Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

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

Takashi Takano[1,†], Kyoko Tsukiyama-Kohara[2,*], Masahiro Hayashi[1], Yuichi Hirata[1], Masaaki Satoh[2], Yuko Tokunaga[1], Chise Tateno[3], Yukiko Hayashi[4], Tsunekazu Hishima[4], Nobuaki Funata[4], Masayuki Sudoh[5], Michinori Kohara[1]

[1]*Department of Microbiology and Cell Biology, Tokyo Metropolitan Institute of Medical Science, 2-1-6 Kamikitazawa, Setagaya-ku, Tokyo 156-8506, Japan;* [2]*Department of Experimental Phylaxiology, Faculty of Medical and Pharmaceutical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto, Kumamoto 860-8556, Japan;* [3]*Phoenix Bio Co., Ltd., Study Service Department, 3-4-1 Kagamiyama, Higashi-Hiroshima 739-0046, Japan;* [4]*Department of Pathology, Tokyo Metropolitan Komagome Hospital, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8677, Japan;* [5]*Kamakura Research Laboratories, Chugai Pharmaceutical Co., Ltd., Kajiwara 200, Kamakura-City, Kanagawa 247-8530, Japan*

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

* Corresponding author. Address: Department of Experimental Phylaxiology, Faculty of Life Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto, Kumamoto 860-8556, Japan. Tel./fax: +81 96 373 5560.
E-mail address: kkohara@kumamoto-u.ac.jp (K. Tsukiyama-Kohara).
† Present address: Division of Veterinary Public Health, Nippon Veterinary and Life Science University, 1-7-1 Kyonan, Musashino, Tokyo 180-8602, Japan.

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.

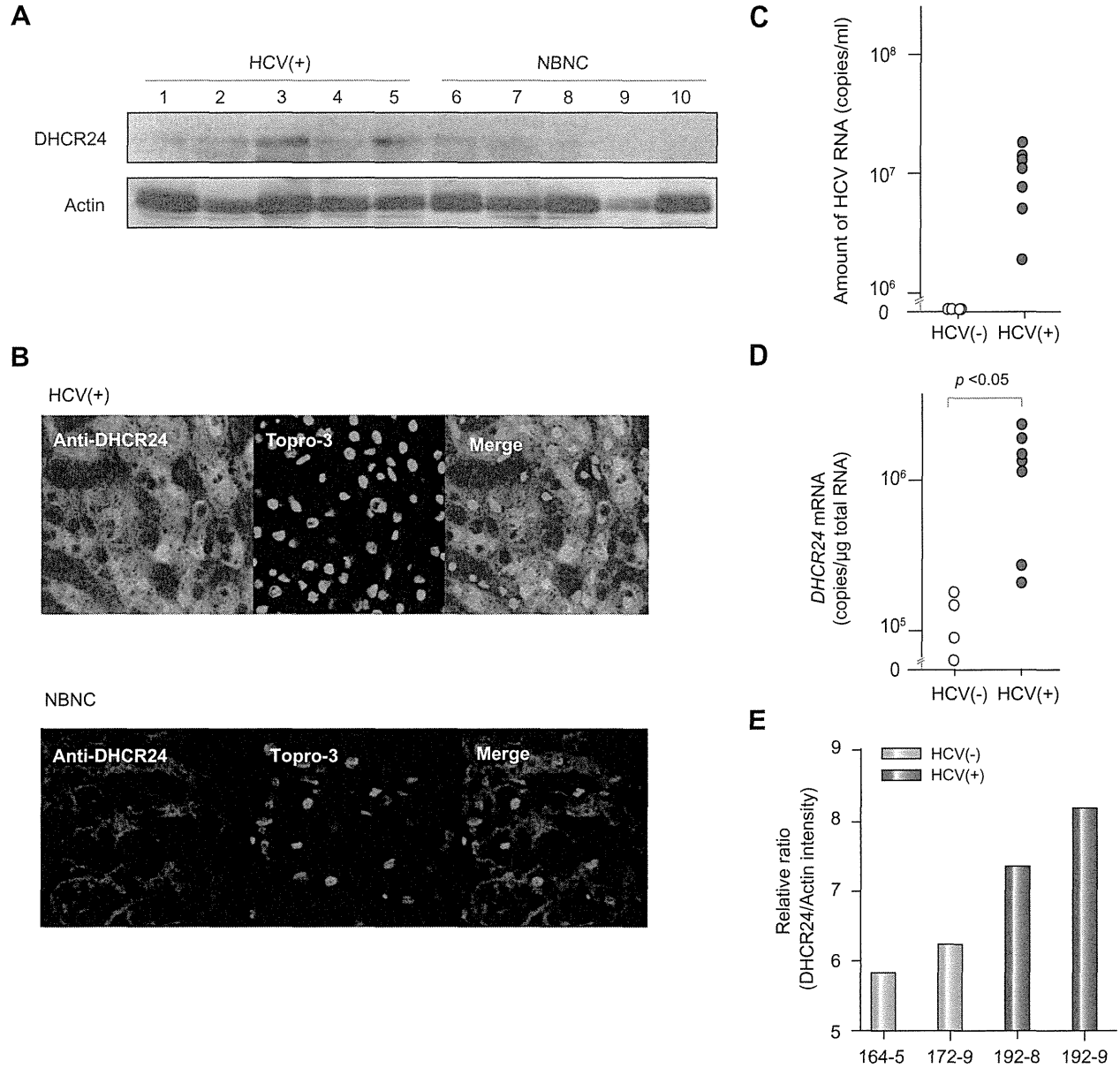

Fig. 1. HCV induces DHCR24 overexpression *in vitro* and *in vivo*. (A) Expression of DHCR24 in non-cancerous regions of livers of HCV-infected (+) and NBNC-HCC patients. Lysates (25 μg/lane) of non-cancerous liver tissues from HCC patients were analyzed by Western blot analysis using MoAb 2-152a. The 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

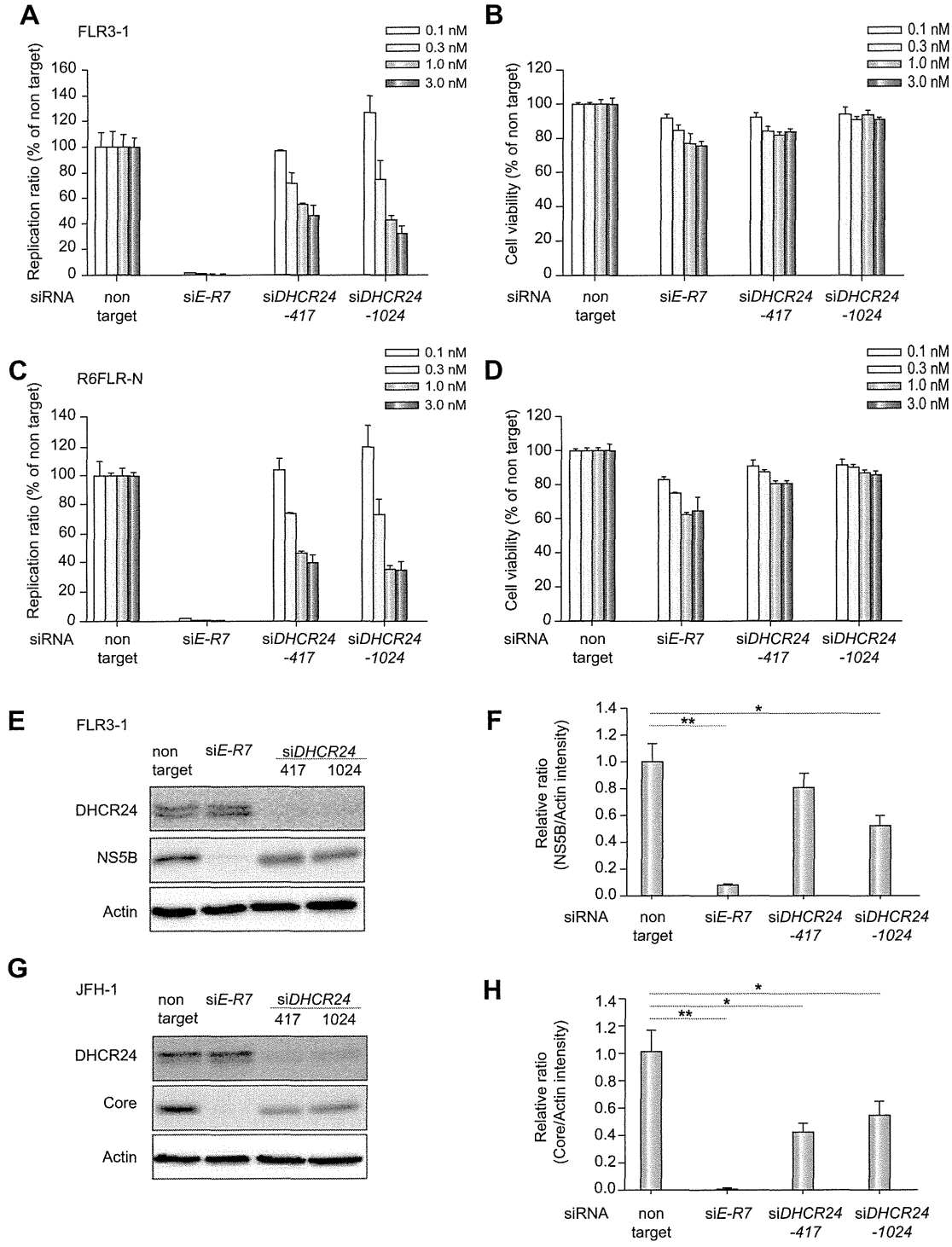

Fig. 2. Effect of DHCR24 knockdown on HCV replication. (A–D) Effect of DHCR24 knockdown on HCV replication in HCV replicon cells (FLR3-1 and R6FLR-N) at 72 h after the anti-*DHCR24* siRNAs (417 and 1024), siRNAs against HCV (siE-R7 for FLR3-1 and JFH-1; siE-R5 for R6-FLR-N), or non-target control siRNAs 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).

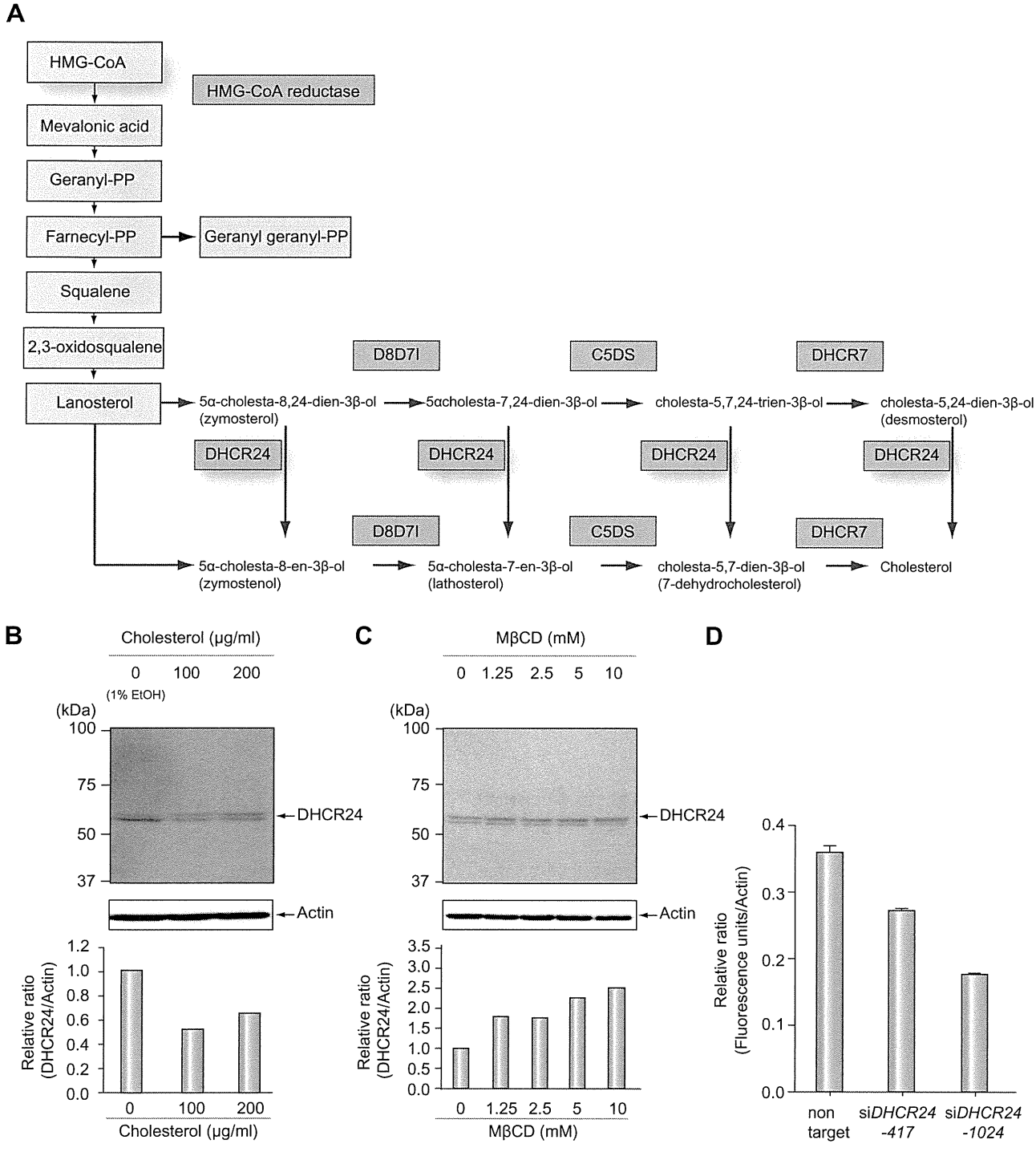

Fig. 3. The level of cholesterol and DHCR24 expression. (A) Cholesterol synthesis pathway, starting from HMG-CoA [26]. The abbreviations used are: D8D7I, 3β-hydroxysterol-delta(8)-delta(7)-isomerase; and C5DS, 3β-hydroxysterol-C^5-desaturase. (B) Cholesterol (0, 100, and 200 μg/ml) was added to HuH-7 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.

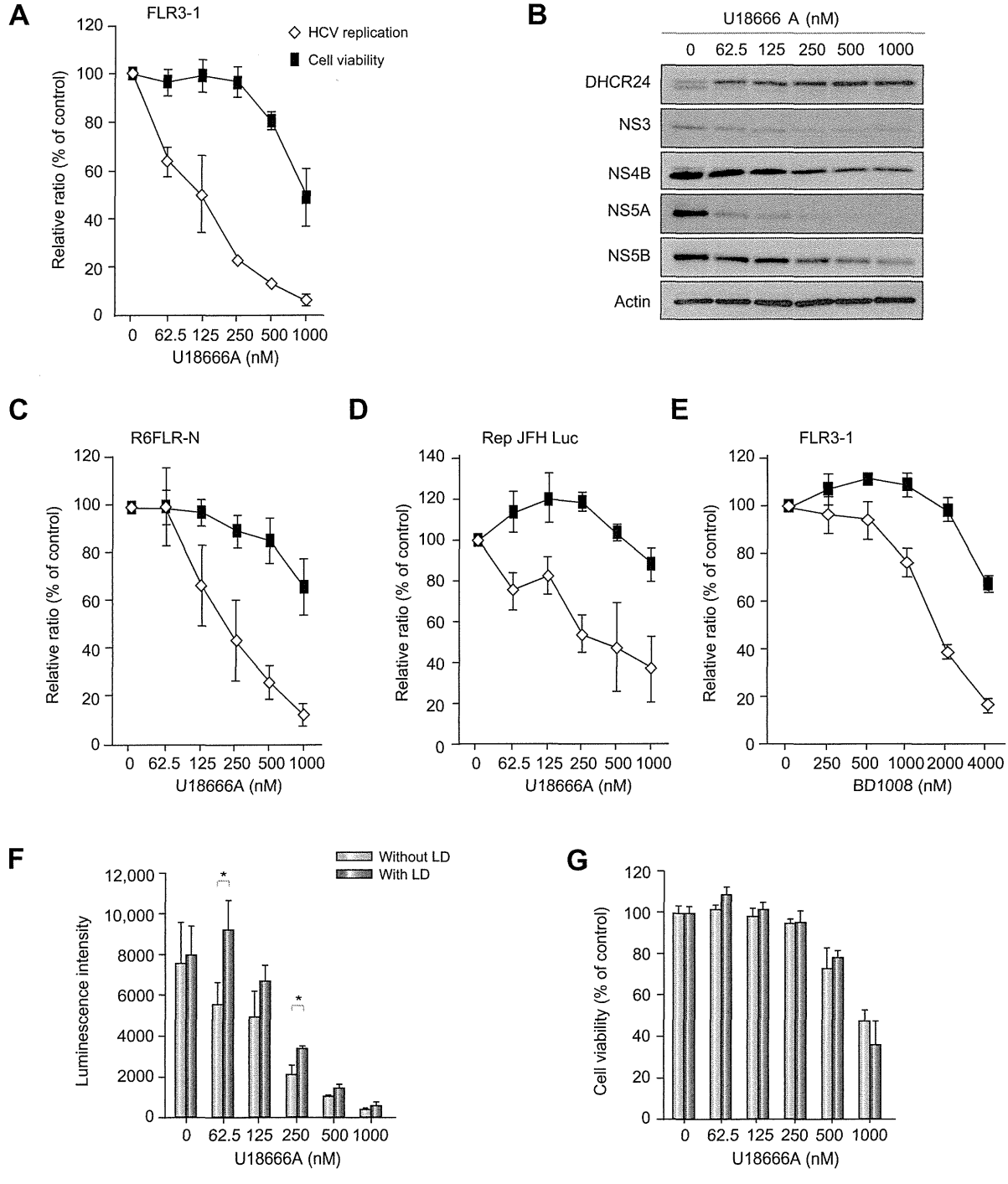

Fig. 4. Effect of U18666A on HCV replication. (A) Addition of U18666A to FLR3-1 cells and subsequent examination of HCV replication by the luciferase assay. Cell viability was measured by WST-8 assay. HCV replication and cell viability were measured 48 h after addition of U18666A. The bars indicate SD 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.

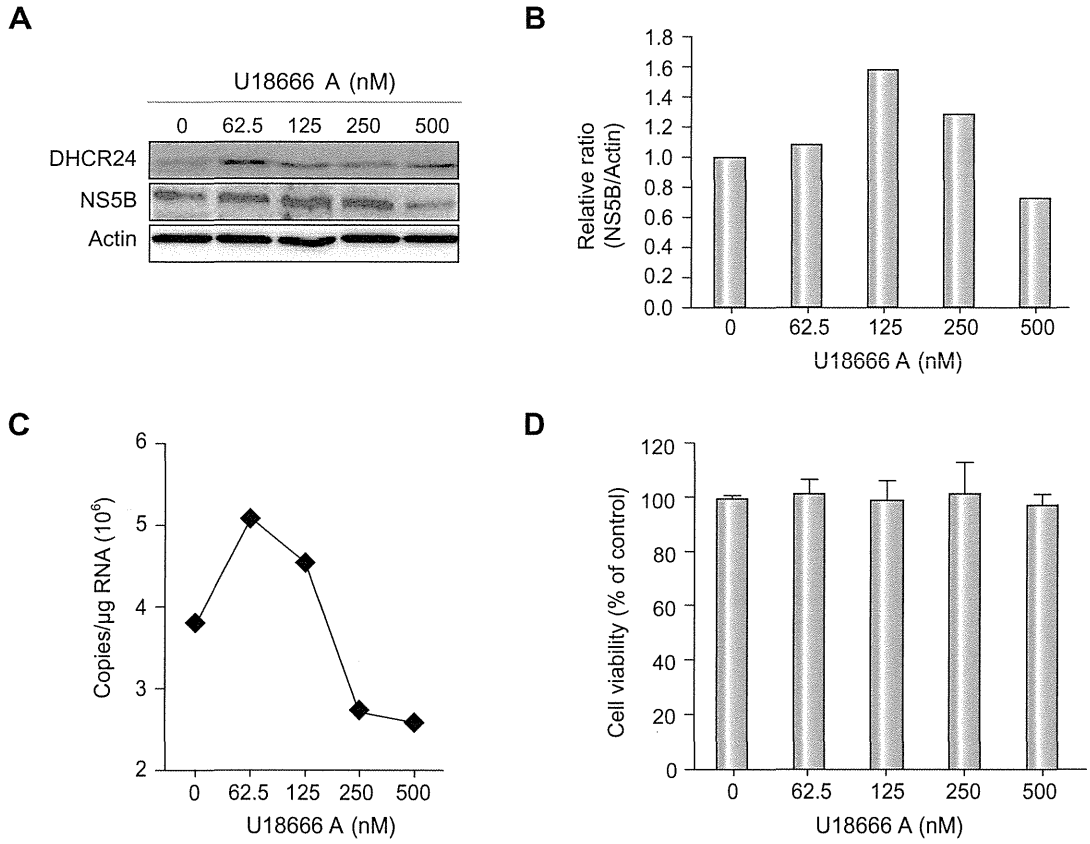

Fig. 5. Effect of U18666A on cells infected with HCV JFH-1. HCV JFH-1-infected cells treated with U18666A were examined 72 h after treatment. (A) Expression of HCV-NS5B protein with or without U18666A treatment, analyzed by Western blot analysis. (B) The intensity of HCV-NS5B protein expression is represented 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

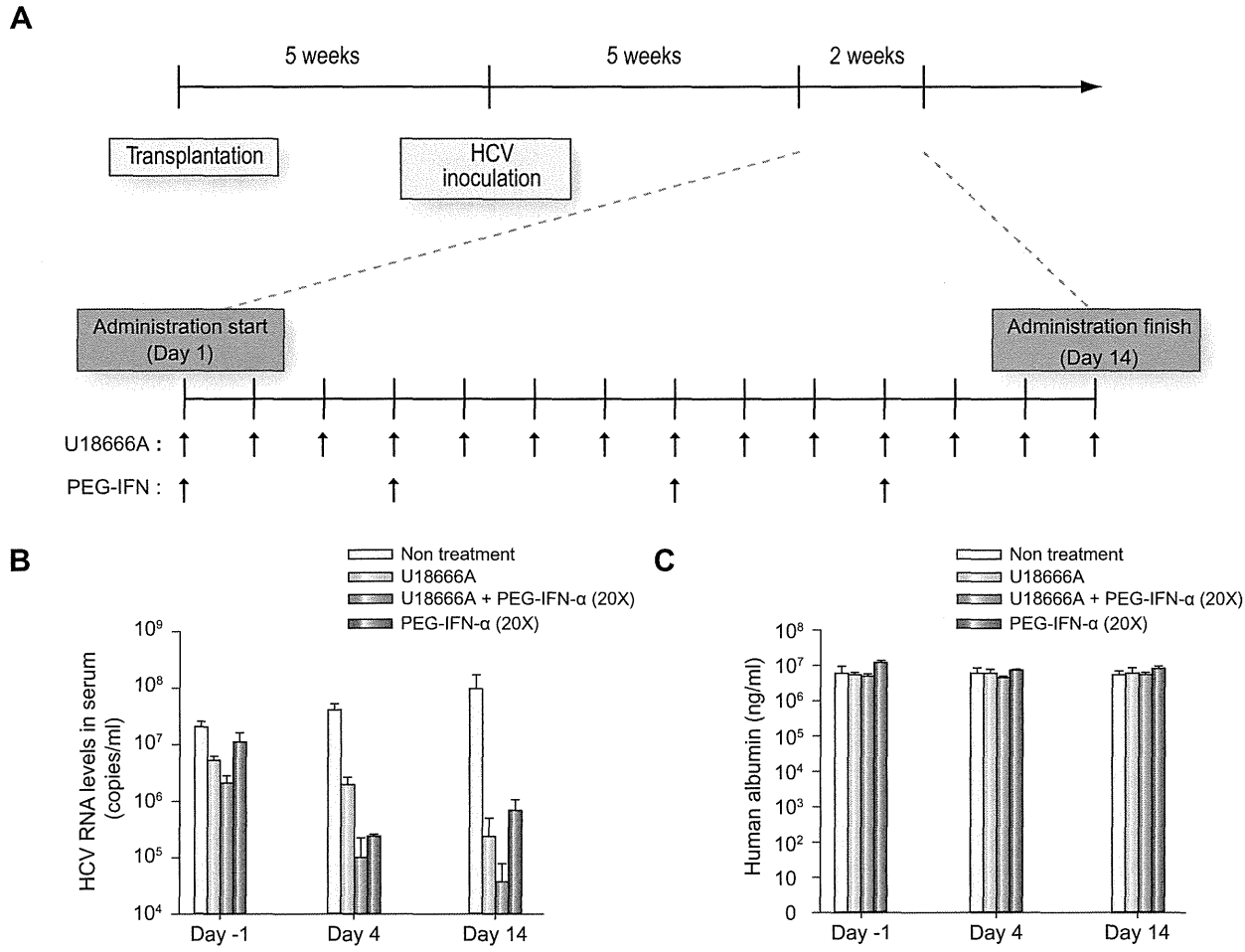

Fig. 6. Evaluation of the anti-HCV effect of U18666A in chimeric mice. (A) Diagram of the schedule that was followed to produce chimeric mice with the humanized liver, perform blood sampling, and administer drugs to chimeric mice infected with HCV. Four groups of three chimeric mice with the humanized 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.

References

[1] Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. Lancet 2003;362:1907–1917.

[2] Tsukuma H, Hiyama T, Tanaka S, et al. Risk factors for hepatocellular carcinoma among patients with chronic liver disease. N Engl J Med 1993;328:1797–1801.

[3] Wasley A, Alter MJ. Epidemiology of hepatitis C: geographic differences and temporal trends. Semin Liver Dis 2000;20:1–16.

[4] Kohara M, Tanaka T, Tsukiyama-Kohara K, et al. Hepatitis C virus genotypes 1 and 2 respond to interferon-alpha with different virologic kinetics. J Infect Dis 1995;172:934–938.

[5] Tsukiyama-Kohara K, Tone S, Maruyama I, et al. Activation of the CKI-CDK-Rb-E2F pathway in full genome hepatitis C virus-expressing cells. J Biol Chem 2004;279:14531–14541.

[6] Nishimura T, Kohara M, Izumi K, et al. Hepatitis C virus impairs p53 via persistent overexpression of 3beta-hydroxysterol Delta24-reductase. J Biol Chem 2009;284:36442–36452.

[7] Greeve I, Hermans-Borgmeyer I, Brellinger C, et al. The human DIMINUTO/DWARF1 homolog seladin-1 confers resistance to Alzheimer's disease-associated neurodegeneration and oxidative stress. J Neurosci 2000;20:7345–7352.

[8] Wu C, Miloslavskaya I, Demontis S, Maestro R, Galaktionov K. Regulation of cellular response to oncogenic and oxidative stress by Seladin-1. Nature 2004;432:640–645.

[9] Waterham HR, Koster J, Romeijn GJ, et al. Mutations in the 3beta-hydroxysterol Delta24-reductase gene cause desmosterolosis, an autosomal recessive disorder of cholesterol biosynthesis. Am J Hum Genet 2001;69:685–694.

[10] Wechsler A, Brafman A, Shafir M, et al. Generation of viable cholesterol-free mice. Science 2003;302:2087.

[11] Aizaki H, Lee KJ, Sung VM, Ishiko H, Lai MM. Characterization of the hepatitis C virus RNA replication complex associated with lipid rafts. Virology 2004;324:450–461.

[12] Di Stasi D, Vallacchi V, Campi V, et al. DHCR24 gene expression is upregulated in melanoma metastases and associated to resistance to oxidative stress-induced apoptosis. Int J Cancer 2005;115:224–230.

[13] Bierkamper GG, Cenedella RJ. Induction of chronic epileptiform activity in the rat by an inhibitor of cholesterol synthesis, U18666A. Brain Res 1978;150:343–351.

[14] Nakabayashi H, Taketa K, Miyano K, Yamane T, Sato J. Growth of human hepatoma cells lines with differentiated functions in chemically defined medium. Cancer Res 1982;42:3858–3863.

[15] Knowles BB, Howe CC, Aden DP. Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. Science (New York, NY) 1980;209:497–499.

[16] Sakamoto H, Okamoto K, Aoki M, et al. Host sphingolipid biosynthesis as a target for hepatitis C virus therapy. Nat Chem Biol 2005;1:333–337.

[17] Watanabe T, Sudoh M, Miyagishi M, et al. Intracellular-diced dsRNA has enhanced efficacy for silencing HCV RNA and overcomes variation in the viral genotype. Gene Ther 2006;13:883–892.

[18] Wakita T, Pietschmann T, Kato T, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 2005;11:791–796.

[19] Inoue K, Umehara T, Ruegg UT, et al. Evaluation of a cyclophilin inhibitor in hepatitis C virus-infected chimeric mice in vivo. Hepatology 2007;45:921–928.

[20] Jensen ON, Wilm M, Shevchenko A, Mann M. Sample preparation methods for mass spectrometric peptide mapping directly from 2-DE gels. Methods Mol Biol (Clifton, NJ) 1999;112:513–530.

[21] Nakagawa S, Umehara T, Matsuda C, et al. Hsp90 inhibitors suppress HCV replication in replicon cells and humanized liver mice. Biochem Biophys Res Commun 2007;353:882–888.

[22] Takeuchi T, Katsume A, Tanaka T, et al. Real-time detection system for quantification of hepatitis C virus genome. Gastroenterology 1999;116:636–642.

[23] Mercer DF, Schiller DE, Elliott JF, et al. Hepatitis C virus replication in mice with chimeric human livers. Nat Med 2001;7:927–933.

[24] Tateno C, Yoshizane Y, Saito N, et al. Near completely humanized liver in mice shows human-type metabolic responses to drugs. Am J Pathol 2004;165:901–912.

[25] Umehara T, Sudoh M, Yasui F, et al. Serine palmitoyltransferase inhibitor suppresses HCV replication in a mouse model. Biochem Biophys Res Commun 2006;346:67–73.

[26] Kedjouar B, de Medina P, Oulad-Abdelghani M, et al. Molecular characterization of the microsomal tamoxifen binding site. J Biol Chem 2004;279:34048–34061.

[27] John CS, Lim BB, Geyer BC, Vilner BJ, Bowen WD. 99mTc-labeled sigma-receptor-binding complex: synthesis, characterization, and specific binding to human ductal breast carcinoma (T47D) cells. Bioconjug Chem 1997;8:304–309.

[28] Ye J, Wang C, Sumpter Jr R, et al. Disruption of hepatitis C virus RNA replication through inhibition of host protein geranyl-geranylation. Proc Natl Acad Sci USA 2003;100:15865–15870.

[29] Kapadia SB, Chisari FV. Hepatitis C virus RNA replication is regulated by host geranyl-geranylation and fatty acids. Proc Natl Acad Sci USA 2005;102:2561–2566.

[30] Aizaki H, Morikawa K, Fukasawa M, et al. Critical role of virion-associated cholesterol and sphingolipid in hepatitis C virus infection. J Virol 2008;82:5715–5724.

[31] Syed GH, Amako Y, Siddiqui A. Hepatitis C virus hijacks host lipid metabolism. Trends Endocrinol Metab 2010;21:33–40.

[32] Crameri A, Biondi E, Kuehnle K, et al. The role of seladin-1/DHCR24 in cholesterol biosynthesis, APP processing and Abeta generation in vivo. EMBO J 2006;25:432–443.

[33] Cenedella RJ. Cholesterol synthesis inhibitor U18666A and the role of sterol metabolism and trafficking in numerous pathophysiological processes. Lipids 2009;44:477–487.

[34] Bader T, Fazili J, Madhoun M, et al. Fluvastatin inhibits hepatitis C replication in humans. Am J Gastroenterol 2008;103:1383–1389.

[35] Ikeda M, Abe K, Yamada M, et al. Different anti-HCV profiles of statins and their potential for combination therapy with interferon. Hepatology (Baltimore, MD) 2006;44:117–125.

[36] Argo CK, Loria P, Caldwell SH, Lonardo A. Statins in liver disease: a molehill, an iceberg, or neither? Hepatology (Baltimore, MD) 2008;48:662–669.

[37] Su AI, Pezacki JP, Wodicka L, et al. Genomic analysis of the host response to hepatitis C virus infection. Proc Natl Acad Sci USA 2002;99:15669–15674.

[38] Honda M, Yamashita T, Ueda T, et al. Different signaling pathways in the livers of patients with chronic hepatitis B or chronic hepatitis C. Hepatology 2006;44:1122–1138.

Journal of Medical Virology 83:801–809 (2011)

Translocase of Outer Mitochondrial Membrane 70 Expression Is Induced by Hepatitis C Virus and Is Related to the Apoptotic Response

Takashi Takano,[1,2,3] Michinori Kohara,[2] Yuri Kasama,[1] Tomohiro Nishimura,[1,4] Makoto Saito,[1] Chieko Kai,[3] and Kyoko Tsukiyama-Kohara[1]*

[1]*Faculty of Life Sciences, Department of Experimental Phylaxiology, Kumamoto University, Kumamoto, Japan*
[2]*Department of Microbiology and Cell Biology, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan*
[3]*Laboratory Animal Research Center, Institute of Medical Science, The University of Tokyo, Tokyo, Japan*
[4]*The Chemo-Sero-Therapeutic Research Institute, Kikuchi Research Center, Kyokushi, Kikuchi, Kumamoto, Japan*

The localization of hepatitis C virus (HCV) proteins in cells leads to several problems. The translocase of outer mitochondrial membrane 70 (TOM70) is a mitochondrial import receptor. In this study, TOM70 expression was induced by HCV infection. TOM70 overexpression induced resistance to tumor necrosis factor-alpha (TNF-α)-mediated apoptosis but not to Fas-induced apoptosis in HepG2 cells. TOM70 was found to be induced by the HCV non-structural protein (NS)3/4A protein, and silencing of TOM70 decreased the levels of the NS3 and Mcl-1 proteins. These results indicate that TOM70 can directly interact with the NS3 protein. In hepatoma cells, silencing of TOM70 induced apoptosis and increased caspase-3/7 activity but did not modify caspase-8 and caspase-9 activity. TOM70 silencing-induced apoptosis was impaired in HCV NS3/4A protein-expressing cells. Thus, this study revealed a novel finding, that is, TOM70 is linked with the NS3 protein and the apoptotic response. ***J. Med. Virol. 83:801–809, 2011.***

KEY WORDS: hepatitis C virus; translocase of outer mitochondrial membrane 70; apoptosis; non-structural protein 3; tumor necrosis factor-alpha

INTRODUCTION

Hepatitis C virus (HCV) infection causes acute and chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) [Seeff, 2002]. HCV easily establishes chronic infection, and localization of HCV proteins is reported to induce several disturbances in cells. One of the major target organelles of HCV is the mitochondrion, and HCV non-structural protein (NS)3/4A protease cleaves the mitochondrial antiviral signaling (MAVS)/IPS-1/VISA/Cardif protein, thereby impairing interferon signaling [Li et al., 2005] and influencing apoptotic responses [Nomura-Takigawa et al., 2006; Deng et al., 2008; Lei et al., 2009].

Most mitochondrial proteins are synthesized in the cytosol as preproteins, targeted to the mitochondria by cytosolic factors such as HSP70 and mitochondrial import stimulation factor (MSF), and transported to the intramitochondrial compartments by the preprotein import machineries of the outer and inner membranes (TOM and TIM complexes, respectively) [Mihara and Omura, 1996; Schatz, 1996; Neupert, 1997; Pfanner and Meijer, 1997]. The TOM machinery consists of two import receptors, namely, TOM20 and TOM70, and several other subunits that are arranged in a tightly bound complex termed the general import pore [Pfanner and Geissler, 2001; Hoogenraad et al., 2002; Stojanovski et al., 2003]. TOM70 was identified in *Saccharomyces cerevisiae* as a 70-kDa protein with no known function [Truscott et al., 2001]. TOM70 is recognized as the primary receptor for proteins with internal targeting signals, such as the F_1-ATPase β-subunit

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: Ministry of Health and Welfare of Japan; Grant sponsor: Clinical and Epidemiological Studies of Emerging and Re-emerging Infectious Diseases (Cooperative Research Project).

Takashi Takano present address is Division of Veterinary Public Health, Nippon Veterinary and Life Science University, 1-7-1 Kyonan, Musashino, Tokyo 180-8602, Japan.

*Correspondence to: Kyoko Tsukiyama-Kohara, Faculty of Life Sciences, Department of Experimental Phylaxiology, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan.
E-mail: kkohara@kumamoto-u.ac.jp

Accepted 13 December 2010
DOI 10.1002/jmv.22046
Published online in Wiley Online Library (wileyonlinelibrary.com).

and cytochrome c_1 [Truscott et al., 2001]. TOM70 interacts with human myeloid cell leukemia-1 (Mcl-1), a Bcl-2 family member, and this interaction facilitates the mitochondrial targeting of Mcl-1 [Chou et al., 2006]. Mcl-1 can interact with the HCV core protein and suppresses core-induced apoptosis [Mohd-Ismail et al., 2009].

In the present study, it was found that TOM70 activity was enhanced by HCV. This study addresses TOM70 modification by HCV and its role in the apoptotic response.

MATERIALS AND METHODS

Cells

WRL68, HepG2, HuH-7, and HepG2 cells expressing non-structural proteins (Lenti-NS3/4A-HepG2, Lenti-NS4B-HepG2, Lenti-NS5A-HepG2, Lenti-NS5B-HepG2, and Lenti-empty-HepG2) were maintained and established as described previously [Tsukiyama-Kohara et al., 2004; Nishimura et al., 2009; Saitou et al., 2009]. The Cre/loxP conditional expression system for full-length HCV cDNA (*HCR6-Rz*) in RzM6 cells [Tsukiyama-Kohara et al., 2004] was induced using 100 nM of 4-hydroxytamoxifen (Sigma–Aldrich, St. Louis, MO) and passaged for 8 days (RzM6-8d) or for more than 44 days (RzM6-LC) [Nishimura et al., 2009] (Supplementary Fig. 1). Cell viability was measured using WST-8 (Dojindo, Kumamoto, Japan).

Purification and Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) Analysis of p70 and TOM70 Expression Vector

p70 was identified using MALDI-TOF-MS. The p70 band was excised, alkylated using 40 mM iodoacetamide/0.1 M NH_4HCO_3, and digested using trypsin. The p70 peptides were purified using an UltiMate capillary high-performance liquid chromatography system (Dionex) and analyzed using a 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA), as described previously [Jensen et al., 1999]. An expression vector with myc and His tags was constructed for TOM70 as follows: Total RNA was isolated from HuH-7 cells (10^6) by using the ISOGEN reagent (Nippon Gene, Tokyo, Japan). Purified RNA (2 μg) was reverse transcribed using SuperScriptIII (Invitrogen, Carlsbad, CA) and oligo$(dT)_{12-18}$ primer (Invitrogen), according to the manufacturer's protocol. The coding region of TOM70 cDNA was amplified by polymerase chain reaction (PCR) with LA *Taq* polymerase (Takara Bio, Shiga, Japan) and TOM70-F2 (5′-GGATCCGCAGAGGACACTTGTCATGGC-3′), which contained a *Bam*HI restriction site (underlined), as the forward primer and TOM70-R2 (5′-GCTGGAGTGCAGTGGCTATTC-3′) as the reverse primer. The amplified TOM70 cDNA was subcloned into the pCR2.1-TOPO vector. *Bam*HI–*Eco*RI-digested TOM70 cDNA was subcloned into pcDNA6/Myc-His(+) (Invitrogen) (TOM70-pcDNA6).

Immunoprecipitation (IP) and Western Blotting (WB)

The cells were solubilized in lysis buffer (20 mM HEPES-NaOH [pH 7.5], 1 mM EDTA [pH 7.5], 1 mM dithiothreitol, 1 μM diisopropylfluorophosphate, 150 mM NaCl, and 1% TritonX-100). Samples were centrifuged at 20,400*g* for 10 min at 4°C, and the supernatants were used for IP. Protein-G sepharose 4B beads (GE Healthcare, Piscataway, NJ; 20 μl) were washed, mixed with 2-243a antibody (2 μg) in 1% BSA-phosphate-buffered saline (PBS), and placed on a rotary shaker at 4°C for 1 hr. Next, the beads were washed three times with lysis buffer and treated with the cell lysate (4°C, overnight). The IP mix was washed four times with lysis buffer and solubilized with 2× SDS sample buffer (150 mM Tris [pH 6.8], 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.2% bromophenol blue). WB was performed as described previously [Nishimura et al., 2009]. Anti-myc monoclonal antibody (mAb) (9E10; Santa Cruz Biotechnology, Santa Cruz, CA), anti-HCV core mouse mAb (31-2), and anti-NS3 rabbit polyclonal antibody (R212) were used to examine the interaction between NS3 and myc-TOM70. Anti-Mcl-1 antibody (S-19; Santa Cruz Biotechnology) and anti-MAVS antibody (ab25084; ChIP grade; Abcam, Cambridge, MA) were also used. Professor Mihara (Kyusyu University) kindly provided anti-rat TOM70 polyclonal antibody (rTOM70).

Immunofluorescence Assay (IFA)

For mitochondrial staining, MitoRed (Dojindo) was added to the cell culture medium and incubated for 1 hr. The cells were fixed in 4% paraformaldehyde. The slides were then washed with PBS, permeabilized with 1% Triton X-100; and reacted with 2-243a mAb (1 μg/ml) and a polyclonal antibody against the endoplasmic reticulum (ER) (anti-PDI; 1:1,000; Stressgen Bioreagent, Kampenhout, Belgium) in 0.025% Tween-20 PBS, followed by reaction with FITC-conjugated goat anti-mouse IgG mAb (1:1,000; Cappel Products, Portland, ME) and Alexa 568-conjugated goat anti-rabbit IgG $(Fab')_2$ fragment (Invitrogen) in 0.025% Tween-20 PBS. The slides were covered with Vector Shield (Vector Laboratories, Burlingame, CA) and observed under an Olympus Fluoview laser-scanning microscope (Olympus, Tokyo, Japan).

Evaluation of Cell Death by Assessing Fas or Tumor Necrosis Factor (TNF)-α

The cells were plated in a 96-well plate (10^4 cells/well; Becton Dickinson, Franklin Lake, NJ) and transfected with empty pcDNA6 or TOM70-pcDNA6 (40 ng/well) by using the Lipofectamine 2000 reagent (Invitrogen). After 48 hr, the cells were treated with anti-Fas

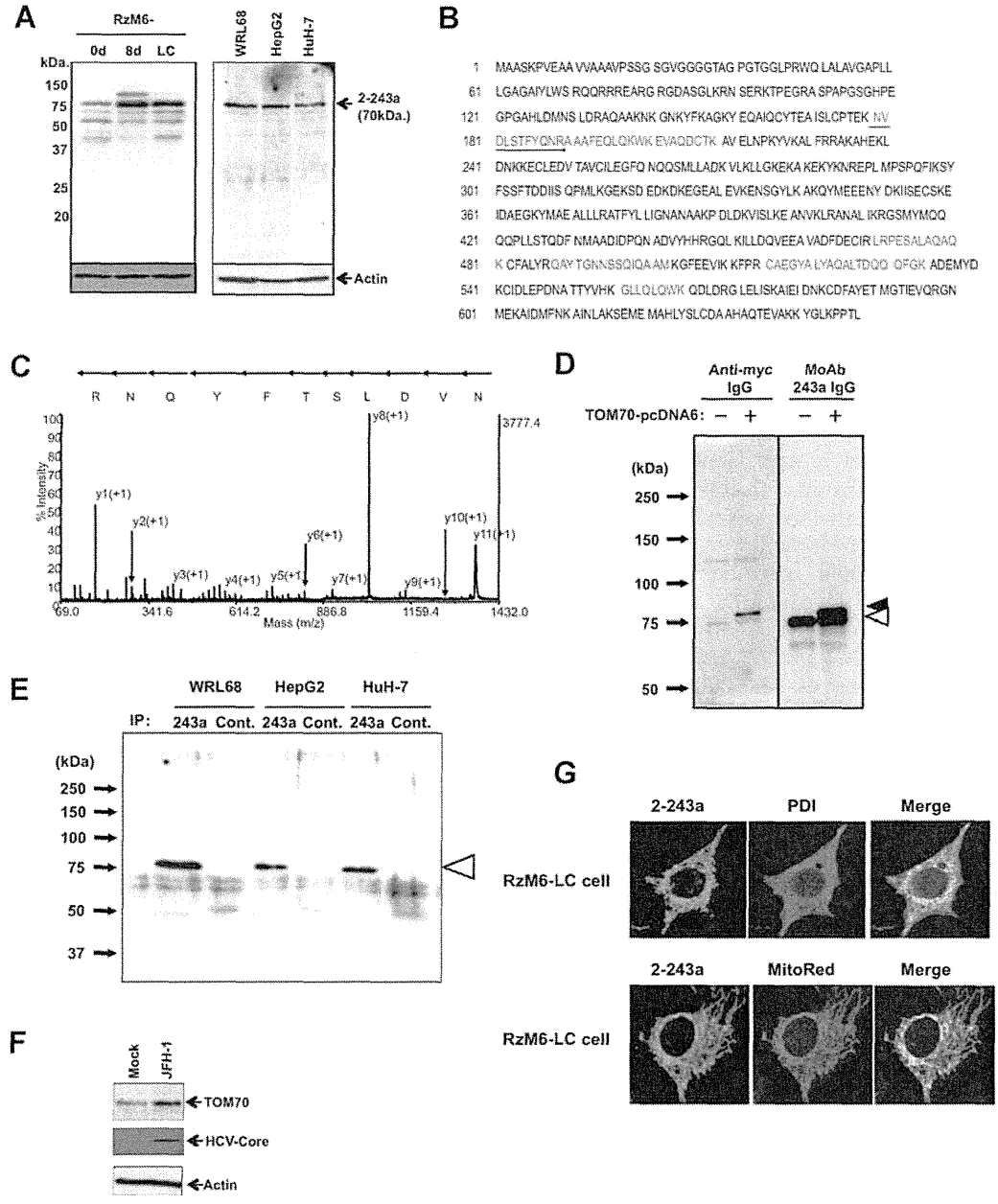

Fig. 1. TOM70 is induced by HCV and is localized in the mitochondria. **A**: TOM70 induction was examined by WB in RzM6-8d and RzM6-LC days (left panel), and TOM70 expression was compared in WRL68, HepG2, and HuH-7 cells (right panel). **B**: Identification of p70 by MALDI-TOF-MS analysis. The sequence of peptides in the amino acid sequence of TOM70 protein was determined using MALDI-TOF-MS analysis (red characters). **C**: MS/MS spectra of the peptide NVDLSTFYQNR (149–159). The sequence covers 14% of the amino acid sequence of TOM70. **D**: Identification of p70 by IP-WB. Expression of TOM70-pcDNA6 in HuH-7. Cell lysates were examined using WB with mAb 2-243a or the anti-myc antibody. myc-TOM70-pcDNA6 expression was recognized by both mAb 2-243a and the anti-myc antibody (black triangle). The expression of cellular TOM70 (empty triangle) was recognized only by mAb 2-243a. **E**: Cell lysates were immunoprecipitated with anti-rat TOM70 antibody and analyzed using WB with mAb 2-243a. The empty triangle indicates TOM70. The molecular weight markers are shown on the left. **F**: Expression of TOM70 and the core protein in mock- and HCV JFH-1-infected HuH-7 cells. **G**: Localization of TOM70 in RzM6-LC cells. The cells were stained with mAb 2-243a and polyclonal antibody against PDI or MitoRed. The magnification is 800×.

antibody (CH-11; 0–20 ng/ml; Beckman Coulter, Murmasaka) or recombinant human TNF-α (0–100 ng/ml; PeproTech, Rocky Hill, NJ), followed by addition of cycloheximide (CHX; 10 μg/ml). After treatment for 24 hr, apoptotic cell death was evaluated by determining cell viability with the WST-8 reagent. Next, the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay was performed using the TMR red in situ cell death detection kit (Roche, Basel, Switzerland).

Generation of Small Interfering Ribonucleic Acid (siRNA) for TOM70

siRNAs for two regions of TOM70, namely, TOM70-d1-siRNA (primer set: TOM70-dicer1-F and TOM70-dicer1-R) and TOM70-d2-siRNA (primer set: TOM70-dicer2-F and TOM70-dicer2-R) were generated.

Gene-specific dsDNA for TOM70 was constructed by PCR using TOM70-pcDNA6 as the template. TOM70-dicer1-F (5′-GCGTAATACGACTCACTATAGGGAGA-TGTTTGGCCTTTAAGTATCC-3′) was used as the forward primer, and TOM70-dicer1-R (5′-GCGTAA-TACGACTCACTATAGGGAGATGATATCATCCGTGA-AAGAAC-3′) was used as the reverse primer; both primers contained a T7 promoter sequence (underlined). PCR performed using these primers yielded a 434-bp product. PCR with the forward primer TOM70-dicer2-F (5′-GCGTAATACGACTCACTATAGGGAGAATGTTTG-CATTGTACCGCC-3′) and the reverse primer TOM70-dicer2-R2 (5′-GCGTAATACGACTCACTATAGGGAGA-TTTGCAACTTCTGTCTGGGC-3′), both of which contained a T7 promoter sequence (underlined), yielded a 474-bp product. Luciferase was amplified from pGL3-Basic (Takara Bio) with Luci-dicer2-F (5′-GCGTAA-TACGACTCACTATAGGGAGACGGTTTTGGAATGTT-TACTAC-3′) as the forward primer and Luci-dicer2-R (5′-GCGTAATACGACTCACTATAGGGAGAGCTGAT-GTAGTCTCAGTGAGC-3′), as the reverse primer, yielding a 309-bp product; both primers contained a T7 promoter sequence (underlined). LA *Taq* polymerase was used for the PCR. All PCR products were analyzed by agarose electrophoresis before purification with the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI).

In vitro transcription was performed with the Dicer siRNA generation kit (Genlantis, San Diego, CA), according to the manufacturer's instructions. Briefly, in vitro transcription reactions were performed in a 20-μl volume with 1 μg PCR product as the template; the reaction mixture was incubated at 37°C for 4 hr, followed by purification with the reagents provided in the Dicer siRNA generation kit. The dsDNA (20 μl) obtained was finally in a 100-μl volume after incubation at 37°C for 27 hr. The siRNAs obtained were purified and quantified according to the manufacturer's instructions.

Next, the cells were plated in 24- or 96-well plates (BD Bioscience, Sparks, MD) at a density of 5×10^4 or 10^4 cells/well, respectively, and left overnight for adherence. The siRNAs (14 nM) generated were transfected to cells by using Lipofectamine RNAiMAX (Invitrogen) and Opti-MEM (Invitrogen). The cells were characterized 48 hr after transfection.

Caspase Assay

The activities of caspase-3/7, caspase-8, and caspase-9 were measured on the basis of the cleavage of a proluminescent substrate containing the DEVD sequence, by using the commercially available Caspase-Glo 9 Assay, Caspase-Glo 8 Assay, and Caspase-Glo 3/7 Assay kits (Promega) and a luminometer (Aloka, Tokyo, Japan). Caspase activity was quantified according to the manufacturer's instructions.

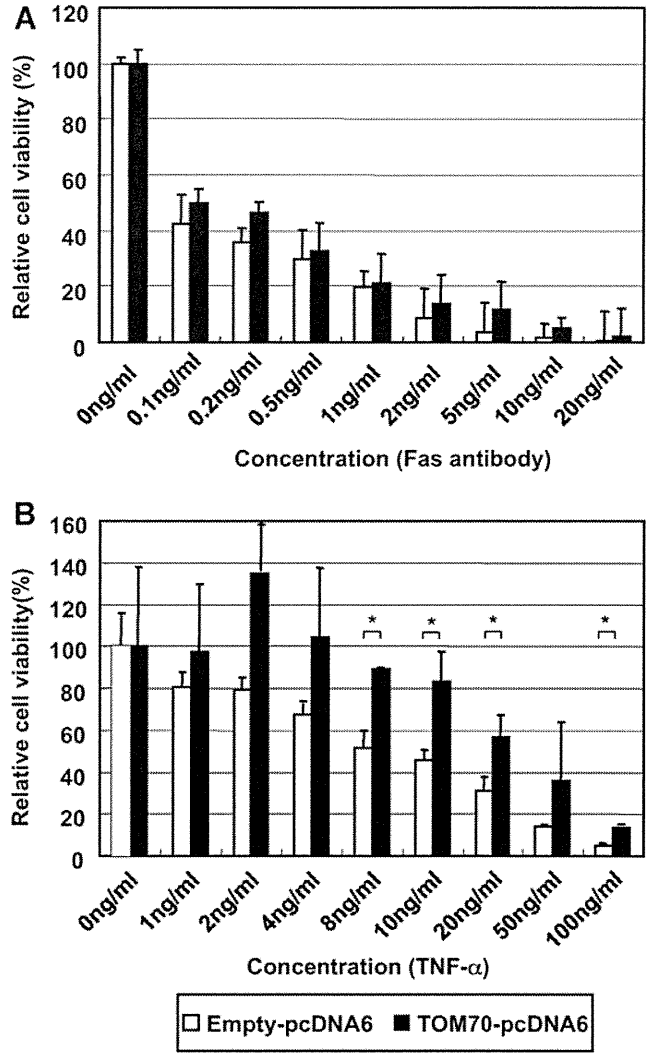

Fig. 2. TOM70 overexpression induced TNF-α-mediated apoptotic resistance. TOM70 overexpression affected TNF-α-mediated apoptosis but not Fas-mediated apoptosis. Cells were transfected with empty pcDNA6 (white bar) or TOM70-pcDNA6 (black bar). After 48 hr, they were treated with (**A**) Fas antibody (0–20 ng/ml) or (**B**) TNF-α (0–100 ng/ml). After 24 hr, cell viability was measured using WST-8. A,B: The data represent the average of the values obtained from triplicate experiments, and the vertical bars indicate the SD. $^*P < 0.05$ (two-tailed Student's *t*-test).

Statistical Analysis

Data were analyzed for statistical significance by using the Student's *t*-test. *P*-values lower than 0.05 were considered statistically significant.

RESULTS

Identification of the p70 Molecule and Induction by HCV

mAbs against RzM6-LC cells were screened, and the clone 2-243a, which recognizes p70, was obtained

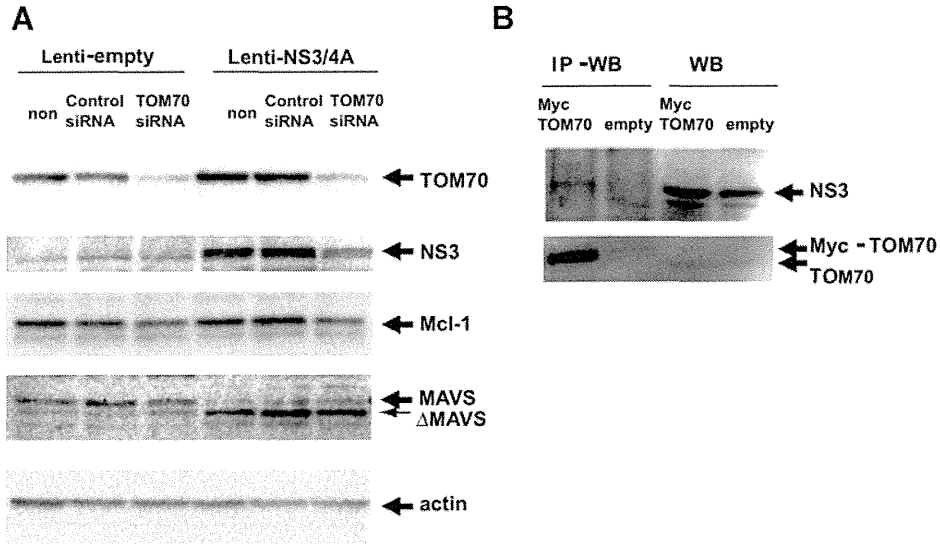

Fig. 3. Interaction between TOM70 and NS3 protein. **A**: The effect of TOM70 siRNA in cells transfected with empty or NS3/4A-containing lentivirus vectors was examined by WB with mAb 2-243a for TOM70; anti-NS3 rabbit polyclonal antibody; anti-Mcl-1 rabbit polyclonal antibody; anti-MAVS rabbit polyclonal antibody; and anti-actin antibody. **B**: The interaction between TOM70 and NS3 was assessed using IP-WB. NS3-expressing HepG2 cells were transfected with pcDNA6-TOM70 (mycTOM70) or pcDNA6 alone (empty) and immunoprecipitated with the anti-myc antibody (9E10). NS3 was detected using polyclonal rabbit anti-NS3 antibody (upper image), and TOM70 was detected using mAb 2-243a (lower image).

(Fig. 1A). p70 was induced to a greater extent by HCV expression after 8 days (RzM6-8d) or more than 44 days (RzM6-LC) than before HCV expression (RzM6-0d). The p70 expression level did not differ among the human hepatic cell lines (WRL68, HepG2, and HuH-7) (Fig. 1A, right panel). p70 was characterized (Fig. 1B–E): The sequence of peptides determined using MALDI-TOF-MS (Fig. 1B) and the MS/MS spectra of the p70 peptide sequence NVDLSTFYQNR (Fig. 1C) are provided. TOM70-pcDNA6 expression in HuH-7 cells was detected by WB with mAb 2-243a (Fig. 1D). Cell lysates were immunoprecipitated with anti-rat TOM70 antibody and detected by WB using mAb 2-243a (Fig. 1E). These results indicate that mAb 2-243a recognizes TOM70. Next, the effect of HCV infection on TOM70 expression was examined (Fig. 1F), and infection with the HCV JFH-1 strain [Wakita et al., 2005] induced TOM70 expression in HuH-7 cells (Fig. 1F). TOM70 localization was characterized using an indirect fluorescence assay (IFA) with 2-243a; anti-PDI, an ER marker; or MitoRed, which is a selective mitochondrial marker (Fig. 1G). TOM70 was associated with the mitochondria in all cells and was a part (~40%) of the ER, indicating that the TOM70 expressions in the mitochondria were higher than those in the ER.

TOM70 Inhibits TNF-α-Mediated Apoptotic Cell Death

The results of previous studies indicate the significant role of mitochondria in the apoptotic response [Hatano, 2007]. TOM70 interacts with Mcl-1 and facilitates mitochondrial targeting by the latter [Chou et al., 2006]. Mcl-1 silencing enhances TNF-related apoptosis-inducing ligand (TRAIL)-mediated cell death [Wirth et al., 2005; Han et al., 2006]. Therefore, the role of TOM70 in the apoptotic response was examined in this study. HepG2 cells were transfected with TOM70-pcDNA6 (Fig. 1D) or empty pcDNA6 (control), and their sensitivity to anti-Fas antibody (Fig. 2A) and TNF-α-mediated apoptotic cell death (Fig. 2B) was examined. When treated with 8 ng/ml of TNF-α, the TOM70-pcDNA6-transfected cells were significantly more viable than those transfected with empty pcDNA6 (Fig. 2B). In contrast, no significant differences were found between the viability of TOM70-pcDNA6 transfected cells and control cells treated with anti-Fas antibody (Fig. 2A). Thus, TNF-α-induced apoptosis was inhibited by TOM70 overexpression.

Interaction of TOM70 With HCV-NS3 and Other Host Factors

To determine the mechanism by which HCV induces TOM70, the TOM70 level in HCV NS3/4A-expressing HepG2 cells was determined (Fig. 3A). The TOM70 level was higher in the NS3/4A-expressing cells than in the control cells. Interestingly, the level of NS3/4A protein as well as Mcl-1 was reduced when TOM70 was silenced. The MAVS protein is cleaved by NS3/4A, as reported previously [Li et al., 2005], and the level of this protein was not influenced by the silencing of TOM70. IP-WB was performed to examine the possible interaction between TOM70 and NS3/4A (Fig. 3B). The pcDNA6-TOM70-myc plasmid was transfected into lenti-NS3/4A vector-transduced HepG2 cells; IP was performed using the anti-myc antibody, and the reaction was detected using the anti-NS3 antibody. The NS3 protein was

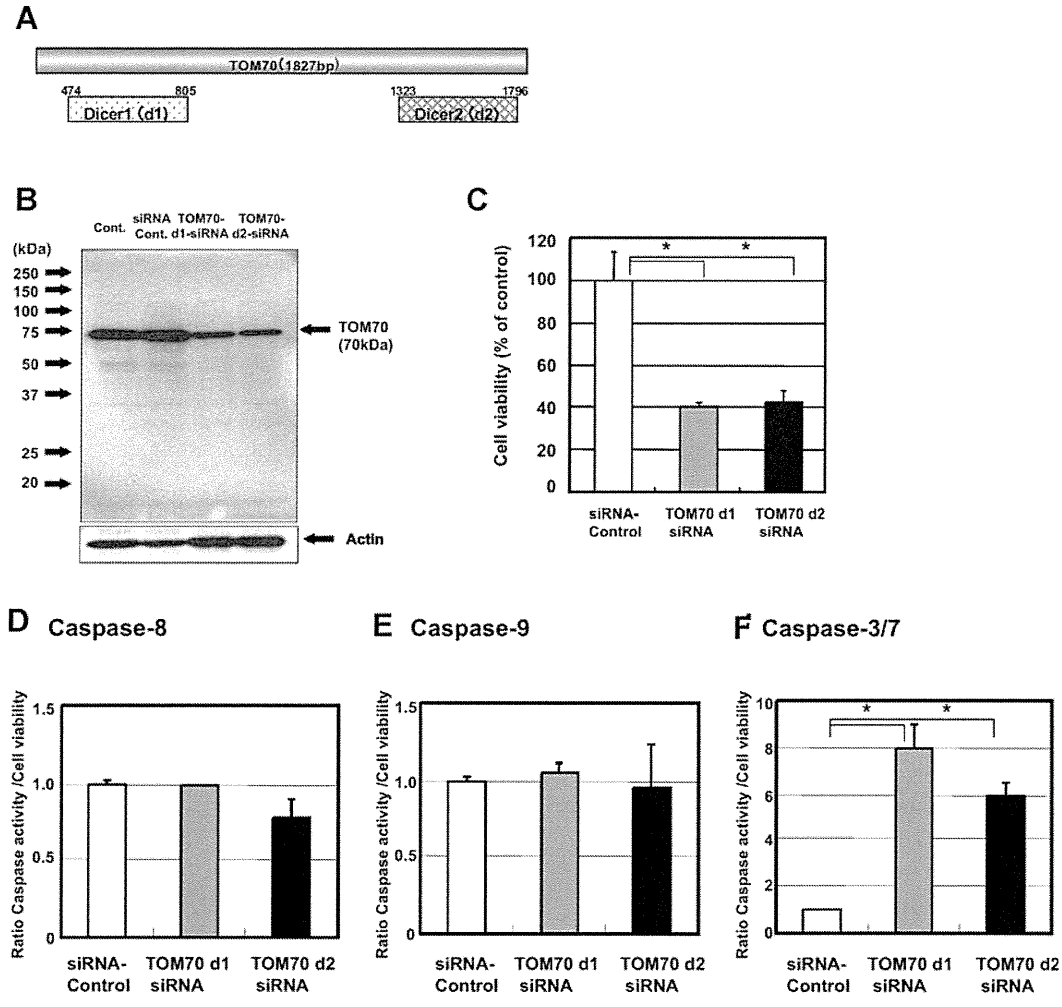

Fig. 4. Silencing of TOM70 induced apoptotic cell death and caspase-3/7 activity. **A**: The positions of TOM70-d1-siRNA and TOM70-d2-siRNA are indicated in the figure. **B**: siRNA-mediated silencing of TOM70 was detected by WB (Cont: no siRNA, siRNA Cont: siRNA control (Luci2-siRNA), TOM70-d1-siRNA, TOM70-d2-siRNA). **C**: TOM70 knockdown-induced cell death was calculated by measuring viability (%) with the WST-8 cell counting kit. The cell viability after 48 hr was scored in HepG2 cells transfected with the siRNA control Luci2-siRNA (□), TOM70-d1-siRNA (■), and TOM70-d2-siRNA (■). The activities of caspase-8 (**D**), caspase-9 (**E**), and caspase-3/7 (**F**) were measured using commercially available assays and a luminometer. The caspase activity was scored after 48 hr in TOM70-knockdown HepG2 cells transfected with control siRNA (□), TOM70-d1-siRNA (■), and TOM70-d2-siRNA (■). C–F: The data represent the average of the values obtained from triplicate experiments, and the vertical bars indicate the SD. $^*P < 0.05$ (two-tailed Student's *t*-test).

specifically precipitated by myc-tagged TOM70. The NS4A protein was not detected in this assay (data not shown). Therefore, the NS3 protein directly interacts with TOM70.

TOM70 Knockdown by RNAi Induces Apoptosis

The effect of TOM70 on the apoptotic response was examined because TOM70 silencing decreased the level of Mcl-1. First, two siRNAs for TOM70 (TOM70-d1-siRNA and TOM70-d2-siRNA) were designed in order to prevent the off-target effect (Fig. 4A). siRNA for luciferase (Luci-d2-siRNA) was used as a control (Fig. 4B). HepG2 cells were transfected with TOM70-d1-siRNA or TOM70-d2-siRNA, and the downregulation of TOM70 expression was confirmed by WB (Fig. 4B). Furthermore, decreased cell viability was observed (Fig. 4C) after 48 hr. Treatment with TOM70-d1-siRNA or TOM70-d2-siRNA significantly decreased the cell viability of HuH-7 cells too (data not shown). These results indicate that TOM70 silencing with siRNA may induce apoptosis.

The activities of caspase-3/7, caspase-8, and caspase-9 in HepG2 cells were examined after TOM70 silencing (Fig. 4D–F). The activities of caspase-8 and caspase-9 in cells transfected with TOM70 siRNA were not significantly different from those in the cells treated with control siRNA (Fig. 4D,E). In contrast, the caspase-3/7 activity in the TOM70-siRNA transfected cells was significantly greater than that in the cells treated with

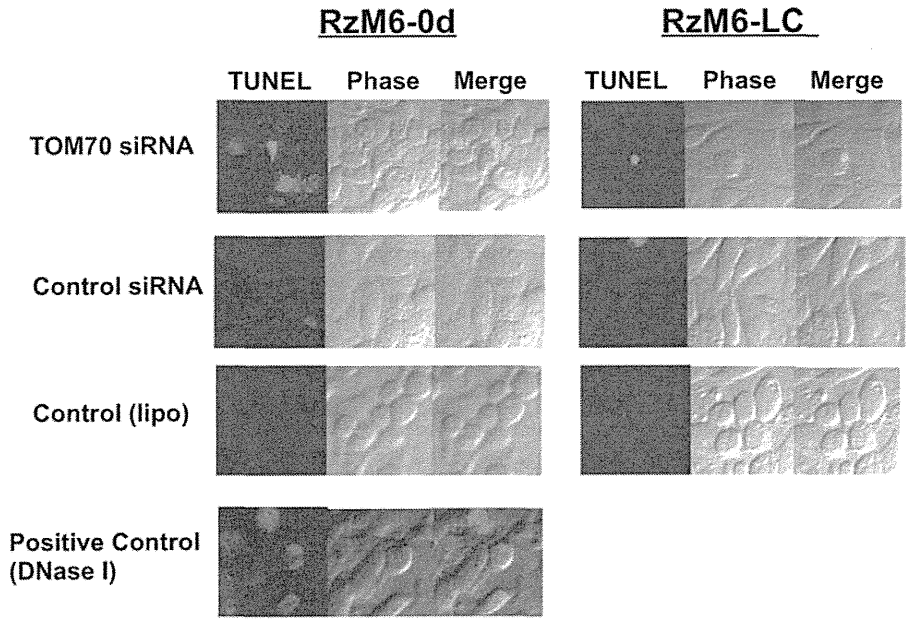

Fig. 5. TUNEL assay in RzM6-0d and RzM6-LC cells transfected with TOM70 siRNA, control siRNA, and control (Lipofectamine). The positive control is RzM6-0d cells treated with DNase I. The magnification is 400×.

control siRNA (Fig. 4F). These results indicate that the siRNA-mediated silencing of TOM70 expression induces apoptosis through caspase-3/7.

TOM70 Silencing-Induced Apoptosis Is Impaired by HCV

Next, the effect of TOM70 silencing-induced apoptosis was examined in RzM6-0d and RzM6-LC cells in order to determine the effect of HCV expression (Fig. 5). The apoptotic response was examined using the TUNEL assay wherein DNA strand breaks are detected and the apoptotic response is thereby detected [Gavrieli et al., 1992]. The DNA strand breaks, which were stained red, were observed using confocal microscopy. Treatment with TOM70 siRNA produced significant DNA strand breaks in the RzM6-0d cells. However, the apoptotic signal was suppressed in the RzM6-LC cells. This indicates the possibility that HCV can impair the apoptotic response induced by TOM70 siRNA.

Silencing of TOM70 Decreases Cell Viability, Whereas HCV-NS3 Restores Cell Viability

The RzM6-0d and RzM6-LC cells were treated with TOM70 siRNA and control siRNA, and the cell viability was measured using the WST-8 assay [Isobe et al., 1999] (Fig. 6A). The viability of the RzM6-LC cells was significantly higher than that of the RzM6-0d cells after treatment with TOM70 siRNA, and this difference increased in a dose-dependent manner. This indicates that the expression of HCV genes may impair the TOM70 siRNA-induced apoptotic response. The responsible HCV protein was identified using the lentivirus vector (Fig. 6B), and TOM70 siRNA-induced cell death was found to be impaired in HCV-NS3/4A-expressing cells.

DISCUSSION

The results of the present study suggest that HCV interacts with TOM70 through the NS3 protein, which indicates the possibility that TOM70 regulates the intracellular localization of HCV NS3; a previous study has reported the mitochondrion to be one of the regions where HCV NS3 is located [Sillanpaa et al., 2008]. The results of a previous study indicate that TOM70 also interacts with the Mcl-1 protein [Chou et al., 2006]. TOM70 silencing decreased the levels of the NS3 and Mcl-1 proteins; therefore, interaction with TOM70 may increase the stability of NS3 and Mcl-1. Recently, it was reported that Mcl-1 is stabilized by the deubiquitinase USP9X and that it can promote tumor cell survival [Schwickart et al., 2010]. Furthermore, Mcl-1 interacts with the HCV core protein through Bcl-2 homology domain 3 (BH3) [Mohd-Ismail et al., 2009], and Mcl-1 overexpression suppresses core-induced apoptosis. Therefore, further studies are required to clarify the relationship between TOM70, Mcl-1, and other host factors in HCV infection. This information may provide novel insights into the mechanism underlying the induction of apoptotic resistance and tumorigenicity in hepatocytes during chronic HCV infection.

The results of this study indicate the regulatory role of TOM70 in apoptosis. TOM70 overexpression was found to suppress the TNF-α-mediated but not the Fas-mediated apoptotic response. TOM70 knockdown

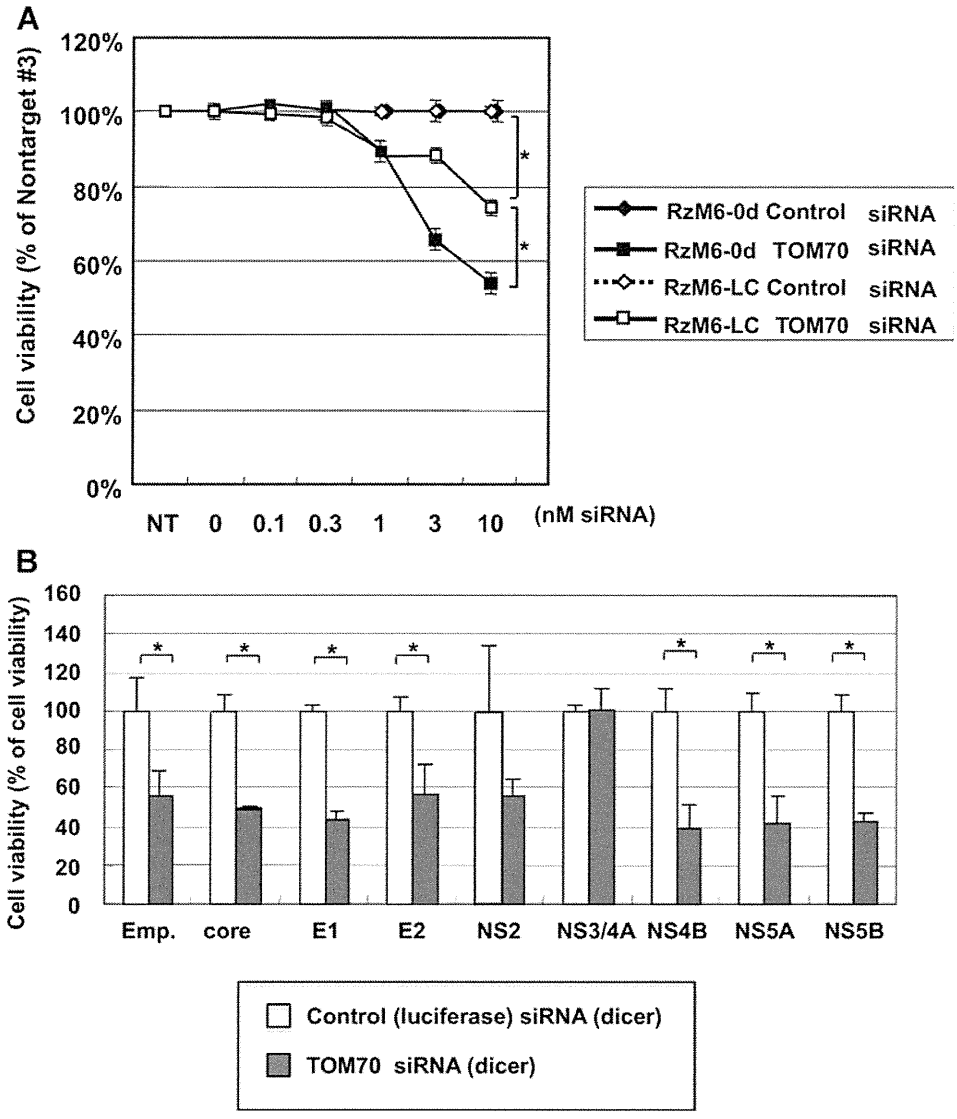

Fig. 6. **A**: Cell viability of RzM6-0d or RzM6-LC cells after treatment with TOM70 siRNA. The viability is given as the ratio (%) of test to control siRNA treatment. **B**: The viability of HepG2 cells transduced with lentivirus vectors expressing the HCV core, E1, E2, NS2, NS3/4A, NS4B, NS5A, and NS5B proteins after treatment with TOM70 siRNA was measured by the WST assay, and the viability is given as the ratio (%) of the test to the control siRNA treatment. The data represents the average of the values obtained from triplicate experiments, and the vertical bars indicate SD. $^{*}P < 0.05$ (two-tailed Student's *t*-test).

increased caspase-3/7 activity, but the activities of caspase-8 and caspase-9 were not significantly affected. This indicates the possibility that TOM70 regulates the TNF receptor-mediated apoptotic pathway. Several reports have indicated that TNF-α-mediated apoptosis is inhibited by HCV proteins. Saito et al. [2006] reported that the HCV core protein inhibited the TNF-α-mediated signaling pathway through the sustained expression of a cellular- FADD-like interleukin-1β-converting enzyme (FLICE) like inhibitory protein (c-FLIP; caspase-8 inhibitor). Majumder et al. [2002] reported that TNF-α-mediated hepatic apoptosis was impaired by the HCV-NS5A protein. The results of the present study revealed an alternative pathway by which HCV can acquire TNF-α-induced apoptotic resistance through TOM70 augmentation. Recently, it has been reported that TOM70 interacts with MAVS, TNFRSF1A-associated via death domain (TRADD), TNF receptor-associated factor 6 (TRAF6), stimulator of interferon genes (STING), and interferon regulatory factor (IRF)-3, and that augmentation of TOM70 activates retinoic acid-inducible gene (RIG)-I signaling [Liu et al., 2010]. The results of recent studies indicate that IRF-3 can activate Bax expression and apoptosis [Chattopadhyay et al., 2004]. Future studies on the modification of the regulatory pathway of TOM70 by HCV may provide further insights on the mechanism underlying persistent HCV infection.

ACKNOWLEDGMENTS

The authors thank Dr. H. Fukuda and Dr. S. Ohmi for their technical support with regard to MALDI-TOF-MS analysis, Dr. F. Yasui and Dr. T. Munakata for their critical comments, Dr. Satoh for his technical support, and Prof. S. Harada for his support.

REFERENCES

Chattopadhyay S, Marques JT, Yamashita M, Peters KL, Smith K, Desai A, Williams BR, Sen GC. 2004. Viral apoptosis is induced by IRF-3-mediated activation of Bax. EMBO J 29:1762–1773.

Chou CH, Lee RS, Yang-Yen HF. 2006. An internal EELD domain facilitates mitochondrial targeting of Mcl-1 via a Tom70-dependent pathway. Mol Biol Cell 17:3952–3963.

Deng L, Adachi T, Kitayama K, Bungyoku Y, Kitazawa S, Ishido S, Shoji I, Hotta H. 2008. Hepatitis C virus infection induces apoptosis through a Bax-triggered, mitochondrion-mediated, caspase 3-dependent pathway. J Virol 82:10375–10385.

Gavrieli Y, Sherman Y, Ben-Sasson SA. 1992. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 119:493–501.

Han J, Goldstein LA, Gastman BR, Rabinowich H. 2006. Interrelated roles for Mcl-1 and BIM in regulation of TRAIL-mediated mitochondrial apoptosis. J Biol Chem 281:10153–10163.

Hatano E. 2007. Tumor necrosis factor signaling in hepatocyte apoptosis. J Gastroenterol Hepatol 22:S43–S44.

Hoogenraad NJ, Ward LA, Ryan MT. 2002. Import and assembly of proteins into mitochondria of mammalian cells. Biochim Biophys Acta 1592:97–105.

Isobe I, Michikawa M, Yanagisawa K. 1999. Enhancement of MTT, a tetrazolium salt, exocytosis by amyloid beta-protein and chloroquine in cultured rat astrocytes. Neurosci Lett 266:129–132.

Jensen ON, Wilm M, Shevchenko A, Mann M. 1999. Sample preparation methods for mass spectrometric peptide mapping directly from 2-DE gels. Methods Mol Biol 112:513–530.

Lei Y, Moore CB, Liesman RM, O'Connor BP, Bergstralh DT, Chen ZJ, Pickles RJ, Ting JP. 2009. MAVS-mediated apoptosis and its inhibition by viral proteins. PLoS One 4:e5466.

Li XD, Sun L, Seth RB, Pineda G, Chen ZJ. 2005. Hepatitis C virus protease NS3/4A cleaves mitochondrial antiviral signaling protein off the mitochondria to evade innate immunity. Proc Natl Acad Sci USA 102:17717–17722.

Liu XY, Wei B, Shi HX, Shan YF, Wang C. 2010. Tom70 mediates activation of interferon regulatory factor 3 on mitochondria. Cell Res 20:994–1011.

Majumder M, Ghosh AK, Steele R, Zhou XY, Phillips NJ, Ray R, Ray RB. 2002. Hepatitis C virus NS5A protein impairs TNF-mediated hepatic apoptosis, but not by an anti-FAS antibody, in transgenic mice. Virology 294:94–105.

Mihara K, Omura T. 1996. Cytoplasmic chaperones in precursor targeting to mitochondria: The role of MSF and hsp 70. Trends Cell Biol 6:104–108.

Mohd-Ismail NK, Deng L, Sukumaran SK, Yu VC, Hotta H, Tan YJ. 2009. The hepatitis C virus core protein contains a BH3 domain that regulates apoptosis through specific interaction with human Mcl-1. J Virol 83:9993–10006.

Neupert W. 1997. Protein import into mitochondria. Annu Rev Biochem 66:863–917.

Nishimura T, Kohara M, Izumi K, Kasama Y, Hirata Y, Huang Y, Shuda M, Mukaidani C, Takano T, Tokunaga Y, Nuriya H, Satoh M, Saito M, Kai C, Tsukiyama-Kohara K. 2009. Hepatitis C virus impairs p53 via persistent overexpression of 3beta-hydroxysterol Delta24-reductase. J Biol Chem 284:36442–36452.

Nomura-Takigawa Y, Nagano-Fujii M, Deng L, Kitazawa S, Ishido S, Sada K, Hotta H. 2006. Non-structural protein 4A of Hepatitis C virus accumulates on mitochondria and renders the cells prone to undergoing mitochondria-mediated apoptosis. J Gen Virol 87:1935–1945.

Pfanner N, Geissler A. 2001. Versatility of the mitochondrial protein import machinery. Nat Rev Mol Cell Biol 2:339–349.

Pfanner N, Meijer M. 1997. The Tom and Tim machine. Curr Biol 7:R100–R103.

Saito K, Meyer K, Warner R, Basu A, Ray RB, Ray R. 2006. Hepatitis C virus core protein inhibits tumor necrosis factor alpha-mediated apoptosis by a protective effect involving cellular FLICE inhibitory protein. J Virol 80:4372–4379.

Saitou K, Mizumoto K, Nishimura T, Kai C, Tsukiyama-Kohara K. 2009. Hepatitis C virus-core protein facilitates the degradation of Ku70 and reduces DNA-PK activity in hepatocytes. Virus Res 144:266–271.

Schatz G. 1996. The protein import system of mitochondria. J Biol Chem 271:31763–31766.

Schwickart M, Huang X, Lill JR, Liu J, Ferrando R, French DM, Maecker H, O'Rourke K, Bazan F, Eastham-Anderson J, Yue P, Dornan D, Huang DC, Dixit VM. 2010. Deubiquitinase USP9X stabilizes MCL1 and promotes tumour cell survival. Nature 463:103–107.

Seeff LB. 2002. Natural history of chronic hepatitis C. Hepatology 36:S35–S46.

Sillanpaa M, Kaukinen P, Melen K, Julkunen I. 2008. Hepatitis C virus proteins interfere with the activation of chemokine gene promoters and downregulate chemokine gene expression. J Gen Virol 89:432–443.

Stojanovski D, Johnston AJ, Streimann I, Hoogenraad NJ, Ryan MT. 2003. Import of nuclear-encoded proteins into mitochondria. Exp Physiol 88:57–64.

Truscott KN, Pfanner N, Voos W. 2001. Transport of proteins into mitochondria. Rev Physiol Biochem Pharmacol 143:81–136.

Tsukiyama-Kohara K, Tone S, Maruyama I, Inoue K, Katsume A, Nuriya H, Ohmori H, Ohkawa J, Taira K, Hoshikawa Y, Shibasaki F, Reth M, Minatogawa Y, Kohara M. 2004. Activation of the CKI-CDK-Rb-E2F pathway in full genome hepatitis C virus-expressing cells. J Biol Chem 279:14531–14541.

Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, Murthy K, Habermann A, Krausslich HG, Mizokami M, Bartenschlager R, Liang TJ. 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 11:791–796.

Wirth T, Kuhnel F, Fleischmann-Mundt B, Woller N, Djojosubroto M, Rudolph KL, Manns M, Zender L, Kubicka S. 2005. Telomerase-dependent virotherapy overcomes resistance of hepatocellular carcinomas against chemotherapy and tumor necrosis factor-related apoptosis-inducing ligand by elimination of Mcl-1. Cancer Res 65:7393–7402.

JLB

Article

Role of interleukin-18 in intrahepatic inflammatory cell recruitment in acute liver injury

Kiminori Kimura,[*,†,1] *Satoshi Sekiguchi,*[†] *Seishu Hayashi,*[*] *Yukiko Hayashi,*[‡] *Tsunekazu Hishima,*[‡] *Masahito Nagaki,*[§] *and Michinori Kohara*[†]

Divisions of *Hepatology and ‡Pathology, Tokyo Metropolitan Cancer and Infectious Diseases Center, Komagome Hospital, Tokyo, Japan; †Department of Microbiology and Cell Biology, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan; and §Department of Gastroenterology, Gifu University Graduate School of Medicine, Gifu, Japan

RECEIVED JULY 20, 2010; REVISED OCTOBER 12, 2010; ACCEPTED NOVEMBER 9, 2010. DOI: 10.1189/jlb.0710412

ABSTRACT

Although the innate immune system has been demonstrated to play important roles as the first line of defense against various infections, little is known about the interactions between intrahepatic inflammatory cells and the cytokine network in the liver. Here, we examined the role of IL-18 in IHL recruitment in acute liver injury. C57BL/6 mice were injected with an αCD40 mAb, and their serum IL-18 levels were observed to increase, with subsequent recruitment of IHLs into the liver. NKT cells were involved in this liver injury, as the serum ALT levels were reduced in NKT KO mice through the suppression of macrophage and monocyte migration and cytokine production. In contrast, depletion of neutrophils exacerbated the liver injury associated with high levels of TNF-α and IL-18 and increased numbers of macrophages and monocytes. Treatment with a neutralizing antibody against IL-18 reduced the serum ALT levels and inflammatory cell accumulation in the liver. Finally, additional administration of rIL-18 with αCD40 injection caused severe liver injury with increased IFN-γ production by NK cells. In conclusion, these findings demonstrate that IL-18 modulates liver inflammation by the recruitment of inflammatory cells, including NKT cells, macrophages, monocytes, and neutrophils. *J. Leukoc. Biol.* 89: 433–442; 2011.

Introduction

Macrophages of the innate immune system are the first line of defense against many pathogens and play a crucial role in the elimination of bacterial infections [1]. The resident liver macrophages, Kupffer cells, are well known to be phagocytic macrophages and account for 80% of the total population of fixed tissue macrophages in the body [2]. These cells are derived from blood monocytes and found mainly in the hepatic sinusoid [3]. They are continuously exposed to various pathogenic components, such as the gram-negative bacteria cell wall constituent LPS, and have the ability to protect their host immediately from the associated bacteria. Activated macrophages can also secrete inflammatory cytokines, such as TNF-α, IL-12, IL-18 [4, 5], and chemokines [6], in response to certain stimuli. These mediators produced by macrophages and the capacity for phagocytosis are essential for protection against microorganisms [7].

In contrast, NKT cells express an invariant TCR chain (V14-J281 in mice) and recognize glycolipid antigens, such as α-galactosylceramide, in association with the MHC class I-like molecule CD1d [8]. APCs, including DCs and macrophages, present antigens to NKT cells, a process that is dependent on CD40 ligation and results in the rapid release of large amounts of Th1 and Th2 cytokines and chemokines. Activated NKT cells can also provide maturation signals for other inflammatory cells, especially DCs, NK cells, and macrophages, thereby involving innate and acquired immunity [9, 10].

IL-18 is a member of the IL-1 family that is produced as a biologically inactive precursor and secreted after activation by cleavage with caspase-1 or other caspases [11]. Originally, IL-18 was identified as an IFN-γ-inducing factor that can act on Th1 cells, nonpolarized T cells, NK cells, B cells, and DCs to produce IFN-γ in the presence of IL-12 [12]. Besides its potent induction of IFN-γ, IL-18 activates NK and T cells, which play central roles in viral clearance [13].

We have already demonstrated that αCD40 mAb injection induces biphasic liver injury by way of inflammatory cytokine and chemokine production [14]. Furthermore, this liver injury requires NK cells and macrophages in the early-phase events, and B cells also contribute to the late-phase liver inflammation [15]. During analyses of this liver injury model, we found that

Abbreviations: αCD40=anti-CD40, ALT=alanine aminotransferase, IHL=intrahepatic leukocyte, KO=knockout (deficient), NLR=NOD-like receptor

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

1. Correspondence: Division of Hepatology, Tokyo Metropolitan Cancer and Infectious Diseases Center, Komagome Hospital, 18-22-3 Honkomagome, Bunkyo-ku, Tokyo 113-8677, Japan. E-mail: kkimura@cick.jp