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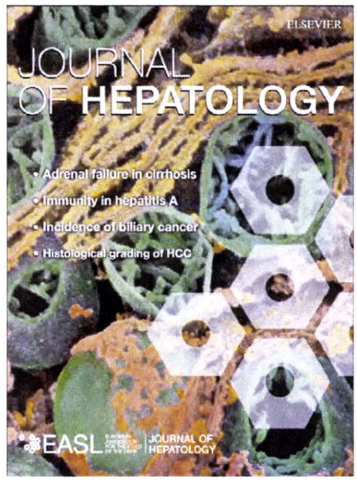

Augmentation of DHCR24 expression by hepatitis C virus infection facilitates viral replication in hepatocytes

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

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Abbreviations; DHCR24, 24-dehydrocholesterol reductase; HCV, hepatitis C virus; MoAb, monoclonal antibody; HCC, Hepatocellular carcinoma; HBV, Hepatitis B virus

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Key words: hepatitis C virus; replication; DHCR24; U18666A

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Abstract

Background & Aims: The role of 24-dehydrocholesterol reductase (DHCR24) in hepatitis C virus infection (HCV) was characterized, because 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 is augmented its expression by HCV infection, and plays a significant role in HCV replication. DHCR24 may serve as a novel anti-HCV drug target.

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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.

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

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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.

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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] were described in *Supplementary data*.

The DHCR24 cDNA was synthesized and amplified by PCR using Phusion *Taq* 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 the *Supplementary data* and [20].

Immunohistochemistry and Western blot analysis.

The detailed procedures are described in the *Online 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 was directed against human DHCR24 mRNA: siDHCR24-417 and siDHCR24-1024. The target sequence of siDHCR24-417 was

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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 hours after transfection.

Inhibition of viral replication by U18666A

U18666A (Calbiochem) was utilized to treat HCV replicon cells at a concentration of 62.5 to 1,000 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 x 10^3/well in a 96-well white plate, SUMILON). Culture medium was replaced after the cells had spread (at 24 hours), and LDL (Calbiochem) was added to reach a final cholesterol concentration of 50 μg/ml. After a 24 hours-incubation, U18666A (62.5, 125, 250, 500, and 1,000 nM) was added to each well, and the cells were incubated for an additional 48 hours. HCV replication activity was measured by the luciferase assay, and cell viability was measured by the WST-8 cell counting kit according to the manufacturer's instructions (Dojindo Laboratories). Cholesterol measurements are described in the *Online Supplementary data.*

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

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For evaluation of the anti-HCV replication effect of the inhibitor U18666A in replicon cells and HCV persistently infected cells were described in the *Online 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^R Gene Expression assays (Applied Biosystems) according to the manufacturer's instructions. PEG-IFN α -2a (Chugai) was administered subcutaneously at a concentration of 30 μ g/kg, at day 1, 4, 8, and 11 (the amount administered to the

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chimeric mice was 20-fold amounts of PEG-IFN 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).

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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 7 times over a 2-week period. Then we fused the splenocytes from the mice that had been immunized with RzM6-LC cells to myeloma cells to establish hybridomas. Characterization of the culture supernatant from more than 1,000 hybridoma cells by ELISA (data not shown) revealed that one MoAb clone (2-152a) recognized a molecule of approximately 60 kDa in various cells (Fig. S1A and S1B). This molecule was more highly expressed in RzM6-LC cells (Fig. S1A), 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) (Fig. S1B). 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 (Fig. S1C, 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 transfected cells (Fig. S1D). We found that the 2-152a antibody specifically recognized DHCR24.

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

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HCV-positive patients than in tissues from HBV- and HCV-negative (NBNC) patients (Fig. 1A, Table S1). The liver tissue from HCV-positive patients was 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 (Fig. S2A) in the chimeric mice after transplantation of hepatocytes indicated that human hepatocytes had engrafted in the mouse livers. At 30 days after transplantation, the mice were infected with HCV, and HCV RNA titers were analyzed both before and after inoculation (Fig. S2B). 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, Fig. S2B). 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), and higher DHCR24 protein levels were present in hepatocytes from HCV-infected mice (No.192-8 and 192-9) than in those of uninfected mice (No.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

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replication or not. We transfected siRNA into HCV replicon cell lines FLR3-1 (Figs. 2A, B) and R6FLR-N (Figs. 2C, D). Treatment with either two different DHCR24 siRNA molecules (siDHCR24-417 or -1024) decreased HCV replication in a dose-dependent manner (Figs. 2A, C) but did not appear to have a significant effect on cell viability (Figs. 2B, D). Western blot analysis using HCV subgenomic replicon cell lines confirmed these findings (Figs. 2E, F). We also transfected the DHCR24 siRNAs into HCV JFH-1 strain [18]-infected HuH7/K4 cell lines and found that the siRNAs inhibited HCV protein expression by western blot analysis (Figs. 2G, 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 level of DHCR24 in HuH-7 cells were decreased approximately 50% by addition of cholesterol compared to that of untreated control (Fig. 3B). On the other hand, that of DHCR24 in HepG2 cells was increased to 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.

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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 of U18666A (62.5, 125, 250, 500, and 1,000 nM) to HCV replicon cells (FLR3-1) decreased HCV replication in a dose-dependent manner 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; Figs. 4C, 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 a much higher concentration was required to suppress replication than was 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 (Figs. 4F, G) by examining low density lipoprotein (LDL)-replaceable dissolved cholesterol levels as described in the Materials and methods. 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 of 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

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of U18666A (Figs. 5A and 5B). 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 humanized liver. The mice were infected with HCV via inoculation of patient serum HCR6 five 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 anti-viral 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).

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Discussion

The results in 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 pathway of cholesterol synthesis pathway (starting from acetyl Co-A) has previously been reported to be involved in HCV replication [28]. The present findings are the first evidence that overexpression of one of the enzymes down stream 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 downregulated 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, may 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 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 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

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mechanism by which HCV infection augments DHCR24 expression in hepatocytes.

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