Statistical Analysis

The results are expressed as the mean ± standard deviation. The significance of differences in the means was determined by Student's *t*-test.

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Author Contributions

Conceived and designed the experiments: MN MT YS ST NA NN AY JT KM. Performed the experiments: YF KAS AF YM OF HT AY. Analyzed the data: MI NK NS SM NE. Wrote the paper: YF AY JT KM. Collected marine organisms: JT. Identified the sponge: NJdV.

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Original Article

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.

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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 phosphotransferase (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 5 M urea, 2 M 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℃ 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 5 M urea, 2 M thiourea, 2% CHAPS, 3% SB3-10, 1% DTT, and 0.2% Bio-Lyte® 3/10 ampholyte (Bio-Rad), the protein

samples, 60 μg 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 (6 M urea, 2% sodium dodecyl sulfate (SDS), 0.375 M Tris-HCl pH 8.8, 20% glycerol, 2% DTT) for 30 min and then in buffer II (6 M urea, 2% SDS, 0.375 M Tris-HCl pH 8.8, 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℃ 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 × 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 (0.1 M 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 (pH 7.6), 137 mM NaCl, and 0.1% Tween 20 (TBS-T) for 1 h at 37℃, and then probed at 4℃ 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℃ for 20 min followed by 99℃ for 5 min using oligo (dT) primer according to the manufacturer's instructions. The resulting cDNA was subjected to PCR using the following primers (forward 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℃ for 30 sec, 60℃ for 2 sec, and 74℃ for 30 sec. PCR temperature conditions for RALDH-1 amplification were as follows: 35 cycles at 94℃ for 30 sec, 51℃ for 2 sec, and 72℃ 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℃ for 30 sec, 61℃ for 2 sec, and 72℃ 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 ± 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

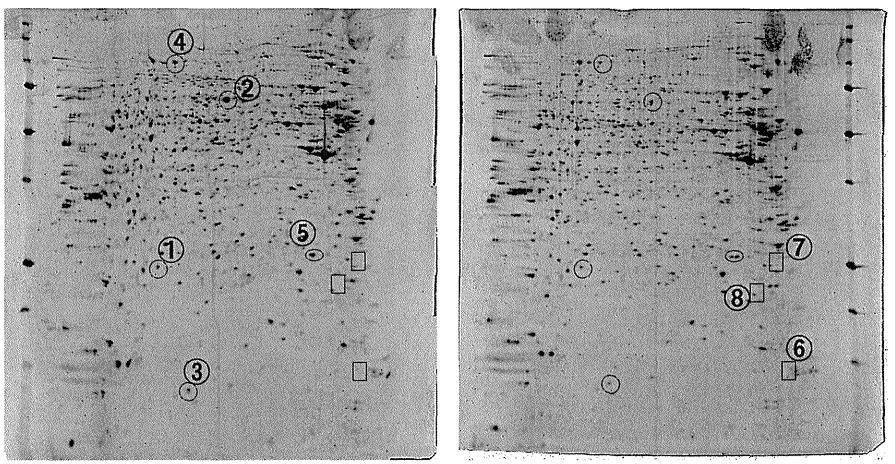

Fig. 1 2-DE analyses of proteins extracted from sO and sOc cells. Total protein extracts from the sO and sOc cells were separated on nonlinear IPG strips (pH 3-10) in the first dimension followed by 12% SDS-PAGE in the second dimension, and then were visualized by SYPRO Ruby staining. The boxed areas 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, Rho GDP-dissociation inhibitor 1 (Rho-GDI alpha), and the plasma retinol-binding protein (RBP) precursor were upregulated in sOc cells.

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

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 (1.6 times) higher than that in sO cells ($p<0.01$).

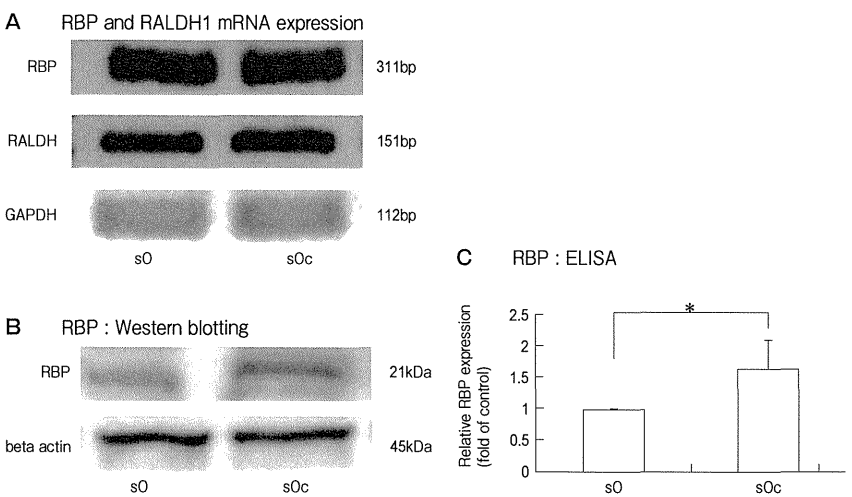

Fig. 2 (A) RT-PCR analysis of RBP and RALDH-1 in sO and sOc cells. The expression of GAPDH was used as an internal control. (B) Western blot analysis of RBP in sO and sOc cells. Actin was used as a loading control. (C) Analysis of RBP levels by ELISA in sO and sOc cells. The results are expressed as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

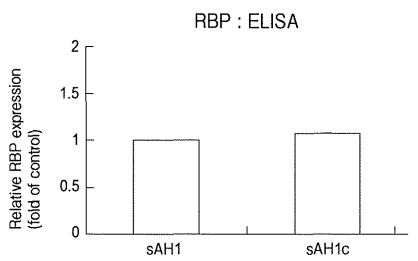

Fig. 3 RBP levels as determined by ELISA in sAH1 and sAH1c cells. The results are expressed as fold increases compared to sAH1 cells. The data represent mean ± SD of triplicate measurements. $^*p = 0.53$

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 2α-chain and 2β-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 12 h 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 expression 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 RXRα 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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ORIGINAL ARTICLE

Genomic polymorphisms in 3β-hydroxysterol Δ24-reductase promoter sequences

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ABSTRACT

It was recently reported by the present team that 3β-hydroxysterol Δ24-reductase (DHCR24) is induced by hepatitis C virus (HCV) infection. In addition, upregulation of DHCR24 impairs p53 activity. In human hepatoma HuH-7 cells, the degree of DHCR24 expression is higher than in normal hepatic cell lines (WRL68) at the transcriptional level. The genomic promoter sequence of DHCR24 was characterized and nucleotide substitutions were observed in HuH-7 cells at nucleotide numbers −1453 (G to A), −1420 (G to T), −488 (A to C) and −200 (G to C). The mutations of these sequences from HuH-7 cell types to WRL68 cell types suppressed DHCR24 gene promoter activity. The sequences were further characterized in hepatocytes from patient tissues. Four tissues from HCV-positive patients with cirrhosis or hepatocellular carcinoma (#1, 2, 3, 5) possessed HuH-7 cell type sequences. Interestingly, one patient with liver cirrhosis (#4) possessed WRL68 cell-type sequences; this patient had been infected with HCV and was HCV negative for 17 years after interferon therapy. Next, the effect of HCV infection on these polymorphisms was examined in humanized chimeric mouse liver and HuH-7 cells. The human hepatocytes possess WRL68 cell type and did not show the nucleotide substitution after HCV infection. The HCV-replicon was removed by interferon treatment and established the cured K4 cells. These cells possess HuH-7 cell type sequences. Thus, this study showed the genomic polymorphism in DHCR24 promoter is not directly influenced by HCV infection.

Key words 3β-hydroxysterol Δ24-reductase, hepatitis C virus, promoter.

Liver cancer is one of the most prevalent forms of cancer (1). More than 80% of cases occur in developing countries; however, Japan also has a remarkably high incidence (2). Among the primary liver cancers, HCC is the most common (3). Its incidence is increasing: between 1975 and 2005, age-adjusted HCC rates tripled (4).

One crucial cause of HCC is HCV infection (5). DHCR24, which functions as an oxidoreductase during cholesterol biosynthesis (6, 7), is linked to HCV-associated hepatocarcinogenesis and development of HCC (8–10). Infection of hepatocytes with HCV results in over-expression of DHCR24. This enzyme protects cells from oxidative stress and inhibits p53 activity (8), thus contributing to the development of HCC (5). These facts prompted us to investigate whether the molecular features of *DHCR24* are linked to HCC development. To this end, we characterized the promoter region of *DHCR24* in HCC cell lines and clinical samples.

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List of Abbreviations: DHCR24, 3β-hydroxysterol Δ24-reductase; DMEM, Dulbecco's modified Eagle's medium; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN, interferon; SVR, sustained viral response.

MATERIALS AND METHODS

Cell lines and growth conditions

HuH-7 and HepG2 cells were cultured in (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FCS (Sigma-Aldrich). WRL68 cells were cultured in DMEM supplemented with 1 mM sodium pyruvate (Invitrogen, Carlsbad, CA, USA), 0.1 mM non-essential amino acids (Invitrogen) and 10% FCS. HuH-7 cell-based HCV replicon harboring cell lines (R6FLR-N) (11) were cured off HCV by interferon treatment (12) and designated as K4 cells.

Northern and western blotting

Northern and western blotting were performed as previously described (8).

Sequencing of genomic DNA and reporter plasmid construction

Genomic DNA was extracted from HuH-7 and WRL68 cells using standard methods. DNA from the promoter region of *DHCR24* (~5 kb) was amplified using PCR (sense primer: 5′-CACTCCTGCTCACCACTGAT-3′; antisense primer: 5′-GTAGTAGATATCGAAGATAAGCGA-GAGCGG-3′). These fragments were individually cloned into the upstream region of the firefly luciferase gene in the pGL3-Basic vector (Promega, Madison, WI, USA) at the *Xho*I and *Nco*I sites (as we had done previously for the HepG2 cell line) (6). DNA sequences were determined using standard methods. Reporter plasmids that possessed chimeric promoters were constructed using restriction enzyme sites for *Tth111I* (position −2160) and *Bss*HII (position −1030).

Dual luciferase reporter assay

Using Lipofectamine LTX (Invitrogen), HepG2 cells (1×10^4 cells/well in a 96-well plate) were transfected with a reporter plasmid (0.25 μg/well) together with an internal control plasmid (phRL-TK; 0.025 μg/well) encoding *Renilla* luciferase (Promega). Forty-eight hours after transfection, the cells were assayed with the Dual-Glo Luciferase Assay System (Promega). Luminescence was measured using a TriStar LB941 microplate reader (Berthold Technologies GmbH, Bad Wildbad, Germany).

Liver tissue samples from chimeric mice or patients infected with hepatitis C virus

Severely combined immunodeficient mice carrying human primary hepatocytes were purchased from BD BioSciences (Franklin Lakes, NJ, USA) and African American, male, 5-year-old, HCV negative mice from PhoenixBio (Hiroshima, Japan) (13). These "human liver chimeric" mice were inoculated or mock-inoculated with plasma collected from an HCV-positive (HCR6 strain (14), GenBank accession #AY045702) patient in accordance with the requirements of the Declaration of Helsinki. HCV infection in the mice thus infected was confirmed by using quantitative PCR for HCV mRNA as previously described (9). The protocols for the animal experiments were pre-approved by the local Ethics Committee, and the animals were maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Informed consent for this clinical study was obtained from five patients with HCV (Table 1) at the Kumamoto University Hospital (Kumamoto, Japan), in accordance with the Helsinki Declaration prior to 2003, and the protocol was approved by the Regional Ethics Committee. LiverPool 20-donor pooled cryopreserved human hepatocytes (Celsis IVT, Baltimore, MD, USA) were purchased and used as the normal human liver tissue control. HCV RNA was detected by the COBAS TaqMan HCV test (Hoffman–La Roche, Basel, Switzerland). Liver

Table 1. Summary of patients with HCC

Patient ID	Sex	Age (years)	Diagnosis	ALT (IU/mL)	Outcome of IFN treatment	HCV RNA detection[†]
#1	F	60	LC	22	NR	+
#2	M	65	LC	31	NT	+
#3	F	57	LC	24	NR	+
#4	M	61	LC, HCC	12	SVR	−[‡]
#5	M	51	LC, HCC	91	NR	+

[†]Serum was tested for HCV RNA using quantitative PCR; [‡]In 1995, #4 was diagnosed with HCV-associated LC and HCC and HCV RNA was detected in his serum. As a result, #4 was treated with IFN. Since then, no HCV RNA has been detected in this patient's serum (>17 years). ALT, alanine aminotransferase; F, female; LC, liver cirrhosis; M, male; NR, no response; NT, not treated.

tissue was obtained from either mice or patients and processed for DNA sequencing. Two DNA fragments (corresponding to positions −1600 to −1292 and −631 to −86) were amplified using PCR with Tth-Bss forward and reverse primers (5′-ATTTCAACATGTCATTAACA-3′ and 5′-TTCTAGCACGGTGCTTTGTG-3′) and Bss-Nco forward and reverse primers (5′-CCAGCCA-TAGCCTTCCATG-3′ and 5′-AATGGCGAGCCGCGC CGG-3′), respectively. The amplified fragments were directly sequenced using the same set of primers.

Statistical analysis

Student's *t*-test was used to test the statistical significance of the results. P values of < 0.05 were considered statistically significant.

RESULTS

First, we measured DHCR24 expression in cell lines of noncancerous hepatocytes (WRL68) and hepatoma cells (HuH-7 and HepG2). Compared with noncancerous hepatocytes, DHCR24 expression in the two hepatoma cell lines was considerably increased with respect to both mRNA and protein (Fig. 1a, b). In addition, the different culture media used for the WRL68 and HuH-7 cells did not significantly influence the degree of expression of DHCR24 protein (Fig. 1c).

To identify the genetic characteristic(s) that govern DHCR24 upregulation, we isolated genomic DNA from these three cell lines and sequenced the *DHCR24* promoter region (nucleotide positions −4976 to +113, where +1 indicates the transcription start site). For this analysis, we sequenced three molecular clones from each cell line. Alignments of WRL68 and HuH-7 sequences showed different nucleotides at four positions: (i) an A to G switch at −1453 (i.e., A in WRL68 and G in HuH-7); (ii) a T to G switch at −1420; (iii) a C to A switch at −488; and (iv) a C to G switch at −200 (Fig. 2). The two hepatoma cell lines (HuH-7 and HepG2) had no nucleotide differences within these regions.

Next, we investigated whether these small changes in the promoter sequence affect gene expression in a heterologous context. We constructed reporter plasmids that placed the firefly luciferase gene under the control of *DHCR24* promoter sequences (either from HuH-7 or WRL68 cells) (Fig. 3a). We measured the promoter activity of each construct in HepG2 cells with dual-luciferase assays. The *DHCR24* promoter derived from HuH-7 cells showed significantly greater activity (i.e., induced greater expression) than the WRL68 promoter (Fig. 3b). We also constructed two reporter plasmids that contained chimeric promoters. In each of these chimeras,

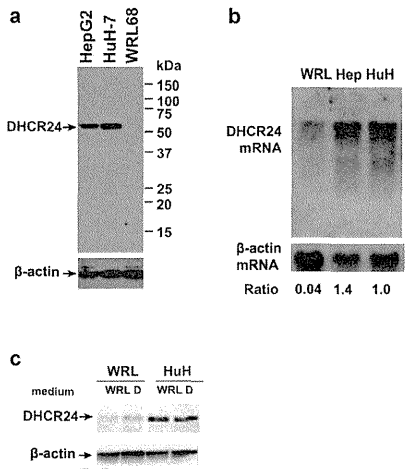

Fig. 1. Expression of DHCR24 in hepatoma cell lines. (a) Lysates from WRL68, HuH-7 and HepG2 cells were subjected to western blot analysis using antibodies directed against DHCR24 (upper panel) and β-actin (lower panel). (b) RNA was extracted from WRL68, HepG2 and HuH-7 cells and subjected to northern blot analysis using probes specific for *DHCR24* (upper panel) and β-actin (lower panel). Band intensities were quantified with a densitometer. Relative band intensity ratios (*DHCR24*/β-actin) are indicated below the gel images (the ratio for HuH-7 cells was set at 1). (c) DHCR24 protein (upper panel) and β-actin (lower panel) were detected in WRL 68 or HuH-7 cells with culture media for WRL68 cells (DMEM, 1 mM sodium pyruvate and 1 mM nonessential amino acids) or HuH-7 cells (DMEM alone).

we replaced HuH-7 fragments containing two polymorphisms with wild-type WRL68 sequences (Fig. 3a). These chimeric promoters had less activity than did intact promoters from both HuH-7 and WRL68 cells (Fig. 3b). These results indicate that the *DHCR24* promoter from HuH-7 cells contributes to the strong degree of *DHCR24* expression. In addition, all four nucleotide sequences of HuH-7 cell type in promoter fragments might be important for strong promoter activity.

Thereafter, we examined whether polymorphisms within the *DHCR24* promoter could be detected in clinical samples. We collected samples of liver tissue from five patients infected with HCV (Table 1) and sequenced the *DHCR24* promoter region (Table 2). Of the five samples tested, four (#1–3 and #5) showed all four of the polymorphisms associated with strong promoter activity (i.e., G, G, A and G nucleotides at positions −1453, −1420, −488, and −200). In contrast, promoter

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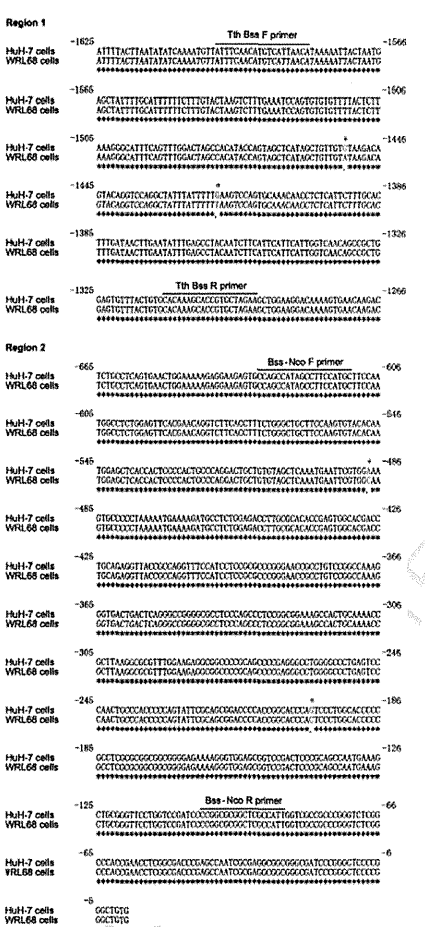

Fig. 2. Alignment of *DHCR24* promoter sequences. Nucleotide sequences from *DHCR24* promoter regions obtained from HuH-7 and WRL68 cells are shown. Cell-type-specific differences between these sequences (at positions −1453, −1420, −488, and −200) are indicated by asterisks and colors (red and blue represent nucleotides in HuH-7 and WRL68 cells, respectively). Positions of primer sequences are indicated.

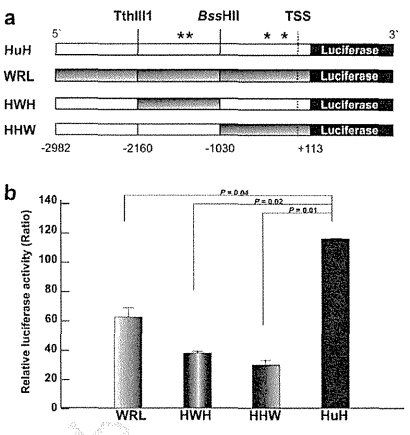

Fig. 3. Effect of nucleotide changes on *DHCR24* promoter activity. (a) Schematic diagrams of reporter constructs. Intact or chimeric *DHCR24* promoter sequences were used to drive the expression of firefly luciferase. Fragments of DNA derived from HuH-7 and WRL68 cells are colored white and grey, respectively. The asterisks indicate the position of each nucleotide polymorphism. Restriction enzyme sites (*Tth111I* and *Bss*HII) and transcription start sites are indicated. (b) Promoter activity of reporter constructs. HepG2 cells were transfected with the indicated reporter construct together with a control plasmid encoding *Renilla* luciferase. The relative ratio of firefly/*Renilla* luciferase activity is shown. Error bars indicate the standard deviation of two independent experiments. Each experiment was performed in triplicate. TSS, transcription start sites.

Table 2. Summary of nucleotide substitutions within the *DHCR24* promoter region

	Nucleotide position			
Origin of DNA sample	−1453	−1420	−488	−200
HuH-7 cells (high)[†]	G	G	A	G
WRL68 cells (low)[‡]	A	T	C	C
Patient #1	G	G	A	G
Patient #2	G	G	A	G
Patient #3	G	G	A	G
Patient #4	A	T	C	C
Patient #5 (NC)	G	G	A	G
Patient #5 (C)	G	G	A	G
20-donor pool[§]	A	T	C	C

[†]DHCR24 was expressed strongly in HuH-7 cells (Fig. 1), [‡]DHCR24 was expressed weakly in WRL68 cells (Fig. 1), [§]Pooled normal human hepatocytes from a 20-donor pool. C, cancerous region; NC, non-cancerous region.

Table 3. Summary of nucleotide substitutions within the *DHCR24* promoter region with or without HCV infection

		Nucleotide position			
Origin of DNA sample	HCV	−1453	−1420	−488	−200
HuH-7 cells (high)	–	G	G	A	G
WRL68 cells (low)	–	A	T	C	C
Chimeric mouse liver	–	A	T	C	C
HCV infected chimeric mouse liver	+[1]	A	T	C	C
HCV replicon cells (R6FLR-N)	+	G	G	A	G
Cured K4 cells	+	G	G	A	G

[1]7.5×10^6 copies/mL of HCV in patient plasma was inoculated.

sequences from patient 4 (#4) had nucleotides associated with weak activity at these positions (i.e., A, T, C, and C). Intriguingly, only #4 exhibited an SVR, which is characterized by the absence of detectable HCV RNA in serum for >24 weeks following IFN treatment. The SVR status of #4 has persisted since 1995. In #5, promoter sequences were the same in cancerous and non-cancerous regions of the liver. These results suggest that the four polymorphisms within the *DHCR24* promoter region may influence the susceptibility to malignancy and IFN responsiveness of hepatoma cells and thus influence the fate of patients with HCC.

To assess the impact of HCV infection on genomic polymorphism in DHCR24 promoter sequences, we determined the sequences in human hepatocytes that had been transplanted into severely combined immunodeficient mice that we infected or mock-infected with HCV We detected markedly high titers of HCV only in the infected mice (Table 3). Sequencing revealed that all four polymorphic nucleotide positions were of the weak activity type. Notably, we detected no nucleotide differences between HCV- and mock-infected mice in the targeted regions (Table 3). We also established cured K4 cells by treating HCV replicon cells R6FLR-N with IFN. Analysis of the genomic sequence of these cell lines showed no nucleotide differences in R6-FLR-N and K4 cells (Table 3). These results suggest that the differences in the DHCR24 promoter sequence are ingenerate rather than induced by HCV infection.

DISCUSSION

In this study, we analyzed the promoter sequences associated with *DHCR24* in hepatocytes and identified polymorphisms that regulate the degree of expression of downstream genes (Figs. 1–3). #4 had an SVR in response to IFN treatment; thus these *DHCR24* promoter sequence polymorphisms are potential biomarkers for predicting patients' responsiveness to IFN treatment.

Genomic polymorphisms within the *DHCR24* promoter region may influence binding of transcription factors (Supplementary Fig. S1). In fact, a T-to-G nucleotide substitution at position −1420 generates a potential binding site for the protein encoded by the caudal homeobox gene (*CdxA*), a homeobox transcription factor responsible for gastrointestinal tract development and epithelial differentiation (15). A C-to-A substitution at position −488 generates potential binding sites for nuclear factor kappa-light-chain enhancer of activated B cells and STATx (16), as well as a low-affinity binding site for Nkx-2 (17). Finally, a C-to-G substitution at position −200 potentially abolishes a p300 binding site (18). These changes in transcription factor binding affinities could upregulate *DHCR24* expression, thereby promote carcinogenesis.

Previously, we discovered that DHCR24 is a host factor involved in HCV-associated development of HCC (8, 9). This protein is upregulated by HCV infection (8), and reduced degrees of expression (via siRNA knockdown) inhibit HCV replication (9). These findings are consistent with the role of DHCR24 in cholesterol biosynthesis (6, 7), which is important for HCV replication (19). Also, because the efficiency of HCV replication might have been lower in #4 than in other patients with strongly active DHCR24 promoter, the weak DHCR24 expression in this patient (Supplementary Fig. S2) might have contributed to the efficacy of IFN treatment.

In conclusion, we have discovered polymorphisms in the promoter region of DHCR24 gene that have not been induced by HCV infection. Future study will clarify their biological significance.

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DISCLOSURE

The authors have no financial relationships to disclosure.

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PLOS ONE

Immunization with a Recombinant Vaccinia Virus That Encodes Nonstructural Proteins of the Hepatitis C Virus Suppresses Viral Protein Levels in Mouse Liver

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Abstract

Chronic hepatitis C, which is caused by infection with the hepatitis C virus (HCV), is a global health problem. Using a mouse model of hepatitis C, we examined the therapeutic effects of a recombinant vaccinia virus (rVV) that encodes an HCV protein. We generated immunocompetent mice that each expressed multiple HCV proteins via a Cre/*loxP* switching system and established several distinct attenuated rVV strains. The HCV core protein was expressed consistently in the liver after polyinosinic acid–polycytidylic acid injection, and these mice showed chronic hepatitis C-related pathological findings (hepatocyte abnormalities, accumulation of glycogen, steatosis), liver fibrosis, and hepatocellular carcinoma. Immunization with one rVV strain (rVV-N25), which encoded nonstructural HCV proteins, suppressed serum inflammatory cytokine levels and alleviated the symptoms of pathological chronic hepatitis C within 7 days after injection. Furthermore, HCV protein levels in liver tissue also decreased in a CD4 and CD8 T-cell-dependent manner. Consistent with these results, we showed that rVV-N25 immunization induced a robust CD8 T-cell immune response that was specific to the HCV nonstructural protein 2. We also demonstrated that the onset of chronic hepatitis in CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice was mainly attributable to inflammatory cytokines, (tumor necrosis factor) TNF-α and (interleukin) IL-6. Thus, our generated mice model should be useful for further investigation of the immunological processes associated with persistent expression of HCV proteins because these mice had not developed immune tolerance to the HCV antigen. In addition, we propose that rVV-N25 could be developed as an effective therapeutic vaccine.

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Introduction

Hepatitis C virus (HCV) is a major public health problem; approximately 170 million people are infected with HCV worldwide [1]. HCV causes persistent infections that can lead to chronic liver diseases such as chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) [2]. Antiviral drugs are not highly effective in individuals with a chronic infection; furthermore, an effective vaccine against HVC has not been developed. A convenient animal model of HCV infection will greatly facilitate the development of an effective HCV vaccine.

Transgenic mice that express HCV proteins have been generated to study HCV expression [3,4]; however, in each of these cases, the relevant transgenes is expressed during embryonic development; therefore, the transgenic mice become immunotolerant to the transgenic products, and consequently, the adult mice are not useful for investigations of the pathogenesis of chronic hepatitis C. To address this problem, we developed a system that can drive conditional expression of an HCV transgene; our system involves the Cre/*loxP* system and a recombinant adenovirus capable of expressing Cre recombinase [5,6]. Concerns have been expressed that an adenovirus and transient expression of HCV proteins could induce immune responses [5] and, therefore, obscure any evidence of the effect of the host immune responses on chronic liver pathology. Therefore, here, we used a Cre/*loxP* switching system to generate an immunocompetent mouse model of HCV protein expression; with this system, we could study the host immune responses against HCV proteins.

Folgori et al. (2006) reported effective vaccination of chimpanzees with an adenoviral vector and plasmid DNA encoding the HCV nonstructural region. This technique protected the liver tissues from acute hepatitis, which results when whole animals are challenged with virus [7]. However, this vaccine has not yet been shown to be effective against chronic HCV infection.

Here, we aimed to address how HCV expression causes chronic liver diseases and to provide new options for HCV vaccine development. Using LC16m8, a highly attenuated strain of vaccinia virus (VV), we generated three recombinant vaccinia viruses (rVVs) that each encoded one of three different HCV proteins and found that one recombinant virus (rVV-N25), which encoded nonstructural HCV proteins, resolved pathological chronic hepatitis C symptoms in the liver. We also found that immunization with rVV-N25 suppressed HCV core protein levels in the livers of transgenic mice; moreover, this suppression was mediated by CD4 and CD8 T cells, as has been previously reported [8].

Results

Generation of a Model of Persistent HCV Protein Expression

To produce adult mice that express an HCV transgene, we bred CN2-29 transgenic mice, which carry an HCV transgene, [5,6,9] with Mx1-Cre transgenic mice [10], which express Cre recombinase in response to interferon (IFN)-α or a chemical inducer of IFN-α, poly(I:C) (Figure 1A). Following poly(I:C) injection, the HCV transgene was rearranged, and HCV sequences were expressed in the livers of F1 progeny (CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice) within 7 days after poly(I:C) injection (Figure 1B).

To evaluate the characteristic features of these CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice, we analyzed serum alanine aminotransferase (ALT) and liver HCV core protein levels after poly(I:C) injection. As illustrated in Figure 1C, serum ALT levels increased and reached a peak at 24 h after the first poly(I:C) injection; this elevation appeared to be a direct result of the poly(I:C) treatment, which causes liver injury [11]. After this peak, serum ALT levels dropped continuously until day 4, and then ALT levels began to increase, as did HCV core protein levels. Thereafter, the HCV core protein was expressed consistently for at least 600 days.

Histological analysis showed HCV core protein expression in most hepatocytes of the transgenic mice; these mice showed evidence of lymphocytic infiltration that was caused by the HCV core proteins (Figure 1D and E). These observations, in addition to the modified histology activity index (HAI) scores, indicated that expression of HCV proteins caused chronic hepatitis in the CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice because a weak, though persistent, immune response followed an initial bout of acute hepatitis (Figure S1). Moreover, we observed a number of other pathological changes in these mice – including swelling of hepatocytes, abnormal architecture of liver-cell cords, abnormal accumulation of glycogen, steatosis, fibrosis, and HCC (Figures 1E and F, Table S1). Steatosis was mild in the younger mice (day 21) and became increasingly severe over time (days 120 and 180; Figure S2). Importantly, none of the pathological changes were observed in the CN2-29$^{(+/-)}$/MxCre$^{(-/-)}$ mice after poly(I:C) injection (Figure 1F).

Recombinant Vaccinia Virus Immunization in HCV Transgenic Mice

To determine whether activation of the host immune response caused the reduction with HCV protein levels in the livers of CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice, we used a highly attenuated VV strain, LC16m8, to generate three rVVs [12]. Each rVV encoded a different HCV protein; rVV-CN2 encoded mainly structural proteins, rVV-N25 encoded nonstructural proteins, and rVV-CN5 encoded the entire HCV protein region (Figure 2A). Because rVVs can express a variety of proteins and induce strong and long-term immunity, they have been evaluated as potential prophylactic vaccines [13].

We used western blots to confirm that each HCV protein was expressed in cell lines. Each of seven proteins – the core, E1, E2, NS3-4A, NS4B, NS5A, and NS5B – was recognized and labeled by a separate cognate antibody directed (Figure S3). To induce effective immune responses against HCV proteins in transgenic mice, we injected an rVV-HCV (rVV-CN2, rVV-CN5, or rVV-N25) or LC16m8 (as the control) intradermally into CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice 90 days after poly(I:C) injection (Figure 2B). Analysis of liver sections 7 days after immunization with rVV-N25 revealed dramatic improvement in a variety of pathological findings associated with chronic hepatitis – including piecemeal necrosis, hepatocyte swelling, abnormal architecture of liver-cell cords, abnormal accumulation of glycogen, and steatosis (Figures 2C–E). Collectively, these results demonstrated that only the rVV-N25 treatment resulted in histological changes indicative of improvement in the chronic hepatitis suffered by the transgenic mice.

To determine whether rVV-N25 treatment induced the same effect in other strains of HCV transgenic mice, we analyzed RzCN5-15$^{(+/-)}$/MxCre$^{(+/-)}$ mice, which express all HCV proteins; in these mice, chronic hepatitis was resolved within 28 days of immunization with rVV-N25. Taken together, these findings indicated that rVV-N25 had a dramatic therapeutic effect on both types of HCV transgenic mice (Figure S4).

Treatment with rVV-N25 Reduced the HCV Core Protein Levels in the Livers

To assess in detail the effects of rVV-HCV immunization on HCV protein clearance from the livers of CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice, we monitored the levels of HCV core protein in liver samples via ELISA. We found that within 28 days after immunization the HCV core protein levels were significantly lower in livers of rVV-N25-treated mice than in those of control mice (Figure 3A). Immunohistochemical analysis indicated that, within 28 days after immunization, levels of HCV core protein were substantially lower in the livers of CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice than in those of control mice (Figure 3B). Importantly, neither resolution of chronic hepatitis nor reduction in the HCV protein levels was observed in the mice treated with LC16m8, rVV-CN2, or rVV-CN5. These results indicated that HCV nonstructural proteins might be important for effects of therapeutic vaccines. In contrast, rVV-CN5 which encoded HCV structural and non-structural proteins did not show any significant effects. These results indicated that HCV structural proteins might have inhibited the therapeutic effects of the non-structural proteins. Therefore, it may be important to exclude the HCV structural proteins (aa 1–541) as antigenic proteins when developing therapeutic vaccines against chronic hepatitis C.

In addition, we measured serum ALT levels in CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice from all four treatment groups 28 days after rVV-HCV immunization. Serum ALT levels were not significant-

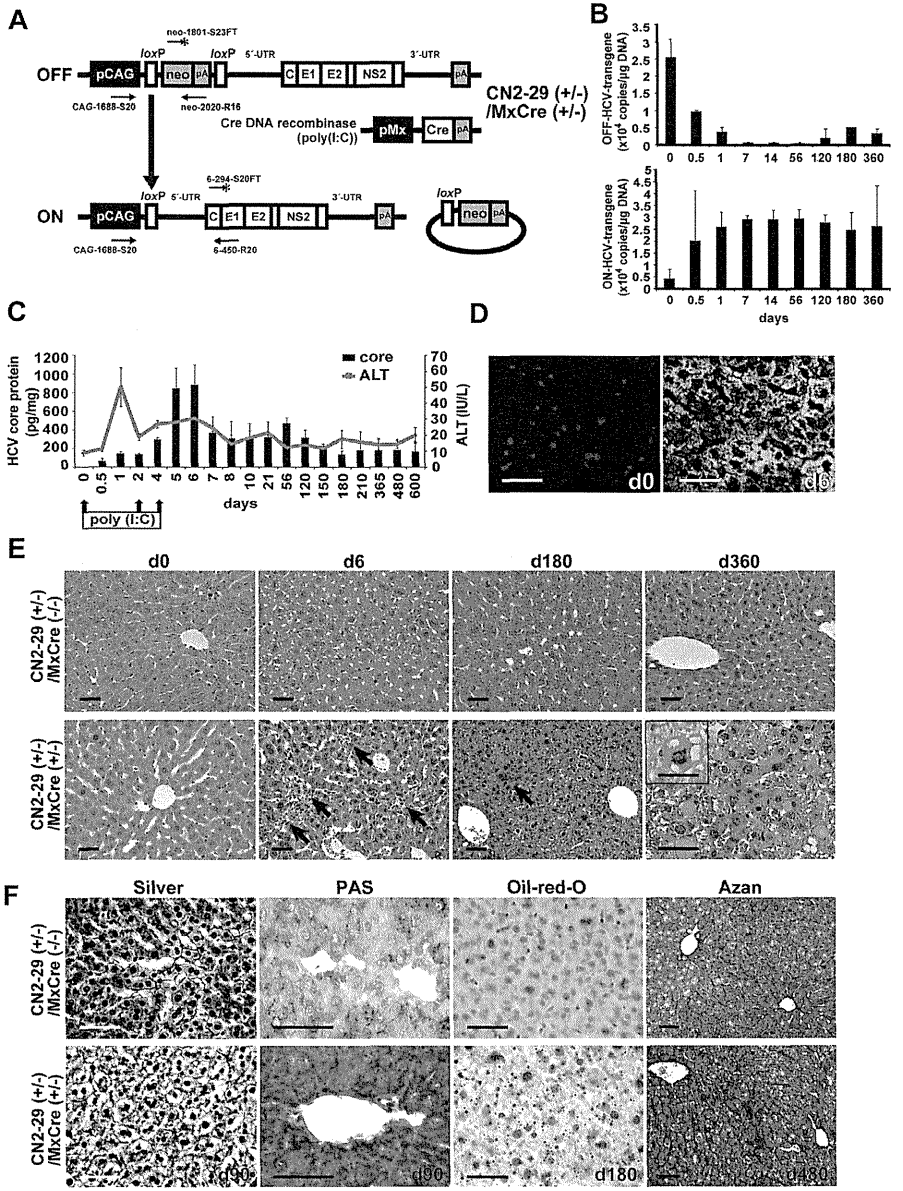

Figure 1. Pathogenesis in immunocompetent mice with persistent HCV expression. (**A**) Structure of CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ and the Cre-mediated activation of the transgene unit. R6CN2 HCV cDNA was cloned downstream of the CAG promoter, neomycin-resistant gene (*neo*), and poly A (pA) signal flanked by two *loxP* sequences. This cDNA contains the core, E1, E2, and NS2 regions. (**B**) Cre-mediated genomic DNA recombination. After poly(I:C) injection, genomic DNA was extracted from liver tissues and analyzed by quantitative RTD-PCR for Cre-mediated transgenic recombination. The transgene was almost fully recombined in transgenic mouse livers 7 days after the injection. In all cases, n = 3 mice per group. (**C**) HCV core protein expression was sustained for at least 600 days after poly(I:C) injection. (**D**) Immunohistochemical analysis revealed that most hepatocytes expressed the HCV core protein within 6 days after injection. (**E**) Liver sections from CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice after the poly(I:C) injection. Infiltrating lymphocytes (arrows) were observed on days 6 and 180; Hepatocellular carcinoma (HCC) was observed on day 360. In contrast, these pathological changes were not observed in CN2-29$^{(+/-)}$/MxCre$^{(-/-)}$ mice after the injection. The inset image shows abnormal mitosis in a tumor cell. (**F**) Hepatocyte swelling and abnormal architecture of liver-cell cords (silver staining), as well as abnormal glycogen accumulation (PAS staining) were observed on day 90 in CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice. We observed steatosis (oil-red-O staining) on day 180 and, subsequently, fibrosis (Azan staining) on day 480. The scale bars indicate 50 μm.
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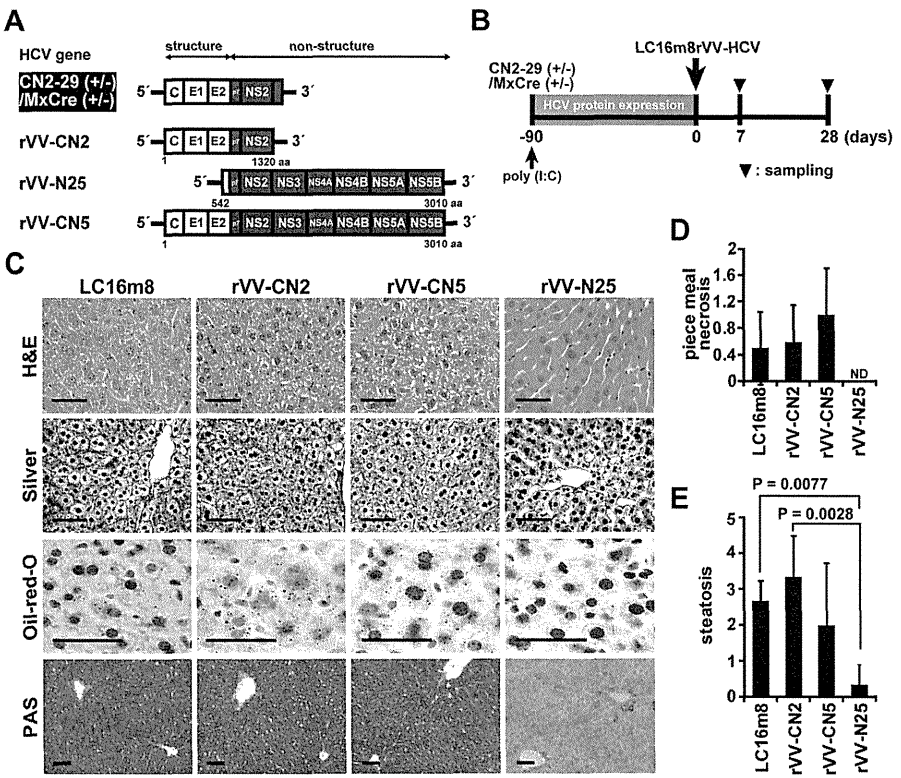

Figure 2. Effects of rVV-HCV treatment on the CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice. (**A**) HCV gene structure in the CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice and recombinant vaccinia viruses (rVV-HCV). MxCre/CN2-29 cDNA contains the core, E1, E2, and NS2 regions. The rVV-CN2 cDNA contains the core, E1, E2, and NS2 regions. The rVV-N25 cDNA contains the NS2, NS3, NS4A, NS4B, NS5A, and NS5B regions. The rVV-CN5 cDNA contains the entire HCV region. (**B**) Four groups of CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice were inoculated intradermally with rVV-CN2, rVV-N25, rVV-CN5, or LC16m8 90 days after the poly(I:C) injection. Blood, liver, and spleen tissue samples were collected 7 and 28 days after the inoculation. (**C**) Liver sections from the four groups of CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice 7 days after the inoculation. The sections were stained with H&E, silver, oil-red-O, or PAS. The scale bars indicate 50 μm. (**D**) Histological evaluation of piecemeal necrosis in the four groups of CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice 7 days after inoculation. (**E**) Histological evaluation of steatosis in the four groups of CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice 7 days after inoculation. Significant relationships are indicated by a P-value.
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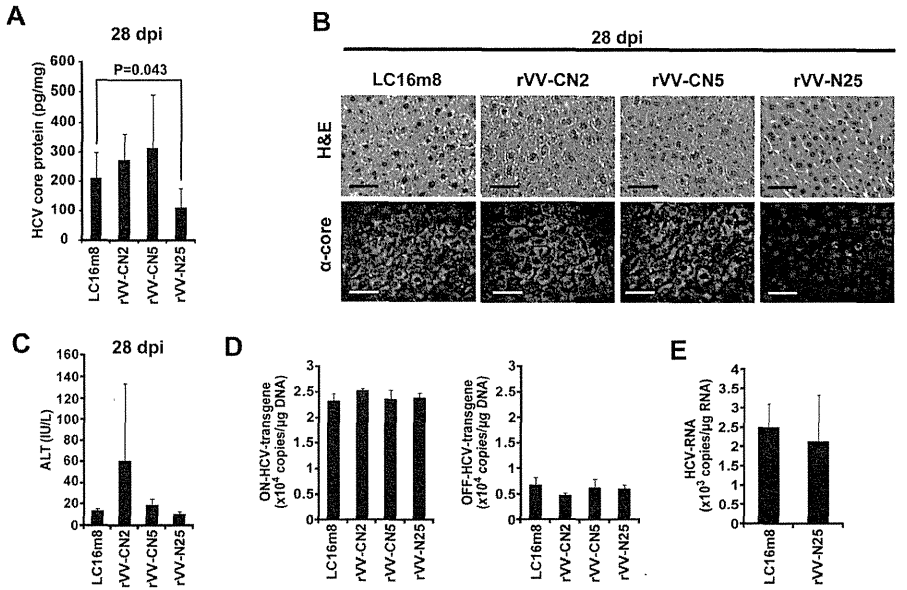

Figure 3. Effects of HCV core protein expression on the livers of CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice inoculated with rVV-HCV. (**A**) Expression of the HCV core protein in the four treatment groups of CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice 28 days after the inoculation. Significant relationships are indicated by a P-value. (**B**) H&E staining and immunohistochemical analysis for HCV core protein in the LC16m8-, rVV-CN2-, rVV-CN5-, or rVV-N25-treated CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice 28 days after the inoculation. Liver sections were stained with the anti-core monoclonal antibody. The scale bars indicate 50 μm. (**C**) Effects of HCV core protein expression on serum ALT levels in the four treatment groups of CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice 28 days after immunization. (**D**) Cre-mediated genomic DNA recombination in the four treatment groups 28 days after immunization. (**E**) Expression of HCV mRNA in the LC16m8- or rVV-N25-treated CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice 28 days after immunization. In all cases, n = 6 mice per group.
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ly different in the rVV-N25-treated mice and control mice (Figure 3C); this finding indicated that rVV-N25 treatment did not cause liver injury and that the antiviral effect was independent of hepatocyte destruction.

We hypothesized that the reduction in the levels of HCV core protein in rVV-HCV-treated mice was not caused by cytolytic elimination of hepatocytes that expressed HCV proteins. To investigate this hypothesis, we conducted an RTD-PCR analysis of genomic DNA from liver samples of CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice. The recombined transgene was similar in rVV-N25-treated and control mice 28 days after immunization (Figure 3D). We also measured the expression of HCV mRNA in LC16m8-treated CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice with that in rVV-N25-treated CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice 28 days after immunization; the HCV mRNA levels did not differ between rVV-N25-treated CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ and control mice (Figure 3E). These results indicated that rVV-N25-induced suppression of HCV core protein expression could be controlled at a posttranscriptional level.

Role of CD4 and CD8 T cells in rVV-N25-treated Mice

Viral clearance is usually associated with CD4 and CD8 T-cell activity that is regulated by cytolytic or noncytolytic antiviral mechanism [14]. To determine whether CD4 or CD8 T-cell activity was required for the reduction in HCV core protein levels in the livers of transgenic mice, we analyzed the core protein levels in CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice immunized with rVV-N25 in the absence of CD4 or CD8 T cells (Figure 4A). As expected, the mice lacking CD4 or CD8 T cells failed to show a reduction in HCV core protein levels (Figure 4B).

However, in mice lacking either CD4 or CD8 T-cells, the pathological changes associated with chronic hepatitis were resolved following rVV-N25 immunization, and the steatosis score of rVV-N25-treated mice was significantly lower than that of control mice (Figures 4C–E). These results indicated that CD4 and CD8 T cells were not responsible for the rVV-N25-induced amelioration of histological findings and that other inflammatory cell types may play an as-yet-unidentified role in the resolution of the pathological changes in these mice.

rVV-N25 Immunization Induced an NS2-specific Activated CD8 T cells Response

Because we found that HCV protein reduction in the liver required CD8 T cells, we tested whether HCV-specific CD8 T cells were present in splenocytes 28 days after immunization. To determine the functional reactivity of HCV-specific CD8$^+$ T cells, we performed a CD107a mobilization assay and intracellular IFN-γ staining. CN2-29 transgenic mice expressed the HCV structural protein and the NS2 region. However, rVV-N25 comprised only

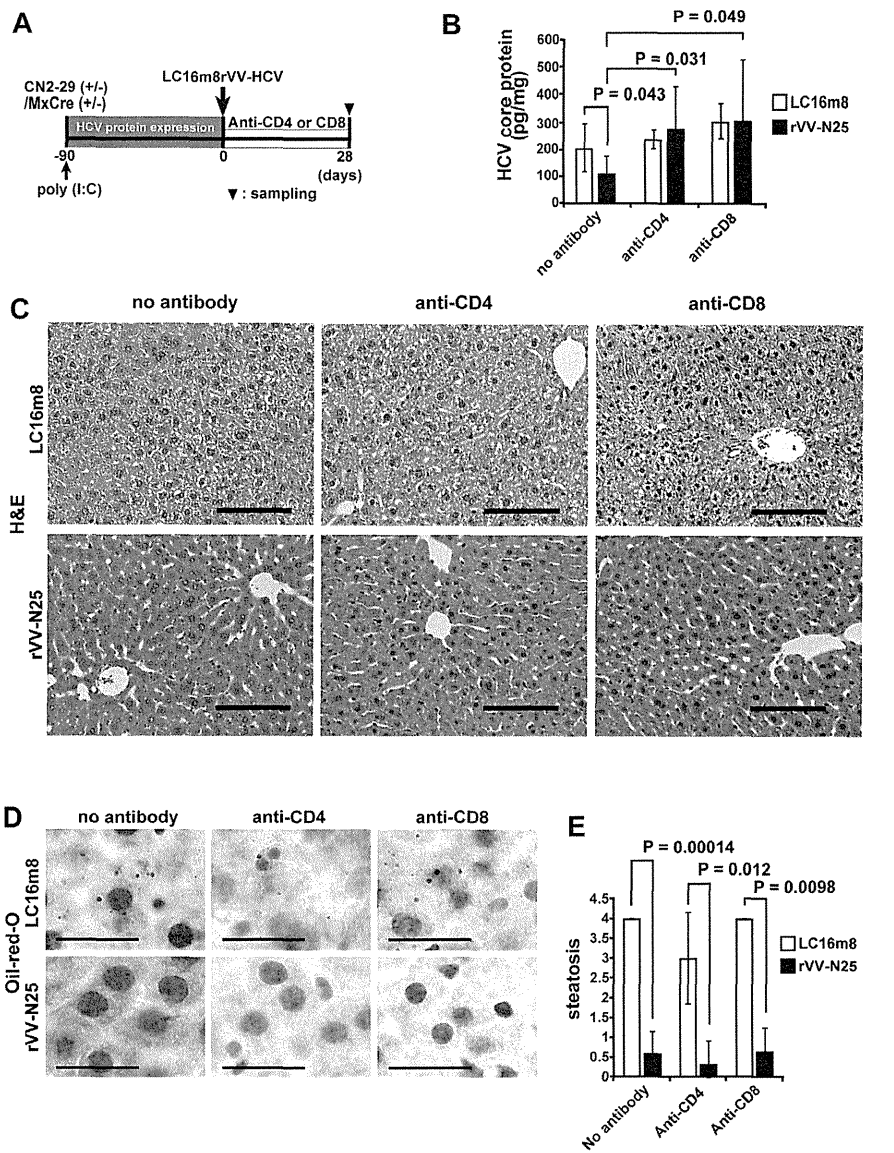

Figure 4. Role of CD4 and CD8 T cells in rVV-N25-treated mice. (**A**) Schematic diagram depicts depletion of CD4 and CD8 T cells via treatment with monoclonal antibodies. (**B**) Comparison of HCV core protein expression in control, CD4-depleted, and CD8-depleted mice 28 days after immunization with LC16m8 or rVV-N25. (**C, D**) Histological analysis of liver samples from CD4-depleted or CD8-depleted CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice

28 days after immunization with LC16m8 or rVV-N25. The scale bars indicate 100 μm (**C**) and 50 μm (**D**). (**E**) Histological evaluation of steatosis in liver samples from CD4-depleted or CD8-depleted CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice 28 days after immunization with LC16m8 or rVV-N25. Significant relationships are indicated by a P-value.
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a HCV nonstructural protein. Thus, we focused on the role of the NS2 region as the target for CD8 T cells and generated EL-4 cell lines that expressed the NS2 antigen or the CN2 antigen.

Isolated splenocytes from immunized mice were co-cultured with EL-4CN2 or EL-4NS2 cell lines for 2 weeks and analyzed.

Cytolytic cell activation can be measured using CD107a, a marker of degranulation [15]. The ratio of CD8$^+$CD107a$^+$ cells to all CD8 T cells significantly increased in rVV-N25-treated splenocytes after co-culture with EL-4CN2 or EL-4NS2 ($P<0.05$), whereas splenocytes that had been treated with any other rVV were not detected (Figure 5A, B and C). These results indicated that rVV-N25 treatment increased the frequency of HCV NS2-specific activated CD8 T cells. Consistent with these results, the ratio of CD8$^+$IFN-γ$^+$ cells to all CD8 T cells for rVV-N25-treated mice was also significantly higher than that for mice treated with any other rVV ($P<0.05$). Taken together, these findings indicated that rVV-N25 induced an effective CD8 T-cell immune response and that NS2 is an important epitope for CD8 T cells.

rVV-N25 Immunization Suppressed Inflammatory Cytokines Production

To determine whether rVV-N25 treatment affected inflammatory cytokine production, we measured serum levels of inflammatory cytokines after rVV immunization. The serum levels of these inflammatory cytokines increased in the CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice (Figure 6A, Figure S5). Immunization with rVV-N25 affected serum levels of inflammatory cytokines in CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice and caused a return to the cytokine levels observed in wild-type untreated mice (Figure 6A). In wild-type mice, the cytokine levels remained unchanged after immunization (Figure 6A). These results indicated that inflammatory cytokines were responsible for liver pathogenesis in the transgenic mice.

To test the hypothesis that inflammatory cytokines were responsible for liver pathogenesis in CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice, we administered transgenic mouse serum intravenously into nontransgenic mice. We observed the development of chronic hepatitis in the nontransgenic mice within 7 days after the serum transfer (Figures 6B and C). This finding was consistent with the

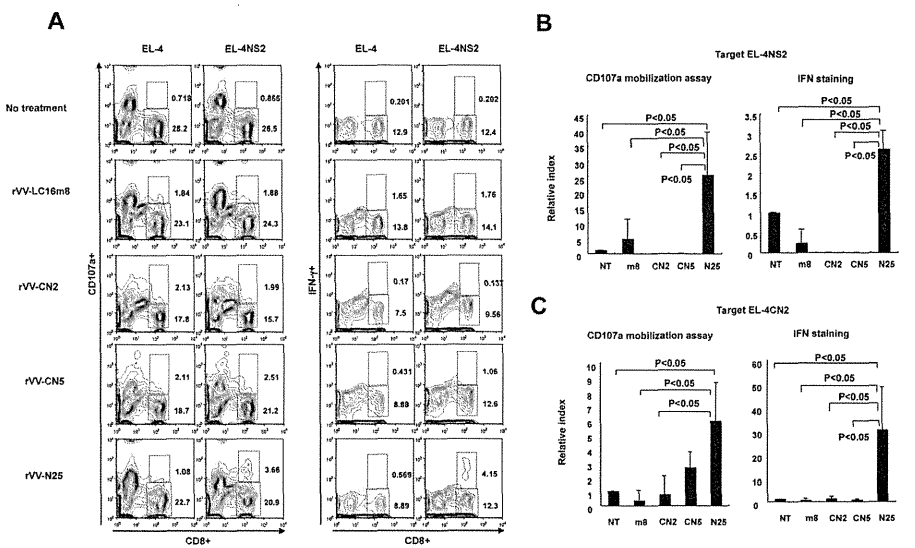

Figure 5. Immunization with rVV-N25 induced CD8 T-cell degranulation, a marker for cytotoxicity, and IFN-γ production. (**A**) The numbers represent the percentage of CD107a positive cells and negative cells (left two columns) and IFN-γ-positive cells and negative cells (right two columns). (**B, C**) The ratio of CD8$^+$IFN-γ$^+$ cells to all CD8 T cells for rVV-N25-treated mice was significantly higher than that for mice treated with any other rVV. Splenocytes (4×10^6 per well) were cultured with EL-4CN2 or EL-4NS2 cell lines in RPMI 1640 complete medium including 3% T-STIM^{TM} with ConA for 2 weeks. Harvested cells were incubated for 4 h with EL-4, EL-4CN2, or EL-4NS2 in combination with PE-labeled anti-CD107a mAb and monensin in RPMI 1640 complete medium with 50 IU/mL IL-2, according to the manufacturer's instruction. After incubation, cell suspensions were washed with PBS, and the cells were further stained with APC-labeled anti-IFN-γ mAb and Pacific blue-labeled anti-CD8 mAb. Harvested cells were stained with anti-CD107a-PE, anti-IFN-γ-APC, or anti-CD8-Pacific blue. Results that are representative of three independent experiments are shown. Significant relationships are indicated by P-value.
doi:10.1371/journal.pone.0051656.g005

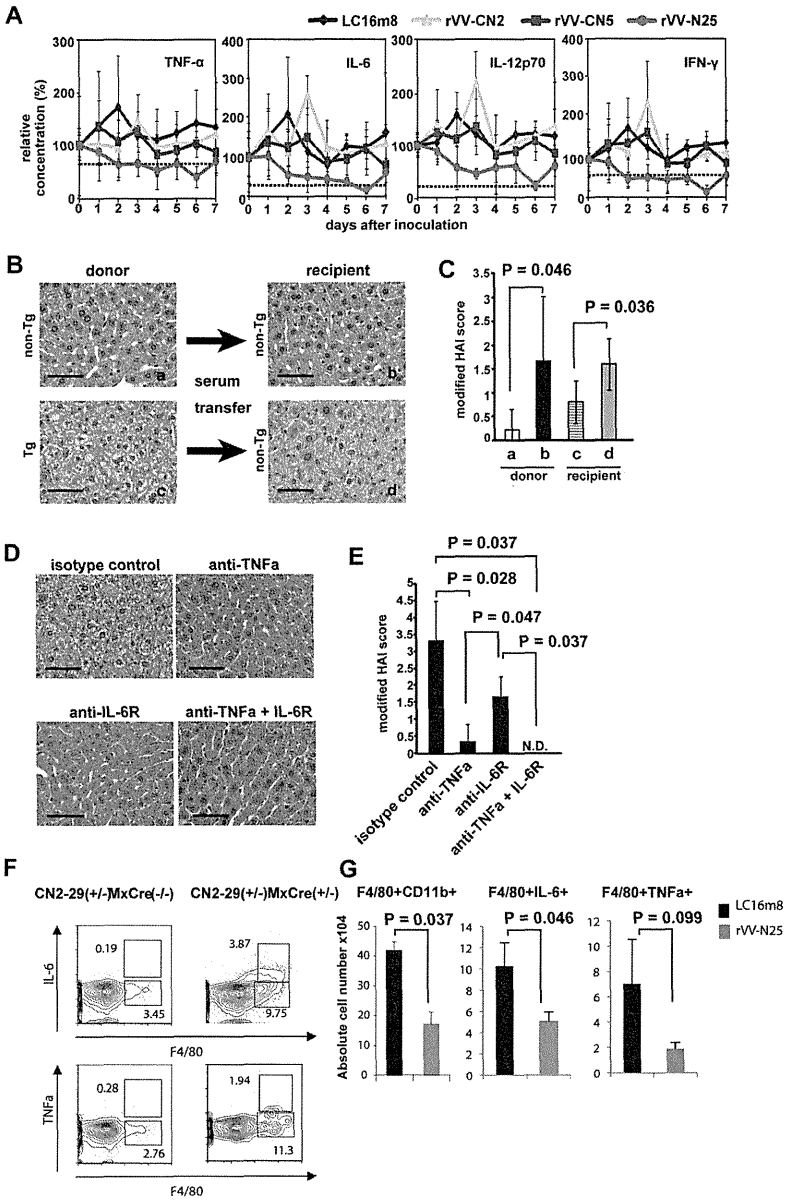

Figure 6. Immunization with rVV-N25 suppresses serum inflammatory cytokine levels. (**A**) Daily cytokine levels in the serum of CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice during the week following immunization with LC16m8, rVV-CN2, rVV-N25, or rVV-CN5. Values represent means ± SD (n = 3) and reflect the concentrations relative to those measured on day 0. The broken lines indicate the baseline data from wild-type mice. In all cases, n = 6 mice per group. (**B**) Liver sections from CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ and CN2-29$^{(+/-)}$/MxCre$^{(-/-)}$ mice. (**C**) Histology activity index (HAI) scores of liver samples taken from CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$, or CN2-29$^{(+/-)}$/MxCre$^{(-/-)}$ mice. (**D**) Liver sections from CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice in which TNF-α was neutralized and the IL-6 receptor was blocked. The scale bars indicate 50 μm. (**E**) HAI scores of liver samples taken from CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ in which TNF-α was neutralized and the IL-6 receptor was blocked. Tg and non-Tg indicate CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ and CN2-29$^{(+/-)}$/MxCre$^{(-/-)}$, respectively. (F) Macrophages were the main producers of TNF-α and IL-6 in CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice following poly(I:C) injection. (G) Immunization with rVV-N25 reduced the number of macrophages in liver samples from CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice and suppressed TNF-α and IL-6 production from macrophages (Figure 6G). Significant relationships are indicated by a P-value.
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hypothesis that inflammatory mediators played a key role in inducing hepatitis. Furthermore, to investigate whether TNF-α and IL-6 played particularly critical roles in the pathogenesis of chronic hepatitis in the transgenic mice, we neutralized TNF-α and blocked the IL-6 receptor in the livers of these mice. As expected, chronic hepatitis did not develop in these mice. (Figure 6D and E).

Next, to determine which cell population(s) produced TNF-α, IL-6, or both during continuous HCV expression in CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice, we isolated intrahepatic lymphocytes (IHLs) and labeled the macrophages (the F4/80^{+} cells) with anti-TNF-α and anti-IL-6 antibodies using an intracellular cytokine detection method. Macrophages in CN2-29$^{(+/-)}$/MxCre$^{(-/-)}$ mice produced small amounts of TNF-α and IL-6, while those in CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice produced much larger amounts of these cytokines (Figure 6F).

Finally, we evaluated whether rVV-N25 treatment affected the number of macrophages, cytokine production by macrophages, or both; specifically, we isolated IHLs from CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice 7 days after immunization with rVV-N25 or with LC16m8. The percentage of macrophages (CD11b^{+}F4/80^{+}) among IHLs and IL-6 production from these macrophages were significantly lower in rVV-N25-treated mice than in control mice (Figure 6G). Though the percentage of TNF-α-producing macrophages was not significantly different in rVV-N25-treated and control mice (P = 0.099), rVV-N25 treatment appeared to suppress these macrophages. These results demonstrated that rVV-N25 had a suppressive effect on activated macrophages, and they indicated that this suppression ameliorated the histological indicators of chronic hepatitis.

Discussion

Various HCV transgenic mouse models have been developed and used to examine immune response to HCV expression and the effects of pathogenic HCV protein on hepatocytes [4,16,17]. However, these transgenic mice develop tolerance to the HCV protein; therefore, examining immune response to HCV protein has been difficult.

To overcome the problem of immune tolerance in mouse models of HCV expression, we developed an HCV model in mice that relies on conditional expression of the core, E1, E2, and NS2 proteins and the Cre/loxP switching system [5,6]; we showed that the injection of an Ad-Cre vector enhanced the frequency of HCV-specific activated CD8 T cells in the liver of these mice and caused liver injury. However, the Ad-Cre adenovirus vector alone causes acute hepatitis in wild-type mice. Nevertheless, the transgenic model was useful for evaluating interactions between the host immune system and viral protein (serum ALT level over 2,000 IU/L) [5]; HCV core protein levels were reduced and expression of this protein was transient (about 2 weeks). Therefore, this Ad-Cre-dependent model cannot be used to effectively investigate immune responses to chronic HCV hepatitis.

Here, we used poly (I:C)-induced expression of Cre recombinase to generate HCV transgenic mice in order to study the effect of HCV protein and confirmed that these mice developed chronic active hepatitis–including steatosis, lipid deposition, and hepatocellular carcinoma. These pathological findings in the transgenic mice were very similar to those in humans with chronic hepatitis C; therefore, this mouse model of HCV may be useful for analyzing the immune response to chronic hepatitis. However, experimental results obtained with this mouse model may not directly translate to clinical findings from patients with HCV infection because the expression of HCV proteins was not liver specific in these mice. Furthermore, poly(I:C) injection can activate innate immune responses and, consequently, might induce temporary liver injury [18]. Additionally, poly(I:C) injection has an adjuvant effect; specifically, it stimulates TLR3 signaling [19].

To evaluate whether poly(I:C) injection caused hepatitis in CN2-29$^{(+/-)}$/MxCre$^{(-/-)}$ mice, we examined serum ALT levels and liver histology following poly(I:C) injection. We found that, following poly(I:C) injection, serum ALT levels in CN2-29$^{(+/-)}$/MxCre$^{(-/-)}$ mice increased, reached a peak one day after injection, declined from day 1 to day 6, and were not elevated thereafter; this time-course indicated that poly(I:C) injection alone did not induced continuous liver injury (figure S6). Based on these findings, we believe that the effects of poly(I:C) injection in these mice did not confound our analysis of chronic hepatitis.

Immunization with rVV-N25 suppressed HCV protein levels in the liver, and this suppression was associated with ameliorated pathological chronic hepatitis findings (see Figure 3). Importantly, rVV-N25 treatment did not cause liver injury based on the serum ALT levels; therefore, this treatment was unlikely to have cytopathic effects on infected hepatocytes. These findings provided strong evidence that rVV-N25 treatment effectively halted the progression of chronic hepatitis. Immunization with plasmid DNA or with recombinant vaccinia virus can effectively induce cellular and humoral immune responses and exert a protective effect against challenge with HCV infection [20,21]. However, findings from these previous studies revealed HCV immunization of both uninfected, naïve animals and immune-tolerant animals induced a HCV-specific immune response. In the model describe here; the animals were immune competent for HCV; therefore, our findings provided further important evidence that rVV-N25 was effective in the treatment of chronic hepatitis.

In addition, we demonstrated that rVV-N25 treatment in the absence of CD4 and CD8 T cells had no effect on HCV clearance. This important observation indicated that rVV-N25-induced HCV clearance was mediated by CD4 and CD8 T cells. Many studies have shown that spontaneous viral clearance during acute HCV infection is characterized by a vigorous, broadly reactive CD4 and CD8 T-cell response. [8,22] HCV clearance and hepatocellular cytotoxicity are both mediated by CD8 antigen-specific (cytotoxic T lymphocyte) CTLs [23]. Consistent with these observations, rVV-N25 treatment effectively induced the accumulation of NS2-specific CD8 T cells, which express high levels of CD107a and IFN-γin the spleen. Notably, even with rVV-N25 immunization, the frequency of activated CD8 T cells was very low, and a minimum of 2-weeks incubation was required to distinguish the difference between rVV treatments. Even if a small population of specific CD8+ T cells played a relevant role in the reduction of core protein, it is difficult to assert that the only NS2-specific CD8+ T cells were important to this reduction. However, based on the results presented in Figure 4B, we are able to conclude that at least CD8+ and/or CD4+ T cells were important to the reduction in HCV core protein. Therefore, to elucidate the mechanism of HCV protein clearance, further investigation of not only the other T cell epitopes but also other immunocompetent cells is required.

Interestingly, rVV-N25 treatment–but not the rVV-CN2 or rVV-CN5 treatment–efficiently induced a HCV-specific activated CD8 T cells response; this difference in efficacy could have one or more possible causes. The HCV structural proteins (core, E1, and E2 proteins) in the rVV-CN construct may cause the difference; Saito et al. reported that injection with plasmid constructs encoding the core protein induced a specific CTL response in BALB/c mice [24]. Reportedly, CTL activity against core or envelope protein is completely absent from transgenic mice immunized with a plasmid encoding the HCV structural proteins, but core-specific CTL activity is present in transgenic mice that were immunized with a plasmid encoding the HCV core [21]. In contrast, when recombinant vaccinia virus expressing different regions of the HCV polyprotein were injected into BALB/c mice, only the HCV core protein markedly suppressed vaccinia-specific CTL responses [25]. Thus, the HCV core protein may have an immunomodulatory function [26]. Based on these reports and our results, we hypothesize that the causes underlying the effectiveness of rVV-N25 treatment were as follows: 1) this rVV construct included the core and envelope proteins and 2) the core protein had an immune-suppressive effect on CTL induction. Therefore, we suggest that exclusion of the core and envelope antigen as immunogen is one important factor in HCV vaccine design.

Interestingly, immunization with rVV-N25 rapidly suppressed the inflammatory response; however, immunization with either of the other rVVs did not (see Figure 6A). This result indicated that rVV-N25 may modulate inflammation via innate immunity, as well as via acquired immunity. Reportedly, Toll-like receptor (TLR)-dependent recognition pathways play a role in the recognition of poxviruses [27]. TLR2 and TLR9 have also been implicated in the recognition of the vaccinia virus [28,29]. These findings indicate that TLR on dendritic cells may modulate the immunosuppressive effect of rVV-N25 in our model of HCV infection; however, further examination of this hypothesis is required. The finding that pathological symptoms in the HCV transgenic mice were completely blocked by intravenous injection of TNF-α and IL-6 neutralizing antibodies indicated that the progression of chronic hepatitis depended on inflammatory cytokines in serum, rather than the HCV protein levels in hepatocytes. Lymphocytes, macrophages, hepatocytes, and adipocytes each produce TNF-α and IL-6 [30,31], and HCV-infected patients have elevated levels of TNF-α and IL-6 [32,33]. Both cytokines also contribute to the maintenance of hepatosteatosis in mice fed a high-fat diet [34], and production of TNF-α and IL-6 is elevated in obese mice due to the low grade inflammatory response that is caused by lipid accumulation [35]. These findings indicate that both cytokines are responsible for HCV-triggered hepatosteatosis, and anti-cytokine neutralization is a potential treatment for chronic hepatitis if antiviral therapy is not successful.

The reduction of macrophages in number might be due to the induction of apoptosis by vaccinia virus *in vitro* infection as previous reported [36]. To understand the mechanisms responsible for the reduction of the number of macrophage, we performed another experiment to confirm whether the macrophages were infected with vaccinia virus inoculation. However, based on PCR analyses; vaccinia virus DNA was not present in liver tissue that contained macrophages (Figure S7). Furthermore, apoptosis of macrophages was not detected in liver samples (Data not shown). Based on these results, it is unlikely that the reduction in the number of macrophages was due to apoptosis induced by vaccinia virus infection. Although rVV-N25 reduced the number of macrophage, precise mechanism is still unknown. Further examination to elucidate the mechanism is required.

In conclusion, our findings demonstrated that rVV-N25 is a promising candidate for an HCV vaccine therapy. Additionally, the findings of this study indicate that rVV-N25 immunization can be used for prevention of HCV infection and as an antiviral therapy against ongoing HCV infection.

Materials and Methods

Ethics Statement

All animal care and experimental procedures were performed according to the guidelines established by the Tokyo Metropolitan Institute of Medical Science Subcommittee on Laboratory Animal Care; these guidelines conform to the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology, Japan, 2006. All protocols were approved by the Committee on the Ethics of Animal Experiments of the Tokyo Metropolitan Institute of Medical Science (Permit Number: 11–078). All efforts were made to minimize the suffering of the animals.

Animals

R6CN2 HCV cDNA (nt 294–3435) [37] and full genomic HCV cDNA (nt 1–9611) [38,39] were cloned from a blood sample taken from a patient (#R6) with chronic active hepatitis (Text S1). The infectious titer of this blood sample has been previously reported [40]. R6CN2HCV and R6CN5HCV transgenic mice were bred with Mx1-Cre transgenic mice (purchased from Jackson Laboratory) to produce R6CN2HCV-MxCre and R6CN5HCV-MxCre transgenic mice, which were designated CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ and RzCN5-15$^{(+/-)}$/MxCre$^{(+/-)}$ mice, respectively. Cre expression in the livers of these mice was induced by intraperitoneal injection of polyinosinic acid–polycytidylic acid [poly(I:C)] (GE Healthcare UK Ltd., Buckinghamshire, England); 300 μL of a poly(I:C) solution (1 mg/mL in phosphate-buffered saline [PBS]) was injected three times at 48-h intervals. All animal care and experimental procedures were performed according to the guidelines established by the Tokyo Metropolitan Institute of Medical Science Subcommittee on Laboratory Animal Care.

Histology and Immunohistochemical Staining

Tissue samples were fixed in 4% paraformaldehyde in PBS, embedded in paraffin, sectioned (4-μm thickness), and stained with hematoxylin and eosin (H&E). Staining with periodic acid–Schiff stain, Azan stain, silver, or Oil-red-O was also performed to visualize glycogen degeneration, fibrillization, reticular fiber degeneration, or lipid degeneration, respectively.

For immunohistochemical staining, unfixed frozen liver sections were fixed in 4% paraformaldehyde for 10 min and then incubated with blocking buffer (1% bovine serum albumin in PBS) for 30 min at room temperature. Subsequently, the sections were incubated with biotinylated mouse anti-HCV core mono-

clonal antibody (5E3) for 2 h at room temperature. After being washed with PBS, the sections were incubated with streptavidin–Alexa Fluor 488 (Invitrogen). The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Fluorescence was observed using a confocal laser microscope (Laser scanning microscope 510, Carl Zeiss).

Generation of rVVs

The pBR322-based plasmid vector pBMSF7C contained the ATI/p7.5 hybrid promoter within the hemagglutinin gene region of the vaccinia virus, which was reconstructed from the pSEJ1-10 plasmid and pBM vector [41,42]. Separate full-length cDNAs encoding either the HCV structural protein, nonstructural protein, or all HCV proteins were cloned from HCV R6 strain (genotype 1b) RNA by RT-PCR. Each cDNA was inserted into a separate pBMSF7C vector downstream of the pBMSF7C ATI/p7.5 hybrid promoter; the final designation of each recombinant plasmid was pBMSF7C-CN2, pBMSF7C-N25, or pBMSF-CN5 (Figure 2). They were then transfected into primary rabbit kidney cells infected with LC16m8 (multiplicity of infection = 10). The virus–cell mixture was harvested 24 h after the initial transfection by scrapping; the mixture was then frozen at −80°C until use. The hemagglutinin-negative recombinant viruses were cloned as previously described [42] and named rVV-CN2, rVV-N25, or rVV-CN5. Insertion of the HCV protein genes into the LC16m8 genome was confirmed by direct PCR, and expression of each protein from the recombinant viruses was confirmed by western blot analysis. The titers of rVV-CN2, rVV-N25, and rVV-CN5 were determined using a standard plaque assay and RK13 cells.

Statistical Analysis

Data are shown as mean ± SD. Data were analyzed using the nonparametric Mann–Whitney or Kruskal–Wallis tests or ANOVA as appropriate; GraphPad Prism 5 for Macintosh (GraphPad) was used for all analyses. P values <0.05 were considered statistically significant.

Supporting Information

Figure S1 HAI score of liver samples taken from CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice.
(EPS)

Figure S2 Lipid degeneration in samples of liver taken from CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice.
(EPS)

Figure S3 HCV protein expression after infection of LC16m8, rVV-CN2, rVV-N25, or rVV-CN5 into HepG2 cells.
(EPS)

Figure S4 Effects of treatment with rVV-N25 in RzCN5-15$^{(+/-)}$/MxCre$^{(+/-)}$ mice.
(EPS)

Figure S5 Daily cytokine profiles of the serum from CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice during the week following inoculation with LC16m8, rVV-CN2, rVV-N25, or rVV-CN5.
(EPS)

Figure S6 The immune response following poly(I:C) injection in the acute phase.
(EPS)

Figure S7 Detection of vaccinia virus DNA in the skin, liver, and spleen after inoculation with attenuated vaccinia virus (Lister strain) or highly attenuated vaccinia virus (LC16m8 strain).
(EPS)

Table S1 Incidence of hepatocellular carcinoma in male and female transgenic mice at 360, 480, and 600 days after poly(I:C) injection.
(EPS)

Text S1 Supporting information including material and methods, and references.
(DOCX)

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Author Contributions

Performed the experiments: SS KK TC Y. Tobita TO FY Y. Tokunaga. Analyzed the data: SS KK TC MK. Contributed reagents/materials/analysis tools: KT-K TW TT MM K. Mizuno YH TH K. Matsushima. Wrote the paper: SS KK MK. Study concept and design: MK.

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Review

Role of Oxidative Stress in Hepatocarcinogenesis Induced by Hepatitis C Virus

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Abstract: Hepatitis C virus (HCV) easily establishes chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC). During the progression of HCV infections, reactive oxygen species (ROS) are generated, and these ROS then induce significant DNA damage. The role of ROS in the pathogenesis of HCV infection is still not fully understood. Recently, we found that HCV induced the expression of 3β-hydroxysterol Δ24-reductase (DHCR24). We also found that a HCV responsive region is present in the 5'-flanking genomic promoter region of DHCR24 and the HCV responsive region was characterized as (−167/−140). Moreover, the transcription factor Sp1 was found to bind to this region in response to oxidative stress under the regulation of ataxia telangiectasia mutated (ATM) kinase. Overexpression of DHCR24 impaired p53 activity by suppression of acetylation and increased interaction with MDM2. This impairment of p53 suppressed the hydrogen peroxide-induced apoptotic response in hepatocytes. Thus, a target of oxidative stress in HCV infection is DHCR24 through Sp1, which suppresses apoptotic responses and increases tumorigenicity.

Keywords: hepatitis C virus; reactive oxygen species; 3β-hydroxysterol Δ24-reductase

1. Introduction

Hepatitis C virus (HCV) is a member of the *Flaviviridae* family of RNA viruses, and possesses a positive-strand RNA genome [1]. HCV mainly replicates in the cytoplasm, but frequently establishes chronic infections, leading to the development of chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) [2,3]. The estimated worldwide prevalence of HCV infections is 2.2%–3.0% [4], and

chronic HCV infection is a major global public health concern. HCV does not possess canonical oncogenes and is unable to integrate into the host genome, but easily establishes chronic infections, resulting in HCC with high frequency. The exact mechanism by which this occurs is not fully understood; however, possible mediators of HCV pathogenesis are reactive oxygen species (ROS). During chronic hepatitis, the immune response induces the production of ROS [5] and nitric oxide (NO) [6]. Furthermore, HCV viral nucleocapsid protein, an HCV core protein, was shown to increase oxidative stress in the liver [7,8]. Moreover, HCV affects the steady-state levels of a mitochondrial protein chaperone known as prohibitin, leading to impaired function of the mitochondrial respiratory chain with the overproduction of ROS [9]. On the other hand, HCV compromises some of the antioxidant systems, including haeme oxygenase-1 [10] and NADH dehydrogenase quinone 1 [9], resulting in the provocation of oxidative stress in the liver during HCV infections. Thus, HCV infections not only induce ROS overproduction, but also hamper the antioxidant system in the liver. The induction of oxidative stress also results in the generation of deletions in mitochondrial and nuclear DNA, which are indicators of genetic damage. NO has been shown to induce oxidative DNA damage and inhibit DNA repair [11–13]. These nucleotide abnormalities may contribute to the development of HCC [14].

2. Survey of HCV-Positive HCC-Related Host Factors

To define the host factors involved in hepatocarcinogenesis during HCV persistent infections, we established a human hepatoblastoma-derived cell line (HepG2), which expresses the full-length HCV genome under the control of a Cre/*lox*P system (RzM6 cells [15]). Using colony-formation assays and nude mice tumor-formation assays, we found that passaging of HCV-expressing cells (RzM6-LC cells) increased their tumorigenicity. To identify which pathway was responsible for the increase in tumorigenicity in RzM6-LC cells, we raised monoclonal antibodies against the RzM6-LC cells and characterized them [16]. We found that one of these clones (2-152a) recognizes 3β-hydroxysterol Δ24-reductase (or dehydrocholesterol reductase 24; DHCR24). DHCR24 functions as an enzyme that catalyzes the conversion of desmosterol to cholesterol in the post-squalene cholesterol biosynthesis pathway [17,18]. The absence of DHCR24 leads to desmosterosis [19]. Furthermore, expression of DHCR24 is down-regulated in areas of the brain affected by Alzheimer's disease [20]. DHCR24 is a multifunctional enzyme, which exerts resistance against oxidative stress and prevents apoptotic cell death when it is expressed at high levels [20–24]. Endogenous DHCR24/seladin-1 levels are up-regulated in response to acute oxidative stress [21,25,26], but the expression levels decline upon chronic exposure to oxidative stress [21,22]. DHCR24 is also reported to function as a hydrogen peroxide scavenger [24]. Thus, DHCR24 plays a crucial role in maintaining cellular physiology by regulating both cholesterol synthesis and cellular defence against oxidative stress, although the biological relevance of the hydrogen peroxide concentration (0.5–2 mM) used in some experiments requires future study.

3. HCV Induces DHCR24 Expression through Oxidative Stress

Since we observed up-regulation of DHCR24 expression in RzM6-LC cells, we decided to characterize the effects of HCV on DHCR24 expression [16,27]. Silencing of HCV by siRNA in RzM6-LC cells down-regulated the expression of DHCR24. By using chimeric mice with humanized

liver [28], HCV infection induced the up-regulation of DHCR24 expression in human hepatocytes, whereas hepatitis B virus (HBV) infection had no significant effect on DHCR24 expression [16]. The regulation of DHCR24 expression was elicited at the transcriptional level. Therefore, we cloned the 5′-flanking region of the predicted genomic promoter region of *DHCR24* (~5 kb) and characterized the promoter activity by construction of promoter reporter plasmids [27]. We transfected each HCV protein (core, E1, E2, NS2, NS3/4A, NS4B, NS5A, and NS5B) or the full-genome HCV. The full-genome HCV induced significantly higher DHCR24 expression than other HCV viral proteins. The serial deletion mutants of the 5′-flanking region of *DHCR24* revealed that the minimum responsive element to the full-genome HCV was between −167 and −140 of the *DHCR24* gene. An electronic mobility shift assay (EMSA) identified that the specific binding factor to this element was the Sp1 transcription factor.

Transcription of *DHCR24* was induced by oxidative stress and impaired by the removal of the HCV minimum responsive element. Furthermore, the augmentation of *DHCR24* expression was impaired by treatment with a ROS scavenger, *N*-acetylcysteine. We then explored the role of the Sp1 transcription factor in the regulation of *DHCR24* expression. Phosphorylation of Sp1 at Ser101 was elevated under oxidative stress and increased by the presence of HCV. This phosphorylation of Sp1 was mediated through ataxia telangiectasia mutated (ATM) kinase [29,30]. Sustained phosphorylation of ATM and delayed de-phosphorylation of histone H2AX at Ser139 (γH2AX) were observed in HCV replicon cells [27,31], indicating that DNA repair was impaired in cells expressing or replicating HCV.

Previous studies revealed that expression of the HCV gene elevates the level of ROS via dysregulation of ER-mediated calcium homeostasis, which results in oxidative stress [32]. Also, the HCV core protein inhibits mitochondrial electron transport and increases ROS [33]. Recently, HCV infection is reported to increase ROS production through NADPH oxidase activity, especially elevated NADPH oxidase 4 (Nox4) [34]. The production of ROS can induce DHCR24 expression [27]. Thus, our results raised the possibility that DHCR24 plays a role in response to ROS generated as a consequence of HCV infection, thereby suppressing DNA repair and promoting tumorigenicity.

4. Overexpression of DHCR24 Results in Impairment of p53 Activity

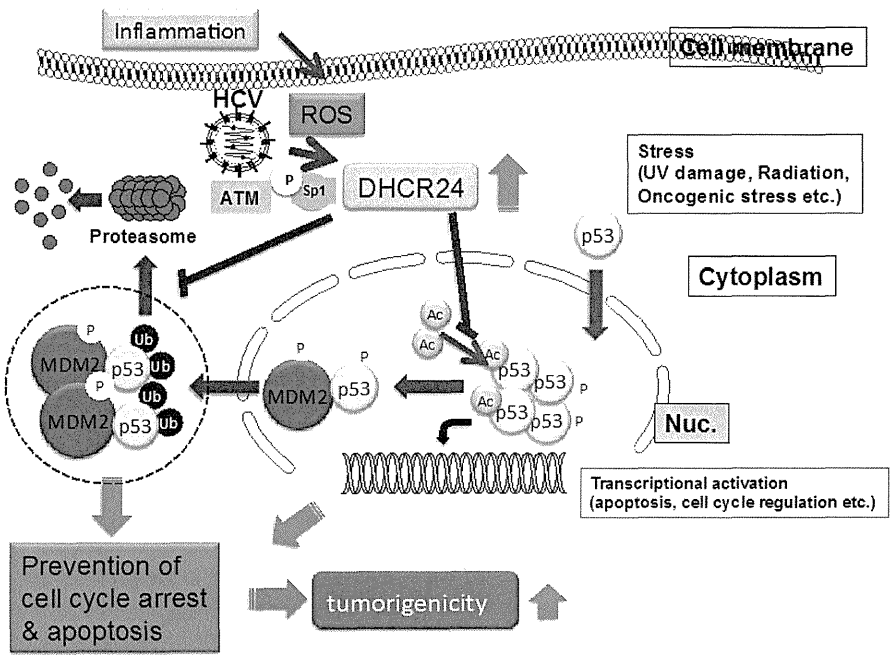
HCV gene expression or infection persistently induces over-expression of DHCR24 [16,27] in its turn induces apoptotic resistance to oxidative stress (Figure 1).

HCV gene expression elevates the levels of ROS through dysregulation of ER-mediated calcium homeostasis. This increases the level of SP1 phosphorylation by ATM kinase, and results in the transcriptional activation of the *DHCR24* gene. The augmentation of DHCR24 by HCV suppresses p53 activity by blocking nuclear p53 acetylation and increasing the interaction between p53 and HDM2 (p53-specific E3 ligase) in the cytoplasm, which may be mediated by inhibition of p53 degradation. This impairment of p53 activity may result in apoptotic resistance and increased tumorigenicity.

To further examine this mechanism, we characterized the regulatory proteins involved in the oxidative stress-induced apoptotic response and found that p53 activity was impaired in response to hydrogen peroxide, which was clarified by a $p21^{WAF1/CIP1}$ promoter reporter assay. The post-translational modification of p53 after hydrogen peroxide treatment was characterized, and we found that the acetylation of p53 at Lys^{373} and Lys^{382} was impaired by the over-expression of DHCR24. The decreased level of p53 acetylation may impair p53 sequence-specific DNA-binding activity [35] and stability [36,37].

Moreover, interaction of p53 with its specific E3 ubiquitin ligase MDM2 (also known as HDM2) in the cytoplasm was augmented. These results strongly suggest that the increased interaction between p53 and MDM2, in the cytoplasm, impaired both the nuclear translocation and the activity of p53. This interaction between p53 and MDM2 was regulated by mitogen-activated protein kinase/extracellular signal-regulated kinase kinase extracellular signal-regulated kinase (MEK-ERK)-induced phosphorylation at Ser^{166} in the MDM2 protein. Interestingly, MEK-ERK phosphorylation of MDM2 was liver specific [38].

Figure 1. Elevation of tumorigenicity in HCV infected hepatocytes through increased oxidative stress and DHCR24.



5. Conclusion

The results of our studies showed a novel HCV-induced pathway that activates DHCR24 in response to oxidative stress. Overexpression of DHCR24 by HCV contributed to the development of HCC during persistent HCV infections. Recently, we found that silencing of DHCR24 by siRNA suppresses HCV replication [39] and an inhibitor of DHCR24 (U18666A) had an anti-viral effect *in vivo*. Monoclonal antibodies to DHCR24 (2-152a) suppress HCV replication through the betaine GABA transporter-1 (BGT-1) [40]. Thus, DHCR24 is involved in HCV replication and pathogenicity. DHCR24 catalyzes the reduction of the delta-24 bond of the sterol intermediate and works further downstream of farnesyl pyrophosphate, and therefore does not influence geranylgeranylation. Our findings may indicate the

possible existence of a regulatory pathway of HCV replication by cholesterol synthesis and trafficking through DHCR24 in addition to protein geranylgeranylation. DHCR24 deficiency reduces cholesterol levels and disorganizes cholesterol-rich detergent-resistant membrane domains (DRMs) in mouse brains. Additionally, the HCV replication complex has been detected in the DRM fraction. Therefore, a deficiency in DRM, induced by silencing of DHCR24, may suppress HCV replication. In addition, BGT-1 plays a role in tonicity regulation and hyper-osmolarity [41], and recent reports show that hyperosmotic shrinkage stimulates duck hepatitis B virus replication [42]. BGT-1 is involved in sodium and chloride coupled betaine uptake and betaine levels affect lipid distribution even to such an extent that low plasma betaine levels correlate with unfavorable lipid profiles [43]. Future study will clarify the regulatory role of DHCR24 and BGT-1 in HCV replication.

In conclusion, the results of our studies suggest that HCV infected cells may become anti-apoptotic and replicate efficiently to establish chronic infection through over-expression of DHCR24. Thus, the HCV-induced oxidative stress responsive protein DHCR24 may play a critical role in the pathogenesis of HCV persistent infections.

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Impairment of interferon regulatory factor-3 activation by hepatitis C virus core protein basic amino acid region 1

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ABSTRACT

Interferon regulatory factor-3 (IRF-3), a key transcriptional factor in the type I interferon system, is frequently impaired by hepatitis C virus (HCV), in order to establish persistent infection. However, the exact mechanism by which the virus establishes persistent infection has not been fully understood yet. The present study aimed to investigate the effects of various HCV proteins on IRF-3 activation, and elucidate the underlying mechanisms. To achieve this, full-length HCV and HCV subgenomic constructs corresponding to structural and each of the nonstructural proteins were transiently transfected into HepG2 cells. IFN-β induction, plaque formation, and IRF-3 dimerization were elicited by Newcastle disease virus (NDV) infection. The expressions of IRF-3 homodimer and its monomer, Ser386-phosphorylated IRF-3, and HCV core protein were detected by immunofluorescence and western blotting. IFN-β mRNA expression was quantified by real-time PCR (RT-PCR), and IRF-3 activity was measured by the levels of IRF-3 dimerization and phosphorylation, induced by NDV infection or polyriboinosinic:polyribocytidylic acid [poly(I:C)]. Switching of the expression of the complete HCV genome as well as the core proteins, E1, E2, and NS2, suppressed IFN-β mRNA levels and IRF-3 dimerization, induced by NDV infection. Our study revealed a crucial region of the HCV core protein, basic amino acid region 1 (BR1), to inhibit IRF-3 dimerization as well as its phosphorylation induced by NDV infection and poly (I:C), thus interfering with IRF-3 activation. Therefore, our study suggests that rescue of the IRF-3 pathway impairment may be an effective treatment for HCV infection.

1. Introduction

Hepatitis C virus (HCV), a flavivirus comprising a positive-sense, single-stranded RNA (ssRNA) of approximately 9.6 kb [1], causes persistent disease in infected individuals, possibly leading to chronic liver injury [2]. Despite the approximately 170 million individuals worldwide suffering from HCV infection that ranges from chronic hepatitis to hepatocellular carcinoma (HCC) [3,4], the exact mechanism by which the virus establishes persistent infection is not fully resolved.

The innate immune system is activated immediately upon infection as the first line of host defense against invading pathogens, with type I interferon (IFN) signaling being the crucial step in the antiviral response [5]. The IFN system is, therefore, a prime target of HCV and other viruses in order to establish persistent infections [6], wherein the disruption of the type I IFN-activation pathway forms the most efficient strategy for HCV. Studies on HCV IFN-interference mechanisms have revealed that the HCV proteins NS5A and E2 selectively inhibit the double-stranded RNA-activated protein kinase (PKR) [7,8], an IFN-inducible antiviral molecule that controls transcription and translation [6]. IFN-β, a crucial molecule in type I IFN signaling, is regulated by several cellular factors associated with the activation of interferon regulatory factor-3 (IRF-3), leading to its rapid induction following viral infection [9,10]. However, IFN-β induction is impaired in HCV-infected cells, thus resulting in the disruption of IFN downstream signaling cascade [11].

IRF-3, a key constitutively expressed transcriptional factor localized in the cytoplasm in its inactive form [9], is activated upon phosphorylation, whereby it translocates to the nucleus to act as a transcriptional factor for positive regulatory domain (PRD) I of the IFN-β promoter. IRF-3 activation also induces phosphorylation of Ser385 and Ser386 or the serine/threonine (Ser/Thr) cluster between amino acids (aa) 396 and 405 (located at the C-terminus of IRF-3), a step that is essential for dimerization and nuclear translocation.

A previous study by Foy et al. showed that the NS3/4A serine protease derived from a subgenomic replicon participates in the suppression of the cellular pathway that activates IRF-3 [12]. The NS3/4A protein of HCV disrupts signaling of the double-stranded RNA (dsRNA) receptors, retinoic acid-inducible gene-I (RIG-I), and Toll-like receptor 3 (TLR3) by inducing proteolysis of interferon promoter stimulator-1 (IPS-1) [13–15] and Toll/interleukin-1 receptor (TIR) domain-containing adaptor protein inducing IFN-β (TRIF) [16], as well as by suppressing the downstream activation of IFN-β [13].

In view of the above observations and the emerging data on the role of HCV in regulating the IRF-3 pathway by additional mechanisms, we aimed to investigate the effects of various HCV proteins on IRF-3 activation, and further elucidate the underlying novel mechanisms.

2. Material and methods

2.1. Transient expression of the HCV core proteins E1, E2, and NS3-4A

HepG2 cells were transfected to express E1, E2, or NS3-4A HCV core protein under the control of EF promoter (Invitrogen). The HCV core expression vectors were derived from HCR6 (genotype 1b), HCR24-12K (genotype 2a), or HCR24-12Q (genotype 2a). The E1, E2, and NS3 clones derived from HCR6 contained either the full-length cDNAs encoding the core protein or 1 of the 3 different deletions (deletion mutants), each of which lacked 1 of the 3 basic amino acid regions (BR), BR1 deletion (aa 4–14), BR2 deletion (aa 37–44), and BR3 deletion (aa 57–72) [17]. HepG2 cells were transfected with 4 μg of the core cDNA (amino acids 1–191), E1 (amino

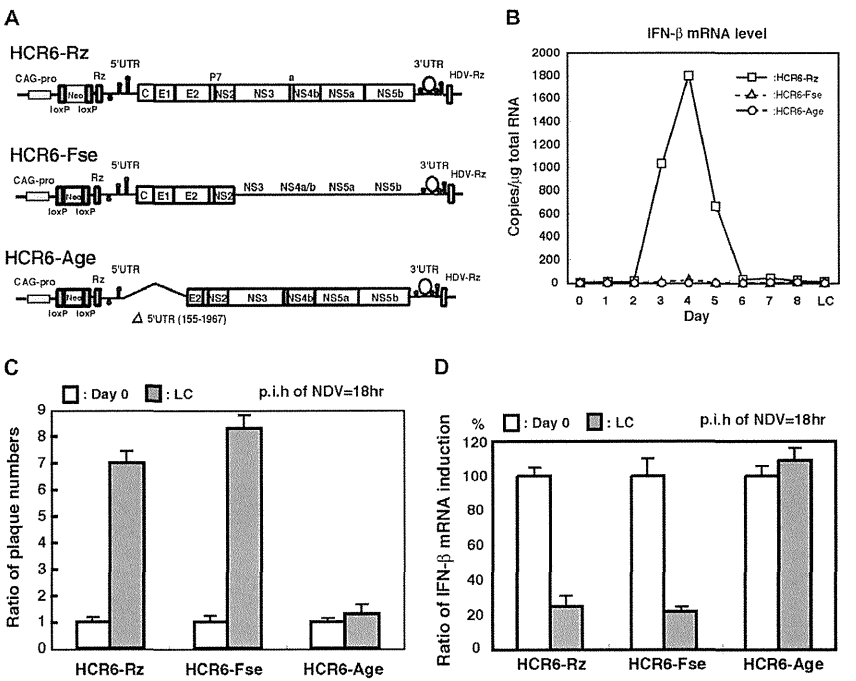

Fig. 1. (A) Structures of the conditional expression vectors for HCV RNAs and proteins. The cDNA clones that displayed highest level of homology to the consensus sequences among the 3 clones were used to construct *HCR6-Rz* (nt 1–9611). *HCR6-Fse* clone harbored a termination codon introduced at nucleotide 3606 and truncated *HCR6-Age* clone lacked nucleotides 155–1967. They were flanked with ribozyme (Rz) and hepatitis D virus ribozyme (HDV-Rz) sequences under the control of the CAG promoter in the Cre/*lox*P switching expression cassette, which consisted of the neomycin resistance gene, as a stuffer region flanked by the *lox*P sequence. (B) IFN-β mRNA levels in the cell lines *HCR6-Rz*, *HCR6-Fse*, and *HCR6-Age* before and after the expression of the HCV protein. The results are expressed as copy numbers per microgram of total RNA, as quantified by RT-PCR. (C) Efficiency of NDV plaque formation. Plaque assays were performed on Vero cells for NDV infectivity in *HCR6-Rz*, *HCR6-Fse*, or *HCR6-Age* before and after the expression of the HCV protein. The plaque numbers were counted 3 days after NDV inoculation. The ratio indicates the plaque numbers after the expression of the HCV genome divided by the plaque numbers before the expression of the HCV genome. (D) Suppression of IFN-β mRNA induction by HCV expression, 18 days after NDV inoculation. Day 0, before the expression of the HCV genome; Day 48, after the expression of the HCV genome. The results are expressed relative to the levels on Day 0 (100%) in each of the 3 HCV-expressing systems. p.i.h., post-inoculation hour.

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acids 192–383), E2 (amino acids 384–809), NS3-4A (amino acids 1027–1711), or the core regions lacking the BR, in 35-mm dishes, by using Lipofectamine 2000 (Invitrogen) at 37 °C for 6 h. The medium was subsequently replaced with normal culture medium, and the cells were harvested after 48 h.

2.2. Newcastle disease virus (NDV) infection and addition of polyriboinosinic:polyribocytidylic acid [poly(I:C)]

NDV (Miyadera strain) was propagated from swabs by using the embryonated egg culture method, as described in the Supplementary methods.

Poly(I:C) (20 μg/well; GE Healthcare) was added to HepG2 cells in 35-mm dishes, 48 h after transfection with the core expression vector derived from HCR6 (genotype 1b).

2.3. Native PAGE of IRF-3 and phosphorylated IRF-3

Cells were lysed in 30 μL of lysis buffer (50 mM Tris–HCl [pH 8.0], 1% NP-40, 150 mM NaCl, 100 μg/mL leupeptin, 1 mM PMSF, 5 mM Na_3VO_4), mixed vigorously, and centrifuged at 15,000 rpm for 10 min; the supernatant was isolated. Total protein samples (10 μg) were electrophoresed on a 7.5% native PAGE gel (Bio-Rad Laboratories) and transferred onto a PVDF membrane. IRF-3 homodimer and its monomer and Ser386-phosphorylated IRF-3 were detected by western blotting using polyclonal rabbit antibodies, anti-human IRF-3 (1:1000) [18] and, anti-human Ser386-phosphorylated IRF-3 (1:10,000), respectively. Detection was achieved by enhanced chemiluminescence (ECL; Amersham, UK) according to the manufacturer's instructions. The rabbit anti-human IRF-3 and anti-human Ser386-phosphorylated IRF-3 antibodies were described previously [19].

2.4. Quantification of IFN-β gene expression

IFN-β mRNA expression was quantified by using real-time PCR (RT-PCR) as described previously [20] and Supplementary methods using the following primers and probes: sense (5′-CCATCTATGA GATGCTCCAGAA-3′), antisense (5′-TTTTCTTCCAGGACTGTCTTCA-GA-3′) and probe (5′-AGCACTGGCTGGAATGAGACTATTGTTG-3′).

3. Results

3.1. Induction of IFN and IRF-3 by HCV-Rz

To evaluate the effect of HCV gene persistent expression, cell lines expressing the HCV genomes in a Cre/*lox*P expression system [20] were established by transfecting the full-genome HCV (*HCR6-Rz*); core, E1, E2, and NS2 (*HCR6-Fse*); and E2~NS5b (*HCR6-Age*) (Fig. 1A) clones into HepG2 cells. Of the 3 HCV expression systems, only *HCR6-Rz* transiently induced endogenous IFN-β expression (Fig. 1B). No endogenous IFN-β was detected by RT-PCR on Day 0 or Day 48 (long culture; LC) [21] in any of the 3 systems (Fig. 1B).

3.2. Effect of HCV expression on NDV infection and IFN-β induction

Further, we examined whether the persistent expression of HCV genome influenced the plaque formation activity of NDV in *HCR6-Rz*-, *HCR6-Fse*-, and *HCR6-Age*-expressing cell lines. Prior to the expression of HCV proteins, the plaque numbers were similar across all the cell lines (Fig. 1C). Expression of HCV proteins for more than 48 days [21] increased plaque numbers in the *HCR6-Rz*- and *HCR6-Fse*-expressing cells from 7- to 8-fold (Fig. 1C); however, plaque numbers in the *HCR6-Age*-expressing cells remained constant. These findings thus indicate that HCV structural proteins interfered with the induction of IFN-β mRNA, after NDV infection (18 h post-infection). Therefore, we measured IFN-β mRNA levels in the 3 cell lines, *HCR6-Rz*, *HCR6-Fse*, and *HCR6-Age*, by RT-PCR before (Day 0) and after (Day 48) inoculation with NDV (Fig. 1D). The mRNA expression of IFN-β was not observed prior to NDV infection in any of the 3 cell lines (Day 0). Notably, after 18 h of NDV inoculation and prior to the expression of various HCV proteins (Day 0), the levels of IFN-β mRNA transcription were similar among *HCR6-Rz*-, *HCR6-Fse*-, and *HCR6-Age*-expressing cell lines (Fig. 1D). Following HCV protein expression, the induced IFN-β mRNA expression was reduced to 20% in both *HCR6-Rz*- and *HCR6-Fse*-expressing cell lines. However, IFN-β mRNA expression remained constant in the *HCR6-Age*-expressing cell line.

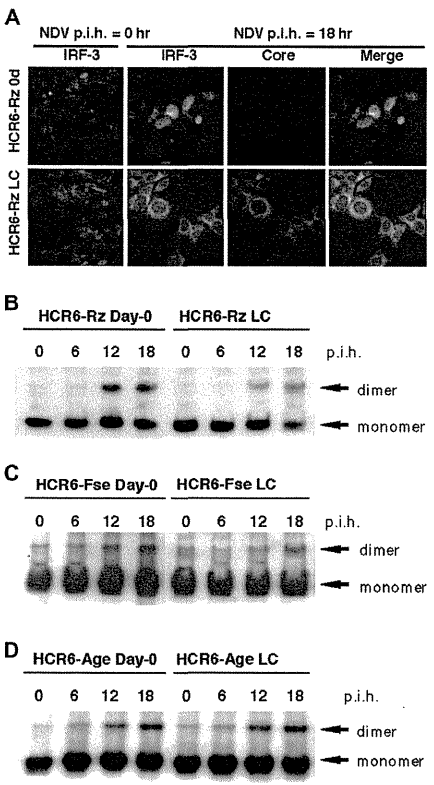

Fig. 2. (A) HCV inhibition of IRF-3 nuclear translocation. IRF-3 is a constitutively expressed transcriptional factor that localizes in the cytoplasm in a diffuse manner, when inactive. After NDV inoculation and prior to the expression of the HCV genome, IRF-3 translocated to the nucleus but was retained at a perinuclear site in the *HCR6-Rz*-expressing cells (Day 48). IRF-3 colocalized with the HCV core protein. (B–D) Suppression of IRF-3 dimerization by HCV expression. The dimeric and monomeric forms of IRF-3 were detected by western blotting on native PAGE gels. The influence of (B) *HCR6-Rz* expression, (C) *HCR6-Fse* expression, and (D) *HCR6-Age* expression on IRF-3 dimerization is shown. NDV was used to induce IRF-3 dimerization.

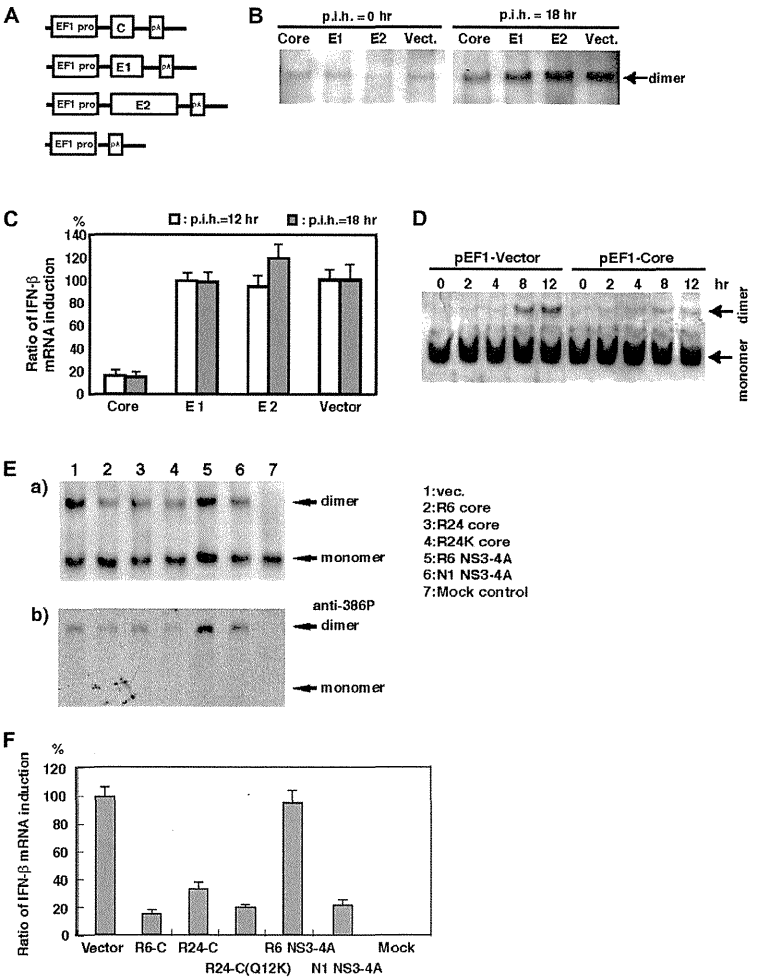

Fig. 3. (A) Structures of the HCR6 core, E1, and E2 expression vectors encoding the HCV core (aa 1–191), E1 (amino acids 192–383), and E2 (amino acids 384–809) proteins, respectively, under the control of the EF1 promoter. (B) Left panel, IRF-3 dimerization induced by NDV before transfection with the expression vectors; right panel, IRF-3 dimerization induced by NDV after transfection with the expression vectors. (C) IFN-β mRNA induction levels at 12 and 18 h after NDV inoculation into HepG2 cells transfected with the vector alone, core, E1, or E2 proteins. The results are expressed relative to the induction levels of IFN-β in HepG2 cells transfected with the vector alone (100%), for post-inoculation, each time. (D) IRF-3 dimerization on administration of 10 mg/mL poly(I:C) before the expression (Day 0), and at 2, 4, 8, and 12 h after the expression of the HCV core (pEF-Core) or vector plasmid DNA. (E) Effects of the expression of vector (lane 1), R6 core (lane 2), R24 core (lane 3), R24 core (Q12K) (lane 4), R6-NS3-4A (lane 5), N1-NS3-4A (lane 6), and mock control (lane 7) on IRF-3 dimerization (upper column a) and phosphorylation of serine residue at amino acid 386 in IRF-3 (lower column b), after infection with NDV for 18 h. F. Effects of the expressions of R6-C, R24-C, R24-C(Q12K), and R6-NS3 on IFN-β induction, 18 h after NDV inoculation. The IFN-β mRNA levels were assayed by RT-PCR. The results are expressed relative to the induction levels of IFN-β in HepG2 cells transfected with the vector alone (100%).

3.3. Effect of HCV on IRF-3 localization, nuclear translocation, and dimerization by NDV

The effect of HCV expression on cellular localization of IRF-3 was analyzed in *HCR6-Rz*-expressing cells infected with NDV before (Day 0) and after LC (Fig. 2A). Prior to NDV infection, IRF-3 was detected in the cytoplasm by immunofluorescence. Notably, after 18 h of NDV inoculation and prior to *HCR6-Rz* protein expression, when IFN-β induction and IRF-3 dimerization were maximal, a substantial amount of IRF-3 translocated to the nucleus.