

Eradication of Hepatitis C Virus Subgenomic Replicon by Interferon Results in Aberrant Retinol-Related Protein Expression

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Hepatitis C virus (HCV) infection induces several changes in hepatocytes, such as oxidative stress, steatosis, and hepatocarcinogenesis. Although considerable progress has been made during recent years, the mechanisms underlying these functions remain unclear. We employed proteomic techniques in HCV replicon-harboring cells to determine the effects of HCV replication on host-cell protein expression. We examined two-dimensional electrophoresis (2-DE) and mass spectrometry to compare and identify differentially expressed proteins between HCV subgenomic replicon-harboring cells and their "cured" cells. One of the identified proteins was confirmed using enzyme-linked immunosorbent assay (ELISA) and Western blot analysis. Full-length HCV genome RNA replicating and cured cells were also assessed using ELISA. Replicon-harboring cells showed higher expression of retinal dehydrogenase 1 (RALDH-1), which converts retinol to retinoic acid, and the cured cells showed higher expression of retinol-binding protein (RBP), which transports retinol from the liver to target tissues. The alteration in RBP expression was also confirmed by ELISA and Western blot analysis. We conclude that protein expression profiling demonstrated that HCV replicon eradication affected retinol-related protein expression.

Key words: hepatitis C virus, retinol-binding protein

Hepatitis C virus (HCV) is the most common hepatitis virus in Japan. Approximately 85% of cases progress to chronic infection, resulting in liver cirrhosis and hepatocellular carcinoma. HCV is a member of the *Flaviviridae* family and possesses a single-stranded, sense RNA genome of about 9.6 kb that encodes a single polyprotein. This precursor protein is cleaved co- and post-transcriptionally into at

least 10 proteins: core, envelope (E) 1, E2, p7, nonstructural protein (NS) 2, NS3, NS4A, NS4B, NS5A, and NS5B [1, 2]. Although many studies have examined the functions of single proteins, the function of the polyprotein has not been sufficiently studied, owing to the lack of reproducible HCV proliferation in cell culture systems.

Recent advances in cell culture have enabled the reproducible implementation of the HCV replication system. An HCV replicon system that contained HCV nonstructural proteins and showed autologous replication of the HCV proteins was first established in

1999 [3]. Recently, a full-length HCV genome containing replication system was introduced, followed by an *in vitro* viral replication system that can produce HCV viral particles, has also been established [4–8].

Recent advances in genomic and proteomic technologies have provided powerful tools for studying the global characteristics of host cell protein responses to HCV *in vitro*. Refined multidimensional liquid chromatographic (LC) separation, coupled with mass spectrometry (MS) for proteome analysis, has enabled global analysis using less protein and with increased sensitivity, throughput, and dynamic range than with previous proteomic techniques. Although the efficient replication of an HCV subgenomic replicon is thought to affect the gene expression profiles of host cells [9, 10], few proteomic analyses of this system have been reported [11].

In the present study, a proteomic approach was utilized to compare global protein expression profiles in HCV subgenomic replicon-harboring cells with “cured” cells, from which the replicons had been eliminated by prolonged treatment with interferon (IFN) alpha.

Materials and Methods

Cell cultures. The sO cells, harboring HCV subgenomic replicons derived from genotype 1b strains, were produced from HuH-7 cells [12]. sAH1 cells harboring the HCV subgenomic replicons were used to establish a cloned cell line, using an HCV replicon RNA library constructed with the HCV AH1 strain [7]. AH1 cells, harboring HCV full genome RNA were established from a full-length HCV genome RNA by the transfection of HCV RNA into sAH1c cells. sAH1c cells were “cured sAH1 cells” which were created by eliminating HCV RNA from transfected subgenomic replicon-harboring sAH1 cells by prolonged IFN alpha treatment. Cured cells were used because these enhanced the colony formation of the subgenomic replicon more than did parental HuH-7 cells [8].

The sO and sAH1 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and G418 (300 µg/ml; Geneticine, Invitrogen, Carlsbad, CA, USA). HCV RNA-replicating cells are G418-resistant due to the production of neomycin phospho-

transferase (Neo^R) from the efficient replication of HCV RNA. The presence of G418 is toxic when HCV RNA is excluded from the cells or its levels are decreased. Therefore, cured cells obtained from sO and sAH1 cells were maintained in the absence of G418.

IFN treatment (establishment of cured cells).

The sO and sAH1 cells are sensitive to IFN [7, 12]. To prepare cured cells, sO and sAH1 cells (1×10^6) were plated onto 10-cm plates and cultured for 1 day immediately prior to IFN treatment. Human IFN alpha (Sigma, St. Louis, MO, USA) was added to the cells at a final concentration of 3,000 IU/ml. The cells were incubated in the absence of G418 for 3 weeks with the addition of IFN alpha (3,000 IU/ml) at 4-day intervals. The cured cells obtained from sO and sAH1 cells were named sOc and sAH1c, respectively. Negativity for HCV-RNA was confirmed by RT-PCR and defined as “cured” cells.

Protein extraction and two-dimensional electrophoresis (2-DE).

Cells were washed with phosphate-buffered saline (PBS), and harvested by mechanical scraping during the exponential growth phase. Cells were centrifuged, and the cell pellets were dissolved in lysis buffer consisting of 5M urea, 2M thiourea, 2% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS), 2% sulfobetaine (SB) 3-10, 1% dithiothreitol (DTT) and a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). After three freeze-thaw cycles, the pellets were sonicated for 30 sec and ultracentrifuged at 75,000 g for 30 min at 10°C using an Optima™ TLF Ultracentrifuge (Beckman Coulter, Brea, CA, USA). The supernatant was transferred to a new tube and treated with a ReadyPrep 2D Cleanup Kit (Bio-Rad, Hercules, CA, USA) to remove ions, DNA, and RNA. The protein concentration was estimated using an RC-DC Protein Assay (Bio-Rad), according to a standard two-washed protocol. Pharmalyte 3-10 for isoelectric focusing (IEF) was formulated to increase the resolution at the basic end of a flatbed isoelectric focusing gel.

The first dimension IEF was performed using a 17-cm immobilized pH gradient (IPG) DryStrip (Bio-Rad), nonlinear pH 3-10. After rehydration for 15 h in 300 µL buffer consisting of 5M urea, 2M thiourea, 2% CHAPS, 3% SB3-10, 1% DTT, and 0.2% Bio-Lyte® 3/10 ampholyte (Bio-Rad), the protein

samples, 60 μ g each, were loaded onto the strips. Focusing was accomplished with the following conditions: 250 V for 40 min, 10,000 V for 4 h, a third step with a total 70,000 V-h, and finally maintenance at 500 V as needed.

The focused strips were then equilibrated in buffer I (6M urea, 2% sodium dodecyl sulfate (SDS), 0.375M Tris-HCl pH8.8, 20% glycerol, 2% DTT) for 30 min and then in buffer II (6M urea, 2% SDS, 0.375M Tris-HCl pH8.8, 20% glycerol, 2.5% iodoacetamide) for 15 min with gentle shaking. The second-dimension separation was carried out on 12% SDS-polyacrylamide gels using PROTEAN II Cell (Bio-Rad) at 20°C using 40 mA/gel constant amps for 4 h. After 2-DE, the gels were stained with SYPRO Ruby (Invitrogen) according to the manufacturer's protocol.

Image analysis of 2-DE gels. Images of SYPRO Ruby-stained gels were obtained using an FLA-3000 (Fujifilm, Tokyo, Japan) image analyzer. Background subtraction, spot detection, and volume normalization were performed with PDQuest Advanced Version 8.0 software (Bio-Rad). The gels were then destained and restained with a silver staining kit (Dodeca Silver Stain kit, Bio-Rad). The spot intensity of each sample was determined and analyzed with PDQuest Advanced Version 8.0 software (Bio-Rad). The intensities of the matched spots were compared, and differences >1.5-fold were confirmed by visual inspection. Three independent experiments were performed.

Protein identification using mass spectrometry. Overexpressed protein spots were selected based on Sypro Ruby staining intensity. Spots of interest were manually excised after silver staining. Gel spots were washed and digested with sequencing-grade trypsin and the resulting peptides were extracted using standard protocols. Peptide sequencing was accomplished by using a nanoflow HPLC, with electronic flow control (1100 Series nanoflow LC system, Agilent Technologies, Palo Alto, CA, USA), interfaced to an ion trap mass spectrometer (LC-MSD Trap SL, Agilent Technologies). A reverse-phase column (75 mm \times 150 mm, C18 Zorbax StableBond) was used as the analytical column. The MS data were searched against a subset of human proteins in the Spectrum Mill for MassHunter Workstation software, protein sequence database.

Positive protein identification was based on a total MS/MS search score of > 21.

Analysis of retinol-binding protein (RBP) levels using ELISA. Conditioned medium was collected from sO and sOc cells. Cell extracts were prepared by treatment with cell lysis buffer (MBL, Nagoya, Japan) and centrifuged at 15,000 rpm for 5 min. Protein concentrations were determined by the colorimetric BioRad protein assay. The protein concentrations in the sO cells and sOc samples were equalized. RBP concentrations in culture supernatants were measured by an RBP ELISA kit (Assaypro, St. Charles, MO, USA) according to the manufacturer's instructions.

Analysis of RBP levels using Western blot analysis. Cultured cells were washed twice with ice-cold PBS and lysed with lysis buffer (0.1M Tris-HCl, 4% SDS, 10% glycerol, 0.004% bromophenol blue, 10% 2-mercaptoethanol). The lysates were collected and boiled for 5 min. Samples were electrophoresed on a 15% SDS-polyacrylamide gel, and transferred to a PVDF transfer membrane (Millipore, Bedford, MA, USA). Membranes were blocked in 5% BSA in 20 mM Tris-HCl (pH7.6), 137 mM NaCl, and 0.1% Tween 20 (TBS-T) for 1 h at 37°C, and then probed at 4°C overnight with antibodies in TBS-T containing 1% BSA. The primary antibodies were rabbit anti-beta actin (Sigma) and goat anti-RBP (Abcam, Cambridge, MA, USA). After washing, the membranes were incubated with a peroxidase-conjugated secondary antibody (anti-rabbit: Amersham Biosciences, Piscataway, NJ, USA) (anti-goat: R&D Systems, Minneapolis, MN, USA) at room temperature for 1 h, and visualized with an enhanced chemiluminescence detection system (Amersham Biosciences).

Analysis of RBP and retinal dehydrogenase 1 (RALDH-1) mRNA expression by reverse transcription polymerase chain reaction (RT-PCR). Expression levels of RBP and RALDH-1 genes were analyzed using RT-PCR. Total RNA was extracted from cell lines by using TRIzol reagent (Life Technologies, Carlsbad, CA, USA). Two micrograms of total RNA was reverse-transcribed into cDNA using ReverTra Ace (Toyobo, Osaka, Japan) at 42°C for 20 min followed by 99°C for 5 min using oligo (dT) primer according to the manufacturer's instructions. The resulting cDNA was subjected to PCR using the following primers (for-

ward PCR and reverse PCR primers, respectively):
 RBP (F: 5'-TTCCGAGTCAAAGAGAACTTCG
 R: 5'-TCATAGTCCGTGTCGATGATCC)
 RALDH-1 (F: 5'-TACTCACCGATTTGAAGATT
 R: 5'-TTGTCAACATCCTCCTTATC)
 GAPDH (F: 5'-CACCCACTCCTCCACCTTTG
 R: 5'-GTCCACCACCCTGTTGCTGT)

PCR reactions were performed using the KOD Dash DNA polymerase (Toyobo). PCR temperature conditions for RBP amplification were as follows: 30 cycles at 94°C for 30 sec, 60°C for 2 sec, and 74°C for 30 sec. PCR temperature conditions for RALDH-1 amplification were as follows: 35 cycles at 94°C for 30 sec, 51°C for 2 sec, and 72°C for 30 sec. To define the best amplification conditions for these genes, we tried 25, 30, 35, and 40 cycles of PCR. We found that 30 cycles for RBP and 35 cycles for RALDH-1 were the best conditions. For each primer set, the sense and antisense primer pairs were located on different exons to avoid amplification of contaminating genomic DNA. The housekeeping gene GAPDH (with the following amplifications: 30 cycles of 94°C

for 30 sec, 61°C for 2 sec, and 72°C for 30 sec) was used as an internal control to confirm the success of the RT-PCR. PCR products were analyzed on 2% agarose gels stained with ethidium bromide.

Statistical analysis. Results were expressed as means \pm standard deviation (SD). All data were compared using Student's *t* test (Stat View, Cary, NC, USA). Data were considered statistically significant at $p < 0.05$.

Results

Proteomic profiling analysis of sO and sOc cells. Fig. 1 shows representative images of proteomic profiling of sO and sOc cells. Five spots were identified as reduced in sOc (spots: 1 to 5) and 3 spots were identified as enhanced in sOc (spots: 6 to 8) (Fig. 1).

Protein identification of detected spots. The results of LC/MS analysis are shown in Table 1. Ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCH-L1), hemoglobin beta subunit, T-complex

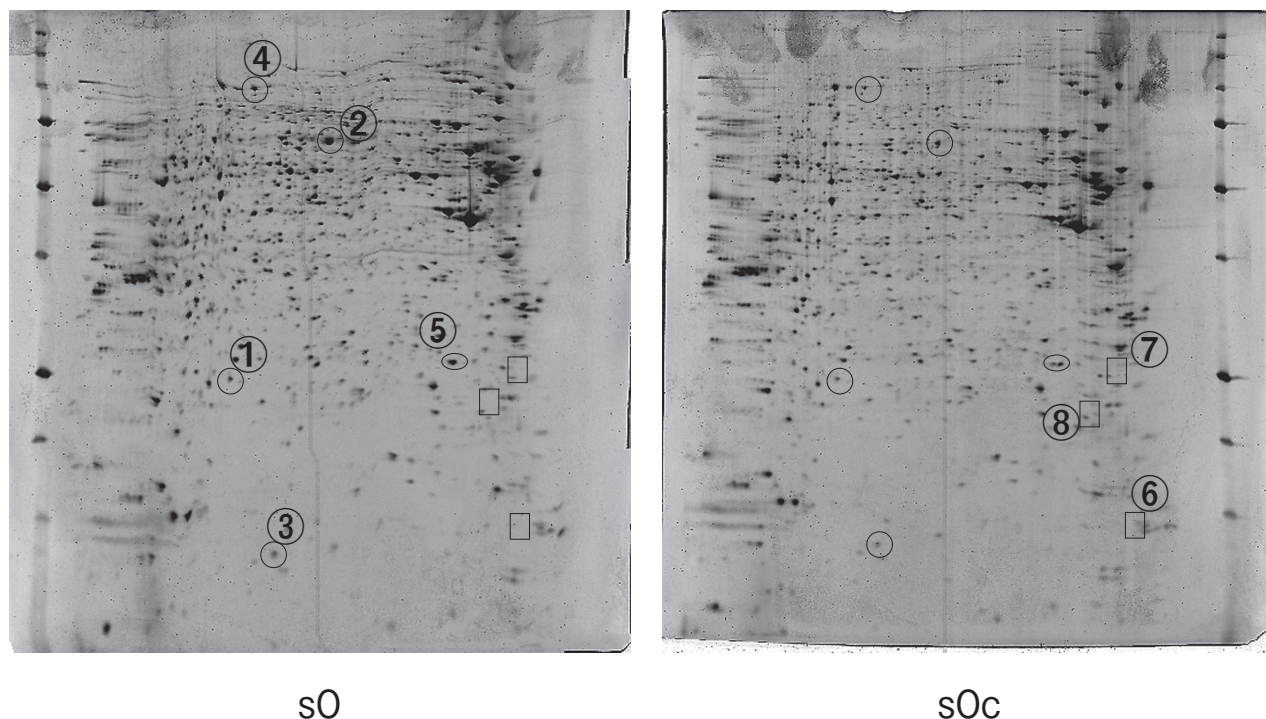


Fig. 1 2-DE analyses of proteins extracted from sO and sOc cells. Total protein extracts from the sO and sOc cells were separated on nonlinear IPG strips (pH3-10) in the first dimension followed by 12% SDS-PAGE in the second dimension, and then were visualized by SYPRO Ruby staining. The boxed areas were identical between sO and sOc.

protein 1 subunit gamma (TCP1-gamma), RALDH-1, and Elongation factor 2 were down-regulated in sOc cells, *i.e.*, interferon-induced or HCV elimination-induced. UCH-L1 was divided into 2 spots that might be modified. Conversely, myosin light polypeptide 6,

Table 1

A) Proteins upregulated in sO

- 1 Ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCH-L1)
- 2 Hemoglobin subunit beta
- 3 T-complex protein 1 subunit gamma (TCP1-gamma)
- 4 Retinal dehydrogenase 1 (RALDH-1)
- 5 Elongation factor 2

B) Proteins upregulated in sOc

- 6 Myosin Light Polypeptide 6
- 7 Rho GDP-dissociation inhibitor 1
- 8 Plasma retinol binding protein

Rho GDP-dissociation inhibitor 1 (Rho-GDI alpha), and the plasma retinol-binding protein (RBP) precursor were upregulated in sOc cells.

Of these proteins, retinol metabolism-related proteins were included in both downregulated (RALDH-1) and upregulated (RBP) by HCV eradication. Thus, we further examined the expression of these proteins.

RBP and RALDH-1 mRNA expression.

We examined the expression levels of RBP and RALDH-1 in sO and sOc cells by RT-PCR in order to explore retinol's role in HCV replicon-harboring cells. No significant differences in RBP and RALDH-1 mRNA expression were found between sO and sOc cell lines (Fig. 2A).

RBP expression. Western blot analysis and ELISA were employed for RBP analysis. The Western blot results, shown in Fig. 2B, indicate that RBP expression was higher in sOc cells than in sO cells. The ELISA results, shown in Fig. 2C, indicated that the RBP level in sOc cells was significantly (1.6times) higher than that in sO cells ($p <$

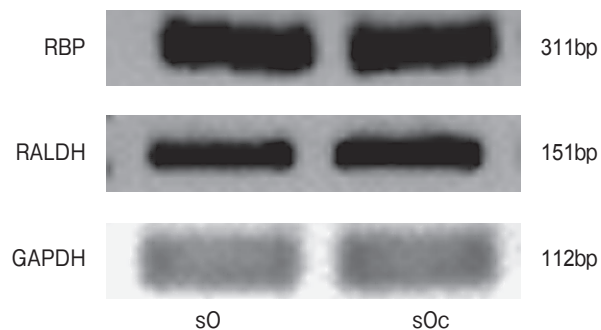
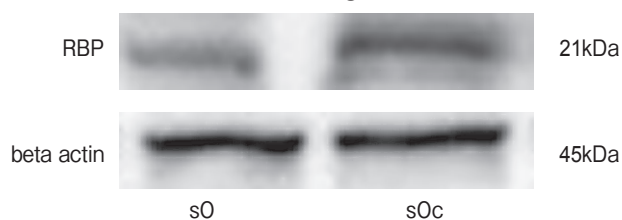
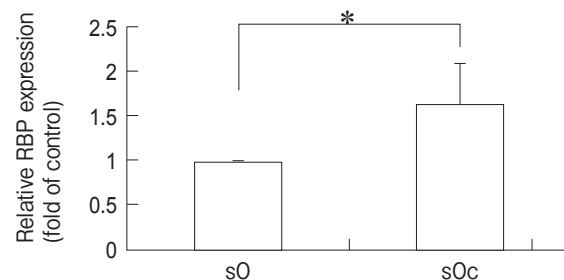
A RBP and RALDH1 mRNA expression

B RBP : Western blotting

C RBP : ELISA


Fig. 2 (A) RT-PCR analysis of RBP and RALDH-1 in sO and sOc cells. The expression of GAPDH was used as an internal control. (B) Western blot analysis of RBP in sO and sOc cells. Actin was used as a loading control. (C) Analysis of RBP levels by ELISA in sO and sOc cells. The results are expressed as fold increases compared to sO cells. The data represent mean \pm SD of triplicate measurements. * $p < 0.01$

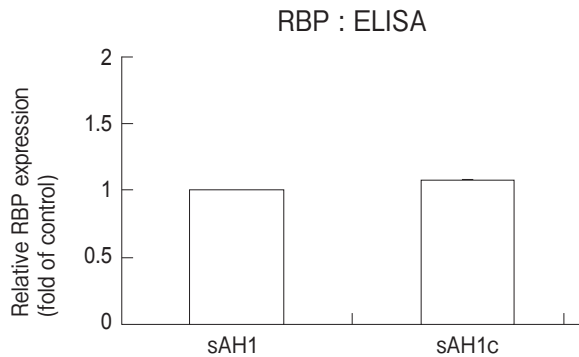


Fig. 3 RBP levels as determined by ELISA in sAH1 and sAH1c cells. The results are expressed as fold increases compared to sAH1 cells. The data represent mean \pm SD of triplicate measurements. * $p = 0.53$

0.01).

Additionally, we employed ELISA to compare RBP levels in sAH1 and sAH1c cells. RBP expression levels did not differ between sAH1 and sAH1c cells (Fig. 3).

Discussion

We have shown that, compared with cured cells, HCV subgenomic replicon-harboring cells bear more Hemoglobin subunit beta, TCP1-gamma, RALDH-1, Elongation factor 2, and UCH-L1. Furthermore, when the replicon-harboring cells were treated with interferon, thereby eliminating HCV proteins, myosin light polypeptide 6, Rho GDP-dissociation inhibitor 1 (Rho-GDI alpha), and plasma RBP precursor were upregulated.

Since these proteins have many functions and do not belong to a single functional category, we concentrated our next step on retinol-related proteins RBP and RALDH-1, which HCV elimination both upregulated and downregulated.

Other molecules were used as follows, and we did not subject them to further analysis. UCH-L1 is a member of a group of deubiquitinating enzymes and is one of the most abundant proteins in the brain [13]. It has been linked to Parkinson's disease, neuronal degeneration, and neuropathic pain. Additional evidence implicates it in other organ cancers. UCH-L1 is expressed in certain lung cancer cell lines and in breast cancer cells, especially in high grade tumors

[14, 15]. Hemoglobin (Hb) is a heterotetramer, consisting of 2 α -chain and 2 β -chain subunits that form 2 semirigid $\alpha\beta$ dimers ($\alpha_1\beta_1$ and $\alpha_2\beta_2$). Hemoglobin beta is a hemoglobin subunit. TCP1-gamma is a member of the group II chaperonin family. The substrates for TCP1-gamma are cytoskeletal proteins such as tubulins, actins, and cyclin E. It is also thought to be involved in cell growth, since TCP1-gamma is strongly upregulated during the G1/S phase transition of the cell cycle through the early S phase [16]. Disruption of the TCP1-gamma function using siRNA results in the inhibition of cell proliferation, decreased cell viability, cell-cycle arrest, and cellular apoptosis [17]. Elongation factor 2 is known to regulate protein synthesis. Elongation factor 2 catalyzes the ribosomal translocation reaction, resulting in movement of ribosomes along mRNA during protein translation, thereby increasing protein synthesis. Elongation factor 2 also has anti-apoptotic effects against TNF-alpha or HIV-1 viral protein R-induced apoptosis [18].

The non-structural HCV proteins in our replicon-harboring cells may increase retinoic acid as a result of RALDH-1 upregulation.

In our proteomic profiling analysis of sO and sOc cells, 2 proteins involved in retinol (vitamin A) metabolism were markedly altered. The liver is the major site of vitamin A-loaded RBP production for the purpose of vitamin A delivery to peripheral organs. Eradication of the HCV replicon resulted in RALDH-1 downregulation and RBP upregulation.

Dietary retinol is absorbed in the intestine, processed into retinyl esters, and transferred into the circulation. The circulating retinyl esters are taken to the liver and converted to retinol. A portion of the hepatic retinol is bound to a cellular binding protein (CRBP) and stored. The remainder of the hepatic retinol is bound to RBP and transported to target cells. Retinol is oxidized to retinal by a subfamily of alcohol dehydrogenase (ADH) enzymes. Retinal is oxidized to retinoic acid (RA) by 3 retinaldehyde dehydrogenases (RALDHs): RALDH-1, RALDH-2, and RALDH-3. These enzymes are expressed in different patterns and play specific roles in various tissues [19]. RA has many functions and can modulate a variety of important processes, such as cell growth and differentiation, the induction of apoptosis, and the prevention of angiogenesis. All-trans- and 9-cis-

retinoic acid regulate transcription and target gene expression through binding to the retinoic acid receptor (RAR) and the retinoid X receptor (RXR), within the nucleus.

Retinol is stored in hepatic stellate cells as retinyl ester and secreted into the blood bound to RBP, which is synthesized mainly in the liver [20]. Therefore, the concentration of plasma vitamin A is strongly related to the synthesis of RBP in the liver. Moreover, RBP synthesis depends on vitamin A levels in the hepatocyte, and the levels of vitamin A and RBP are correlated. RBP is eliminated by the kidney with a half-life of 12h in the circulation.

The parental HuH-7 cells may not be appropriate for use as control cells in proteomic analysis, because the HCV subgenomic replicon cells used are derived from a single cloned cell. Therefore, it is very important to avoid clone-based differences for the proteomic analysis. From this point of view, we compared HCV replicon-harboring cells and their cured cells. In HCV replicon-harboring cells, RALDH-1 was increased and might induce retinoic acid production. When the HCV is eliminated, the activation of retinol metabolism is reduced and may result in an increase in retinol that could induce RBP precursor upregulation. Although RALDH-1 was increased, proteins downstream from retinol metabolism, such as retinoic acid or retinoic acid receptors, were not upregulated in our experiment. This might be explained by either 1) the effects being minimal, or 2) the downstream pathway being compromised. In infection with another hepatotropic virus, hepatitis B, HBx protein is known to promote oncogenesis. The HBx gene has been reported to induce promoter hypermethylation in the retinoic acid receptor $\beta 2$ (RAR- $\beta 2$) gene, resulting in decreased susceptibility to retinoic acid-induced cell growth inhibition [21]. Both the hepatitis B and C viruses have pathologic effects on the liver, resulting in chronic hepatitis and hepatocarcinogenesis. Retinol metabolism dysfunction could be involved in this common pathology.

In our experiment, RBP expression in full-length HCV genome-containing sAH1 cells was not different from that in cured cells, whereas the subgenomic replicon-harboring sO cells containing non-structural proteins exhibited decreased RBP levels. The HCV core protein has been reported to affect retinol metabolism. A comprehensive analysis of gene expres-

sion in the liver of core gene-expressing transgenic mice revealed the downregulation of RBP [22]. The HCV core protein is reported to bind Sp110b, a transcriptional suppressor of retinoic acid receptor (RAR), and to suppress the function of Sp110b, which results in the activation of retinoic acid-related functions, such as apoptosis [23]. The core protein has been found to interact with RXR α in its DNA-binding domain [24]. These results led us to understand that the core protein and the non-structural proteins both decrease RBP. However, their co-expression might interfere with these effects. Chronic infection with hepatitis C virus results in hepatocarcinogenesis 30 to 40 years post-infection. This may illustrate that this virus's carcinogenic effects are not strong enough to directly induce early cell proliferation. Our present results indicate that the carcinogenic effect of individual HCV proteins may be controlled by mutual interference.

Proteomic analysis of hepatitis B virus (HBV) replicon-harboring cells, compared with parent cells, revealed that retinol metabolism-related proteins were differentially expressed [25]. In the HBV replicon-harboring cells, ALDH, RBP and CRBP1 were upregulated. Although the expression pattern was different, HBV, another chronic hepatitis and hepatocarcinogenic virus, induced alterations in retinol metabolism-related proteins. DNA microarray analysis experiments comparing the same HCV subgenomic replicon cells (sO) with cured cells indicated that there was no effect on retinol metabolism-related genes [26]. This illustrates that the HCV replicon's effects on retinol metabolism-related proteins are post-transcriptional.

In conclusion, we have employed proteomic techniques to elucidate the mechanisms underlying the replication and pathogenesis of HCV in HCV replicon-harboring cells. By comparing the protein expression profiles of HCV subgenomic replicon-harboring cell lines with cured cells, we observed several alterations in proteins that are correlated with cell proliferation and apoptosis control, including retinol metabolism. Such an analysis of the protein expression profile in HCV replicon-harboring cells has extended our understanding of the mechanisms underlying HCV pathogenesis.

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