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25-Hydroxyvitamin D_3 Suppresses Hepatitis C Virus Production

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Because the current interferon (IFN)-based treatment for hepatitis C virus (HCV) infection has a therapeutic limitation and side effects, a more efficient therapeutic strategy is desired. Recent studies show that supplementation of vitamin D significantly improves sustained viral response via IFN-based therapy. However, mechanisms and an active molecular form of vitamin D for its anti-HCV effects have not been fully clarified. To address these questions, we infected HuH-7 cells with cell culture-generated HCV in the presence or absence of vitamin D_3 or its metabolites. To our surprise, 25-hydroxyvitamin D_3 [25(OH)D_3], but not vitamin D_3 or 1,25-dihydroxyvitamin D_3, reduced the extra- and intracellular levels of HCV core antigen in a concentration-dependent manner. Single-cycle virus production assay with a CD81-negative cell line reveals that the inhibitory effect of 25(OH)D_3 is at the level of infectious virus assembly but not entry or replication. Long-term 25(OH)D_3 treatment generates a HCV mutant with acquired resistance to 25(OH)D_3, and this mutation resulting in a N1279Y substitution in the nonstructural region 3 helicase domain is responsible for the resistance. *Conclusion:* 25(OH)D_3 is a novel anti-HCV agent that targets an infectious viral particle assembly step. This finding provides insight into the improved efficacy of anti-HCV treatment via the combination of vitamin D_3 and IFN. Our results also suggest that 25(OH)D_3, not vitamin D_3, is a better therapeutic option in patients with hepatic dysfunction and reduced enzymatic activity for generation of 25(OH)D_3. (HEPATOLOGY 2012;56:1231-1239)

Hepatitis C virus (HCV) infection affects about 200 million people worldwide.[1,2] The majority of HCV-infected patients fail to clear the virus and develop chronic liver diseases, including cirrhosis and hepatocellular carcinoma. Standard treatment for chronic hepatitis C is currently based on a combination of pegylated interferon (IFN) and ribavirin.[2] However, the therapy is accompanied by substantial side effects and is only effective in about half of patients.[3,4] Thus, it is critical to provide a new therapeutic modality against chronic hepatitis C. Recently, vitamin D supplementation has been shown to improve the efficacy of combination therapy with IFN and ribavirin.[5,6] However, mechanisms of this effect have not yet been fully elucidated.

Vitamin D absorbed in the intestine from diet or synthesized in the skin is converted to 25-hydroxyvitamin D [25(OH)D] in the liver. Released 25(OH)D is bound to α-globulin and transported to proximal tubules of the kidney,[7] where 25(OH)D is hydroxylated either by 25(OH)D-1 alpha-hydroxylase to generate the active form, 1,25-dihydroxyvitamin D [1,25$(OH)_2$D], or by 25(OH)D-24-hydroxylase to form the biologically inactive form, 24,25-dihydroxyvitamin D [24,25$(OH)_2$D]. 1,25$(OH)_2$D is a key hormone for calcium and bone homeostasis, and its production is tightly regulated by plasma levels of calcium and phosphorus and parathyroid hormone, In addition, vitamin D has nonskeletal actions, and vitamin D deficiency is associated with many diseases including cancer, autoimmune disorder, cardiovascular disease, insulin resistance, and infectious diseases.[8-13] Thus it is not surprising that vitamin D status also

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Abbreviations: 1,25$(OH)_2$D, 1,25-dihydroxyvitamin D; 24,25$(OH)_2$D, 24,25-dihydroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D; Ag, antigen; DMEM, Dulbecco's modified Eagle's medium; HCV, hepatitis C virus; HCVcc, cell culture-generated HCV JFH-1 virus; HCVpp, HCV pseudoparticles; IFN, interferon; ISG, IFN-stimulated gene; JFH-1/wt, wild-type JFH-1; MLV, murine leukemia virus; NS3, nonstructural region 3; PCR, polymerase chain reaction; WST-8, water-soluble tetrazolium salt 2-(2-methoxy-4- nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium, monosodium salt.

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Development of a drug assay system with hepatitis C virus genome derived from a patient with acute hepatitis C

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Abstract We developed a new cell culture drug assay system (AH1R), in which genome-length hepatitis C virus (HCV) RNA (AH1 strain of genotype 1b derived from a patient with acute hepatitis C) efficiently replicates. By comparing the AH1R system with the OR6 assay system that we developed previously (O strain of genotype 1b derived from an HCV-positive blood donor), we demonstrated that the anti-HCV profiles of reagents including interferon-γ and cyclosporine A significantly differed between these assay systems. Furthermore, we found unexpectedly that rolipram, an anti-inflammatory drug, showed anti-HCV activity in the AH1R assay but not in the OR6 assay, suggesting that the anti-HCV activity of rolipram differs depending on the HCV strain. Taken together, these results suggest that the AH1R assay system is useful for the objective evaluation of anti-HCV reagents and for the discovery of different classes of anti-HCV reagents.

Keywords HCV · Acute hepatitis C · Anti-HCV drug assay system · Anti-HCV activity of rolipram

Introduction

Hepatitis C virus (HCV) infection frequently causes chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma. HCV is an enveloped virus with a positive single-stranded 9.6 kb RNA genome, which encodes a large polyprotein precursor of approximately 3,000 amino acid (aa) residues [1, 2]. This polyprotein is cleaved by a combination of the host and viral proteases into at least 10 proteins in the following order: Core, envelope 1 (E1), E2, p7, non-structural 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B [1].

Human hepatoma HuH-7 cell culture-based HCV replicon systems derived from a number of HCV strains have been widely used for various studies on HCV RNA replication [3, 4] since the first replicon system (based on the Con1 strain of genotype 1b) was developed in 1999 [5]. Genome-length HCV RNA replication systems (see Fig. 2 for details) derived from a limited number of HCV strains (H77, N, Con1, O, and JFH-1) are also sometimes used for such studies, as they are more useful than the replicon systems lacking the structural region of HCV, although the production of infectious HCV from the genome-length HCV RNA has not been demonstrated to date [3, 4]. Furthermore, these RNA replication systems have been improved enough to be suitable for the screening of anti-HCV reagents by the introduction of reporter genes such as luciferase [3, 4, 6]. We also developed an HuH-7-derived cell culture assay system (OR6) in which genome-length HCV RNA (O strain of genotype 1b derived from an HCV-positive blood donor) encoding renilla luciferase (RL) efficiently replicates [7]. Such reporter assay systems could save time and facilitate the mass screening of anti-HCV reagents, since the values of luciferase correlated well with the level of HCV RNA after treatment with anti-HCV reagents. Furthermore, OR6 assay system became more useful as a drug assay system than the HCV subgenomic replicon-based reporter assay systems developed to date [3, 4], because the older systems lack the Core-NS2 regions containing structural proteins likely to be involved in the events that take place in the HCV-infected human liver.

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Exploitation of lipid components by viral and host proteins for hepatitis C virus infection

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Hepatitis C virus (HCV), which is a major causative agent of blood-borne hepatitis, has chronically infected about 170 million individuals worldwide and leads to chronic infection, resulting in development of steatosis, cirrhosis, and eventually hepatocellular carcinoma. Hepatocellular carcinoma associated with HCV infection is not only caused by chronic inflammation, but also by the biological activity of HCV proteins. HCV core protein is known as a main component of the viral nucleocapsid. It cooperates with host factors and possesses biological activity causing lipid alteration, oxidative stress, and progression of cell growth, while other viral proteins also interact with host proteins including molecular chaperones, membrane-anchoring proteins, and enzymes associated with lipid metabolism to maintain the efficiency of viral replication and production. HCV core protein is localized on the surface of lipid droplets in infected cells. However, the role of lipid droplets in HCV infection has not yet been elucidated. Several groups recently reported that other viral proteins also support viral infection by regulation of lipid droplets and core localization in infected cells. Furthermore, lipid components are required for modification of host factors and the intracellular membrane to maintain or up-regulate viral replication. In this review, we summarize the current status of knowledge regarding the exploitation of lipid components by viral and host proteins in HCV infection.

Keywords: HCV, hepatitis, lipid droplets, host factor

INTRODUCTION

Hepatitis C virus (HCV) is a major causative agent of chronic liver disease including steatosis, cirrhosis, and hepatocellular carcinoma. Epidemiological studies indicate that HCV is also associated with extrahepatic manifestations including type 2 diabetes mellitus, B-cell non-Hodgkin lymphoma, mixed cryoglobulinemia, and Sjögren's syndrome (Jacobson et al., 2010). It has been estimated that there are 170 million patients worldwide, of whom most are infected with HCV. Combination therapy with pegylated interferon (PEG-IFN) and ribavirin has been the standard treatment but it fails to cure ∼50% of treated patients (Soriano et al., 2009).

Hepatitis C virus belongs to the genus *Hepacivirus* of the family Flaviviridae. The viral genome of HCV is characterized by a single positive strand RNA with a nucleotide length of 9.6 kb and it encodes a single polypeptide (**Figure 1**). This polyprotein is cleaved by host and viral proteases into structural and non-structural proteins (Harada et al., 1991; Hijikata et al., 1991; Grakoui et al., 1993a,b). Structural proteins, including the core protein and two envelope proteins, and the viroporin p7 are located within one-third of the N-terminal, while the remaining viral proteins are classified as non-structural proteins which form a replication complex with host factors (Grakoui et al., 1993c). HCV core protein is cleaved by signal peptide cleavage and then released from E1 (Santolini et al., 1994). After cleavage by signal peptidase (SP), the C-terminal transmembrane region of the core protein is further cleaved by signal peptide peptidase (SPP; Hussy et al., 1996; McLauchlan et al., 2002). The nucleocapsid, composed of matured core proteins and the viral genome, is surrounded by an envelope composed of host lipids and viral envelope proteins (Wakita et al., 2005). The life cycle of HCV is shown in **Figure 2**. The viral envelope proteins play a role in the binding to host receptors and membrane fusion for uncoating. Recently, several groups reported that the viral particle binds to a very low-density lipoprotein (VLDL), including apolipoprotein E (apoE), which is required for the binding step (Andre et al., 2002; Nielsen et al., 2006; Chang et al., 2007; Benga et al., 2010) as described below. The virus infects hepatocytes via entry factors known as receptors and co-receptors. The viral particle complex composed of the enveloped nucleocapsid and VLDL including apoE (Merz et al., 2011), is reported to bind to heparin sulfate (HS; Barth et al., 2003) and the low-density lipoprotein (LDL) receptor (LDLR; Agnello et al., 1999), although Albecka et al. (2012) recently reported that LDLR is required for optimal replication of the HCV genome rather than entry of the infectious viral particle. Other host factors may be involved in apoE-mediated entry. The HCV viral particle is transferred to the scavenger receptor class B type I (SR-BI; Scarselli et al., 2002; Bartosch et al., 2003) and CD81 (Pileri et al., 1998) through E2 binding and then enters cells with claudin-1 (CLDN1; Evans et al., 2007) and occludin (OCLN; Ploss et al., 2009) by endocytosis. The Niemann–Pick C1-like 1 cholesterol absorption receptor has recently been reported to be an HCV cell entry factor that is involved in the entry step between post-binding and

A Subclone of HuH-7 with Enhanced Intracellular Hepatitis C Virus Production and Evasion of Virus Related-Cell Cycle Arrest

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Abstract

Hepatitis C virus (HCV) cell culture system with JFH-1 strain and HuH-7 cells enabled us to produce infectious HCV particles *in vitro*, and such system is useful to explore the anti-HCV compounds and to develop the vaccine against HCV. In the present study, we describe the derivation of a cell line that permits improved production of HCV particles. Specifically, we characterized several subclones that were isolated from the original HuH-7 cell line by limiting dilution. These HuH-7 subclones displayed a notable range of HCV production levels following transfection by full-genome JFH-1 RNA. Among these subclones, HuH-7T1 produced HCV more efficiently than other subclones and Huh-7.5.1 that is known to be highly permissive for HCV replication. Upon transfection with full-genome RNA, HCV production was increased ten-fold in HuH-7T1 compared to Huh-7.5.1. This increase in viral production correlated with increased efficiency of intracellular infectious virus production. Furthermore, HCV replication did not induce cell cycle arrest in HuH-7T1, whereas it did in Huh-7.5.1. Consequently, the use of HuH-7T1 as host cells could provide increased population of HCV-positive cells and elevated viral titer. In conclusion, we isolated a HuH-7 subclone, HuH-7T1, that supports efficient HCV production. High efficiency of intracellular infectious virus production and evasion of cell cycle arrest were important for this phenotype. We expect that the use of this cell line will facilitate analysis of the underlying mechanisms for HCV particle assembly and the cell cycle arrest caused by HCV.

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Introduction

Hepatitis C virus (HCV) is a major cause of chronic liver disease [1,2]. Currently, approximately 200 million people are infected with HCV worldwide and are at continued risk of developing chronic liver diseases such as chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [3,4]. Historically, the lack of a cell culture system capable of producing virus particles hampered progress in the field of HCV research. Subsequently, a robust HCV cell culture system was developed using HCV JFH-1 strain that had been cloned from a fulminant hepatitis patient [5,6,7]. JFH-1 was the first HCV strain that could replicate and produce HCV particles autonomously *in vitro*, thereby facilitating investigation of the entire life cycle of the virus. This HCV cell culture system employed HuH-7 cell line, which was established from a hepatocellular carcinoma [5,8], as a host. Since the HCV replicon system enabling HCV subgenomic RNA replication was originally developed using HuH-7 [9], this cell line has been used in the research field of HCV most frequently. However, HuH-7 is known to be heterogeneous. Notably, Saintz et al. reported that HuH-7 cell lines obtained from various laboratories exhibit distinct morphological, cell growth, and HCV susceptibility properties [10]. We also found that single-cell cloning of HuH-7 maintained in our laboratory yielded multiple subclones that exhibited different characteristics of HCV infection and replication [11]. In the present study, we derived cell lines from original HuH-7 obtained from the cell bank and screened to identify a cell line with improved production of infectious HCV particles. As we report here, we obtained one such clone (HuH-7T1) and performed an initial characterization of the HCV life cycle in this host.

Materials and Methods

Cell culture

The original HuH-7 cell line (catalog number; JCRB0403) was purchased from Health Science Research Resources Bank (Osaka, Japan). The cured cell line, Huh-7.5.1, was a kind gift from Dr. Francis V. Chisari (Scripps Research Institute, La Jolla, CA) [6]. These cell lines were cultured at 37°C in a 5% CO_2 environment using Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum.

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Japanese Reference Panel of Blood Specimens for Evaluation of Hepatitis C Virus RNA and Core Antigen Quantitative Assays

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An accurate and reliable quantitative assay for hepatitis C virus (HCV) is essential for measuring viral propagation and the efficacy of antiviral therapy. There is a growing need for domestic reference panels for evaluation of clinical assay kits because the performance of these kits may vary with region-specific genotypes or polymorphisms. In this study, we established a reference panel by selecting 80 donated blood specimens in Japan that tested positive for HCV. Using this panel, we quantified HCV viral loads using two HCV RNA kits and five core antigen (Ag) kits currently available in Japan. The data from the two HCV RNA assay kits showed excellent correlation. All RNA titers were distributed evenly across a range from 3 to 7 log IU/ml. Although the data from the five core Ag kits also correlated with RNA titers, the sensitivities of individual kits were not sufficient to quantify viral load in all samples. As calculated by the correlation with RNA titers, the theoretical lower limits of detection by these core Ag assays were higher than those for the detection of RNA. Moreover, in several samples in our panel, core Ag levels were underestimated compared to RNA titers. Sequence analysis in the HCV core region suggested that polymorphisms at amino acids 47 to 49 of the core Ag were responsible for this underestimation. The panel established in this study will be useful for estimating the quality of currently available and upcoming HCV assay kits; such quality control is essential for clinical usage of these kits.

Hepatitis C virus (HCV) is a major cause of chronic liver disease worldwide (15). There is no protective vaccine against this virus, and once an individual is infected, HCV often establishes persistent infection and leads to chronic hepatitis, cirrhosis, and hepatocellular carcinoma (9). The most widely used therapy for HCV infection is the combined administration of pegylated alpha interferon and ribavirin (29). However, this treatment is problematic, as it has limited efficacy, high cost, and severe adverse effects (8, 25). To estimate the outcome of antiviral therapy, and to understand the state of viral propagation, it is important to determine the HCV viral load in chronic hepatitis C patients by the use of accurate and reliable HCV quantitative assays (9, 14). For this purpose, several commercial assay kits for HCV RNA and core antigen (Ag) quantification are currently used in Japan. For quantification of HCV RNA levels, two real-time quantitative reverse transcription-PCR (qRT-PCR)-based assay kits are available, including the COBAS AmpliPrep/COBAS TaqMan HCV test (CAP/CTM-RNA; Roche Diagnostics, Tokyo, Japan) and the Abbott RealTime HCV test (ART-RNA; Abbott Japan, Tokyo, Japan). These assays are known to have high sensitivity and a wide dynamic range, but they require technical skill and attention to maintaining the specified conditions (4–6, 16, 24, 33–35). Alternatively, HCV viremia can be quantified by assessment of HCV core Ag level (1–3, 7, 10, 12, 13, 17–22, 27, 30–32). Five HCV core Ag assay kits are commercially available in Japan, including Architect HCV Ag (Architect-Ag; Abbott Japan), Lumipulse Ortho HCV Ag (Lumipulse-Ag; Fujirebio, Tokyo, Japan), Lumispot Eiken HCV Ag (Lumispot-Ag; Eiken Chemical, Tokyo, Japan), the Ortho HCV Ag ELISA test (ELISA-Ag; Ortho Clinical Diagnostics, Tokyo, Japan), and the Ortho HCV Ag IRMA test (IRMA-Ag; Ortho Clinical Diagnostics, Tokyo, Japan). These assays have some disadvantages compared to those measuring HCV RNA (notably, low sensitivity and narrow range of quantification) but also have some advantages (including ease of use, reduced risk of contamination, reduced cost, and reliability even with samples stored at room temperature for extended periods of time [1, 32]). Although core Ag levels are thought to be related closely to HCV RNA titers, the correlation and linearity of core Ag levels have not yet been fully evaluated. In addition, these quantitative parameters are known to be affected by nucleotide and amino acid sequences at the target regions of the assays (5, 6, 28, 34), and this sequence variation depends on genotypes or predominant strains in specific geographical regions.

In this study, we established a Japanese reference panel of samples for evaluation of HCV RNA and core Ag levels by collecting donated blood specimens that tested positive for HCV RNA and anti-HCV antibodies. Using this reference panel, we evaluated the HCV loads in these specimens with two HCV RNA assay kits and five core Ag assay kits and assessed correlations among the data generated by these kits.

MATERIALS AND METHODS

Preparation of reference panel. To establish a reference panel for HCV quantitative assays, a total of 80 donated plasma samples were selected. All of these specimens, supplied by the Japanese Red Cross Blood Centers, tested positive for the presence of HCV RNA and anti-HCV antibodies. These samples, collected in Japan from May to September of 2007, were obtained from Japanese blood donor volunteers in various regions of

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Production of Infectious Chimeric Hepatitis C Virus Genotype 2b Harboring Minimal Regions of JFH-1

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To establish a cell culture system for chimeric hepatitis C virus (HCV) genotype 2b, we prepared a chimeric construct harboring the 5′ untranslated region (UTR) to the E2 region of the MA strain (genotype 2b) and the region of p7 to the 3′ UTR of the JFH-1 strain (genotype 2a). This chimeric RNA (MA/JFH-1.1) replicated and produced infectious virus in Huh7.5.1 cells. Replacement of the 5′ UTR of this chimera with that from JFH-1 (MA/JFH-1.2) enhanced virus production, but infectivity remained low. In a long-term follow-up study, we identified a cell culture-adaptive mutation in the core region (R167G) and found that it enhanced virus assembly. We previously reported that the NS3 helicase (N3H) and the region of NS5B to 3′ X (N5BX) of JFH-1 enabled replication of the J6CF strain (genotype 2a), which could not replicate in cells. To reduce JFH-1 content in MA/JFH-1.2, we produced a chimeric viral genome for MA harboring the N3H and N5BX regions of JFH-1, combined with a JFH-1 5′ UTR replacement and the R167G mutation (MA/N3H+N5BX-JFH1/R167G). This chimeric RNA replicated efficiently, but virus production was low. After the introduction of four additional cell culture-adaptive mutations, MA/N3H+N5BX-JFH1/5am produced infectious virus efficiently. Using this chimeric virus harboring minimal regions of JFH-1, we analyzed interferon sensitivity and found that this chimeric virus was more sensitive to interferon than JFH-1 and another chimeric virus containing more regions from JFH-1 (MA/JFH-1.2/R167G). In conclusion, we established an HCV genotype 2b cell culture system using a chimeric genome harboring minimal regions of JFH-1. This cell culture system may be useful for characterizing genotype 2b viruses and developing antiviral strategies.

Hepatitis C virus (HCV) is a major cause of chronic liver disease (5, 13), but the lack of a robust cell culture system to produce virus particles has hampered the progress of HCV research (2). Although the development of a subgenomic replicon system has enabled research into HCV RNA replication (15), infectious virus particle production has not been possible. Recently, an HCV cell culture system was developed using a genotype 2a strain, JFH-1, cloned from a fulminant hepatitis patient (14, 29, 32), thereby allowing investigation of the entire life cycle of this virus. However, several groups of investigators have reported genotype- and/or strain-dependent effects of some antiviral reagents (6, 17) and neutralizing antibodies (7, 25). Therefore, efficient virus production systems using various genotypes and strains are indispensable for HCV research and the development of antiviral strategies.

The JFH-1 strain is the first HCV strain that can efficiently produce HCV particles in HuH-7 cells (29). Other strains can replicate and produce infectious virus by HCV RNA transfection, but the efficiency is far lower than that of JFH-1 (24, 31). In the case of replication-incompetent strains, chimeric virus containing the JFH-1 nonstructural protein coding region is useful for analyses of viral characteristics (6, 9, 14, 23, 30, 31).

In this study, we developed a genotype 2b chimeric infectious virus production system using the MA strain (accession number AB030907) (19) harboring minimal regions of JFH-1 and cell culture-adaptive mutations that enhance infectious virus production.

MATERIALS AND METHODS

Cell culture. Huh7.5.1 cells (a kind gift from Francis V. Chisari) (32) and Huh7-25 cells (1) were cultured at 37°C in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum under 5% CO_2 conditions. For follow-up study, RNA-transfected cells were passaged every 2 to 5 days depending on cell status.

Full-length genomic HCV constructs. Plasmids used in the analysis of genomic RNA replication were constructed based on pJFH1 (29) and pMA (19). For convenience, an EcoRI recognition site was introduced upstream of the T7 promoter region of pMA by PCR, and an XbaI recognition site was introduced at the end of the 3′ untranslated region (UTR). To construct MA/JFH-1, the EcoRI-BsaBI (nucleotides [nt] 1 to 2570; 5′ UTR to E2) fragment of pMA was substituted into pJFH1 (Fig. 1A). Replacement of the 5′ UTR was performed by exchanging the EcoRI-AgeI (nt 1 to 159) fragment. A point mutation in the core region (R167G) was introduced into MA chimeric constructs by PCR using the following primers: sense, 5′-TTA TGC AAC GGG GAA TTT ACC CGG TTG CTC T-3′; antisense, 5′-GGT AAA TTC CCC GTT GCA TAA TTT ATC CCG TC-3′. G167R substitution in the JFH-1 construct was performed by PCR using the following primers: sense, 5′-ATT ATG CAA CAA GGA ACC TAC CCG GTT TCC C-3′; antisense, 5′-GGT AGG TTC CTT GTT GCA TAA TTA ACC CCG TC-3′. Point mutations (L814S, R1012G, T1106A, and V1951A) were introduced into MA chimeric constructs by PCR using the following primers: L814S, 5′-GCT TAC GCC TCG GAC GCC GCT GAA CAA GGG G-3′ (sense) and 5′-AGC GGC GTC CGA GGC GTA AGC CTG CTG CGG C-3′ (antisense); R1012G, 5′-GAG GCT AGG TGG

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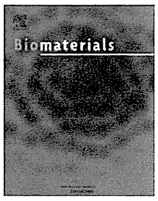

The promotion of hepatic maturation of human pluripotent stem cells in 3D co-culture using type I collagen and Swiss 3T3 cell sheets

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ABSTRACT

Hepatocyte-like cells differentiated from human embryonic stem cells (hESCs) or human induced pluripotent stem cells (hiPSCs) are known to be a useful cell source for drug screening. We recently developed an efficient hepatic differentiation method from hESCs and hiPSCs by sequential transduction of FOXA2 and HNF1α. It is known that the combination of three-dimensional (3D) culture and co-culture, namely 3D co-culture, can maintain the functions of primary hepatocytes. However, hepatic maturation of hESC- or hiPSC-derived hepatocyte-like cells (hEHs or hiPHs, respectively) by 3D co-culture systems has not been examined. Therefore, we utilized a cell sheet engineering technology to promote hepatic maturation. The gene expression levels of hepatocyte-related markers (such as cytochrome P450 enzymes and conjugating enzymes) and the amount of albumin secretion in the hEHs or hiPHs, which were 3D co-cultured with the Swiss 3T3 cell sheet, were significantly up-regulated in comparison with those in the hEHs or hiPHs cultured in a monolayer. Furthermore, we found that type I collagen synthesized in Swiss 3T3 cells plays an important role in hepatic maturation. The hEHs or hiPHs that were 3D co-cultured with the Swiss 3T3 cell sheet would be powerful tools for medical applications, such as drug screening.

1. Introduction

Several studies have recently shown the ability of human embryonic stem cells (hESCs) [1] and human induced pluripotent stem cells (hiPSCs) [2] to differentiate into hepatocyte-like cells [3–6]. Although primary human hepatocytes are generally employed for drug toxicity screening in the early phase of pharmaceutical development, these cells have some drawbacks, such as their limited range of sources, difference in variability and functions from batch to batch, and de-differentiation. Because hESC- or hiPSC-derived hepatocyte-like cells (hEHs or hiPHs, respectively) have potential to resolve these problems, they are expected to be applied to drug screening. The hepatic differentiation processes from hESCs and hiPSCs are divided into three-stages, differentiation into definitive endoderm (DE) cells, hepatoblasts, and mature hepatocytes. Hepatic differentiation methods based on the treatment of growth factors have been widely used to generate hepatocyte-like cells from hESCs or hiPSCs [5–9]. However, the hepatic differentiation efficiency is not high enough for medical applications such as drug screening [10]. To promote the efficiency of hepatic differentiation and hepatic maturation, we have developed hepatic differentiation methods that combine the transduction of transcription factor genes involved in liver development

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ORIGINAL ARTICLE

Association of *ITPA* gene variation and serum ribavirin concentration with a decline in blood cell concentrations during pegylated interferon-alpha plus ribavirin therapy for chronic hepatitis C

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Abstract

Background Genetic variation leading to inosine triphosphatase (*ITPA*) deficiency protects chronic hepatitis C patients receiving ribavirin against hemolytic anemia. The relationship between *ITPA* gene variation and serum ribavirin concentration was analyzed in association with a reduction in blood cells and dose reduction of pegylated interferon (PEG-IFN) or ribavirin.

Patients and methods A total of 300 hepatitis C patients treated with PEG-IFN plus ribavirin were analyzed. Genetic polymorphisms were determined in *ITPA* and the quantitative reduction in blood cells from the baseline was analyzed every 4 weeks for the duration of treatment and after the end of therapy. The decline in hemoglobin (Hb) or platelet (PLT) level at week 4 compared to baseline was also assessed according to ribavirin concentrations.

Results Patients with the *ITPA-CA/AA* genotypes showed a lower degree of Hb reduction throughout therapy than those with the *ITPA-CC* genotype and a marked difference in mean Hb reduction was found at week 4 (*CA/AA* −1.0 vs. *CC* −2.8, $p < 0.001$). The *ITPA-CC* genotype had significantly less reduction in the mean platelet count than the *ITPA-CA/AA* genotypes early during treatment ($p < 0.001$ for weeks 4 and 8). Patients with the *ITPA-CA/AA* genotypes were less likely to develop anemia, regardless of the concentration of ribavirin. Patients with baseline PLT counts below $130 \times 10^3/\mu l$ had a significantly lower tendency to achieve sustained virological response (SVR), especially those with the *ITPA-CA/AA* genotypes. ITPA gene variation was not extracted by multivariable analysis as an important predictor of SVR.

Conclusions Despite the fact that ITPA variants were less likely to develop anemia, patients with low baseline PLT counts were difficult to treat, especially those with the *ITPA-CA/AA* genotype. These results may give a valuable pharmacogenetic diagnostic tool for the tailoring of dosing to minimize drug-induced adverse events.

Keywords Hepatitis C virus (HCV) · Pegylated interferon plus ribavirin therapy · *ITPA* (inosine triphosphatase)

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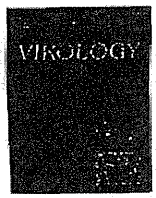

Novel monoclonal antibodies for identification of multicentric Castleman's disease; Kaposi's sarcoma-associated herpesvirus-encoded vMIP-I and vMIP-II

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vMIP-I
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Chemokine
KSHV
HHV-8

ABSTRACT

Recent studies have indicated that vMIP-I and vMIP-II play important roles in the pathogenesis of Kaposi's sarcoma-associated herpesvirus (KSHV)-related diseases due to the effects of these proteins on vascularization. We developed monoclonal antibodies against KSHV-encoded viral macrophage inflammatory protein-I (vMIP-I) and vMIP-II to study these expression profiles and reveal the pathogenesis of KSHV-related diseases. The MAbs against vMIP-I and vMIP-II reacted to KSHV-infected cell lines after lytic induction. Both vMIP-I and the vMIP-II gene products were detected 24 h post-induction with 12-O-tetradecanoylphorbol-13-acetate until 60 h in the cytoplasm of primary effusion lymphoma cell lines. In clinical specimens, both vMIP-I and vMIP-II gene products were detected in the tissues of patients with multicentric Castleman's disease. On the other hand, only vMIP-II was detected in a subset of Kaposi's sarcoma. We concluded that these antibodies might be powerful tools to elucidate the pathogenesis of KSHV-related diseases.

Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV-8), is a gammaherpesvirus originally identified in HIV-positive Kaposi's sarcoma (KS) tissues (Chang et al., 1994). KSHV is responsible for AIDS associated cancers such as Kaposi's sarcoma (KS), primary effusion lymphoma (PEL), and multicentric Castleman's disease (MCD) (Cesarman et al., 1995; Schalling et al., 1995; Soulier et al., 1995). As is the case for all herpesviruses, KSHV has two life cycles, one latent and the other lytic. Lytic gene expression can be induced by the treatment of latently infected cells with chemical agents such as 12-O-tetradecanoylphorbol-13-acetate (TPA), sodium butyrate (Arvanitakis et al., 1996; Miller et al., 1997). It has been demonstrated that two KSHV-encoded chemokines, K6 (which encodes a vMIP-I) and K4 (which encodes a vMIP-II), are expressed in the course of lytic infection (Moore et al., 1996; Sun et al., 1999). Previous reports showed that both vMIP-I and vMIP-II induced Ca^{2+} signal transduction via certain chemokine receptors and the receptor-dependent migration of cells (Benelli et al., 2000; Chen et al., 1998; Endres et al., 1999; Kledal et al., 1997). In addition, in a chick chorioallantoic membrane assay, the both proteins showed strong angiogenic properties (Boshoff et al., 1997). However, little is known about the contribution of vMIPs to KSHV malignancy under physiologic conditions.

In this report, we generated new monoclonal antibodies against vMIP-I and vMIP-II, and confirmed the detection of both vMIP-I and vMIP-II in histological sections of tissues from MCD patients as well as in KSHV-infected PEL cell lines. In cases of KS, vMIP-II was detected, but not vMIP-I. These results suggest that the expression properties of vMIP-I and vMIP-II might be related to KSHV-associated diseases, and may even be involved in the generation of diseases. Thus, antiviral chemokine MAbs could potentially become useful tools for the diagnosis of KSHV-related diseases.

Materials and methods

Cells

Kaposi's sarcoma-associated herpesvirus-positive cell lines (BC-1, BC-3, BCBL-1 and TY-1 cells) and a negative cell line (BJAB cells) were

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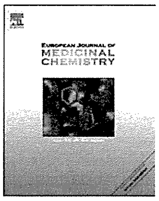

Original article

Inhibition of hepatitis C virus NS5B polymerase by S-trityl-L-cysteine derivatives

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ABSTRACT

Structure-based studies led to the identification of a constrained derivative of S-trityl-L-cysteine (STLC) scaffold as a candidate inhibitor of hepatitis C virus (HCV) NS5B polymerase. A panel of STLC derivatives were synthesized and investigated for their activity against HCV NS5B. Three STLC derivatives, **9**, F-3070, and F-3065, were identified as modest HCV NS5B inhibitors with IC_{50} values between 22.3 and 39.7 μM. F-3070 and F-3065 displayed potent inhibition of intracellular NS5B activity in the BHK-NS5B-FRLuc reporter and also inhibited HCV RNA replication in the Huh7/Rep-Feo1b reporter system. Binding mode investigations suggested that the STLC scaffold can be used to develop new NS5B inhibitors by further chemical modification at one of the trityl phenyl group.

1. Introduction

Hepatitis C virus (HCV) infection represents a major public-health concern. It is estimated that over 200 million people, ~3% of the world population, are chronically infected with the virus [1–3]. HCV has an array of immune evasion strategies and can persist in the host for years. Individuals with chronic HCV infection are at increased risk of developing cirrhosis and hepatocellular carcinoma [3–7]. Currently, HCV infections are treated by a combination of pegylated-interferon, the nucleoside analog ribavirin, and one of two recently approved HCV protease inhibitors, Boceprevir or Telaprevir [8–13]. However, this therapy is limited in efficacy against the various HCV genotypes. Furthermore, in addition to its high cost, the current treatment is associated with severe side effects and a complicated dosing regimen that may limit patient compliance [11,12]. Also the possibility of selecting drug resistant HCV variants remains [12,13]. Therefore, the development of additional, efficacious and more cost effective HCV antiviral therapies that target viral proteins and have limited effects on host biological processes is a priority.

HCV is a member of the *Flaviviridae* family. The positive sense, 9.6 kb RNA genome is translated into a single 3000 amino acid polyprotein via an IRES sequence located within the 5′ non-translated region (NTR) of the viral genome [14,15]. The viral polyprotein is processed by both host and viral proteases into individual viral proteins consisting of four structural (core, E1, E2, and p7) and six nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [16]. HCV replicates exclusively in the cytoplasm of host cells. Replication of the viral RNA genome is mediated by the RNA-dependent RNA polymerase (RdRp) activity of the HCV nonstructural protein NS5B [17–19]. Because of the absolute requirement of NS5B to synthesize nascent HCV RNA, NS5B represents an attractive target for the development of anti-HCV inhibitors [20,21]. Furthermore, host cells lack RdRp. Therefore, an inhibitor that blocks RdRp activity should, in theory, have minimal or no effect on host biological processes. Though, a number of NIs and NNIs with potent in vitro anti-NS5B activity have been identified in recent years, they have presented challenges of toxicity and selection of resistant viruses, thus necessitating identification of better NS5B inhibitor scaffolds.

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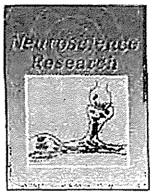

Overexpression of HGF attenuates the degeneration of Purkinje cells and Bergmann glia in a knockin mouse model of spinocerebellar ataxia type 7

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ABSTRACT

Spinocerebellar ataxia type 7 (SCA7) is an autosomal dominant disorder associated with cerebellar neurodegeneration caused by expansion of a CAG repeat in the ataxin-7 gene. Hepatocyte growth factor (HGF), a pleiotrophic growth factor, displays highly potent neurotrophic activities on cerebellar neurons. A mutant c-met/HGF receptor knockin mouse model has revealed a role for HGF in the postnatal development of the cerebellum. The present study was designed to elucidate the effect of HGF on cerebellar neurodegeneration in a knockin mouse model of SCA7 (SCA7-KI mouse). SCA7-KI mice were crossed with transgenic mice overexpressing HGF (HGF-Tg mice) to produce SCA7-KI/HGF-Tg mice that were used to examine the phenotypic differences following HGF overexpression. The Purkinje cellular degeneration is thought to occur *via* cell-autonomous and non-cell autonomous mechanisms mediated by a reduction of glutamate transporter levels in Bergmann glia. The Purkinje cellular degeneration and reduced expression of glutamate transporters in the cerebellum of SCA7-KI mice were largely attenuated in the SCA7-KI/HGF-Tg mice. Moreover, phenotypic impairments exhibited by SCA7-KI mice during rotarod tests were alleviated in SCA7-KI/HGF-Tg mice. The bifunctional nature of HGF on both Purkinje cells and Bergmann glia highlight the potential therapeutic utility of this molecule for the treatment of SCA7 and related disorders.

1. Introduction

Spinocerebellar ataxia type 7 (SCA7) is a progressive inherited disorder characterized by ataxia and neurodegeneration of the cerebellum and retina (Ieraci et al., 2002). The disease is the result of an abnormal CAG repeat expansion in the ataxin-7 gene. SCA7 patients display ataxia in addition to neurodegeneration and neuronal death of Purkinje cells (Ieraci et al., 2002). SCA7 knockin mice also show a neurodegeneration of Purkinje cells (Yoo et al., 2003). Therefore, it is thought that the protection of Purkinje cells may represent a therapeutic strategy to combat SCA7.

Additionally, following findings suggest that Bergmann glial cells have been considered as another therapeutic target of the disease. Bergmann glia are cerebellum-specific astrocytes that are located around synapses between Purkinje cells and cerebellar granule cells or climbing fibers. The glial cells play a role in removing excess glutamate from synapses *via* two primary glutamate transporters, glutamate/aspartate transporter (GLAST) and glutamate transporter-1 (GLT-1) (Huang and Bordey, 2004). A mouse model that expresses expanded ataxin-7 specifically in Bergmann glia displays a neurodegeneration of Purkinje cells, indicating that a dysfunction of Bergmann glia contributes to the degeneration of Purkinje cells in SCA7 mice and thereby progression of the disease in a non-cell autonomous manner (Custer et al., 2006). Therefore, prevention of cell degeneration and concomitant increase of glutamate transporter function may represent a valid therapeutic strategy for SCA7.

Hepatocyte growth factor (HGF), which was first identified as a potent mitogen for mature hepatocytes (Nakamura et al., 1984, 1989), exhibits neurotrophic activities in a wide variety of neurons in the hippocampus, the cerebral cortex, the cerebellum, the brainstem (midbrain dopaminergic neurons) and the spinal cord (sensory and motor neurons) (Funakoshi and Nakamura, 2011). Recent experiments have indicated that HGF exerts neuroprotective effects on various neurons in animal models of cerebral ischemia, amyotrophic lateral sclerosis (ALS) and spinal cord injury (Funakoshi and Nakamura, 2011; Sun et al., 2002; Ishigaki et al., 2007; Kitamura et al., 2011; Miyazawa et al., 1998). In the cerebellum, HGF is expressed in Purkinje cells and granular cells, and plays a role in the cerebellum during both developmental and adult

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Kaposi's sarcoma-associated herpesvirus genome replication, partitioning, and maintenance in latency

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Kaposi's sarcoma-associated herpesvirus (KSHV) is thought to be an oncogenic member of the γ-herpesvirus subfamily. The virus usually establishes latency upon infection as a default infection pattern. The viral genome replicates according to the host cell cycle by recruiting the host cellular replication machinery. Among the latently expressing viral factors, LANA plays pivotal roles in viral genome replication, partitioning, and maintenance. LANA binds with two LANA-binding sites (LBS1/2) within a terminal repeat (TR) sequence and is indispensable for viral genome replication in latency. The nuclear matrix region seems to be important as a replication site, since LANA as well as cellular replication factors accumulate there and recruit the viral replication origin in latency (ori-P) by its binding activity to LBS. KSHV ori-P consists of LBS followed by a 32-bp GC-rich segment (32GC). Although it has been reported that LANA recruits cellular pre-replication complexes (pre-RC) such as origin recognition complexes (ORCs) to the ori-P through its interaction with ORCs, this mechanism does not account completely for the requirement of the 32GC. On the other hand, there are few reports about the partitioning and maintenance of the viral genome. LANA interacts with many kinds of chromosomal proteins, including Brd2/RING3, core histones, such as H2A/H2B and histone H1, and so on. The detailed molecular mechanisms by which LANA enables KSHV genome partitioning and maintenance still remain obscure. By integrating the findings reported thus far on KSHV genome replication, partitioning, and maintenance in latency, we will summarize what we know now, discuss what questions remain to be answered, and determine what needs to be done next to understand the mechanisms underlying viral replication, partitioning, and maintenance strategy.

Keywords: Kaposi's sarcoma-associated herpesvirus, human herpesvirus 8, latency-associated nuclear antigen, ori-P, DNA replication, genome maintenance, pre-replication complex, nuclear matrix

INTRODUCTION

Kaposi's sarcoma (KS)-associated herpesvirus (KSHV) is a gamma-2 herpesvirus discovered from KS specimens in 1994 (Chang et al., 1994). KSHV is closely associated with KS and several non-Hodgkin lymphomas, including primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD; Cesarman et al., 1995, 1996; Soulier et al., 1995). While KS is the most common cancer in acquired immune deficiency syndrome patients (Potthoff et al., 2010), KSHV is detected in about 95% of all types of KS lesions by PCR analysis (Dupin et al., 1995; Huang et al., 1995; Moore and Chang, 1995). PEL is a rare B cell lymphoma originated from preterminal B cells, and PEL in AIDS patients is often associated with KSHV as well as EBV. Several KSHV-infected PEL cell lines have been established, and EBV is frequently lost in the course of establishment (Arvanitakis et al., 1996; Gaidano et al., 1996; Renne et al., 1996; Said et al., 1996; Carbone et al., 1997, 1998; Katano et al., 1999). MCD is a plasmacytic lymphadenopathy with polyclonal hyper-immunoglobulinemia and high levels of serum IL-6 (Frizzera et al., 1983; Yoshizaki et al., 1989).

Like all herpesviruses, KSHV has two life cycles: latent and lytic replication phases (for review, see Boshoff and Chang, 2001). Whereas KSHV is usually in latency when it infects KS and PEL cells, in MCD some cells express lytic genes (Katano et al., 2000; Parravicini et al., 2000). On the other hand, it has been reported that KSHV infection itself and/or viral lytic proteins promote cell proliferation and angiogenesis as well as lymphatic reprogramming (Ciufo et al., 2001; Gao et al., 2003; Carroll et al., 2004; Hong et al., 2004; Naranatt et al., 2004; Pan et al., 2004; Wang et al., 2004; Sharma-Walia et al., 2006; Qian et al., 2007, 2008; Sadagopan et al., 2007; Ye et al., 2007).

In latency, the KSHV genome is present as an episome, which is capable of autonomously replicating during S phase of the host cell cycle without integration into host chromosomes, and only limited genes are expressed during latency. Therefore, there is no generation of progeny virions. It is very important to elucidate and learn the virus's survival strategy in order to control infection and to formulate treatment for KSHV-related diseases.

In this review, we would like to focus on studies on the mechanisms underlying viral DNA replication, genome segregation and maintenance, and gene expression regulation in latency, and to discuss these topics in the light of studies on cellular mechanisms.

GENE EXPRESSION CONTROL IN KSHV LATENCY

The KSHV genome is a double-stranded linear DNA in the virion. It is circularized upon infection and is maintained as an episome in the infected nucleus. The complete genome is about 160–170 kbp,

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Multiple Intra-Familial Transmission Patterns of Hepatitis B Virus Genotype D in North-Eastern Egypt

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The transmission rate of intra-familial hepatitis B virus (HBV) and mode of transmission were investigated in north eastern Egypt. HBV infection was investigated serologically and confirmed by molecular evolutionary analysis in family members (N = 230) of 55 chronic hepatitis B carriers (index cases). Hepatitis B surface antigen (HBsAg) and hepatitis B core antibody (anti-HBc) prevalence was 12.2% and 23% among family members, respectively. HBsAg carriers were prevalent in the age groups; <10 (16.2%) and 21–30 years (23.3%). The prevalence of HBsAg was significantly higher in the family members of females (19.2%) than males (8.6%) index cases ($P = 0.031$). HBsAg and anti-HBc seropositive rates were higher significantly in the offspring of females (23%, 29.8%) than those of the males index cases (4.3%, 9.8%) ($P = 0.001$, 0.003), as well as higher in the offspring of an infected mother (26.5, 31.8%) than those of an infected father (4.7%, 10.5%) ($P = 0.0006$, 0.009). No significant difference was found in HBsAg seropositive rates between vaccinated (10.6%) and unvaccinated family members (14.8%). Phylogenetic analysis of the preS2 and S regions of HBV genome showed that the HBV isolates were of subgenotype D1 in nine index cases and 14 family members. HBV familial transmission was confirmed in five of six families with three transmission patterns; maternal, paternal, and sexual. It is concluded that multiple intra-familial transmission routes of HBV genotype D were determined; including maternal, paternal and horizontal. Universal HBV vaccination should be modified by including the first dose at birth with (HBIG) administration to the newborn of mothers infected with HBV. ***J. Med. Virol. 84:587–595, 2012.*** © 2012 Wiley Periodicals, Inc.

KEY WORDS: HBV genotype D; intra-familial transmission; vaccine

INTRODUCTION

Chronic hepatitis B virus (HBV) infection is a major health problem worldwide and is affecting approximately 350 million individuals [Lee, 1997]. Infection with HBV may lead to chronic state of hepatitis in 5–10% of patients who acquired the infection in the adult life and in 80–90% of patients who acquired the infection in the infancy [Chen, 1993]. Infection with HBV can lead to a progressive liver disease including liver cirrhosis and hepatocellular carcinoma (HCC) with approximately 1 million HBV-associated deaths from HCC every year [Seeger and Mason, 2000; Kao and Chen, 2002].

Based on the proportion of the population who are seropositive for hepatitis B surface antigen (HBsAg),

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Mostafa Ragheb and Abeer Elkady contributed equally to this study.

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Short communication

Suppression of hepatitis C virus replicon by adenovirus vector-mediated expression of tough decoy RNA against miR-122a

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ABSTRACT

Recent studies have demonstrated that the liver-specific microRNA (miRNA) miR-122a plays an important role in the replication of hepatitis C virus (HCV). Antisense nucleotides against miR-122a, including locked nucleic acid (LNA), have shown promising results for suppression of HCV replication; however, a liver-specific delivery system of antisense nucleotides has not been fully developed. In this study, an adenovirus (Ad) vector that expresses tough decoy (TuD)-RNA against miR-122a (TuD-122a) was developed to suppress the HCV replication in the liver hepatocytes. Ad vectors have been well established to exhibit a marked hepatotropism following systemic administration. An in vitro reporter gene expression assay demonstrated that Ad vector-mediated expression of TuD-122a efficiently blocked the miR-122a in Huh-7 cells. Furthermore, transduction with the Ad vector expressing TuD-122a in HCV replicon-expressing cells resulted in significant reduction in the HCV replicon levels. These results indicate that Ad vector-mediated expression of TuD-122a would be a promising tool for treatment of HCV infection.

Hepatitis C virus (HCV) is a hepatotropic human virus belonging to a member of the family *Flaviviridae* and possessing a 9.6-kb positive-sense RNA genome. HCV infection causes chronic hepatic inflammation and fibrosis, leading to hepatocellular carcinoma (Hoofnagle, 2002). Currently, 170 million people worldwide are infected with HCV, and suffering from or at risk for the diseases described above. In order to suppress the replication of HCV, PEGylated interferon alpha and ribavirin, which is a nucleotide analogue, have been used as standard-of-care therapy; however, the therapeutic efficiency has been limited, in spite of relatively severe side effects, including fever and malaise (Chisari, 2005; Feld and Hoofnagle, 2005). Another therapeutic strategy should be developed to efficiently suppress the HCV infection and HCV-caused diseases.

Among several host factors involved in HCV infection, the abundant liver-specific microRNA (miRNA), miR-122a has been demonstrated to be crucial for efficient replication and/or translation of the HCV genome (Henke et al., 2008; Jopling et al., 2005; Randall et al., 2007). The HCV genome has two closely spaced miR-122a-binding sites in the 5′-untranslated region (UTR), which contains overlapping *cis*-acting signals involved in translation and RNA synthesis (Jopling et al., 2005). Although the mechanism of the miR-122a-mediated enhancement of HCV replication is controversial (Henke et al., 2008; Jopling et al., 2005; Machlin et al., 2011; Roberts et al., 2011; Wilson et al., 2011), antisense oligonucleotides complementary to miR-122a, including locked nucleic acid (LNA) oligonucleotides, have been shown to significantly inhibit miR-122a and reduce the HCV genome, and thereby to exhibit superior therapeutic effects (Henke et al., 2008; Jopling et al., 2005; Krutzfeldt et al., 2005; Lanford et al., 2010). Intravenous administration of LNA oligonucleotides against miR-122a into HCV-infected chimpanzees resulted in the long-lasting suppression of HCV viremia without viral resistance or severe side effects (Lanford et al., 2010). In addition, the 5′-UTR of the HCV genome is composed of highly conserved structural domains, suggesting that a mutant lacking the miR-122a-binding sites in the genome is unlikely to appear. These results indicate that miR-122a is a promising target for the treatment of HCV-related diseases; however, LNA oligonucleotides accumulate in the kidney immediately after intravenous administration and are excreted into the urine (Fluiter et al., 2003).

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Trans-complemented hepatitis C virus particles as a versatile tool for study of virus assembly and infection

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ABSTRACT

In this study, we compared the entry processes of *trans*-complemented hepatitis C virus particles (HCVtcp), cell culture-produced HCV (HCVcc) and HCV pseudoparticles (HCVpp). Anti-CD81 antibody reduced the entry of HCVtcp and HCVcc to almost background levels, and that of HCVpp by approximately 50%. Apolipoprotein E-dependent infection was observed with HCVtcp and HCVcc, but not with HCVpp, suggesting that the HCVtcp system is more relevant as a model of HCV infection than HCVpp. We improved the productivity of HCVtcp by introducing adapted mutations and by deleting sequences not required for replication from the subgenomic replicon construct. Furthermore, blind passage of the HCVtcp in packaging cells resulted in a novel mutation in the NS3 region, N1586D, which contributed to assembly of infectious virus. These results demonstrate that our plasmid-based system for efficient production of HCVtcp is beneficial for studying HCV life cycles, particularly in viral assembly and infection.

Introduction

Over 170 million people worldwide are chronically infected with hepatitis C virus (HCV), and are at risk of developing chronic liver diseases (Hoofnagle, 2002). HCV is an enveloped virus of the family *Flaviviridae*, and its genome is a positive-strand RNA consisting of the 5′-untranslated region (UTR), an open reading frame encoding viral proteins (core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) and the 3′-UTR (Suzuki et al., 2007).

Host–virus interactions are required during the initial steps of viral infection. It was previously reported that CD81 (Bartosch et al., 2003a, b; McKeating et al., 2004; Pileri et al., 1998), scavenger receptor class B type I (Bartosch et al., 2003a, b; Scarselli et al., 2002), claudin-1 (Evans et al., 2007; Liu et al., 2009) and occludin (Benedicto et al., 2009; Evans et al., 2007; Liu et al., 2009; Ploss et al., 2009) are critical molecules for HCV entry into cells. CD81 interacts with HCV E2 via a second extracellular loop (Bartosch et al., 2003a, b; Hsu et al., 2003) and its role in the internalization process was confirmed (Cormier et al., 2004; Flint et al., 2006). It has also been shown that infectious HCV particles produced in cell cultures (HCVcc) exist as apolipoprotein E (ApoE)-enriched lipoprotein particles (Chang et al., 2007) and that ApoE is important for HCV infectivity (Owen et al., 2009).

Investigation of HCV had been hampered by difficulties in amplifying the virus in vitro before development of robust cell culture systems based on JFH-1 isolates (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). Retrovirus-based HCV pseudoparticles (HCVpp), in which cell entry is dependent on HCV glycoproteins, have been used to study virus entry (Bartosch et al., 2003a; Hsu et al., 2003). Vesicular stomatitis virus (VSV)-based pseudotypic viruses bearing HCV E1 and E2 and replication-competent recombinant VSV encoding HCV envelopes have also been available as surrogate models for studies of HCV infection (Mazumdar et al., 2011; Tani et al., 2007).

It was recently shown that HCV subgenomic replicons can be packaged when structural proteins are supplied in *trans* (Adair et al., 2009; Ishii et al., 2008; Masaki et al., 2010; Steinmann et al., 2008). These *trans*-complemented HCV particles (HCVtcp) are infectious, but support only single-round infection and are unable to spread. Establishment of flexible systems to efficiently produce HCVtcp should contribute to studying HCV assembly, in particular encapsidation of the viral genome, and entry to cells with less stringent biosafety and biosecurity measures. Although single-round infection can be achieved by using the HCVcc system with receptor knock-out

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Hepatology Research

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Original Article

Factors responsible for the discrepancy between *IL28B* polymorphism prediction and the viral response to peginterferon plus ribavirin therapy in Japanese chronic hepatitis C patients

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Aim: *IL28B* polymorphisms serve to predict response to pegylated interferon plus ribavirin therapy (PEG IFN/RBV) in Japanese patients with chronic hepatitis C (CHC) very reliably. However, the prediction by the *IL28B* polymorphism contradicted the virological response to PEG IFN/RBV in some patients. Here, we aimed to investigate the factors responsible for the discrepancy between the *IL28B* polymorphism prediction and virological responses.

Methods: CHC patients with genotype 1b and high viral load were enrolled in this study. In a case–control study, clinical and virological factors were analyzed for 130 patients with rs8099917 TT genotype and 96 patients with rs8099917 TG or GG genotype who were matched according to sex, age, hemoglobin level and platelet count.

Results: Higher low-density lipoprotein (LDL) cholesterol, lower γ-glutamyltransferase and the percentage of wild-type phenotype at amino acids 70 and 91 were significantly associated with the rs8099917 TT genotype. Multivariate analysis showed that rs8099917 TG or GG genotype, older age and lower LDL cholesterol were independently associated with the non-virological responder (NVR) phenotype. In patients with rs8099917 TT genotype (predicted as virological responder [VR]), multivariate analysis showed that older age was independently associated with NVR. In patients with rs8099917 TG or GG genotype (predicted as NVR), multivariate analysis showed that younger age was independently associated with VR.

Conclusion: Patient age gave rise to the discrepancy between the prediction by *IL28B* polymorphism and the virological responses, suggesting that patients should be treated at a younger age.

Key words: aging, genotype, IL28B, low-density lipoprotein cholesterol, single nucleotide polymorphism

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INTRODUCTION

HEPATITIS C VIRUS (HCV) infection is a global health problem with worldwide estimates of 120–130 million carriers.[1] Chronic HCV infection, the leading cause of liver transplantation, can lead to progressive liver disease, resulting in cirrhosis and complications, including decompensated liver disease and hepatocellular carcinoma.[2] The current standard-of-care treatment for suitable patients with chronic HCV infection consists of pegylated interferon-α-2a or -2b (PEG IFN) given by injection in combination with oral ribavirin (RBV) for 24 or 48 weeks, depending on HCV genotype. Large-scale treatment in the USA and Europe showed that 42–52% of patients with HCV genotype 1

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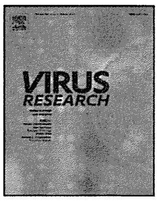

Identification of host genes showing differential expression profiles with cell-based long-term replication of hepatitis C virus RNA

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ABSTRACT

Persistent hepatitis C virus (HCV) infection frequently causes hepatocellular carcinoma. However, the mechanisms of HCV-associated hepatocarcinogenesis and disease progression are unclear. Although the human hepatoma cell line, HuH-7, has been widely used as the only cell culture system for robust HCV replication, we recently developed new human hepatoma Li23 cell line-derived OL, OL8, OL11, and OL14 cells, in which genome-length HCV RNA (O strain of genotype 1b) efficiently replicates. OL, OL8, OL11, and OL14 cells were cultured for more than 2 years. We prepared cured cells from OL8 and OL11 cells by interferon-γ treatment. The cured cells were also cultured for more than 2 years. cDNA microarray and RT-PCR analyses were performed using total RNAs prepared from these cells. We first selected several hundred highly or moderately expressed probes, the expression levels of which were upregulated or downregulated at ratios of more than 2 or less than 0.5 in each set of compared cells (e.g., parent OL8 cells versus OL8 cells cultured for 2 years). From among these probes, we next selected those whose expression levels commonly changed during a 2-year culture of genome-length HCV RNA-replicating cells, but which did not change during a 2-year culture period in cured cells. We further examined the expression levels of the selected candidate genes by RT-PCR analysis using additional specimens from the cells cultured for 3.5 years. Reproducibility of the RT-PCR analysis using specimens from recultured cells was also confirmed. Finally, we identified 5 upregulated genes and 4 downregulated genes, the expression levels of which were irreversibly altered during 3.5-year replication of HCV RNA. These genes may play roles in the optimization of the environment in HCV RNA replication, or may play key roles in the progression of HCV-associated hepatic diseases.

1. Introduction

Hepatitis C virus (HCV) is a causative agent of chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma (HCC) (Choo et al., 1989; Saito et al., 1990; Thomas, 2000). However, the mechanisms of HCV-associated hepatocarcinogenesis and disease progression are still unclear. HCV is an enveloped virus with a positive single-stranded 9.6 kb RNA genome, which encodes a large polyprotein precursor of approximately 3000 amino acid residues. This polyprotein is cleaved by a combination of the host and viral proteases into at least 10 proteins in the following order: Core, envelope 1 (E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (Hijikata et al., 1991, 1993; Kato et al., 1990).

The initial development of a cell culture-based replicon system (Lohmann et al., 1999) and a genome-length HCV RNA-replication system (Ikeda et al., 2002) using genotype 1b strains enabled the rapid progression of investigations into the mechanisms underlying HCV replication (Bartenschlager, 2005; Lindenbach and Rice, 2005). Furthermore, these RNA replication systems have been improved such that they have become suitable for the screening of anti-HCV reagents by the introduction of reporter genes such as luciferase (Ikeda et al., 2005; Krieger et al., 2001). Moreover, in 2005, an efficient virus production system using the JFH1 genotype 2a strain was developed using human hepatoma cell line HuH-7-derived cells (Wakita et al., 2005). However, to date, HuH-7-derived cells are used as the only cell culture

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Abbreviations: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; E1, envelope 1; EGF, epidermal growth factor; RT-PCR, reverse transcription-polymerase chain reaction; IFN, interferon; ACSM3, acyl-CoA synthetase medium-chain family member 3; ANGPT1, angiopoietin 1; CDKN2C, cyclin-dependent kinase inhibitor 2C; PLA1A, phospholipase A1 member A; SEL1L3, Sel-1 suppressor of lin-12-like 3; SLC39A4, solute carrier family 39 member 4; TBC1D4, TBC1 domain family member 4; WISP3, WNT1 inducible signaling pathway protein 3; ANXA1, annexin A1; AREG, amphiregulin; BASP1, brain abundant, membrane attached signal protein 1; CIDEC, cell death activator CIDE-3; CPB2, carboxypeptidase B2; HSPA6, heat-shock 70 kDa protein B'; PI3, peptidase inhibitor 3; SLC1A3, solute carrier family 1 member 3; THSD4, thrombospondin type-1 domain-containing protein 4; ICAM-1, intercellular adhesion molecule-1; ALXR, ANXA1 receptor.

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Research Article

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Generation of metabolically functioning hepatocytes from human pluripotent stem cells by FOXA2 and HNF1α transduction

Kazuo Takayama[1,2], Mitsuru Inamura[1,2], Kenji Kawabata[2,3], Michiko Sugawara[4], Kiyomi Kikuchi[4], Maiko Higuchi[2], Yasuhito Nagamoto[1,2], Hitoshi Watanabe[1,2], Katsuhisa Tashiro[2], Fuminori Sakurai[1], Takao Hayakawa[5,6], Miho Kusuda Furue[7,8], Hiroyuki Mizuguchi[1,2,9,*]

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Background & Aims: Hepatocyte-like cells differentiated from human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs) can be utilized as a tool for screening for hepatotoxicity in the early phase of pharmaceutical development. We have recently reported that hepatic differentiation is promoted by sequential transduction of SOX17, HEX, and HNF4α into hESC- or hiPSC-derived cells, but further maturation of hepatocyte-like cells is required for widespread use of drug screening.
Methods: To screen for hepatic differentiation-promoting factors, we tested the seven candidate genes related to liver development.
Results: The combination of two transcription factors, FOXA2 and HNF1α, promoted efficient hepatic differentiation from hESCs and hiPSCs. The expression profile of hepatocyte-related genes (such as genes encoding cytochrome P450 enzymes, conjugating enzymes, hepatic transporters, and hepatic nuclear receptors) achieved with FOXA2 and HNF1α transduction was comparable to that obtained in primary human hepatocytes. The hepatocyte-like cells generated by FOXA2 and HNF1α transduction exerted various hepatocyte functions including albumin and urea secretion, and the uptake of indocyanine green and low density lipoprotein. Moreover, these cells had the capacity to metabolize all nine tested drugs and were successfully employed to evaluate drug-induced cytotoxicity.
Conclusions: Our method employing the transduction of FOXA2 and HNF1α represents a useful tool for the efficient generation of metabolically functional hepatocytes from hESCs and hiPSCs, and the screening of drug-induced cytotoxicity.

Introduction

Hepatocyte-like cells differentiated from human embryonic stem cells (hESCs) [1] or human induced pluripotent stem cells (hiPSCs) [2] have more advantages than primary human hepatocytes (PHs) for drug screening. While application of PHs in drug screening has been hindered by lack of cellular growth, loss of function, and de-differentiation *in vitro* [3], hESC- or hiPSC-derived hepatocyte-like cells (hESC-hepa or hiPSC-hepa, respectively) have potential to solve these problems.

Hepatic differentiation from hESCs and hiPSCs can be divided into four stages: definitive endoderm (DE) differentiation, hepatic commitment, hepatic expansion, and hepatic maturation. Various growth factors are required to mimic liver development [4] and to promote hepatic differentiation. Previously, we showed that transduction of transcription factors in addition to treatment with optimal growth factors was effective to enhance hepatic differentiation [5–7]. An almost homogeneous hepatocyte population was obtained by sequential transduction of SOX17, HEX, and HNF4α into hESC- or hiPSCs-derived cells [7]. However, further maturation of the hESC-hepa and hiPSC-hepa is required for widespread use of drug screening because the drug metabolism capacity of these cells was not sufficient.

In some previous reports, hESC-hepa and hiPSC-hepa have been characterized for their hepatocyte functions in numerous ways, including functional assessment such as glycogen storage and low density lipoprotein (LDL) uptake [7]. To make a more precise judgment as to whether hESC-hepa and hiPSC-hepa can be applied to drug screening, it is more important to assess cytochrome P450 (CYP) induction potency and drug metabolism capacity rather than general hepatocyte function. Although Duan *et al.* have examined the drug metabolism capacity of hESC-hepa, drug metabolites were measured at 24 or 48 h [8]. To precisely

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Keywords: FOXA2; HNF1α; Hepatocytes; Adenovirus; Drug screening; Drug metabolism; hESCs; hiPSCs.
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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Geranylgeranylacetone has anti-hepatitis C virus activity via activation of mTOR in human hepatoma cells

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Abstract

Background Geranylgeranylacetone (GGA), an isoprenoid compound which includes retinoids, has been used orally as an anti-ulcer drug in Japan. GGA acts as a potent inducer of anti-viral gene expression by stimulating ISGF3 formation in human hepatoma cells. This drug has few side effects and reinforces the effect of IFN when administered in combination with peg-IFN and ribavirin. This study verified the anti-HCV activity of GGA in a replicon system. In addition, mechanisms of anti-HCV activity were examined in the replicon cells.

Methods OR6 cells stably harboring the full-length genotype 1 replicon containing the *Renilla* luciferase gene, ORN/C-5B/KE, were used to examine the influence of the anti-HCV effect of GGA. After treatment, the cells were harvested with Renilla lysis reagent and then subjected to a luciferase assay according to the manufacturer's protocol.

Result The results showed that GGA had anti-HCV activity. GGA induced anti-HCV replicon activity in a time- and dose-dependent manner. GGA did not activate the tyrosine 701 and serine 727 on STAT-1, and did not induce HSP-70 in OR6 cells. The anti-HCV effect depended on the GGA induced mTOR activity, not STAT-1 activity and PKR. An additive effect was observed with a combination of IFN and GGA.

Conclusions GGA has mTOR dependent anti-HCV activity. There is a possibility that the GGA anti-HCV activity can be complimented by IFN. It will be necessary to examine the clinical effectiveness of the combination of GGA and IFN for HCV patients in the future.

Keywords mTOR · STAT-1 · Interferon · HCV · GGA

Abbreviations

IFN	Interferon
HCV	Hepatitis C virus
STAT	Signal transducers and activators of transcription
ISGF-3	IFN-stimulated gene factor 3
ISRE	IFN-stimulated regulatory element
PKR	Double-stranded RNA-dependent protein kinase
Rapa	Rapamycin
PI3-K	Phosphatidylinositol 3-kinase
mTOR	Mammalian target of rapamycin
GGA	Geranylgeranylacetone
siRNA	Small interfering RNA

Introduction

Currently, chronic hepatitis C virus (HCV) infection is the major cause of hepatocellular carcinoma worldwide [1]. Therefore, an anti-HCV strategy is important for prevention of carcinogenesis. The treatment of HCV with a combination of pegylated interferon (IFN) and ribavirin is effective in 80% of HCV genotype 2 or 3 cases, but less than 50% of genotype 1 cases. New anti-HCV agents have been developed to inhibit the life cycle of HCV and are

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Proteomic Analysis of Hepatitis C Virus (HCV) Core Protein Transfection and Host Regulator PA28γ Knockout in HCV Pathogenesis: A Network-Based Study

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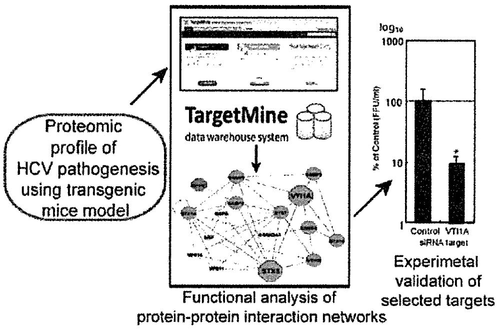
S *Supporting Information*



ABSTRACT: Hepatitis C virus (HCV) causes chronic liver disease worldwide. HCV Core protein (Core) forms the viral capsid and is crucial for HCV pathogenesis and HCV-induced hepatocellular carcinoma, through its interaction with the host factor proteasome activator PA28γ. Here, using BD-PowerBlot high-throughput Western array, we attempt to further investigate HCV pathogenesis by comparing the protein levels in liver samples from Core-transgenic mice with or without the knockout of PA28γ expression (abbreviated PA28$\gamma^{-/-}$CoreTG and CoreTG, respectively) against the wild-type (WT). The differentially expressed proteins integrated into the human interactome were shown to participate in compact and well-connected cellular networks. Functional analysis of the interaction networks using a newly developed data warehouse system highlighted cellular pathways associated with vesicular transport, immune system, cellular adhesion, and cell growth and death among others that were prominently influenced by Core and PA28γ in HCV infection. Follow-up assays with *in vitro* HCV cell culture systems validated VTI1A, a vesicular transport associated factor, which was upregulated in CoreTG but not in PA28$\gamma^{-/-}$CoreTG, as a novel regulator of HCV release but not replication. Our analysis provided novel insights into the Core-PA28γ interplay in HCV pathogenesis and identified potential targets for better anti-HCV therapy and potentially novel biomarkers of HCV infection.

KEYWORDS: *CoreTG, GO, HCC, HCV, KEGG, OMIM, PA28$\gamma^{-/-}$CoreTG, PPI, siRNA, TargetMine*

■ INTRODUCTION

Hepatitis C virus (HCV) is a prime cause of chronic liver disease frequently characterized by liver inflammation with accompanying steatosis, progressive fibrosis, and hepatocellular carcinoma (HCC) and infects nearly 3% of the world's population. HCV contains a single-stranded RNA genome encoding a 3000-amino-acid polyprotein, which is processed by host and viral factors to yield 10 viral proteins, Core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B.[1−4] HCV variants are classified into six major genotypes with multiple subtypes characterized by phylogenetic heterogeneity, differences in infectivity, and interferon sensitivity.[5,6] The availability of cell-culture-based systems for HCV infection has provided an increased understanding of HCV pathogenesis.[5,7−9] Transgenic mice (preferably C57BL strain) expressing HCV proteins in the liver are also a preferred choice for the investigation of HCV pathogenesis.[10] However, despite considerable research efforts, precise molecular mechanisms underlying HCV pathology remain unclear.

HCV Core protein (hereafter referred to as Core) is spliced from the polyprotein by the signal peptidase and further processed into a highly conserved 21-kDa mature form by the signal peptide peptidase; this processing facilitates its transfer to the detergent-resistant membrane fraction where virus replication and assembly take place. Core is a multifunctional protein implicated in RNA binding and as a pathogenic factor; it induces steatosis and HCC and, thus, liver failure.[1,10] The ubiquitin-proteasome pathway, the premier intracellular protein degradation system in eukaryotes, is a key regulator of cellular processes and is also associated with the evasion of host immune response by many viruses, viral maturation, and progeny release.[11] Core binds to the proteasome activator PA28γ in the nucleus and is degraded via a PA28γ-dependent pathway. PA28γ plays a crucial role in Core-induced insulin resistance, steatogenesis, and hepatocarcinogenesis and in HCV propagation; PA28γ knockout in Core transgenic mice disrupts

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