Exp. Anim. 60(2), 93–100, 2011

—Review—
Review Series: Frontiers of Model Animals for Human Diseases

An Experimental Mouse Model for Hepatitis C Virus

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Abstract: Chronic hepatitis C virus (HCV) infection affects approximately 170 million people and is a major global health problem because infected individuals can develop liver cirrhosis and hepatocellular carcinoma. Despite significant improvements in antiviral drugs, only around 50% of treated patients with genotype 1 and 4 demonstrate HCV clearance. Unfortunately, an anti-HCV vaccine is still not available. To progress treatment of HCV, it is necessary to understand the mechanism(s) by which HCV infects hepatocytes, and how the host immune response prevents the spread of the virus. Because HCV infects only humans and chimpanzees, it is difficult to evaluate immune response mechanisms, and the effects of chemicals and new technologies on these response mechanisms. These difficulties underline the importance of establishing a small HCV-infected animal model. This review focuses on the progress made in recent years towards the development of an experimental mouse model for HCV.

Key words: apoptosis, B cell lymphoma, HCV, immune response, transgenic mice

Introduction

Hepatitis C virus (HCV) is a non-cytopathic, hepatotropic member of the *Flaviviridae* family, causing acute and chronic necroinflammatory liver diseases [25]. Chronic HCV infection has caused an epidemic with approximately 170 million people infected worldwide and three to four million people newly infected each year [25, 35]. Natural history studies show that 5–20% of patients develop cirrhosis after about 20 years of infection [1, 42, 49]. An increasing number of patients with cirrhosis will develop hepatocellular carcinoma. End-stage liver disease due to chronic HCV infection is the leading cause of liver transplantation in the western world [36]. Furthermore, co-infection with HCV and human immunodeficiency virus (HIV) results in more serious liver cirrhosis than HCV infection alone and the mortality of HIV-infected HCV patients is a serious problem in the USA [44. 55].

The HCV genome is a 9.6-kb, uncapped, linear, single-strand RNA molecule with positive polarity that serves as a template for both translation and replication. Translation of the plus-strand RNA initiates at an internal ribosomal entry site, resulting in the production of a single polyprotein precursor that is processed into structural (C, E1, E2, p7) and non-structural (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) protein subunits by host and viral proteases [8, 9, 37, 46]. Because of the lack of a proofreading func-

(Received 20 December 2010 / Accepted 29 January 2010)
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tion for the RNA-dependent RNA-polymerase (NS5B), HCV has a high mutation rate and exists as a genetically heterogeneous quasispecies in individual patients [8].

It is well established that the pathogenesis of HCV infection is controlled by host-virus interactions mediated by the immune response [4, 5, 13]. It has been difficult to clarify the relationship between HCV and the host immune response because of the fact that HCV is only infectious in humans and chimpanzees [3, 11]. It has also been demonstrated that viral clearance during self-limited acute HCV infection is characterized by a vigorous, polyclonal CD4 and CD8 T-cell response [6, 12, 29, 38, 43]. However, the lack of activity in inducing an effective antigen-specific CD4 and CD8 T-cell response in chronically infected HCV cases has been studied in chimpanzees [50], and also in humans infected via needle stick accidents [47]. These studies suggest that a poor immunological response to HCV probably leads to persistent infection. Failure to effectively produce antigen-specific T-cells is possibly the result of viral overload [15–17] and the high level of regulatory T cells present [40]. However, it remains unknown why HCV infection causes persistent infection. It has also been demonstrated that viral replication is inhibited by antigen-specific T cells as well as natural killer (NK) cells, natural killer T (NKT) cells and macrophages in the liver of hepatitis B virus (HBV) transgenic mice [18, 21, 22], indicating that viral clearance requires innate and acquired immune responses.

In this review, we describe an experimental mouse model for HCV infection which allows us to analyze the mechanisms of chronic persistent infection.

HCV Transgenic Mice Models

Generation of HCV transgenic mice using the Cre/loxp switching system

A transgenic mouse model using a stable expression system generates immunotolerance to transgene products. Therefore, an HCV protein switching expression system may be suitable for *in vivo* assays of HCV protein effects, as HCV is thought to infect humans with a mature immune function [1]. Using the *Cre/loxP* system, we developed a transgenic mouse model with efficient conditional transgene activation of HCV cDNA (core, E1, E2, and NS2) (Fig. 1), and two transgenic lines, CN2-8 and CN2-29, were generated [48]. The *Cre/loxP* system has been used in combination with a recombinant adenovirus vector expressing Cre to alter gene expression in the livers of transgenic mice [34]. Temporal control of viral gene expression using a conditional transgene activation system enables detailed analysis of the immune responses in the host, and observation of cytopathic effects due to viral proteins. HCV proteins were mainly detected in the livers of conditionally expressing transgenic mice. Efficient recombination was observed in transgenic mice livers upon intravenous administration of adenoviruses expressing Cre DNA recombinase. After transgene activation, most hepatocytes were stained with anti-core polyclonal antibody, and 21-, 37-, and 64-kDa proteins were detected by western blot analysis in liver lysates using anti-core, -E1, and -E2 monoclonal antibodies, respectively. Serum core protein was detected in transgenic mice 7 days after transgene activation with concurrent increases in serum alanine aminotransferase levels. Importantly, we observed no significant histopathological changes between infected and uninfected CN2 transgenic mice when CD4- and CD8-positive cells were depleted in the infected mice (Fig. 2). These results suggest that HCV structural proteins are not strongly cytopathic to hepatocytes in the absence of an immune response.

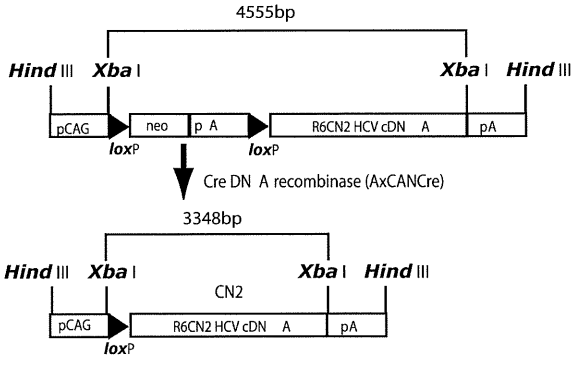

Fig. 1. Structure of CALNCN2, the Cre-mediated activation transgene unit. R6CN2 HCV cDNA (nucleotides 294–3435) was cloned downstream of the CAG promoter, neomycin-resistant gene (neo) and poly(A) signal that was flanked by two *loxP* sequences. R6CN2 HCV cDNA contains the core, E1, E2, and NS2 regions.

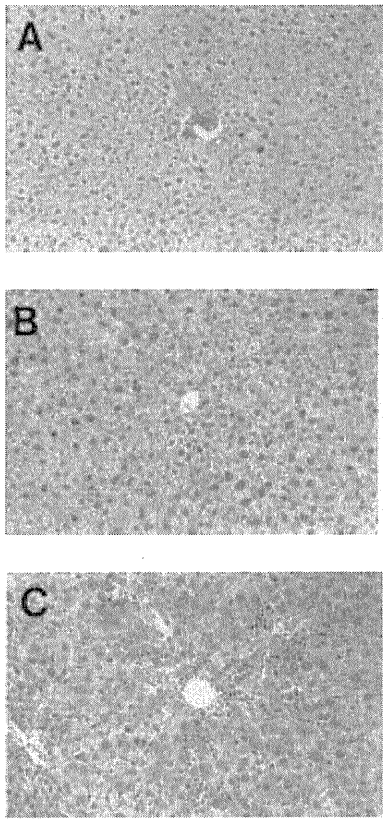

Fig. 2. Histopathology of livers of HCV Tg mice. A, uninfected CN2-8 mouse. B, $CD4^+$ and $CD8^+$ cell-depleted CN2-8 mouse infected with AxCANCre and analyzed at day 7. C, CN2-8 mouse infected with AxCANCre and analyzed at day 7. (×250 magnification).

HCV transgenic mice are resistant to Fas antibody-mediated lethality

The role of apoptosis in HCV infection is not well defined. Furthermore, the kinetics and extent of hepatocyte apoptosis as well as the pro- and anti-apoptotic mechanisms involved remain unclear. We demonstrated that transgene expression in HCV transgenic mice causes resistance to Fas antibody-stimulated lethality [27]. Apoptotic cell death in the livers of HCV protein-expressing mice was significantly reduced compared to non-expressing mice. Histopathological analysis and DNA fragmentation analysis revealed that the HCV proteins suppressed *Fas*-mediated apoptotic cell death. To identify the target pathway of HCV proteins, we characterized caspase activity and showed that the activation of caspase-9 and caspase-3/7, but not caspase-8, was inhibited by HCV proteins. In addition, cytochrome *c* release from mitochondria was inhibited in HCV protein-expressing mice. These results indicate that the expression of HCV proteins may have directly or indirectly inhibited Fas-mediated apoptosis and cell death in mice by repressing the release of cytochrome *c* from mitochondria, thereby suppressing caspase-9 and caspase-3/7 activation. Furthermore, HCV might cause persistent infection as a result of suppression of *Fas*-mediated cell death and inhibition of HCV-infected hepatocyte rejection in the liver, given that numerous viruses have been reported to evade apoptotic mechanisms resulting persistent infection [23].

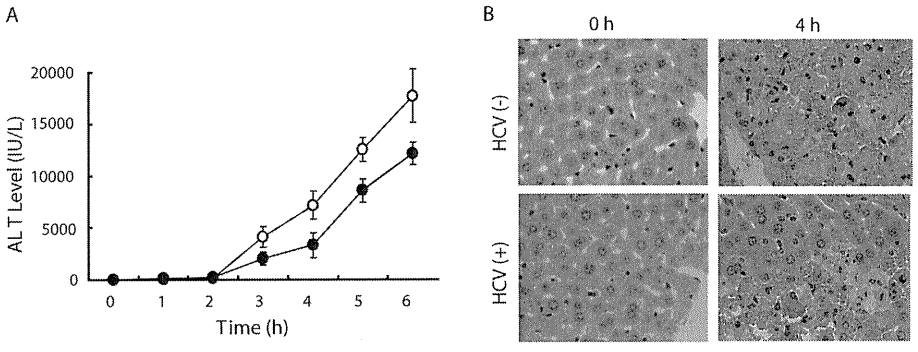

Fig. 3. Serum ALT level and histological analysis of livers after anti-Fas antibody administration. A, serum ALT levels of anti-Fas antibody-injected HCV non-expressing (*open circles*) and expressing (*closed circle*) mice. ALT levels are expressed as the mean ± SD of three individual experiments. B, hematoxylin and eosin staining of liver sections from transgenic mice at pretreatment and 4 h after anti-Fas antibody injection.

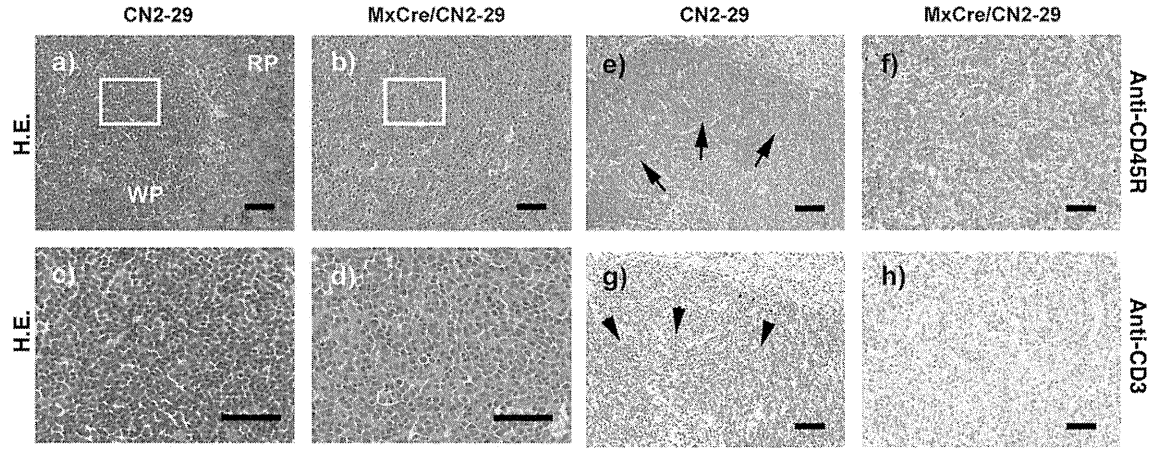

Fig. 4. Stable expression of HCV viral proteins induces lymphoproliferative diseases. The white pulp (WP) and red pulp (RP) comprise the components of the spleen in wild-type (WT) mice. The neoplastic cells replace the normal structures, such as the white pulp and red pulp. (c and d) The neoplastic cells are larger than lymphocytes (c), and the nuclei are irregular, round, oval, elongated, and polygonal (d). (e and g) The white pulp in WT mice consisted of both a B-cell-rich area (arrows, e) and a T-cell-rich area (arrowheads, g). (f and h) The neoplastic cells show staining for the B cell marker, CD45R, thereby supporting the diagnosis of B-cell lymphoma (f), while they do not show staining for the T-cell marker CD3 (h). Frames c and d are higher-magnification views of the boxed areas (white) in a and b, respectively.

The relationship between chronic HCV infection and lymphoma during interferon (IFN) disruption

It has been demonstrated that HCV infection causes lymphoproliferative diseases, such as B cell non-Hodgkin's lymphomas and mixed cryoglobulinemia [25, 39]. We established IFN regulatory factor-1-null (*irf-1*$^{-/-}$) mice with inducible and persistent expression of HCV structural proteins (*irf-1*/CN2 mice), in order to evaluate the molecular mechanisms of lymphoproliferation associated with the disruption of IFN signaling and chronic HCV infection [28]. *Irf-1*/CN2 mice had extremely high incidences of lymphomas and lymphoproliferative disorders and displayed increased mortality. Disruption of *irf-1* reduced their sensitivity to *Fas*-induced apoptosis and decreased the levels of caspase-3/7 and caspase-9 mRNA species and associated enzymatic activities. Furthermore, the *irf-1*/CN2 mice showed decreased activation of caspase-3/7 and caspase-9 and increased levels of interleukin (IL)-2, IL-10, and Bcl-2, which promote oncogenic transformation of lymphocytes. Disruption of IFN signaling resulted in the development of lymphomas, indicating that differential signaling occurs in lymphocytes rather than in the hepatocyte. *IRF-1*-inducible genes probably play essential roles in suppressing HCV-induced lymphomas and in eliminating HCV protein-expressing cells. Our transgenic mice provide evidence that the overexpression of apoptosis-related proteins, including Bcl-2, and/or aberrant cytokine production are primary events in HCV-induced lymphoproliferation.

HCV proteins expressed in B cells cause lymphoma

To extend the above-mentioned study with regard to the interaction of lymphoma and HCV infection, we established HCV transgenic mice that expressed the full HCV genome in B cells (RzCD19Cre mice) and observed a 25.0% incidence of diffuse large B cell non-Hodgkin's lymphomas within 600 days after birth [19]. The incidence of B cell lymphoma significantly correlated with the level of soluble IL-2 receptor alpha subunit (sIL-2Ralpha) in RzCD19Cre mouse serum. All RzCD19Cre mice with substantially elevated serum sIL-2Ralpha levels (>1,000 pg/ml) developed B cell lymphomas. Compared with tissues from control animals, the B cell lymphoma tissues of RzCD19Cre mice expressed significantly higher levels of sIL-2Ralpha. We showed that the expression of HCV in B cells promotes non-Hodgkin's-type diffuse B cell lymphoma, and therefore, the RzCD19Cre mouse is an appropriate model for studying

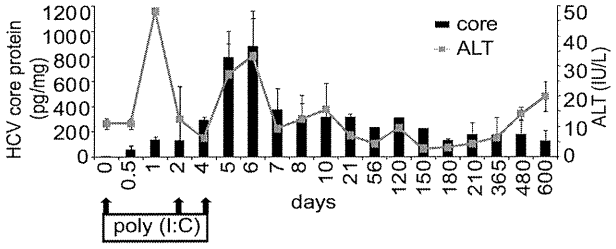

Fig. 5. Pathogenesis of the immunocompetent HCV persistent infection mouse model. HCV core protein expression was sustained for at least 600 days after poly(I:C) injection. Serum ALT levels also continued to elevate.

the mechanisms related to the development of HCV-associated B cell lymphoma [19].

Chronic persistent expression in HCV transgenic mice

We generated another switching system to study the expression of HCV proteins using Mx promoter-driven Cre recombinase with poly(I:C) induction. The *Mx* promoter is active in hepatocytes as well as in hematopoietic cells. We crossed CN2 mice with *Mx1-Cre* transgenic mice, in which Cre recombinase is expressed by the IFN-inducible *Mx1* promoter. Injection of *Mx1-Cre*/CN2-29 mice with poly(I:C) induces IFN production and the expression of CN2 gene products in hematopoietic cells (mainly in Kupffer cells and lymphocytes), livers, and spleens but not in most other tissues. As illustrated in Fig. 5, the serum alanine aminotransferase (ALT) levels increased, peaking at 24 h after the first poly(I:C) injection. These serum ALT levels then decreased until day 4, when they increased again until day 6, along with HCV core protein levels. Thereafter, HCV core protein was observed consistently for at least 600 days. We also showed that the serum ALT levels gradually increased after day 210 despite no change in the HCV core protein levels. Histological analysis showed that the HCV core protein was expressed in most hepatocytes of transgenic mice which also exhibited lymphocytic infiltration by the core protein (Fig. 5). These observations indicate that the expression of the HCV proteins caused chronic hepatitis in the CN2-$29^{(+/-)}$/$MxCre^{(+/-)}$ mice because of a weak and persistent immune response. 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 hepatocellular carcinoma. We are convinced that HCV transgenic mice are suitable for evaluating the mechanisms of persistent HCV infection and for assisting with the design of HCV vaccines.

Role of NK cells in the antiviral effect of HCV transgenic mice

The liver is enriched with NK cells and this intrahepatic population is embedded in the endothelial lining of the liver sinusoids. These NK cells were originally described as 'pit' cells [20]. Intrahepatic NK cells may behave differently to NK cells in other areas because of the 'tolerogenic' environment of the liver, with murine intrahepatic NK cells known to be hyporesponsive. They are less cytotoxic and have an altered cytokine profile producing lower levels of IFN-γ and greater levels of immunoregulatory cytokines, such as IL-10, than peripheral blood and splenic NK cells [24]. This hyporesponsive state has been described in the early stages of HBV infection and may contribute to the establishment of chronic viral infection [7]. Peripheral blood NK cell frequencies, both the absolute number and the percentage of the total lymphocyte population, are reduced in chronic HCV compared to healthy individuals [30]. In individuals with chronic HCV infection, NK cell frequency increases following successful antiviral therapy, while a reduction in peripheral blood NK cell frequency in individuals with chronic HCV as compared to spontaneous resolvers has also been noted [10]. Thus, NK cells may play key roles in suppressing HCV replication. We actually observed much higher levels of HCV core proteins in Tg mice with a depleted population of NK cells. Furthermore, Cre-mediated genomic DNA recombination efficiency in HCV-Tg mice was strong in NK cell-depleted mice between 0.5 and 1 day compared to untreated mice. These data indicate that NK cells participate in the elimination of core expressing hepatocytes during the innate immune response in the acute phase of HCV infection [41].

Chimeric Human Liver Mice Model

Mercer *et al.* generated mice with chimeric human livers by transplanting normal human hepatocytes into

SCID mice carrying a plasminogen activator transgene (*Alb-uPA*). Homozygosity of *Alb-uPA* was associated with significantly higher levels of human hepatocyte engraftment, and these mice developed prolonged HCV infections with high viral titers after inoculation with infected human serum [31].

We used the chimeric mice as they were a vast improvement over the originals, which had a high substitution rate of human hepatocytes [45], and examined the inhibitory effect of DEBIO-025, a novel non-immunosuppressive cyclophilin inhibitor derived from cyclosporin A, on naïve HCV genotypes 1a or 1b *in vivo* [14]. Collectively, this small animal model is useful for assessing the activity of antiviral compounds [33] and for evaluating protection and passive immunization studies of HCV [26, 32], but because they lack an immune system, this model is not suitable for studies of HCV pathogenesis.

A recent study showed that in $Fah^{-/-}Rag2^{-/-}Il2rg^{-/-}$ mice, the selection pressure for transplanted human hepatocytes can be regulated by the drug 2-(2-nitro-4-trifluoro-methylbenzoyl)-1,3-cyclohexanedione. In the absence of this drug, mouse hepatocytes die because of the accumulation of toxic tyrosine catabolites and a lack of fumarylacetoacetate hydrolase, while human hepatocytes remain healthy. These mice have a high level of human liver chimerism, they propagate both HBV and HCV, and the HCV-infected mice are responsive to antiviral treatment. It seems that this human liver chimeric mouse model will be useful for studying HBV and HCV infection, and it has already proven valuable in antiviral drug testing [2].

The development of molecular biological techniques has allowed us to generate transgenic mice. Using these techniques we are able to analyze the immune responses to various viral proteins in mice, even though the virus does not normally infect murine species. It is essential to generate an infectious HCV mouse model for a more precise analysis of the interaction between host and virus. The chimeric human liver mouse model would appear to be a powerful tool for evaluating the effects of antiviral drugs. It is hoped that an experimental mouse model for HCV will yield a number of useful insights into the immunopathogenesis of this viral infection, and assist in the development of antiviral drugs.

Acknowledgments

This study was supported by a grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan and a grant-in-aid for specially promoted research on viral diseases from the Tokyo metropolitan government to K. K., a grant from the Ministry of Health, Labour and Welfare of Japan, and Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation of Japan and National Institutes of Health research grants to M.K.

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Augmentation of DHCR24 expression by hepatitis C virus infection facilitates viral replication in hepatocytes

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Background & Aims: We characterized the role of 24-dehydrocholesterol reductase (DHCR24) in hepatitis C virus infection (HCV). DHCR24 is a cholesterol biosynthetic enzyme and cholesterol is a major component of lipid rafts, which is reported to play an important role in HCV replication. Therefore, we examined the potential of DHCR24 as a target for novel HCV therapeutic agents.
Methods: We examined DHCR24 expression in human hepatocytes in both the livers of HCV-infected patients and those of chimeric mice with human hepatocytes. We targeted *DHCR24* with siRNA and U18666A which is an inhibitor of both DHCR24 and cholesterol synthesis. We measured the level of HCV replication in these HCV replicon cell lines and HCV infected cells. U18666A was administrated into chimeric mice with humanized liver, and anti-viral effects were assessed.
Results: Expression of DHCR24 was induced by HCV infection in human hepatocytes *in vitro*, and in human hepatocytes of chimeric mouse liver. Silencing of *DHCR24* by siRNA decreased HCV replication in replicon cell lines and HCV JFH-1 strain-infected cells. Treatment with U18666A suppressed HCV replication in the replicon cell lines. Moreover, to evaluate the anti-viral effect of U18666A *in vivo*, we administrated U18666A with or without pegylated interferon to chimeric mice and observed an inhibitory effect of U18666A on HCV infection and a synergistic effect with interferon.
Conclusions: DHCR24 is an essential host factor which augmented its expression by HCV infection, and plays a significant role in HCV replication. DHCR24 may serve as a novel anti-HCV drug target.

Introduction

Extensive epidemiological studies have identified multiple risk factors for hepatocellular carcinoma (HCC), including chronic infection with hepatitis C virus (HCV), and hepatitis B virus (HBV), and cirrhosis due to non-viral etiologies, such as alcohol abuse and aflatoxin B1 exposure [1,2]. Of these factors, HCV appears to be the dominant causative factor for HCC in many developed countries. The World Health Organization estimates that 170 million people worldwide are infected with HCV and are, therefore, at risk of developing liver cirrhosis and HCC [3]. The combination of pegylated interferon-α (PEG-IFN-α) and ribavirin is currently the standard treatment regimen for patients with chronic HCV infection. However, viral clearance is achieved in only 40% to 60% of patients and depends on the HCV genotype with which the patient is infected [4].

We previously established the RzM6 cell line, a HepG2 cell line in which the full-length HCV genome (HCR6-Rz) can be conditionally expressed under control of the Cre/*lox*P system and is precisely self-trimmed at the 5′ and 3′-termini by ribozyme sequences [5]. Anchorage-independent growth of these cells accelerates after 44 days of continuous passaging, during which the Cdk-Rb-E2F pathway is activated [5]. In a previous study, we developed monoclonal antibodies (MoAbs) against cell surface antigens on HCV-expressing cells that had been passaged for over 44 days [6]. One of the targets of these MoAbs was 24-dehydrocholesterol reductase (DHCR24 is also called 3-β-hydroxysterol-Δ-24-reductase, seladin-1, desmosterol delta-24-reductase), a molecule that is frequently overexpressed in the hepatocytes of HCV-infected patients.

Keywords: Hepatitis C virus; Replication; DHCR24; U18666A.
Received 18 April 2010; received in revised form 11 November 2010; accepted 2 December 2010; available online 22 December 2010
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Abbreviations: DHCR24, 24-dehydrocholesterol reductase; HCV, hepatitis C virus; MoAb, monoclonal antibody; HCC, hepatocellular carcinoma; HBV, hepatitis B virus.

DHCR24 confers resistance to apoptosis in neuronal cells [7]. It also regulates the cellular response to oxidative stress by binding to the amino terminus of p53, thereby displacing mouse double minute 2 homolog isoform MDM2 (*Homo sapiens*) (MDM2) from p53 and inducing the accumulation of p53 in human embryonic fibroblasts [8].

DHCR24 is a cholesterol biosynthetic enzyme that is also called desmosterol reductase [9,10]. Cholesterol is a major component of lipid rafts, which are reported to play an important role in HCV replication [11]. Therefore, we characterized the role of DHCR24 in HCV replication and evaluated its potential as a target for novel HCV therapeutic agents. We also examined the synergistic antiviral effect of U18666A which is an inhibitor of both DHCR24 [12] and cholesterol synthesis [13] with IFN-α in the treatment of HCV.

Materials and methods

Cells and plasmids

Cell culture methods of the HuH-7 [14], HepG2 [15], hybridoma and myeloma PAI cells, RzM6 cells [5], and the HCV subgenomic replicon cells lines FLR3-1 (genotype 1b, strain Con-1; [16]), R6FLR-N (genotype 1b, strain N; [17]), and Rep JFH Luc3-13) genotype 2a, strain JFH-1 [18]) were utilized to evaluate HCV replication [19] are described in Supplementary data.

The *DHCR24* cDNA was synthesized and amplified by PCR using Phusion™ DNA polymerase (Finnzymes) and cloned into the pcDNA3.1 vector (Invitrogen) or lentivirus vector, as described previously [6].

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry analysis

The detailed procedures are described in Supplementary data [20].

Immunohistochemistry and Western blot analysis

The detailed procedures are described in Supplementary data.

The antibodies used in this experiment were: anti-Core, anti-NS3, anti-NS4B, anti-NS5B [5], and anti-NS5A (kindly provided by Dr. Matsuura, Osaka University), and anti-actin (Sigma).

Inhibition of DHCR24 by siRNA

We synthesized two siRNAs that were directed against human *DHCR24* mRNA: siDHCR24-417 and siDHCR24-1024. The target sequence of siDHCR24-417 was 5′-GUACAAGAAGACACACAAATT-3′, while that of siDHCR24-1024 was 5′-GAGAACUAUCUGAAGACAATT-3′. Additionally, we used siRNAs targeted against the HCV genome (siE-R7 and siE-R5) [17,21]. The siCONTROL Non-Targeting siRNA #3 (Dharmacon RNA Technologies) was used as the negative control siRNA. The chemically synthesized siRNAs were transfected into cells using Lipofectamine RNAiMAX (Invitrogen) and Opti-MEM (Invitrogen) by reverse-transfection. Cells were characterized 72 h after transfection.

Inhibition of viral replication by U18666A

U18666A (Calbiochem) was utilized to treat HCV replicon cells at a concentration of 62.5–1000 nM and chimeric mice at a concentration of 10 mg/kg (i.p.).

To determine whether cholesterol can reverse the U18666A treatment by the addition of cholesterol, we performed the experiments using HCV replicon cells (4×10^3 cells/well in a 96-well white plate, SUMILON). Culture medium was replaced after the cells had spread (at 24 h), and LDL (Calbiochem) was added to reach a final cholesterol concentration of 50 μg/ml. After a 24 h-incubation, U18666A (62.5, 125, 250, 500, and 1000 nM) was added to each well, and the cells were incubated for an additional 48 h. HCV replication activity was measured by luciferase assay, and cell viability was measured with the WST-8 cell counting kit according to the manufacturer's instructions (Dojindo Laboratories). Cholesterol measurements are described in Supplementary data.

Inhibition assay of HCV replication in replicon cells and persistent infected cells

For evaluation of the anti-HCV replication effect of the inhibitor U18666A in replicon cells and HCV persistently infected cells are described in Supplementary data.

Real-time detection (RTD)-PCR

Total RNA was purified from JFH-K4 cells that had been treated with siRNA or U18666A by the acid guanidium–phenol–chloroform method. HCV RNA was quantified by RTD-PCR as previously described [22].

HCV infection of chimeric mice with humanized liver and mRNA quantification by RTD-PCR

We used chimeric mice that were created by transplanting human primary hepatocytes into severe combined immunodeficient mice carrying a urokinase plasminogen activator transgene [23,24] that was controlled by the albumin promoter. These hepatocytes had been infected with plasma from a HCV-positive patient HCR6 (genotype 1b) [19]. The HCV 1b RNA level reached 2.9–18.0×10^6 copies/ml in mouse sera after 1–2 months of infection. HCV RNA in the mouse serum or total RNA from liver tissue from humanized chimeric mice with/without HCV infection was extracted using the acid guanidium–phenol–chloroform method. HCV RNA and *DHCR24* mRNA levels were quantified by RTD-PCR [22]. The primers and probes for HCV were prepared as previously described [22], and the primers and probes for *DHCR24* were prepared using TaqMan® Gene Expression assays (Applied Biosystems) according to the manufacturer's instructions. PEG-IFNα-2a (Chugai) was administered subcutaneously at a concentration of 30 μg/kg, at day 1, 4, 8, and 11 (the amount of PEG-IFN-α administered to the chimeric mice was 20-fold relative to that used in humans), and U18666A was administered intraperitoneally at a concentration of 10 mg/kg, every day for 2 weeks (Fig. 6A). The protocols for the animal experiments were approved by the local ethics committee.

Human serum albumin in the blood of humanized chimeric mice was measured using a commercially available kit, according to the manufacturer's instructions (Alb-II kit; Eiken Chemical).

Results

Identification of DHCR24

We inoculated mice (BALB/c) with RzM6 cells that expressed HCV protein and had been cultured for over 44 days (denoted as RzM6-LC cells); mice were inoculated at least seven times over a 2-week period. We then fused the splenocytes from mice that had been immunized with RzM6-LC cells to myeloma cells to establish hybridomas. Characterization of the culture supernatant from more than 1000 hybridoma cells by ELISA (data not shown) revealed that one MoAb clone (2-152a) recognized a molecule of approximately 60 kDa in various cells (Supplementary Fig. 1A and B. This molecule was more highly expressed in RzM6-LC cells (Supplementary Fig. 1A), HeLa cells, and HCC cell lines (HepG2, HuH-7, Hep3B, and PLC/PRF/5) than in HEK293 cells and several normal liver cell lines (NKNT, TTNT, and WRL68) (Supplementary Fig. 1B). To further characterize this molecule, we performed matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and obtained seven peptide sequences (Supplementary Fig. 1C, underlined). These peptide sequences suggested that the molecule that was recognized by the 2-152a antibody was DHCR24. We constructed a lentivirus expression vector containing myc-tagged DHCR24 (DHCR24-myc) and transduced it into HepG2 cells. By western blot analysis with 2-152a and anti-Myc antibody, we then confirmed that DHCR24 was expressed in the transduced cells (Supplementary Fig. 1D). We found that the 2-152a antibody specifically recognized DHCR24.

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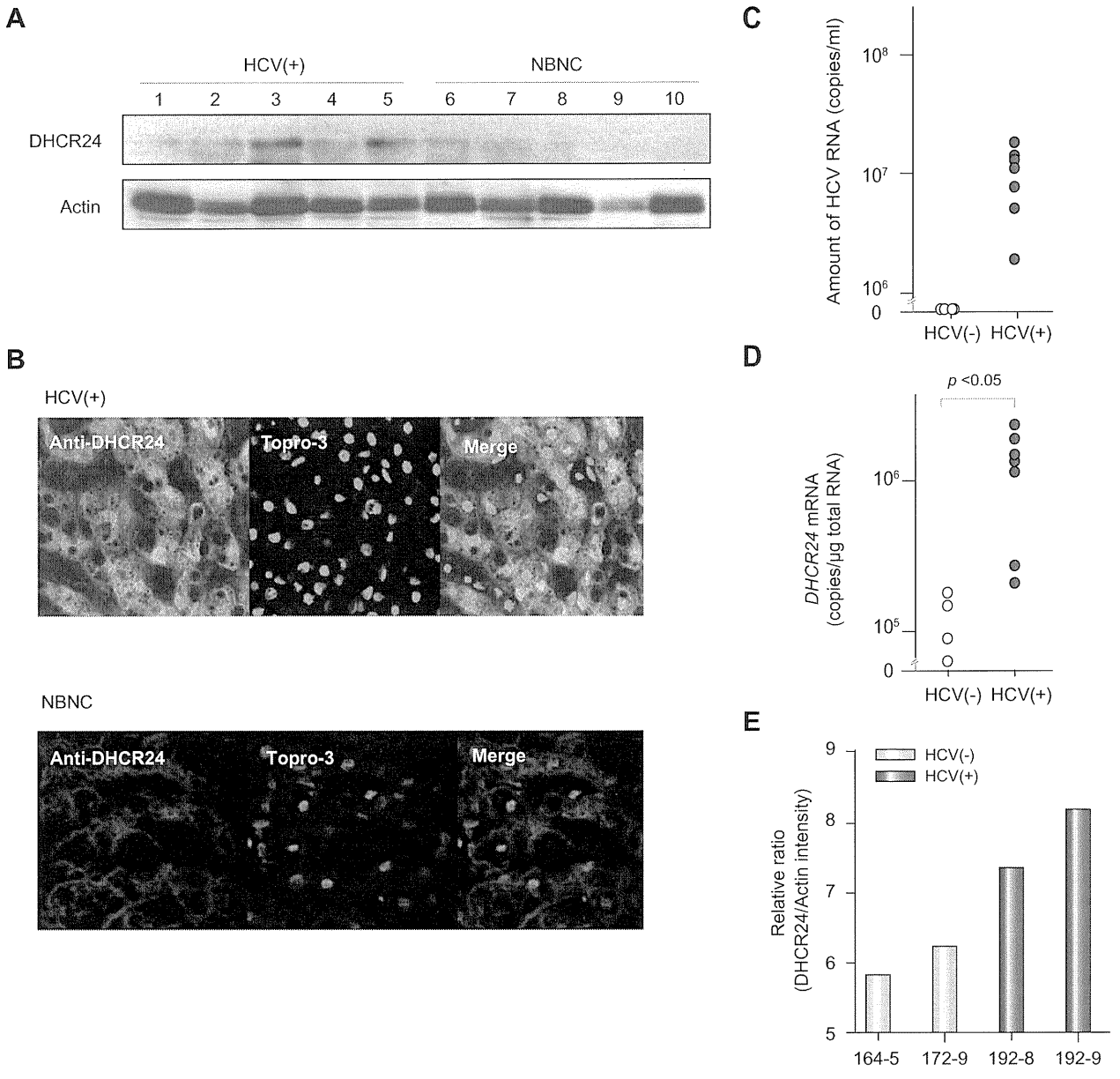

Fig. 1. HCV induces DHCR24 overexpression *in vitro* and *in vivo*. (A) Expression of DHCR24 in non-cancerous regions of livers of HCV-infected (+) and NBNC-HCC patients. Lysates (25 μg/lane) of non-cancerous liver tissues from HCC patients were analyzed by Western blot analysis using MoAb 2-152a. The patient numbers (Supplementary Table 1) are indicated at the top of the blot. (B) Immunohistochemical staining of HCV-infected non-cancerous tissues derived from an HCC patient using the monoclonal antibody 2-152a (Alexa488), anti-TO-PRO-3, or a merge (600× magnification) (upper panel). Tissues from an NBNC patient stained with the monoclonal antibody 2-152a (Alexa488) as well as TO-PRO-3 (640× magnification) (lower panel). (C) The amount of HCV RNA that was present in the HCV-R6 (genotype 1b)-infected chimeric mice with the humanized liver was quantified using RTD-PCR. The results of HCV uninfected ($n = 4$) and infected ($n = 7$) is indicated. (D) The amount of *DHCR24* mRNA present in total RNA isolates of HCV-R6 (genotype 1b)-infected chimeric mice with the humanized liver was quantified using RTD-PCR. $^*p < 0.05$ (Mann–Whitney test). The results of HCV uninfected ($n = 4$) and infected ($n = 7$) are indicated. (E) DHCR24 protein was detected by Western blot analysis using MoAb 2-152a as a probe, and quantitated by LAS3000. Protein levels are normalized to actin and ratio is indicated.

HCV infection in vivo induces persistent overexpression of DHCR24

We next examined whether HCV infection could induce DHCR24 expression in human hepatocytes. DHCR24 was overexpressed more frequently in liver tissues from HCV-positive patients than in tissues from HBV- and HCV-negative (NBNC) patients (Fig. 1A and Supplementary Table 1). The liver tissue from HCV-positive patients stained more strongly for DHCR24 expression than the liver tissue from NBNC patients (Fig. 1B). We inoculated chimeric mice [19,23,25] with HCV ($10^{6.2}$ copies/ml) that had been isolated from the plasma of HCV-infected patients (patient R6, HCV genotype 1b). The serum concentration of human albumin (Supplementary Fig. 2A) in the chimeric mice after transplantation of hepatocytes indicated that human hepatocytes had engrafted in the mouse livers. Thirty days after transplantation, mice were infected with HCV, and HCV and RNA titers were analyzed both

before and after inoculation (Supplementary Fig. 2B). The average amount of HCV RNA that was present in the serum of the infected chimeric mice at 28 days post-infection was 1.1×10^7 copies/ml (Fig. 1C and Supplementary Fig. 2B). The *DHCR24* mRNA levels in the livers of the chimeric mice were also quantified at 28 days post-infection by real-time detection (RTD)-PCR [22]. The results revealed that there was a significant increase in *DHCR24* expression as measured by mRNA levels in HCV infected chimeric mice (Fig. 1D). Next, we examined the extent to which translation of DHCR24 occurred in the chimeric mice (Fig. 1E), higher DHCR24 protein levels were present in hepatocytes from HCV-infected mice (Nos. 192-8 and 192-9) than in those of uninfected mice (Nos. 164-5 and 172-9). These findings indicate that expression of DHCR24 is significantly up-regulated by HCV infection in human hepatocytes.

Role of DHCR24 in HCV replication

Since augmentation of DHCR24 expression was observed by HCV infection in humanized chimeric mice, we next examined whether DHCR24 was involved in HCV replication or not. We transfected siRNA into HCV replicon cell lines FLR3-1 (Fig. 2A and B) and R6FLR-N (Fig. 2C and D). Treatment with either two different *DHCR24* siRNA molecules (siDHCR24-417 or -1024) decreased HCV replication in a dose-dependent manner (Fig. 2A and C) but did not appear to have a significant effect on cell viability (Fig. 2B and D). Western blot analysis using HCV subgenomic replicon cell lines confirmed these findings (Fig. 2E and F). We also transfected the *DHCR24* siRNAs into HCV JFH-1 strain [18]-infected HuH7/K4 cell lines and found, by Western blot analysis, that the siRNAs inhibited HCV protein expression (Fig. 2G and H). These results indicate that DHCR24 may play a role in HCV replication.

The expression level of DHCR24 is linked to intracellular cholesterol levels

Human DHCR24 is involved in cholesterol biosynthesis [10]. It participates in multiple steps of cholesterol synthesis from lanosterol [26] (Fig. 3A). To examine the effect of cholesterol on the DHCR24 expression level in HuH-7 cells, we added cholesterol to cultured cells and determined the DHCR24 expression level (Fig. 3B). Expression levels of DHCR24 in HuH-7 cells were decreased approximately 50% by addition of cholesterol compared to that of the untreated control (Fig. 3B). On the other hand, that of DHCR24 in HepG2 cells was increased 2.5-fold by depletion of cholesterol using methyl-β-cyclodextrin (M-β-CD) (Fig. 3C).

These results indicate that the expression of DHCR24 in a cell correlates with the cholesterol level in that cell. Furthermore, silencing *DHCR24* reduced the cholesterol level in cells compared to control cells (Fig. 3D), suggesting that DHCR24 is essential for cholesterol synthesis.

Effect of U18666A on HCV replication in vitro

We further examined the role that DHCR24 plays in HCV replication by treating cells with U18666A. Treatment with U18666A (62.5, 125, 250, 500, and 1000 nM) of HCV replicon cells (FLR3-1) decreased HCV replication in a dose-dependent manner as shown by luciferase assay (Fig. 4A) and Western blot analysis (Fig. 4B). Notably, DHCR24 protein appeared as doublet bands in the absence of U18666A, but the lower band shifted to the upper band after treatment with U18666A (Fig. 4B). U18666A also suppressed HCV replication in other replicon cell lines (R6FLR-N and Rep JFH Luc 3-13; Fig. 4C and D). Treatment with U18666A (<250 nM) suppressed viral replication without producing significant cytotoxicity. We also examined the effect of 7-dehydrocholesterol reductase (DHCR7) (Fig. 3A) on HCV replication using the specific inhibitor BD1008 [26]. Treatment with BD1008 also suppressed HCV replication, but the concentration required was much higher than that needed in the U18666A assays (Fig. 4E); the concentration also greatly exceeded the intrinsic IC_{50} value for inhibition of σ-receptor binding (47 ± 2 nM) [27]. Therefore, DHCR24 may play a more significant role than DHCR7 in HCV replication. We next evaluated the compensatory effect that the addition of cholesterol had on cells treated with U18666A (Fig. 4F and G) by examining low density lipoprotein (LDL)-replaceable dissolved cholesterol levels as described in Supplementary data. Treatment with cholesterol led to partial restoration of HCV replication (Fig. 4F). These results suggest that U18666A suppresses HCV replication by depleting cellular cholesterol stores.

Next, we characterized the effect that U18666A had on HCV JFH-1 infection. Adding U18666A (62.5, 125, 250, and 500 nM) to HCV JFH-1-infected cell lines for 72 h, reductions of NS5B protein level were observed in cells treated more than 500 nM of U18666A (Fig. 5A and B). Additionally, the HCV RNA copy number in infected cells was suppressed by addition of 250 or 500 nM of U18666A (Fig. 5C). Examination of the cytotoxicity that U18666A (62.5–500 nM) had on infected cells revealed that it had little effect on cell viability (Fig. 5D). These results demonstrate that inhibition of DHCR24 by U18666A suppresses viral replication in HCV replicon cells and HCV-infected cells.

Evaluation of the anti-HCV effect of U18666A in vivo

To examine the effect of U18666A on HCV infection *in vivo*, we administered U18666A to HCV-infected chimeric mice with the humanized liver. The mice were infected with HCV via inoculation of patient serum HCR6 5 weeks after transplantation of human hepatocytes. U18666A (10 mg/kg) and PEG-IFN-α (30 μg/kg) were then administrated to these mice for 2 weeks (Fig. 6A). HCV RNA quantity (Fig. 6B) and serum human albumin levels (Fig. 6C) were measured in the mice after 1, 4, and 14 days of HCV infection. Treatment with U18666A alone significantly decreased HCV RNA levels in the serum (from 1×10^8 to 3×10^5 copies/ml) after 2 weeks, and its suppressive effect was more pronounced than that of PEG-IFN-α alone (8×10^5 copies/ml; Fig. 6B). Moreover, co-administration of U18666A and PEG-IFN-α synergistically (combination index <1) enhanced the antiviral effect of PEG-IFN-α (5×10^4 copies/ml). Treatment with these drugs did not significantly affect the serum human albumin concentrations in treated mice (Fig. 6C).

Discussion

The results of this study revealed that DHCR24, an enzyme that participates in cholesterol synthesis (last step; Fig. 3A), also plays a significant role in HCV replication. To our knowledge, this is the first report that this molecule is involved in HCV infection. The mevalonate route of the cholesterol synthesis pathway (starting from acetyl Co-A) has previously been reported to be involved in

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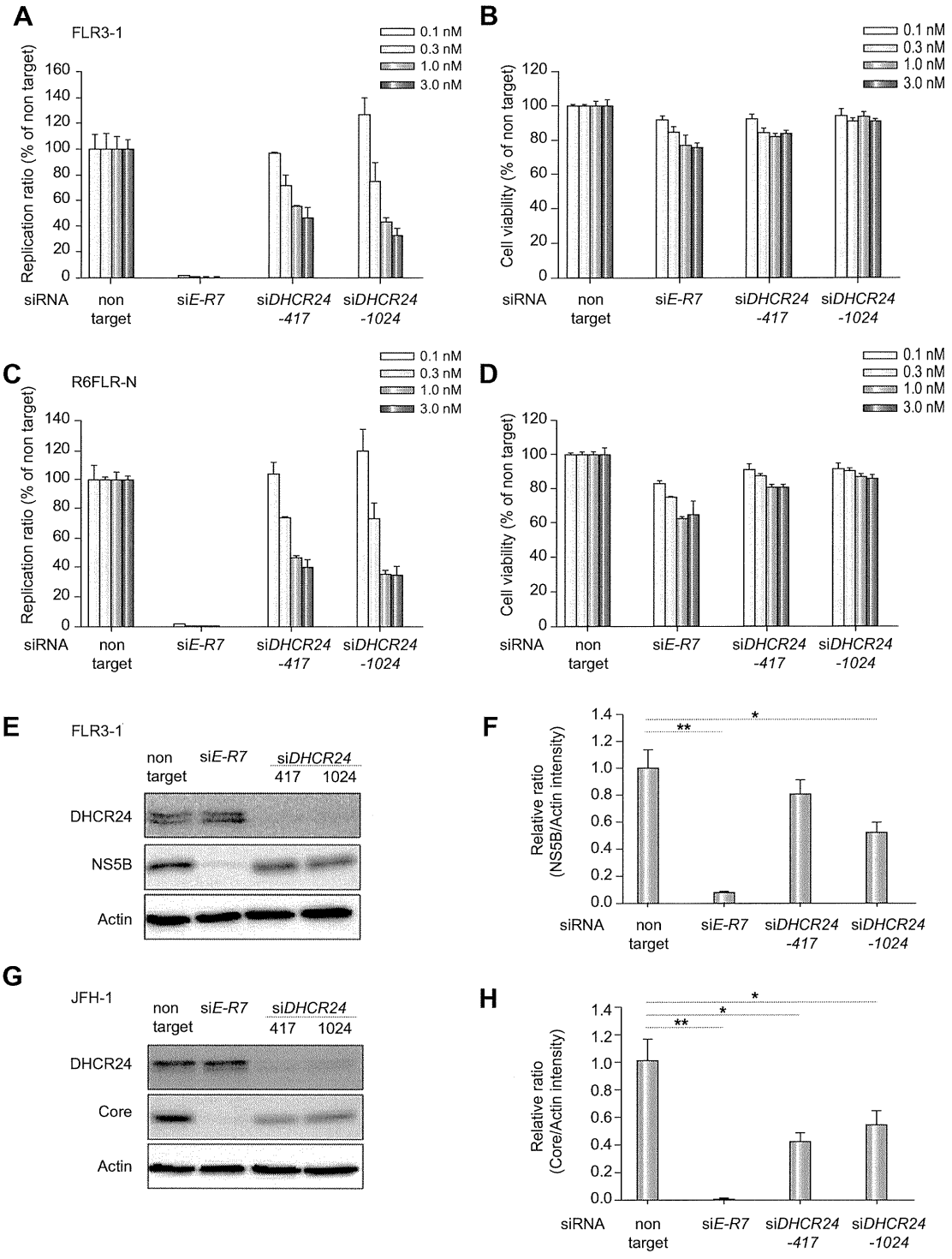

Fig. 2. Effect of DHCR24 knockdown on HCV replication. (A–D) Effect of DHCR24 knockdown on HCV replication in HCV replicon cells (FLR3-1 and R6FLR-N) at 72 h after the anti-*DHCR24* siRNAs (417 and 1024), siRNAs against HCV (siE-R7 for FLR3-1 and JFH-1; siE-R5 for R6-FLR-N), or non-target control siRNAs were transfected into HCV replicon cells. Replication activity was examined by luciferase assay (A and C), and cell viability was measured by the WST-8 assay (B and D). The data represent the mean of three experiments, and the bars indicate SD values. The Western blot analysis (E) and relative intensity of HCV-NS5B protein band was measured by LAS3000 and normalized with that of actin (F) after the treatment with siRNAs targeted against *DHCR24* (siDHCR24-417 and 1024) or HCV (si*E-R7*) in FLR3-1 replicon cells. (G and H) In HCV JFH-1-infected cells, DHCR24 knockdown by si*DHCR*24-417 and 1024 and HCV knockdown by siE-R7 were performed, and DHCR24 and HCV core protein expressions were confirmed by Western blot analysis. The relative intensity ratio of core protein to actin is indicated (H). The data represent the mean of three experiments, and the bars indicate SD values. $^{*}p < 0.05$, $^{**}p < 0.01$ (two-tailed Student's *t* test).

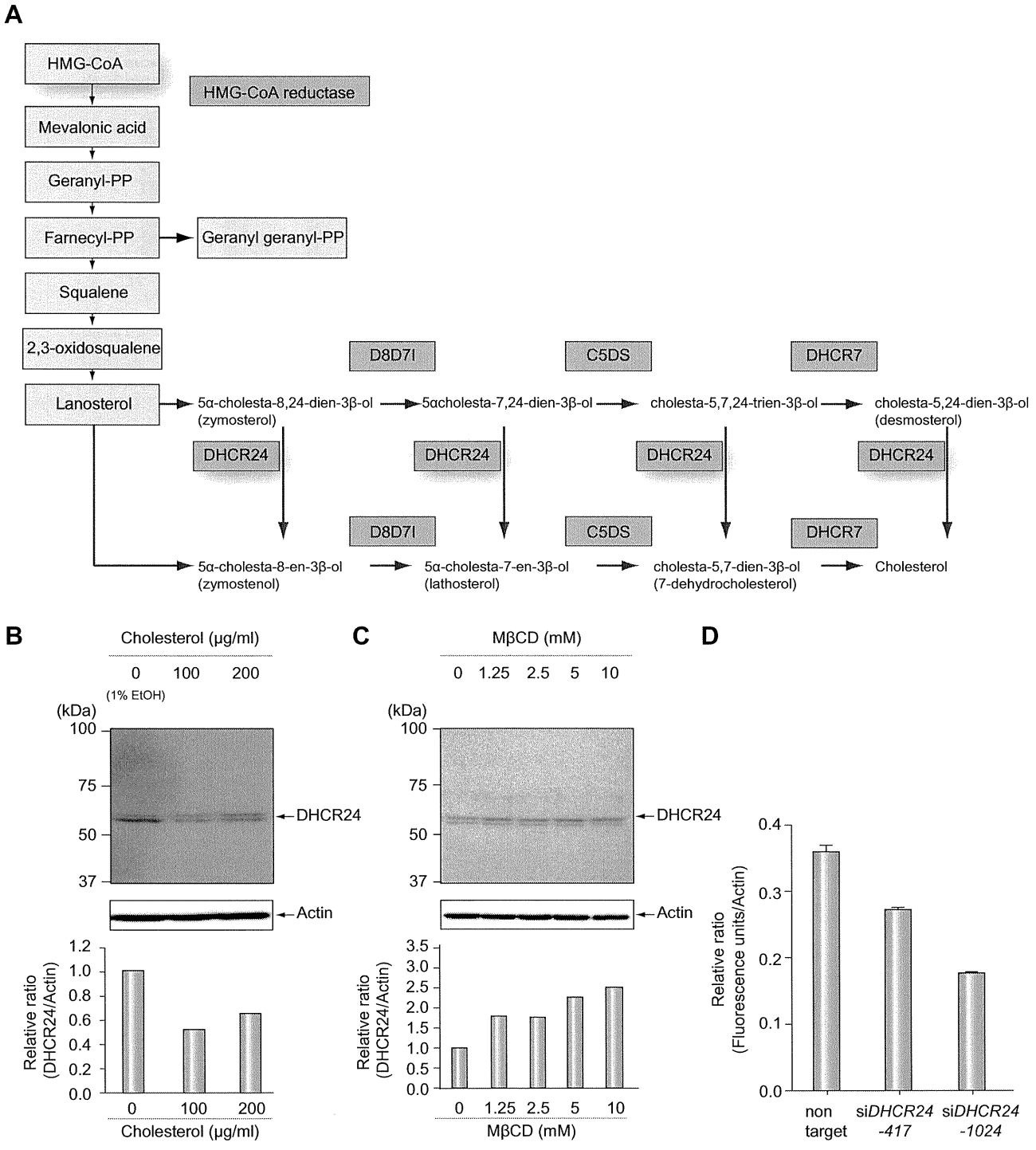

Fig. 3. The level of cholesterol and DHCR24 expression. (A) Cholesterol synthesis pathway, starting from HMG-CoA [26]. The abbreviations used are: D8D7I, 3β-hydroxysterol-delta(8)-delta(7)-isomerase; and C5DS, 3β-hydroxysterol-C^5-desaturase. (B) Cholesterol (0, 100, and 200 μg/ml) was added to HuH-7 cells, and, after 24 h, DHCR24 protein was detected by Western blot analysis using anti-DHCR24 MoAb and protein band intensity was measured and normalized to actin (lower panel). (C) HepG2 cells were treated with MβCD (0, 1.25, 2.5, 5, and 10 mM) for 30 min. After 72 h, these cells were harvested and examined by Western blot analysis with the anti-DHCR24 MoAb and relative intensity was measured as described in (B) (lower panel). (D) Cholesterol concentration in R6FLR-N cells was measured after treatment with non-targeting siRNA and *DHCR24* siRNA (417 and 1024). The cholesterol contents were measured by Amplex Red cholesterol assay, plotted based on fluorescence units and normalized to actin which was measured by Western blot analysis, and the relative ratio was then calculated. The data represent the mean of three experiments, and the bars indicate the SD values.

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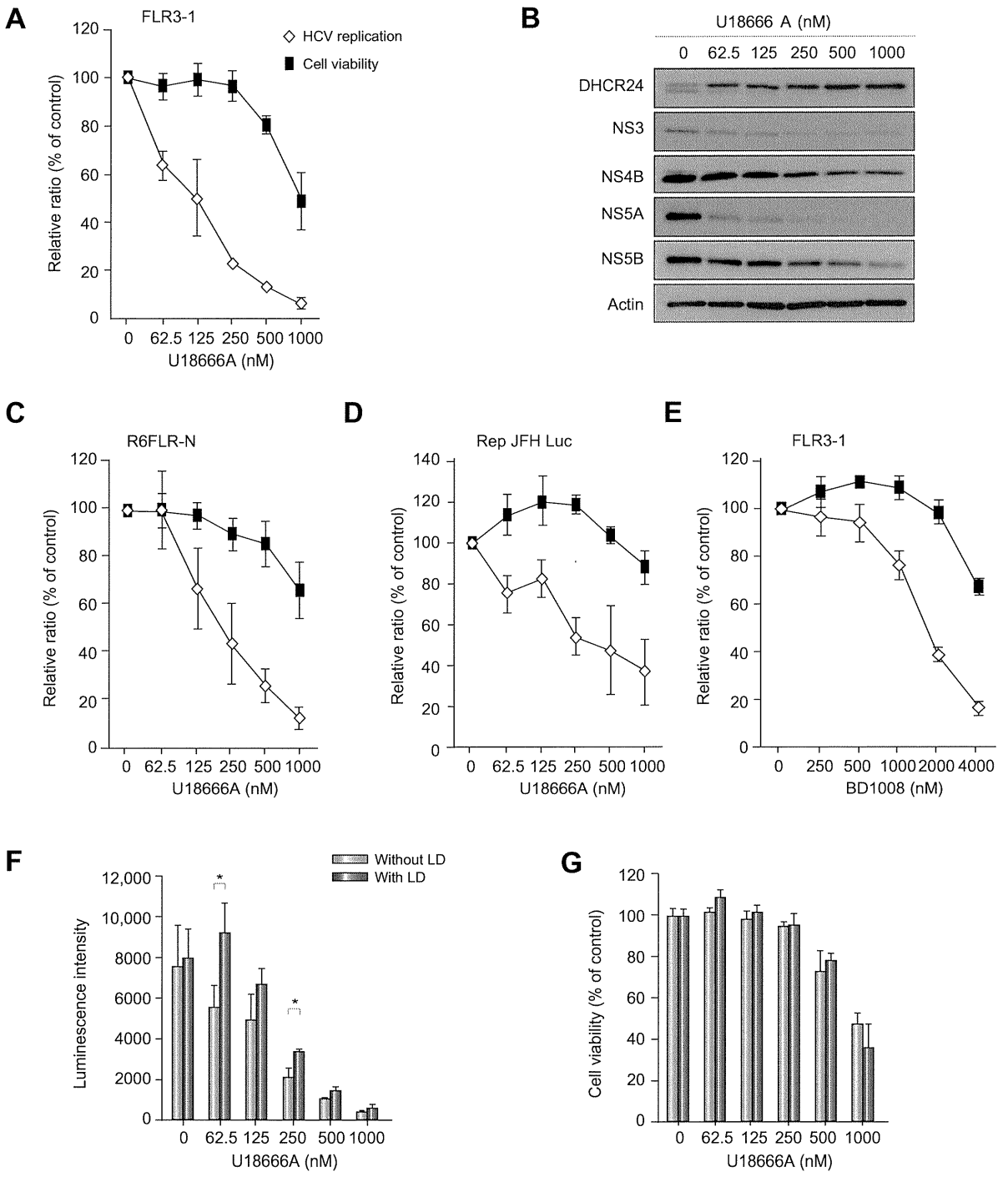

Fig. 4. Effect of U18666A on HCV replication. (A) Addition of U18666A to FLR3-1 cells and subsequent examination of HCV replication by the luciferase assay. Cell viability was measured by WST-8 assay. HCV replication and cell viability were measured 48 h after addition of U18666A. The bars indicate SD values. Open diamonds indicate the relative ratio of viral replication, and black squares indicate the cell viability in relation to untreated controls (A and C–E). (B) Treatment of FLR3-1 cells with U18666A decreased the expression of HCV proteins in a dose-dependent manner, as determined by Western blot analysis. (C and D) Effect of U18666A on HCV replication in other HCV replicon cells (C, R6FLR-N cells; D, Rep JFH Luc 3-13 cells). HCV replication and cell viability analyses were performed as described above. (E) The effect of the DHCR7 inhibitor BD1008 on HCV replicon cells (FLR3-1). Replication activity was examined by the luciferase assay, and cell viability was measured by the WST-8 assay. HCV replication and cell viability analyses were performed 48 h after the addition of U18666A. (F and G) FLR3-1 cells (5×10^3 cells/well) were treated with U18666A alone (light blue, or), low density lipoprotein (LDL) (final cholesterol concentration, 50 μg/ml), and U18666A (dark blue). HCV replication was determined by the luciferase assay 48 h later (F), and cell viability was measured by the WST-8 assay (G). *p <0.05 (two-tailed Student's t-test). The data represent the mean of three experiments, and the bars indicate SD values.

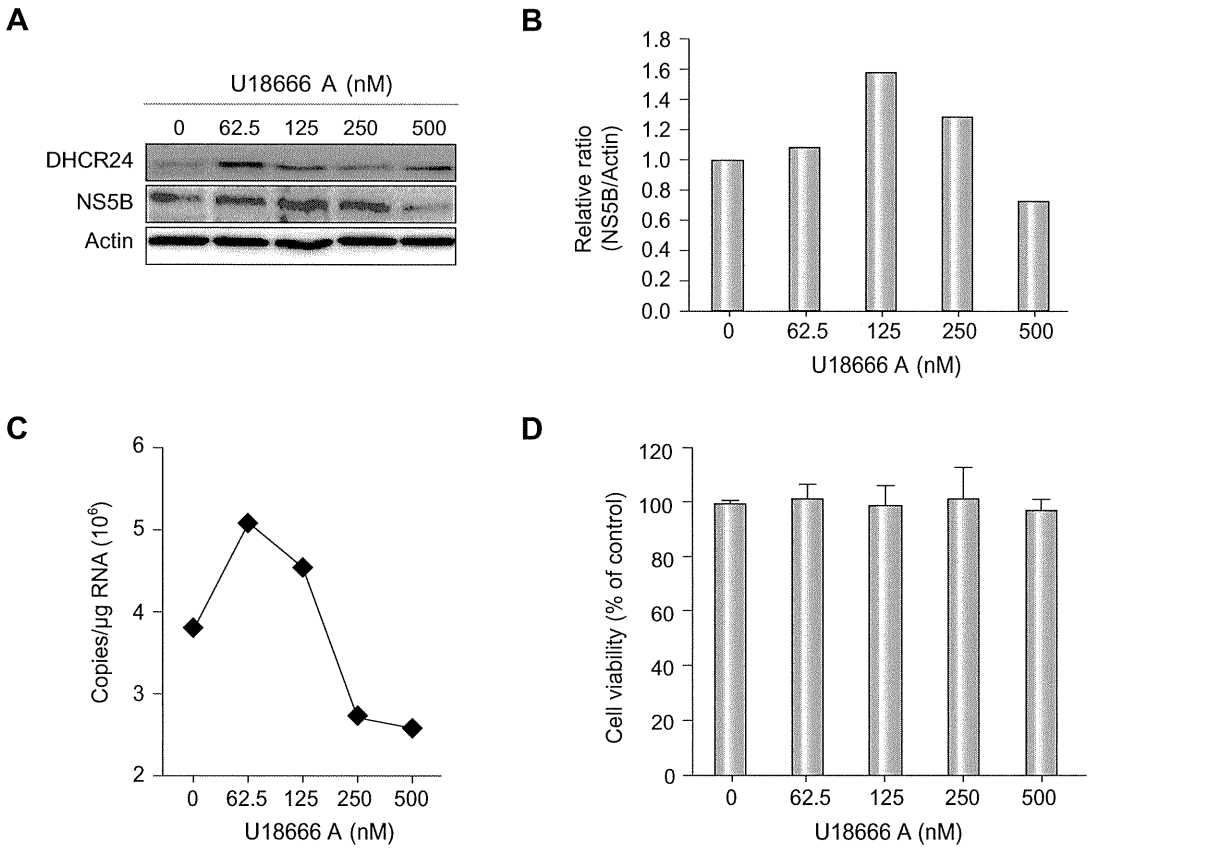

Fig. 5. Effect of U18666A on cells infected with HCV JFH-1. HCV JFH-1-infected cells treated with U18666A were examined 72 h after treatment. (A) Expression of HCV-NS5B protein with or without U18666A treatment, analyzed by Western blot analysis. (B) The intensity of HCV-NS5B protein expression is represented graphically. (C) HCV RNA in HCV JFH-1-infected cells with or without U18666A treatment was measured by RTD-PCR as described in Materials and methods. (D) Cell viability was measured by the WST-8 assay.

HCV replication [28]. The present findings are the first evidence that overexpression of one of the enzymes downstream of the mevalonate pathway, i.e., DHCR24, can be induced by HCV infection. In a previous study, 3-hydroxy 3-methyl-glutaryl Co-A (HMG-CoA) reductase was found to be inhibited by lovastatin, subsequently resulting in suppression of HCV replication [28]. The product of the mevalonate pathway that is required for HCV replication is reported to be a geranyl geranyl lipid [29]. Many lipids are crucial to the viral life cycle, and inhibitors of the cholesterol/fatty acid biosynthetic pathway inhibit viral replication, maturation, and secretion [30,31]. We found that inhibition of DHCR24 down-regulated HCV replication. DHCR24 catalyzes the reduction of the delta-24 bond of the sterol intermediate and works further downstream of farnesyl pyrophosphate (Fig. 3A) and, therefore, does not influence geranyl–geranylation. Thus, our findings indicate the existence of regulatory pathway of HCV replication by cholesterol synthesis and trafficking through DHCR24 rather than by protein geranyl–geranylation. DHCR24 deficiency reduces the cholesterol level and disorganizes cholesterol-rich detergent-resistant membrane domains (DRMs) in mouse brains [32]. Additionally, the HCV replication complex has been detected in the DRM fraction [11]. Therefore, a deficiency in DRM, induced by silencing *DHCR24*, may suppress HCV replication.

We demonstrated that the addition of cholesterol to HCV-infected hepatocytes treated with U18666A led to partial recovery of HCV replication, which suggests that cholesterol may be an important factor in HCV replication. U18666A impairs the intracellular biosynthesis and transport of cholesterol and inhibits the action of membrane-bound enzymes, including DHCR24, during sterol synthesis [33]. Moreover, the DHCR7 inhibitor BD1008 also suppresses HCV replication. Thus, the findings in this study further substantiate the fact that cholesterol plays an important role in HCV replication and infection.

Although monotherapy with statins is reportedly insufficient to induce anti-viral activity in HCV-infected patients [34], a synergistic action between statins and IFN has been observed [35]. The effect of the statin is thought to be mainly mediated by the depletion of geranyl geranyl lipids. It is important to note that higher doses of statins may increase the risk of myopathy, liver dysfunction, and cardiovascular events [36]. Moreover, the EC_{50} values of the statins that are associated with a reduction in HCV replication are reported to be 0.45–2.16 μM, while the IC_{50} of U18666A was estimated to be 125 nM in the present study. Therefore, U18666A may serve as a novel anti-HCV drug that could be utilized with IFN as a combined therapeutic regimen.

In summary, we demonstrated that the expression of DHCR24 is induced by infection with HCV and that DHCR24 is an essential host factor that is required for HCV replication. HCV may increase cholesterol synthesis in cells via the action of a host regulatory factor, such as DHCR24, that is correlated with cholesterol

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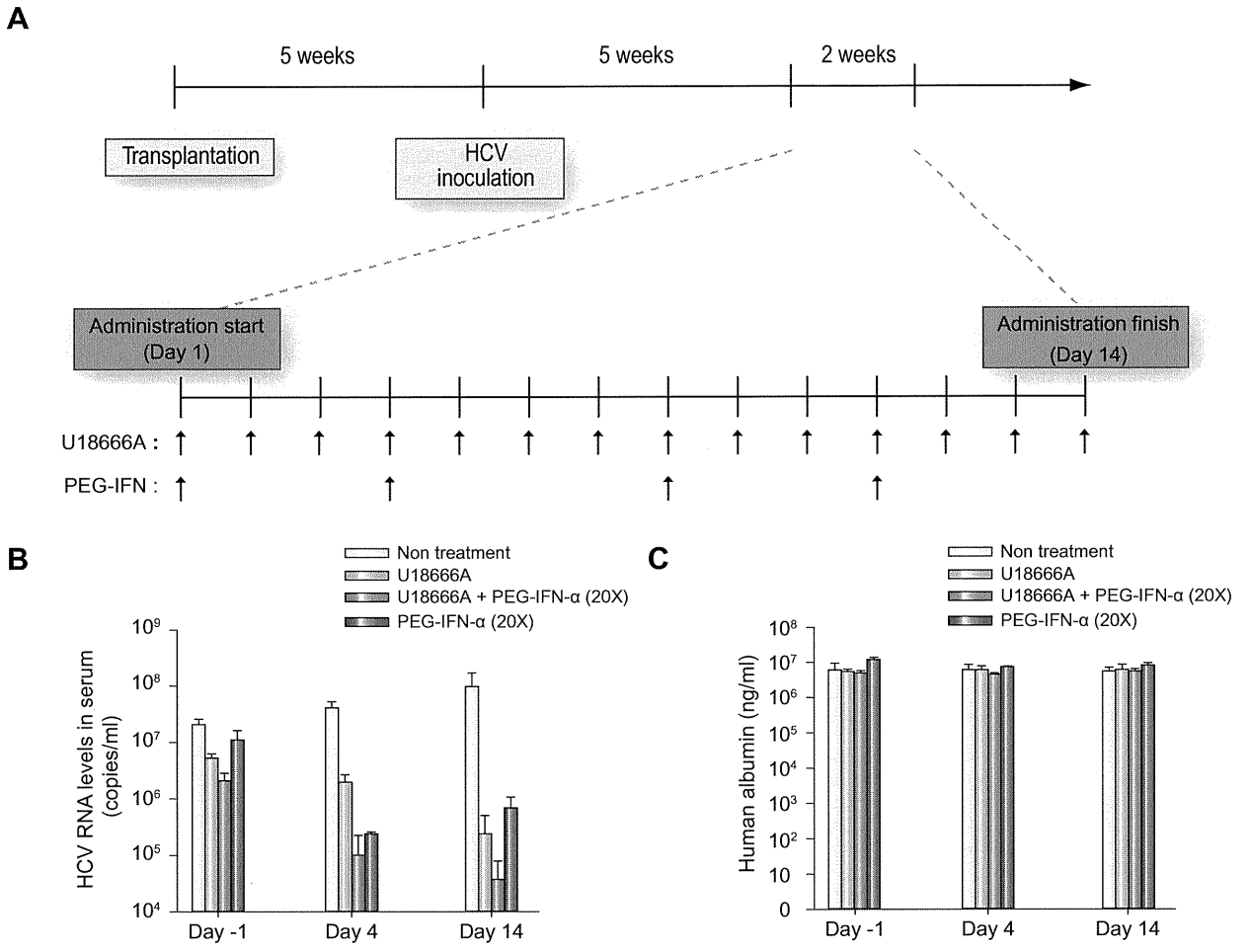

Fig. 6. Evaluation of the anti-HCV effect of U18666A in chimeric mice. (A) Diagram of the schedule that was followed to produce chimeric mice with the humanized liver, perform blood sampling, and administer drugs to chimeric mice infected with HCV. Four groups of three chimeric mice with the humanized liver were treated intraperitoneally with U18666A (10 mg/kg) and/or subcutaneously with PEG-IFN-α (30 μg/kg) at 2-day intervals for 2 weeks. (B) The effect of U18666A and/or PEG-IFN-α on HCV replication in chimeric mice with the humanized liver was determined by quantification of HCV-RNA using RTD-PCR. The bars indicate SD values ($n = 12$). (C) Human albumin concentrations in the sera of chimeric mice with the humanized liver. The bars indicate SD values ($n = 12$).

synthesis and is also directly involved in replication. Genome-wide analysis of the host response to HCV infection revealed the upregulation of genes related to lipid metabolism [37]. DHCR24 expression was found to be upregulated in the cDNA microarray analysis of chronic hepatitis C cases [38]. Future studies are needed to examine the detailed mechanism by which HCV infection augments DHCR24 expression in hepatocytes.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Financial support

This study was supported in part by a grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan, a grant from the Ministry of Health, Labor and Welfare of Japan, and the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation of Japan and the Cooperative Research Project on Clinical and Epidemiological Studies of Emerging and Re-emerging Infectious Diseases.

Acknowledgments

We are very grateful to Dr. T. Watanabe, Dr. S. Nakagawa, Mr. T. Nishimura and Dr. K. Tanaka for technical support, and Dr. S. To'ne, Dr. T. Wakita, Dr. S. Sekiguchi, and Dr. F. Yasui for helpful discussions.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2010.12.011.

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Virus Research 160 (2011) 89–97

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Conditional gene expression in hepatitis C virus transgenic mice without induction of severe liver injury using a non-inflammatory Cre-expressing adenovirus

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ARTICLE INFO

Article history:
Received 29 December 2010
Received in revised form 19 May 2011
Accepted 20 May 2011
Available online 30 May 2011

Keywords:
HCV transgenic mouse
Cre/loxP system
Recombinant adenovirus vector
EF1α promoter
Hepatitis

ABSTRACT

We previously established inducible-hepatitis C virus (HCV) transgenic mice, which expressed the HCV gene (nucleotides 294–3435) encoding the core, E1, E2, and NS2 proteins. The expression of these proteins is regulated by the Cre/loxP system and an adenovirus vector (AdV) that expresses Cre DNA recombinase (Cre) controlled by the CAG promoter (AxCANCre). Recent studies have demonstrated that AxCANCre injection alone results in severe liver injury by induction of the adenovirus protein IX (Ad-pIX) gene. As a result, HCV protein expression in transgenic mice livers was only short-term. In contrast, the EF1α promoter-bearing AdV induces slight Ad-pIX gene expression without inducing severe liver injury. Therefore, in the present study, we developed a Cre-expressing AdV that bears the EF1α promoter (AxEFCre) to express HCV protein in the transgenic mouse livers. In the non-transgenic mice injected with AxCANCre, alanine aminotransferase (ALT) levels were elevated and severe liver inflammation occurred; this was not observed in AxEFCre-injected mice. In contrast, AxEFCre-injected HCV transgenic mice showed milder liver inflammatory responses that were clearly due to HCV protein expression. Moreover, the AxEFCre injection enabled the transgenic mice to persistently express HCV protein. These results indicate that use of AxEFCre efficiently promotes Cre-mediated DNA recombination *in vivo* without a severe hepatitis response to AdV. This inducible-HCV transgenic mouse model using AxEFCre should be useful for research on HCV pathogenesis.

1. Introduction

Infection with the hepatitis C virus (HCV) is a major global health problem, as persistent viral infection leads to liver cirrhosis and hepatocellular carcinoma (Goodman and Ishak, 1995; Shepard et al., 2005). The chimpanzee is the only validated animal model for *in vivo* studies of HCV infection, while *in vivo* studies on the pathogenesis of HCV have been conducted using new animal models (Kremsdorf and Brezillon, 2007). Several groups have established transgenic mice that constitutively express single or multiple HCV protein(s) in the liver (Lerat et al., 2002; Moriya et al., 1997). However, in these mice, HCV protein expression begins *in utero*; as a result, they develop immune tolerance to the HCV antigens, and HCV-specific cellular responses or liver inflammation cannot be induced. To overcome these obstacles, we previously developed immunocompetent HCV transgenic mice in which HCV protein expression was tightly regulated by the Cre/*lox*P system (Wakita et al., 1998).

The E1- and E3-deleted adenovirus vector (AdV) has been widely used for both basic studies of gene function and for gene therapy *in vivo*. To deliver the *Cre* gene into the livers of HCV transgenic mice, we used an AdV that carries the CAG promoter linked to a nuclear localization signal-tagged Cre (AxCANCre), which has been used for Cre-mediated DNA recombination (Baba et al., 2005; Kobayashi et al., 2000; Shintani et al., 1999; Wakita et al., 1998). While AdV is relatively efficient in inducing transgene expression, several studies have shown that the viral vector itself can induce strong inflammatory responses in murine livers (Kafri et al., 1998; Wakita et al., 2000). Moreover, expression of transgenes via AdVs persists for only 2–4 weeks due to elimination of infected cells through immune responses directed against the AdVs (Akagi et al., 1997; Bangari and Mittal, 2006; Kafri et al., 1998; Sun et al., 2005; Wakita et al., 2000). To address these problems, the viral

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doi:10.1016/j.virusres.2011.05.019

genes that cause cellular immune responses have been investigated. Recently, Nakai et al. (2007) reported that co-expression of adenovirus protein IX (Ad-pIX) resulted in AdV-induced immune responses. However, AdVs that carried the EF1α promoter did not induce Ad-pIX or increase the alanine aminotransferase (ALT) level, facilitating long-term transgene expression in mice.

In the present study, we generated a Cre-expressing AdV bearing the EF1α promoter (AxEFCre) to enable the persistent expression of HCV protein in the livers of inducible-HCV transgenic mice regulated by the Cre/*lox*P recombination system. When this AdV was used to express Cre in the HCV transgenic mouse livers, it induced less severe inflammatory responses and improved the long-term expression of HCV proteins compared to CAG promoter-bearing AdVs. Thus, AxEFCre efficiently promotes Cre-mediated DNA recombination *in vivo* without a severe hepatitis response to AdV and should be useful for HCV gene expression in the HCV transgenic mice.

2. Materials and methods

2.1. Cells

The 293 cells [CRL-1573, a human embryonic kidney cell line that contains the Ad5 E1 region; American Type Culture Collection (ATCC)] and HepG2 cells (HB-8065, a human hepatocellular carcinoma cell line; ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical) supplemented with 10% fetal bovine serum (FBS; JRH Biosciences), 100 U/mL penicillin, and 100 μg/mL streptomycin (GIBCO) (10% FBS-DMEM). In addition, HepG2 cells containing the Cre reporter unit CALNLZ (Baba et al., 2005), termed Hep-CALNLZ cells, were selected for resistance to G418 (300 μg/mL; Sigma) and cultured in 10% FBS-DMEM.

2.2. Adenovirus vectors

E1- and E3-deleted AdVs derived from human adenovirus type 5 encoding expression units with a leftward orientation were used in this study. As expression units, untagged Cre or NLS-tagged Cre under the control of the CAG promoter (AxCANCre or AxCACre), untagged Cre or NLS-tagged Cre under the control of the EF1α promoter (AxEFNCre or AxEFCre), and untagged β-galactosidase (LacZ) under the control of the EF1α promoter (AxEFLacZ) were constructed (Fig. 1A). AxCANCre and AxCACre were generated as described previously (Kanegae et al., 1995). AxEFNCre, AxEFCre, and AxEFLacZ were constructed using pAxEFwtit2 DNA/RE Treatment (Nippon Gene). All of the AdVs were purified using two rounds of CsCl gradient centrifugation, and the titers of the concentrated and purified virus stocks were determined as described previously (Kanegae et al., 1994).

2.3. Animal procedures

HCV transgenic mice CN2-29 (C57BL/6 background) and normal C57BL/6 mice were used in the experiments. The CN2-29 transgenic mice express HCV genotype 1b proteins (core, E1, E2, and NS2 proteins) under the regulation of the Cre/*lox*P conditional switching system (Wakita et al., 1998). The transgenic mice were intravenously injected with each AdV at a dose of 1.0×10^9 plaque-forming units (PFU), and sacrificed 0.5, 7, or 21 days after injection for liver histology and biochemical analysis. All mice were bred in a pathogen-free facility and tested routinely for mouse hepatitis virus and other pathogens. All experiments using mice were approved by The Tokyo Metropolitan Institute of Medical Science Animal Experiment Committee and were performed in accordance with the animal experimentation guidelines of The Tokyo Metropolitan Institute of Medical Science.

2.4. Western blot detection of Cre and Ad-pIX

The HepG2 cells were placed in collagen-coated, 12-well plates and infected with the AdVs at a multiplicity of infection (MOI) of 20 or 100 for Western blot detection of Cre or Ad-pIX, respectively. After 24 h, the cells were washed with phosphate-buffered saline (PBS) and resolved in radioimmunoprecipitation assay (RIPA) buffer [10 mM Tris–HCl (pH 7.5), 0.15 M NaCl, 1% sodium dodecyl sulfate (SDS), 0.5% Nonidet P-40, protease inhibitor cocktail (Complete; Roche Molecular Biochemicals)]. The protein concentrations of the cell lysates were measured using the DC protein assay (Bio-Rad Laboratories). The cell lysates were electrophoresed on SDS–polyacrylamide gel, transferred to polyvinylidene difluoride membrane (GE Healthcare) activated with methanol, and blocked with 5% skim milk in PBS containing 0.1% Tween-20 (PBST). After washing with PBST, the membrane was incubated overnight at 4 °C in the presence of anti-Cre rabbit polyclonal antibody or anti-Ad-pIX rabbit polyclonal antibody (Nakai et al., 2007) prepared from hyper-immune rabbit sera, or anti-β-actin mouse monoclonal antibody (Sigma), followed by incubation with horseradish peroxidase (HRP)-conjugated $F(ab')_2$ of anti-rabbit or mouse IgG (GE Healthcare) for 1 h at room temperature. The expression levels of these proteins were visualized using the ECL system (GE Healthcare) and an LAS3000 imager (Fujifilm).

2.5. LacZ gene activation and cytotoxicity of Cre-expressing AdVs

The Hep-CALNLZ cells were cultured on collagen-coated, 96-well plates and infected with AdVs at various MOIs in four-fold serial dilutions. After 48 h, cytotoxicity assays were performed using the Cell Counting Kit-8 (Dojindo Molecular Technologies), according to the manufacturer's instructions. To detect *LacZ* expression, the cells were fixed with 4% paraformaldehyde in PBS for 10 min, washed with PBS, and incubated in X-Gal solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM $MgCl_2$ in PBS) containing 0.5 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; WAKO Pure Chemicals) at 37 °C overnight.

2.6. Extraction of total RNA and quantification of Ad-pIX mRNA levels

The HepG2 cells were infected with the AdVs at an MOI of 100 and were harvested after 24 h. The CN2-29 transgenic mice were injected with the AdVs at a dose of 1.0×10^9 PFU and were sacrificed to obtain their liver samples after 12 h. Total RNA was extracted from the cells or mouse livers using the RNeasy Mini Kit (Qiagen) and RNase-free DNase (Qiagen). Reverse transcription was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Copy numbers of the Ad-pIX cDNA were assessed by quantitative real-time detection polymerase chain reaction (RTD-PCR) with the specific probe AdIX-354-S25FT (5′-[FAM]-TCAGCAGCTGTTGGATCTGCGCCAC-[TAMRA]-3′); AdIX-327-S24 (5′-TTTGACCCGGGAACTTAATGTCGT-3′) and AdIX-387-R19 (5′-GGAGGAAGCCTTCAGGGCA-3′) were used as primers. The standard curve was generated using pAxEFLacZ. Analyses were conducted using an ABI PRISM 7700 Sequence Detection System with TaqMan Universal PCR Master Mix (Applied Biosystems).