicon molecules in order to determine whether mutations associated with RBV resistance were generated. As shown in Table 2, there were numerically more synonymous and non-synonymous mutations in the RBV-resistant variant replicon cells (RBV treatment) when compared with untreated replicative conditions (No-treatment) across most regions examined. Mutation frequencies of NS3, NS4B and NS5A regions of RBV treatment were significantly higher than those of No-treatment. The total number of synonymous mutations in the RBV-resistant variant replicon cells was 3 times higher than that under untreated replicative conditions, and the number of non-synonymous mutations in the RBV-resistant variant replicon cells was 1.5 times higher than that under untreated replicative conditions. The number of both synonymous and non-synonymous mutations (NS3, NS4B, NS5A and NS5B regions) in the RBV-resistant replicon cells was greater than that in the control cells. We also found a large number of transition

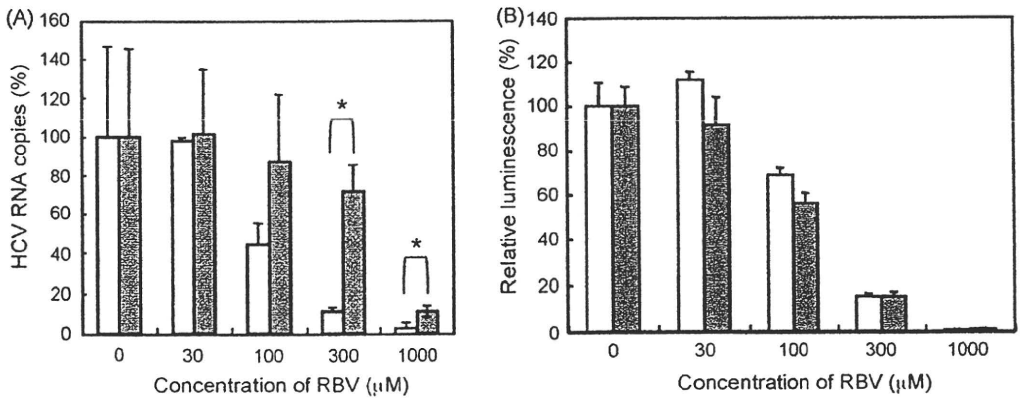

Fig. 2. Testing for replicon-derived resistance (A) or for cell-derived resistance (B). (A) Total RNA from RBV-resistant- or wild-type replicon cells was transfected into naïve Huh7 cells. After selection in 1 mg/ml G418 for 4 weeks, re-established replicon cells, wild-type derived (clear bars) and RBV resistance derived (gray bars), were treated with increasing concentrations of RBV in the absence of G418 for 3 days. HCV RNA copies per microgram total RNA were assessed and the levels from wild-type cells without RBV treatment were set at 100%. Data are indicated as means with standard deviations. $^*p < 0.05$. (B) RBV-resistant- or wild-type replicon cells were cured by passage in IFN-α in the absence of G418. Cured cells were transiently transfected with the replicon RNA derived from pSGR-JFH1/luc. Transient replication assay of transfectants derived from wild-type (clear bars) and RBV resistance (gray bars) was performed after treatment with various concentrations of RBV for 72 h. The values for wild-type-derived cells without RBV treatment were set at 100%. Data are indicated as means with standard deviations.

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Table 2
Mutation frequencies in HCV NS regions after 20-weeks culture with or without RBV treatment.

Region	nt length	No-treatment			RBV treatment		
		No. of non-synonymous mutations[a]	No. of synonymous mutations[a]	Mutation frequency (10^{-3})	No. of non-synonymous mutations[a]	No. of synonymous mutations[a]	Mutation frequency (10^{-3})
NS3	1893	1.7 ± 2.1	2.3 ± 1.5	2.1	4.7 ± 2.4	6.5 ± 2.5	5.9[b]
NS4A	165	1.0 ± 1.0	0.3 ± 0.6	8.1	0.3 ± 0.5	0.5 ± 0.9	4.4
NS4B	780	1.3 ± 1.2	0.3 ± 0.6	2.1	2.3 ± 1.5	2.5 ± 1.2	4.7[c]
NS5A	1380	4.0 ± 1.2	2.0 ± 1.2	4.3	5.9 ± 1.2	6.2 ± 2.4	12.2[c]
NS5B	1773	4.5 ± 1.5	2.3 ± 1.5	3.8	4.8 ± 1.8	4.2 ± 1.1	9.0
NS3–NS5B	5991	12.5 ± 2.7	7.3 ± 2.7	–	17.8 ± 4.5	20.1 ± 4.6	–

[a] Values are means ± standard deviations.
[b] $p < 0.05$ relative to No-treatment by the unpaired t-test.
[c] $p < 0.01$ relative to No-treatment by the unpaired t-test.

mutations in RBV-resistant cells, particularly G-to-A and C-to-U transitions, as expected from previous studies. Although mutations were distributed throughout nonstructural regions, four major amino acid substitutions; T1134S in the NS3 region, P1969S in NS4B, V2405A in NS5A, and Y2471H in NS5B, not seen in wild-type cells were observed in most of the subclones among RBV-resistant replicon cells. T1134S, P1969S, V2405A, and Y2471H were present, respectively, in 7 of 11, 6 of 11, 8 of 13, and 7 of 13 PCR subclones sequenced.

3.4. Effects of T1134S, P1969S, V2405A, and Y2471H on RBV susceptibility

To test the possibility that any of the four mutations as identified confer resistance to RBV, we introduced these mutations individually into the JFH-1 subgenomic replicon containing a luciferase reporter gene. Cells transfected with mutant- or wild-type replicon RNA grown in the presence of various concentrations of RBV for 2 or 3 days. As demonstrated in Fig. 3A, the replication levels of all four mutant replicons (SGR-JFH1/Luc-T1134S, -P1969S, -V2405A, and -Y2471H) in the presence of 125 or 500 μM RBV were higher than those of the wild-type replicon. In particular, the Y2471H mutant significantly reduced susceptibility to RBV; replication levels of SGR-JFH1/Luc-Y2471H were 3–5-fold higher when compared to those of wild-type under the present assay conditions.

The relative replication activity of these mutant replicons was further determined in 3-day replication assay without drug treatment (Fig. 3B). All mutant replicons exhibited reduced efficiency relative to the wild-type replicon. Levels of the Y2471H-mutated replicon were approximately 30% of those of the wild-type, thus suggesting that replicon mutants with reduced sensitivity to RBV are associated with decreased replication fitness.

4. Discussion

It is generally accepted that, during chemotherapy against viral infection, high rates of viral replication and high frequencies of mutation lead to generation of drug-resistant mutants. Although several potential mechanisms for the inhibition of HCV replication by RBV have been proposed, the molecular mechanisms involved in the generation of RBV-resistant HCV remain poorly understood.

This study found that long-term treatment of HCV JFH-1-derived replicon cells with RBV leads to selection of preferential mutations in NS3 (T1134S), NS4B (P1969S), NS5A (V2405A) and NS5B (Y2471H) genes. Each mutation only required a single nucleotide change, and P1969S, V2405A and Y2471H are transition mutations, which are known to be commonly caused by incorporated RBV. Site-directed mutagenesis of these mutations into the replicon demonstrated that Y2471H plays a role in reduced susceptibility to RBV.

Crystal structure information revealed that HCV RdRp is organized into an arrangement with palm, fingers, and thumb subdomains (Lesburg et al., 1999). Residue 2471 (the 33rd position of NS5B) is present in the N-terminal loop region that bridges the fingers. Although this site is apparently distant from the active site of the polymerase in the palm region, it has been reported

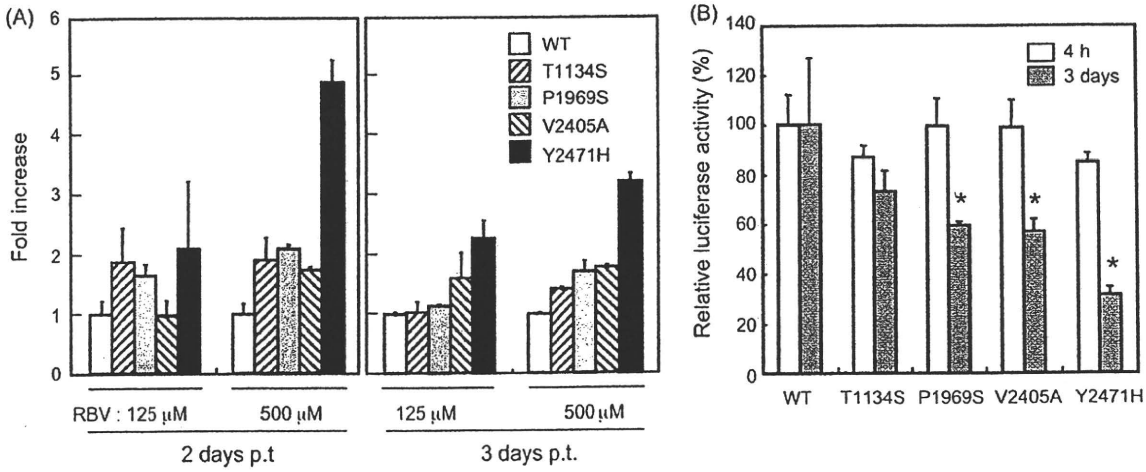

Fig. 3. Impact of major mutations in NS3–NS5B regions on RBV susceptibility (A) and replication capacity (B). Mutated replicons carrying single residue substitutions (T1134S, P1969S, V2405A, and Y2471H) were constructed and used for transient replication assay. Cells were transfected with either wild-type (WT) or with mutant replicon RNA in the absence or presence (125, 500 μM) of RBV. Luciferase activity was assessed at 4 h, 2 days and 3 days post-transfection (p.t.). (A) Luciferase activities of WT were set at 1, and the fold increases in the activities of mutants were plotted. (B) Luciferase activities in the absence of RBV at 4 h and 3 days post-transfection were shown. The activities of mutants were normalized as percentages of the WT activities. Data from triplicate samples were averaged and indicated with standard deviations. *$p < 0.05$ against WT.

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that small molecules, such as benzimidazole compounds, are able to specifically bind the fingers-thumb interface and inhibit polymerase activity (Herlihy et al., 2008), thus suggesting that amino acid substitutions in the loop region may affect RNA polymerization. The involvement of tyrosine residue at position 415 of HCV NS5B in RBV resistance has been previously described for patients with genotype 1a infection and for the genotype 1b replicon (Young et al., 2003). Although the mechanism for resistance remains elusive, it has been hypothesized that RBV interacts with RdRp around this residue, which is located in the thumb subdomain, thus affecting RNA polymerization (Young et al., 2003).

Based on analysis of available sequences from Genbank, tyrosine at the 33rd residue of NS5B is conserved in all isolates of genotype 2a, but not in other genotypes. In genotype 1a and 1b isolates, 96% contain histidine and only a small population contains tyrosine or asparagine at the site. All the isolates of genotypes 3, 4, 5 and 6 contain histidine, whereas phenylalanine is conserved for genotype 2b. It should be noted that V2405 and P1969 are also completely conserved for genotype 2a but not for other genotypes. Therefore, it is likely that the identified HCV variants with reduced susceptibility to RBV are genotype-specific. It will be of interest to determine whether HCV genotype 2a is intrinsically more sensitive to RBV when compared with other genotypes.

At present, at least 4 mechanisms of action of RBV are proposed (Lau et al., 2002). They include (1) direct inhibition of the HCV replication machinery, (2) as an RNA mutagen that drives a rapidly mutating RNA virus over the threshold to "error catastrophe", (3) inhibition of the host enzyme inosine monophosphate dehydrogenase (IMPDH), and (4) enhancement of host T-cell-mediated immunity against viral infection. In addition to the direct inhibition, it is also possible that other mechanisms such as error-prone and IMPDH-inhibition are involved in HCV escape from RBV treatment. Further investigation of the interaction of HCV variants with the viral and cellular factors involved in viral resistance may improve understanding of the mechanism(s) of RBV resistance.

In conclusion, RBV encountered resistance from the HCV genotype 2a replicon largely mediated by mutations in the N-terminal region of NS5B. Although whether these mutagenic effects are also demonstrable in IFN-RBV combination therapy will require further studies, the mutations identified in this study represent the first drug-resistant variants belonging to HCV genotype 2a. The drug resistance patterns found in this study may be of benefit in prediction in vivo resistance profiles and the development of next-generation nucleoside analogues as anti-HCV drugs.

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特集◇HCV検査と治療の最前線

HCVの最新療法 ―DFPP療法を中心に―

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Key words：DFPP療法，早期ウイルス低下，IFN再治療

はじめに

近年，肝癌死亡は年3万人を越え，癌死亡の男性第4位，女性第5位を占めている。また肝疾患死亡も1万5千人近くであり，毎年約5万人近くが慢性肝疾患を背景として死亡しているが，このうちC型肝炎ウイルスが原因と考えられるのが約70%，3万～3万5千人ほどと推定される。C型肝炎は第二の国民病といわれ，国は薬害肝炎訴訟での敗訴などを受け，多方面で肝炎に対する対策を執っている。

C型慢性肝炎の原因治療としてインターフェロン(IFN)療法が開始されて20年近くになる。ここ数年の治療の進歩は目覚ましく，本特集の他稿に述べられているペグ（PEG）IFN，リバビリン併用療法により1型高ウイルス量症例でも約半数にウイルス学的著効が得られるし，新規薬剤としてHCVプロテアーゼ，ポリメラーゼを標的とした抗ウイルス剤の開発も進んでいる。今後C型慢性肝炎の治療成績の大幅な向上にはこれら新規薬剤に期待するところが大であるが，一方，新規薬剤には副作用の問題もあり高齢者が特に多い本邦のC型慢性肝炎の現状を考えると，新規薬剤を使用できるか，あるいは認可まで待てるかといった問題がある。

二重濾過血漿交換療法（double filtration plasmapheresis：DFPP）は従来より，主に疾患関連の免疫グロブリンをはじめとする蛋白を除去することによりその疾患を治療する手段として用いられてきた（表1）。2008年4月より

表1　二重濾過血漿交換療法（DFPP）の保険適応疾患

多発性骨髄腫
マクログロブリン血症
重症筋無力症
悪性関節リウマチ
全身性エリテマトーデス
血栓性血小板減少性紫斑病
重度血液型不適合妊娠
術後肝不全
急性肝不全
多発性硬化症
慢性炎症性脱髄性多発根神経炎
ギラン・バレー症候群
天疱瘡
類天疱瘡
巣状糸球体硬化症
溶血性尿毒症症候群
家族性高コレステロール血症
閉塞性動脈硬化症
中毒性表皮壊死症
スティーブンス・ジョンソン症候群
血友病（インヒビターを有する）
同種腎移植（ABO不適合または抗リンパ球抗体陽性）
C型慢性肝炎（1型高ウイルス量）

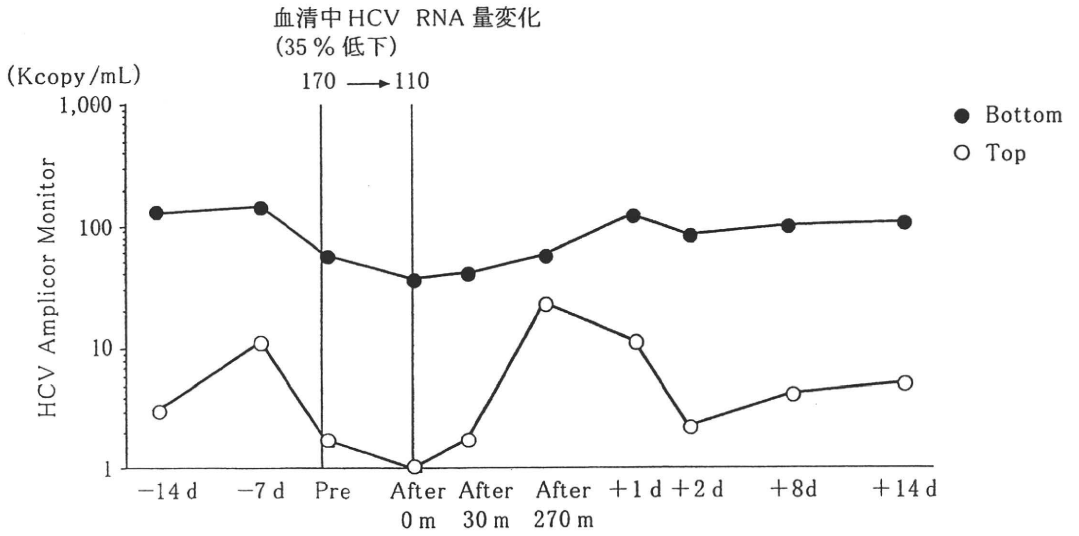

図1　免疫吸着療法によるHCV感染チンパンジーのHCV RNA量変化

DFPPはC型慢性肝炎に対しても保険適応となった。今まで新規あるいは従来薬剤の併用といったかたちでC型慢性肝炎に対するIFN療法効果の向上を目指していた中で，発想の違った方向でIFN療法の効果を上げることが期待されるDFPP療法を本稿では解説する。

Ⅰ．発想の経緯

1型高ウイルス量症例ではIFN療法のウイルス学的著効（sustained virus response：SVR）率が低く，併用療法，投与期間などさまざまな工夫がなされてきた。高ウイルス量状態が自然経過ではあまり変化することはなく[1]，この高ウイルス状態を何らかの方法で是正できないかと考えた。またC型慢性肝炎患者血清中のHCV粒子はその浮遊密度により低比重分画（top）およびIgGと免疫複合体を形成する高比重分画（bottom）の2つの分画が存在していると考えられ，感染価や病態との関連が報告されてきた[2][3]。

われわれはこの浮遊密度で分けられる分画とIFN療法の効果に着目し，同じウイルス量であってもbottomが多い症例ではtopが多い症例に比べIFN療法の有効性が低いことを報告している[4]。この結果からわれわれはbottomのHCV粒子を減らすことができれば，IFN療法の効きやすい状態にできないかと考えた。基礎実験の結果，免疫複合体を特異的に吸着する吸着体にてbottomのHCVを吸着できることを確認し，実際の生体に応用することを考えてHCV感染モデルであるチンパンジーに体外循環併用免疫吸着療法を行った[5]。免疫吸着療法を行う前後で血清中のHCV RNAは170 Kcopy/mLから110 Kcopy/mLと35％の減少がみられたが，その後270分後には前値に回復し，翌日にはさらに上昇していた。このHCV RNAの推移をtop，bottomについて検討すると免疫吸着後まずtopのHCV RNA量が270分後に急速に増え，Top：Bottom比は前値より10倍上昇していた。BottomのHCV RNA量は翌日をピークに増加し，その後定常状態になっていた（**図1**）。

このように免疫吸着による選択的HCV粒子除去にてHCV RNA量の減少およびHCV粒子の比重分画比を変化させることがある程度可能であることがわかったが，IFN療法の効果を上げるにはさらに高率の良いHCV粒子の除去が必要であると考えられた。

Ⅱ．DFPP開発試験の成績

腎不全合併C型慢性肝炎症例では透析療法前後でHCV RNA量が減少するという報告が

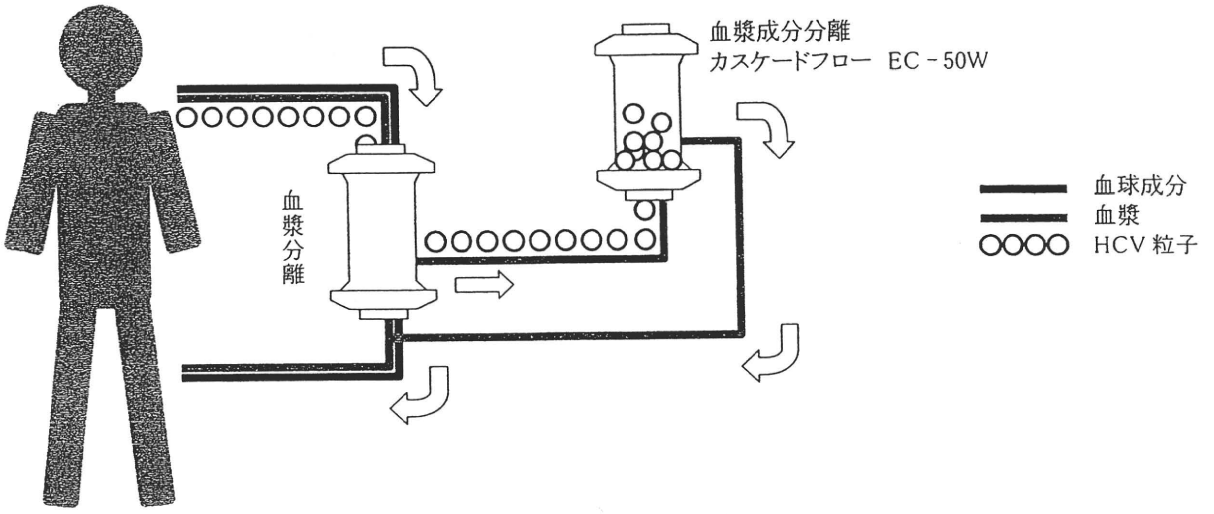

図 2　DFPP フロー図

以前からあった[6]が，使用する透析膜の種類に依存する可能性もあり，効率も良くはなかった[7]。クリオグロブリン血症合併C型慢性肝炎2症例に血漿交換を行った報告では血漿交換前後で最大93.3%のHCV RNAの低下が得られている[8]。この報告でも血漿交換後4～6時間でHCV RNA量は再上昇している。理論的には全血漿交換が一番HCV粒子除去の効率が良さそうであるが，大量の血漿が必要であること，繰り返し続けて行うことが困難であることなどの問題がある。そこで特定の大きさの物質を除去することが可能で，基本的に血液製剤を必要としないDFPP療法に着目した。HCV粒子はおよそ直径55～65 nmとされているので[9]，この大きさの物質を選択的に除去することができればHCV粒子を効率よく除去することが可能と考えられる。すでにDFPP療法に使用されている旭化成クラレメディカル㈱製のカスケードフローEC-50Wは最大膜孔が30 nmであり理論的にはHCV粒子が通過できないと考えられ，さらに体外循環機器として安全性が確立されている。

1．DFPP併用IFN/ribavirin併用療法：予備試験

腎臓内科の協力のもと，9例のgenotype 1b，高ウイルス症例C型慢性肝炎症例にDFPP併用IFNα2b/ribavirin併用療法（24週）を行った[10]。DFPP療法は大腿静脈にダブルルーメンカテーテルを挿入し，血球成分と血漿の分離をプラズマフローOP（旭化成クラレメディカル㈱製）で行い，分離された血漿をカスケードフローEC-50Wにて処理し，処理後血漿を血球成分とともに返血した（**図2**）。50 mL/kgを血漿処理目標とするとおよそ80 mL/分の血流量で約3時間を要した。1本目のIFNはDFPP終了1時間以内，ribavirinはDFPP終了後ただちに投与した。DFPPは1日1回計5日間を目標にしたが，フィブリノーゲン値が100 mg/dL以下となった時には安全性を考えDFPP療法は行わなかった。IFNα2bは2週間連日投与後週3回22週間投与を行った。

まず実際にカスケードフローEC-50WでHCV粒子が効率良く取れているかをみると，カスケードフローEC-50W流出口ではHCV RNA量がアンプリコアオリジナル法（限界：0.5 KIU/mL）で全例感度以下であり，HCV粒子は非常に効率良く血漿から除去できることが確認された。治療開始2週間後のHCV RNA低下量は2.45±1.12 Log IU/mL，2週間後に2 Log以上の低下達成率は9例中6例（66.7%）であり，同時期に当科で通常のIFNα2b/ribavirin併用療法を行った11例の低下量1.57±0.95 Log IU/mL（p＝0.073），2 Log以上の低下達成率4例（36.4%：p＝0.178）と比べると

より初期のHCV RNA量低下が得られる傾向にあった。SVRはDFPP併用で2例（全症例中22.2%，治療完遂例中33.3%），コントロールで2例（全症例中18.2%，治療完遂例中20.0%）であった。体外循環，DFPP療法中の重篤な有害事象は認めなかった。

2．DFPP療法全国試験の成績

SVR率には差を認めなかったもののDFPP療法＋IFNα2b/ribavirin併用療法にてより早期のHCV RNA量の低下が得られることが確認され，現在の主流であるPEG-IFNα2b/ribavirin併用療法にDFPP療法を加えてその治療効果を全国15施設で検討した[11]。

ここでは1型高ウイルス量C型慢性肝炎にDFPP療法＋PEG-IFNα2b/ribavirin併用療法を行った群（n＝30：DFPP群）と通常のPEG-IFNα2b/ribavirin併用療法を行った群（n＝74：コントロール群）の成績を示す。DFPP群は5回を上限として前述のごとく行い，PEG-IFNの初回投与はDFPP終了1時間以内に行った。まず2週間後のHCV RNA減少量はDFPP群では1.48 Log，コントロール群1.35 Log，4週間後ではそれぞれ2.43 Log，2.09 Logであり，DFPP群がコントロール群より低下しているが有意差はなかった。

より詳細に背景を検討するためにIFN初回治療例と再治療で分けると，DFPP群では初回8例，再治療22例，コントロール群でそれぞれ45例，29例であった。特に再治療例でみると2週間後のHCV RNA減少量はDFPP群で1.58 Log，コントロール群1.17 Log，4週間後ではそれぞれ2.47 Log，1.52 Logであり，4週間後のHCV RNA減少量はDFPP群がコントロール群より有意に低下していた（p＝0.010）。またそのうち前治療で無効（前回IFN治療期間中にHCV RNA陰性化得られず）例でも統計的差はないものの，2週間後DFPP群で1.20 Log，コントロール群0.77 Log，4週間後でそれぞれ2.13 Log，1.46 LogとDFPP群で高いウイルス量低下が得られていた。治療完遂例のSVR率はDFPP群24例中17例（70.8%），コントロール群58例中29例（50.0%）と有意差はないものの（p＝0.094），DFPP群で高いSVR率が得られた。特に再治療症例でみると再燃例ではDFPP群11例中9例（81.8%），コントロール群15例中9例（60.0%），無効例でもDFPP群7例中5例（71.4%），コントロール群7例中2例（28.6%）とコントロール群よりDFPP群が前回治療時の反応を問わず高いSVR率が得られた。

III．DFPP療法の市販後調査成績

2008年春にDFPP療法が保険収載後約1,000症例のC型慢性肝炎にDFPP療法が行われている。安全性・有効性を評価するために市販後調査が行われ，2009年5月までに133例の情報が集積されている。これによると男女比はほぼ1:1で平均年齢56.6歳に行われている。まず安全性であるが，副作用は62件，37例（27.8%）に発生したがいずれも重篤なものはなかった。件数の多いものでは嘔吐7件，嘔気7件，発熱7件であり次いでDFPP療法特有のものと思われる皮下出血6件，血圧低下6件，フィブリノーゲン低下3件であった。またブラッドアクセス部感染，穿刺部血腫も各1件ずつ報告されている。有効性についてはいまだSVR率を評価するには至らないが4週，12週後のHCV RNAについては検討されている。前治療歴の明らかな76症例でみると治療開始4週間後のHCV RNA減少量は未治療例（n＝20）3.0±1.6 Log，前治療無効例（n＝33）2.3±2.1 Log，再燃例（n＝23）2.9±1.5 Logと治療歴にかかわらず4週間で2 Log以上のウイルス量低下が得られていた。

DFPP開発時と市販後調査の有効性の大きな違いは開発時症例の無効・再燃例の多くは前治療IFN単独療法に対する評価であったが，現在の無効・再燃例の多くはPEG-IFN/ribavirin併用療法施行症例である。そこで今回の市販後調査症例で前治療をPEG-IFN/ribavirin併用療法に限ってみると，再燃例では4週後（n＝15）2.8±1.2 Logの低下が得られていたが，無

表 2　前治療 PEG-IFN/ribavirin 療法例における DFPP 併用再治療の効果

		前治療無効	前治療再燃
DFPP 療法 4 週後 平均 HCV RNA 減少量 (Log IU/mL)		−1.4±1.6 (n=19)	−2.8±1.2 (n=15)
4 週後	>2 Log 減少 うち 1.2 Log 未満	6/19 (31.6%) 1/19 (5.3%)	10/15 (66.7%) 1/15 (6.7%)
12 週後	>2 Log 減少 うち 1.2 Log 未満	8/17 (47.1%) 1/17 (5.9%)	10/11 (90.9%) 5/11 (45.5%)

効例では（n=19）1.4±1.6 Log と 2 Log 低下は得られていない（**表 2**）。また 12 週間後では再燃例では 90.9% で 2 Log 低下，45.5% で TaqMan 1.2 Log 未満が得られているが，無効例では 2 Log 低下 47.1%，TaqMan 1.2 Log 未満 5.9% であった。十分な PEG-IFN/ribavirin 併用療法が行われたうえで無効であった症例には DFPP 療法を併用しても効果は限定されている可能性があり，さらなる工夫が必要と考えられる。しかしながら PEG-IFN/ribavirin 併用療法再燃例に対しては DFPP 療法を併用することで早くウイルス陰性化が得られ，SVR に結びつく可能性があると考えられる。

ここで PEG-IFN/ribavirin 併用療法再燃例に対して DFPP 併用にて PEG-IFN/ribavirin 療法を行った症例を示す（**図 3**）。第 1 回治療時は 68 歳で，肝生検では F3A1 であった。合併症として心疾患がある。PEG-IFN α 2b/ribavirin 療法を開始し，アンプリコアハイレンジ法にて投与開始前 3,750 KIU/mL から 5 週後には 2.7 KIU/mL と 2 Log 低下は得られていた。8 週，12 週後にはハイレンジ法<5 KIU/mL しか確かめられていないが，14 週後アンプリコア定性法陰性が確認されている。48 週で投与終了し，投与期間中 PEG-IFN，ribavirin ともにほとんど減量していないが再燃が確認された。DFPP が保険収載となった後，希望にて再治療を行っている。第 2 回治療時は 71 歳である。PEG-IFN α 2a/ribavirin 開始時より DFPP を併用，計 5 回を特に問題なく行えた。DFPP 終了時（1 週後）には 1,059 KIU/mL から 8.7 KIU/mL，4 週後には TaqMan 法 1.5 Log IU/mL（およそ 0.1 KIU/mL）まで低下している。8 週後には TaqMan 法で陰性が確認され前回同様 48 週にて投与終了とした。まだ最終治療効果を確認されていないので DFPP 併用にて SVR となるかは不明だが，同じ PEG-IFN α/ribavirin 併用療法下で早期のウイルス量低下が得られている。

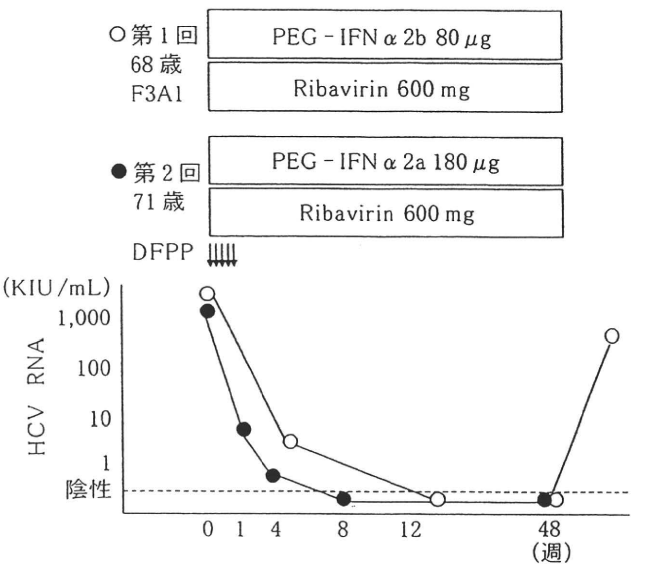

図 3　PEG-IFN α/ribavirin 併用療法再燃に DFPP 併用にて再治療例の経過

おわりに

C 型慢性肝炎に対する保険認可された新たな治療として DFPP 療法を解説した。特に再治療例に早期のウイルス量の低下，優れた SVR が示されているがいまだ症例数が少なく，また