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Hepatitis C Virus NS3/4A Protease Inhibits Complement Activation by Cleaving Complement Component 4

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Abstract

Background: It has been hypothesized that persistent hepatitis C virus (HCV) infection is mediated in part by viral proteins that abrogate the host immune response, including the complement system, but the precise mechanisms are not well understood. We investigated whether HCV proteins are involved in the fragmentation of complement component 4 (C4), composed of subunits C4α, C4β, and C4γ, and the role of HCV proteins in complement activation.

Methods: Human C4 was incubated with HCV nonstructural (NS) 3/4A protease, core, or NS5. Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then subjected to peptide sequencing. The activity of the classical complement pathway was examined using an erythrocyte hemolysis assay. The cleavage pattern of C4 in NS3/4A-expressing and HCV-infected cells, respectively, was also examined.

Results: HCV NS3/4A protease cleaved C4γ in a concentration-dependent manner, but viral core and NS5 did not. A specific inhibitor of NS3/4A protease reduced C4γ cleavage. NS3/4A protease–mediated cleavage of C4 inhibited classical pathway activation, which was abrogated by a NS3/4A protease inhibitor. In addition, co-transfection of cells with C4 and wild-type NS3/4A, but not a catalytic-site mutant of NS3/4A, produced cleaved C4γ fragments. Such C4 processing, with a concomitant reduction in levels of full-length C4γ, was also observed in HCV-infected cells expressing C4.

Conclusions: C4 is a novel cellular substrate of the HCV NS3/4A protease. Understanding disturbances in the complement system mediated by NS3/4A protease may provide new insights into the mechanisms underlying persistent HCV infection.

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Introduction

Hepatitis C virus (HCV) is a single-stranded positive-strand RNA virus of the Flaviviridae family. The viral genome encodes four structural proteins and six non-structural (NS) proteins [1]. NS3/4A, a complex consisting of NS3 with serine protease activity and cofactor NS4A, plays an essential role in processing of HCV proteins. NS3/4A is a target of direct-acting antiviral agents (DAA) [2,3], and use of an NS3/4A protease inhibitor as a DAA markedly increases the therapeutic effect of other anti-HCV agents. Thus, NS3/4A protease may play an important role in interfering with the antiviral response.

HCV has been hypothesized to block the host immune response against persistent infection [4]. Furthermore, the time required for HCV-infected patients to develop hepatic cirrhosis varies among individuals; in particular, the progression of

hepatic fibrosis seems to be slower in HCV carriers with persistent normal alanine aminotransferase (ALT) levels than in chronic hepatitis patients with elevated ALT levels [5]. These differences in clinical features might be caused by variations in the host immune response, but the underling mechanism is unclear.

In the course of proteomic analyses aimed at identifying proteins potentially involved in the pathophysiology of hepatic diseases, we found that a specific peptide fragment of complement component 4 (C4) was significantly more abundant in HCV carriers with persistent normal ALT than in patients with chronic hepatitis [6], as well as more abundant in HCV carriers, regardless of ALT levels, compared to healthy controls. Assuming that C4 expression levels are similar among these groups, this C4 fragment may be generated by post-translational processing in HCV-infected individuals.

The complement system is part of the innate immune system, which can be activated through three pathways: the classical pathway, the mannose-binding lectin pathway, and the alternative pathway. C4, which is involved in the classical- and mannose-binding lectin pathways, can be cleaved by certain cellular protease(s), leading to a cascade of C4 activation [7]. In this study, we provide the first evidence that HCV NS3/4A cleaves C4, and that this cleavage attenuates activation of the classical pathway of complement system.

Materials and Methods

Materials

HCV NS3/4A protease (217 amino acid [aa] fusion protein with NS4A co-factor fused to the N-terminus of NS3 protease domain) with His-tag, HCV core (aa 1–102) with GST-tag, and HCV NS5 (aa 2061–2302) with GST-tag were purchased from AnaSpec (Fremont, CA) or ProSpec (Rehovot, Israel). Isolated human-derived complement components (C1, C2) were obtained from Hycult Biotech (Uden, Netherlands), and C4 and C4-deficient guinea pig serum (C4d-GPS) were purchased from Sigma-Aldrich (St. Louis, MO). VX950, a HCV NS3/4A serine protease inhibitor, was obtained from Selleck Chemicals (Houston, TX). Veronal buffer, sheep erythrocytes, and hemolysin were purchased from Wako (Osaka, Japan), Nippon Biotest Laboratories Inc. (Tokyo, Japan), and Denka Seiken Co. (Tokyo, Japan), respectively.

NS3/4A protease cleavage assay

HCV NS3/4A protease, core, or NS5 (3 µl) and 9 µl of Assay buffer (SensoLyte® 490 HCV Protease Assay Kit, AnaSpec) containing 30 mM dithiothreitol (DTT) were added to C4 (3 µl), and the mixture was incubated at 30°C for 30 min. The solution was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and resolved proteins were stained with Coomassie brilliant blue (CBB). In a separate experiment, VX950 was pre-incubated with NS3/4A protease at 30°C for 30 min, and then incubated with C4 at 30°C for 30 min. Proteins detected by CBB staining were subjected to N-terminal peptide sequence analysis at Nippi Inc. (Tokyo, Japan).

Hemolytic analysis

The method used for hemolytic analysis has been described previously [8,9]. Briefly, intermediates of complement components were sequentially added to sheep erythrocytes sensitized by hemolysin (Ab-sensitized sheep erythrocytes, EA). Dilute erythrocytes and complement components were prepared in Veronal buffer containing 2% gelatin (GVB). To prepare EA, hemolysin was added to 10 ml of erythrocytes (5×10^8 cells/ml) and incubated at 37°C for 30 min. C1 (10 µg) was added to 5 ml of EA, incubated at 30°C for 15 min, and washed twice with GVB (EAC1). NS3/4A protease was prepared in a solution containing 20 mM Tris-HCl (pH 8.0), 20% glycerol, 100 mM KCl, 1 mM DTT, and 0.2 mM EDTA, adjusted to pH 7.5. The reaction solution was adjusted to 2 mM DTT to ensure a uniform effect on C4 activity. C4 was incubated with the NS3/4A protease, and then mixed with 100 µl of EAC1 and incubated at 30°C for 15 min (EAC1-C4). After washing twice with GVB, 1 µl of C2 (0.1 mg/ml) was added and the mixture was incubated at room temperature for 4 min (EAC1-C4-C2). After washing twice again with GVB, 150 µl of 80-fold diluted C4d-GPS was added to 30 µl of EAC1-C4-C2, and the mixture was incubated at 37°C for 30 min. The optical absorbance of the centrifuged supernatant was determined at 415 nm, and the level of hemolysis was calculated using the following formula: Hemolysis (%) = (sample OD_{415} – no C4 OD_{415})/(total hemolysis in distilled water OD_{415} – no C4 OD_{415}) ×100. "No C4" refers to a control sample containing EAC1 not incubated with C4. In a separate experiment, VX950 was first pre-incubated with NS3/4A protease at 30°C for 30 min, and then incubated with C4 at 30°C for 30 min.

Cell culture and transfection

Human hepatoma-derived Huh7.5.1 cells (a kind gift from Dr. F. V. Chisari, The Scripps Research Institute, La Jolla, CA) and human embryonic kidney (HEK) 293T cells were cultured at 37°C under 5% CO_2 in DMEM containing 10% FBS, 100 units/ml penicillin, and 100 g/ml streptomycin. DNA transfections of Huh7.5.1 cells and 293T cells were performed using Lipofectamine LTX/PLUS Reagent (Invitrogen, Carlsbad, CA) and polyethylenimine (Alfa Aesar, Heysham, Lancashire, UK), respectively. The transfection complex was formed at a DNA:reagent ratio of 1:1 (w/w) in OptiMEM (Invitrogen) with incubation for 15 min at room temperature before it was added to the culture.

Preparation of virus stock

The pJ6/JFH1 plasmid was generated by replacing the structural region of the JFH-1 strain with that of the J6CF strain, as described [10]. Cell culture–derived infectious HCV particles (HCVcc) were produced by introducing *in vitro* transcribed RNA from pJ6/JFH1 into Huh-7.5.1 cells by electroporation. The culture supernatant was concentrated using a 100-kDa MWCO Amicon Ultra Centrifugal Filter (Millipore, Bedford, MA). Virus infectivity was measured by indirect immunofluorescence analysis. Virus stocks (1×10^7 focus-forming units/ml) were divided into small aliquots and stored at −80 °C until use.

Plasmids

The C4 expression plasmid pFN21-C4A was purchased from Kazusa DNA Research Institute (Kisarazu, Japan). To create pFN21-C4A delH-Tag, the N-terminal Halo-Tag of pFN21-C4A was removed by digestion with *HindIII* and *PvuI*, followed by blunt-ending with KOD FX neo (Toyobo, Osaka, Japan). pCAG-HA-NS3/4A, which expresses full-length NS3 and NS4 (derived from HCV genotype 1b, Con-1 strain) with an HA tag at the N-terminus of NS3 was generated as described [11]. Point mutation of serine to alanine at position 139 (S139A) in pCAG-HA-NS3/4A was achieved by site-directed mutagenesis using two primers: 5'-TAC TTG AAG GGC TCT GCG GGC GGT CCA CTG C-3' and 5'-GCA GTG GAC CGC CCG CAG AGC CCT TCA AGT A-3'. The point mutation was confirmed by DNA sequencing.

Immunoprecipitation and immunoblotting

Goat anti-human complement C4 antibody (MP Biomedicals, Santa Ana, CA) was bound to protein G–agarose beads (Thermo Scientific, Rockford, IL) in binding buffer (0.5% Nonidet P-40, 25 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA and protease inhibitor cocktail [Roche, Basel, Switzerland]) for 1 h at room temperature. Culture supernatants were incubated with the beads for 1 h at room temperature, and the immunoprecipitated proteins were eluted by heat treatment for 5 min at 100°C with 2× sample buffer. Culture supernatants were directly mixed with 3× sample buffer at a ratio of 1 volume supernatant to 2 volumes sample buffer (1:2 [v/v]). Cells were solubilized in lysis buffer (1% Triton X-100, 25 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA and protease inhibitor cocktail) on ice. Cell debris was removed by centrifugation, and the resultant supernatants were diluted 1:2 (v/v) with 3× sample buffer. Precipitated proteins, culture supernatants, and cell lysates were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore). After blocking in 4% BlockAce (DS Pharma Biomedical, Osaka, Japan), the blots were incubated with the indicated primary antibodies, followed by the secondary antibody in TBST (25 mM Tris [pH 7.5], 150 mM NaCl, and 0.1% Tween 20). The primary antibodies used were anti-C4γ (clone H-291, Santa Cruz Biotechnology, Dallas, TX), anti-human complement C4, anti-HA (Sigma, St. Louis, MO), anti-HCV core (clone 2H9) and anti-GAPDH (clone 6C5, Santa Cruz Biotechnology). Donkey polyclonal Secondary Antibody to Goat IgG-H&L (HRP) (Abcam, Cambridge, UK), HRP-linked anti-mouse IgG, and HRP-linked anti–rabbit IgG (Cell Signaling Technology, Danvers, MA) were used as secondary antibodies. Finally, proteins were visualized using an enhanced chemiluminescence (ECL) reagent (ECL Select Western Blotting Detection Reagent, GE Healthcare, Little Chalfont, UK).

Statistical analysis

The concentration of proteins detected by Western blots was determined by densitometric analysis using the ImageJ software [12]. Statistical analysis was performed with the SPSS software (SPSS Inc., Chicago, IL) using the Tukey test, with $P < 0.05$ considered to indicate a significant difference.

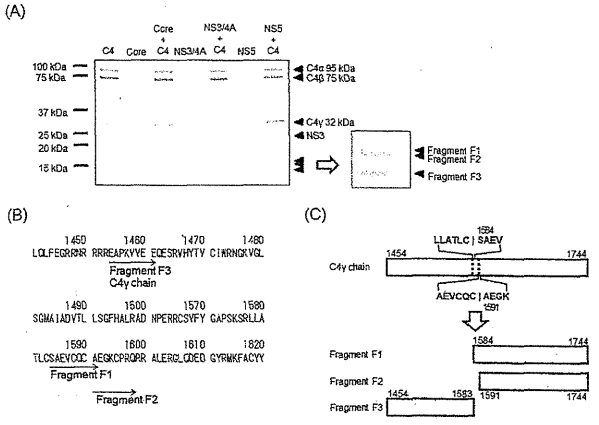

Figure 1. C4 is cleaved by HCV NS3/4A protease at Cys-1583/Ser-1584 or Cys-1590/Ala-1591. (A) HCV NS3/4A protease, core, or NS5 was added to C4, and the products were separated by SDS-PAGE and subjected to CBB staining. Two approximately 17-kDa proteins (Fragment F1 and F2) and a 15-kDa protein (Fragment F3) were detected after incubation of C4 with HCV NS3/4A protease, but not after incubation with core or NS5. (B) Amino acid sequence of aa 1451-1620 region of C4. Protein fragments were analyzed by N-terminal peptide sequencing. The sequences of the N-termini of the 17-kDa proteins (Fragment F1 and F2) were SAEVCQCA and AEGKCPRQ, which are located at aa 1584–1591 and 1591–1598 in C4, respectively. The sequence of the N-terminus of the 15-kDa protein (Fragment F3) was EAPKVVEE, which is located at aa 1454–1461 in C4. (C) Schematic representation of C4γ chain, and Fragment F1, F2 and F3.

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Results

HCV NS3/4A protease cleaves C4 in vitro

To test cleavage of C4 mediated by HCV proteins, C4 (containing subunits C4α, C4β, and C4γ) was mixed with NS3/4A protease, core, or NS5, followed by incubation at 30°C for 30 min. As shown in Figure 1A, doublet bands at 17 kDa (fragments F1 and F2 in the enlarged view) and one band at 15 kDa (fragment F3) were detected in the presence of NS3/4A protease and C4. These bands were not detected after incubation of C4 with core or NS5, or when either core or NS5 were incubated alone.

N-terminal sequence analyses revealed that the bands at approximately 100, 75, and 32 kDa (Figure 1A) represented C4α (N-terminus sequence identified: NVNFQKAI), C4β (KPRLLLFS), and C4γ (EAPKVVEE), respectively. As shown in Figure 1B, the N-terminal sequences of the doublet proteins at 17 kDa were identical to sequences found in C4γ: SAEVCQCA (aa 1584–1591 of C4) and AEGKCPRQ (aa 1591–1598). In addition, the N-terminal sequence of the 15-kDa fragment was EAPKVVEE (aa 1454–1461), indicating that the 15-kDa fragment is the N-terminal region of the C4γ. These results demonstrate that HCV NS3/4A protease cleaves C4 between

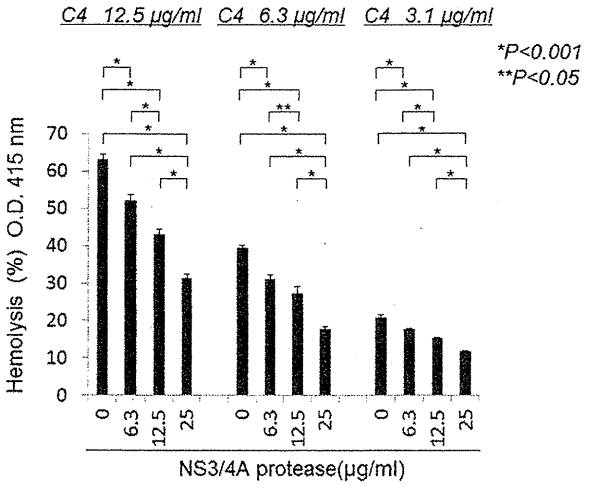

Figure 2. HCV NS3/4A protease inhibits the classical pathway, as assessed by hemolysis. C4 was incubated in the presence or absence of HCV NS3/4A protease, and then C1-sensitized EA (EAC1) was added (EAC1-C4). After washing, C2 was added to form EAC1-C4-C2, and the complex was resuspended in C4d-GPS. The absorbance of the centrifuged supernatant was determined at 415 nm. The grade of hemolysis decreased in the presence of NS3/4A protease in a dose-dependent manner. All measurements were performed in triplicate, and data are expressed as means ± SD.

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either Cys-1583 and Ser-1584 or Cys-1590 and Ala-1591, consistent with the consensus sequence of HCV NS3 proteinase cleavage sites [3,13]. Possible locations for the 15- and 17-kDa fragments of C4γ are shown in Figure 1B and 1C.

HCV NS3/4A protease decreases the activity of the classical pathway of the complement system in a concentration-dependent manner

To examine the functional significance of C4 cleavage by NS3/4A protease, complement components were serially added to EA to reproduce the classical pathway of the complement system. C4, untreated or treated with various concentrations of NS3/4A, was added at various concentrations to the EA-C1 mixture, followed by addition of C2 and C4d-GPS, which were used as sources of C3 and C5-C9. Erythrocyte hemolysis, which is caused by the complement-mediated fusion of erythrocytes, was quantified (Figure 2). NS3/4A treatment significantly decreased hemolysis levels in a concentration-dependent manner. This result, together with those in Figure 1, suggests that the C4 cleavage mediated by NS3/4A protease may contribute to inhibition of complement activation via the classical pathway.

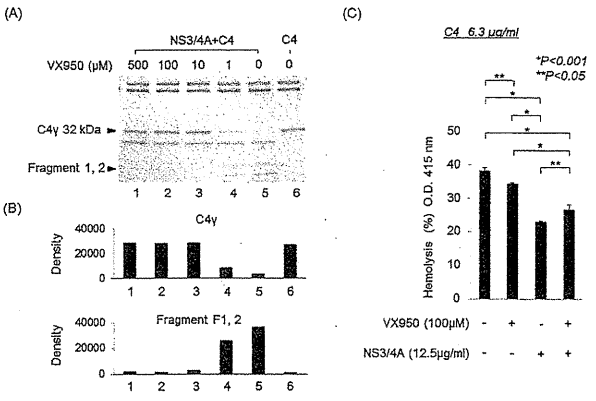

Figure 3. VX950, a HCV NS3/4A protease inhibitor, abrogates cleavage of C4 induced by HCV NS3/4A protease. (A) VX950 was added to HCV NS3/4A protease at the indicated concentrations, and then C4 was added. Proteins were separated by SDS-PAGE for CBB staining. The three C4-derived fragments of 17 kDa and 15 kDa produced by NS3/4A protease action could not be detected after pretreatment with VX950, and this change was accompanied by an increased concentration of the 32-kDa C4γ chain. (B) The C4γ, 17-kDa, and 15-kDa bands were quantified by densitometric analysis using the Image J software. (C) C4 was incubated in the presence or absence of HCV NS3/4A or VX950, and then C1-sensitized EA (EAC1) was added (EAC1–C4). C2 and C4d-GPS were then added, and the absorbance of the supernatant was determined at 415 nm. Hemolysis was inhibited by NS3/4A protease and this inhibition was blocked by VX950. All measurements were made in triplicate, and data are expressed as means ± SD.

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HCV protease inhibitor reduces inactivation of complement by blocking C4 cleavage by NS3/4A protease

We tested the effect of VX950, a specific inhibitor of NS3/4A protease, on C4 cleavage by NS3/4A protease and inhibition of complement activation. As shown in Figure 3A and 3B, under a condition in which more than 80% of 32-kDa C4γ was processed into 17- and 15-kDa fragments in the presence of NS3/4A protease (lanes 5), pretreatment of the protease with 1 μM VX950 moderately inhibited the cleavage of C4γ (lanes 4). The NS3/4A-mediated processing of C4γ into 17- and 15-kDa fragments was almost completely blocked by VX950 at ≥10 μM (lanes 1–3). In the erythrocyte hemolysis assay, the reduction in hemolysis level mediated by NS3/4A significantly recovered in the presence of VX950 (Figure 3C). These results confirmed cleavage of C4γ by NS3/4A and the involvement of the protease in the classical complement pathway.

Cleavage of C4γ in NS3/4A-expressing cells and HCV-infected cells

To determine whether HCV NS3/4A protease cleaves C4 in cells, we analyzed 32-kDa C4γ and its processed fragments in

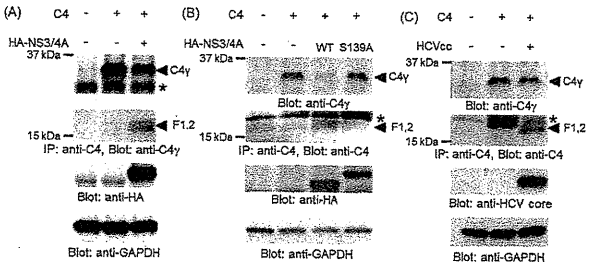

Figure 4. C4 is cleaved by HCV NS3/4 protease in cell cultures. (A) 293T cells were transfected with the indicated plasmids. Anti-C4 immunoprecipitates (IP) of supernatants were separated by SDS-PAGE and analyzed by immunoblotting with anti-C4γ antibody. Detergent-soluble cell lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-HA and anti-GAPDH antibodies. (B) 293T cells were transfected with the indicated plasmids. Culture supernatants were analyzed by immunoblotting with anti-C4γ antibody. Anti-C4 immunoprecipitates (IP) of supernatants were analyzed by immunoblotting with anti-C4 antibody. Detergent-soluble cell lysates were analyzed by immunoblotting with anti-HA and anti-GAPDH antibodies. (C) Huh7.5.1 cells were mock-infected or infected with HCVcc at a multiplicity of infection of 2 for 6 h, followed by mock-transfection or transfection with C4 expression plasmid. Culture supernatants and cell lysates were analyzed as described in (A) and (B). The anti-C4γ antibody was not appropriate for immunoblotting of IP samples derived from Huh7.5.1 cultures because of unavoidable nonspecific cross-reaction. * indicates non-specific reactions in (A) – (C).

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culture medium from 293T cells cotransfected with expression plasmids encoding C4 (pFN21-C4A delH-Tag) and NS3/4A protease (pCAG-HA-NS3/4A). Co-expression of C4 and NS3/4A derived from HCV genotype 1b led to production of the 17-kDa C4γ fragment and reduction in the level of 32-kDa C4γ (Figure 4A). In contrast, the 17-kDa fragment was not detected, and the 32-kDa C4γ level was not changed, when a mutant NS3 with an amino-acid substitution at the catalytic-site (S139A)/4A was co-expressed with C4 (Figure 4B). Next, we investigated C4 cleavage in HCV-infected cultures. In the culture medium of Huh7.5.1 cells infected with HCVcc of strain J6/JFH-1 (genotype 2a) expressing of C4 from pFN21-C4A delH-Tag, the 17-kDa fragment was produced, and the level of 32-kDa C4γ was reduced accordingly (Figure 4C). These data demonstrate that C4γ can be cleaved by HCV NS3/4A, either expressed from a plasmid or in HCV-infected cells, and that proteases of both genotypes 1b and 2a are functional in this cleavage.

Discussion

The results of this study show that C4γ is cleaved by HCV NS3/4A protease *in vitro* and in cell culture. Cleavage of C4 by HCV NS3/4A protease leads to inhibition of activity of the classical complement pathway. C4 cleavage and abrogation of complement activation are blocked by an inhibitor of NS3/4A protease.

HCV NS3/4A protease plays an important role in the replication of non-structural regions [2,3], and might also directly act on the IFN signaling system to inhibit the host immune response and prevent viral clearance, thereby contributing to persistent HCV infection. However, a direct relationship between HCV infection and complement components has not been previously established. Levels of functional C3 or C4 assessed by hemolysis assay are reduced after infection by flaviviruses such as Dengue virus and West Nile virus (WNV) [9,14]. In mice infected with γ-herpesvirus or WNV, genetic deletion of complement C3 or C4 not only enhances mortality but also increases persistent replication of γ-herpesvirus or WNW RNA levels [14,15]. Furthermore, Moulton et al. reported that mousepox virus dissemination was more severe, and viral loads in tissues were higher, in C3-deficient mice; leading to higher mortality than in wild-type mice; those authors concluded that the complement system is critical for slowing viral spread and decreasing tissue titer and damage [16]. Thus, it is likely that the complement system is widely associated with development of viral infection. Further investigation of the role of complement activation mediated by HCV proteins such as HCV NS3/4A protease may provide new insights into development of persistent HCV infection.

Our results indicated that the C4 cleavage site of HCV NS3/4A protease is between either Cys-1583 and Ser-1584 or Cys-1590 and Ala-1591 of C4, both of which are located in the C4γ chain (Figure 1). HCV NS3/4A protease has previously been suggested to cleave at Cys/Thr and Ala/Ser sites [3,13], which is broadly consistent with our results. C4 was also cleaved by HCV NS3/4A protease in HCV-infected cells (Figure 4C), in which unprocessed 32-kDa C4γ and cleaved 17-kDa fragment in the culture medium were observed. In cultures of human hepatoma HepG2 cells, the major fraction of C4α, C4β, and C4γ were present in the culture medium rather than in cells [17,18]. In good agreement with that finding, we detected little C4 in Huh-7–derived cells (data not shown). We speculate that immediately after synthesis, at least a fraction of C4γ can be quickly cleaved by NS3/4A in virally replicating cells, followed by secretion into the culture medium. However, we cannot rule out the possibility that HCV NS3/4A protease is present extracellularly and is functional under some particular conditions, because addition of recombinant antigens derived from the NS3 region to NS4 improves the sensitivity of the anti-HCV test in serum and shortens the window period for seroconversion in patients infected with HCV [19].

Complement components are involved in innate immunity and are responsible for one of the major immunological mechanisms mediated by antibodies [7]. In viral and bacterial infection, these components cause lysis of the outer membrane of virus particles [20] and infected cells [21] by the membrane attack complex C5–C9, ultimately resulting in elimination of the pathogen. Some viruses, such as cytomegalovirus, induce expression of cellular complement inhibitors, for example, decay-accelerating factor and monocyte chemoattractant protein, leading to increased levels of these proteins on the

surfaces of infected cells. Human immunodeficiency virus may incorporate the complement inhibitors into the viral envelope [22,23]. NS1 protein secreted from flaviviruses, such as dengue virus, West Nile virus, and yellow fever virus, not only attenuates activation of the classical and lectin pathways by directly interacting with C4, but also inactivates C4b by interacting with C4-binding protein [9,24]. Thus, NS1 of flaviviruses is considered to play a role in protecting the virus from complement-dependent neutralization. To our knowledge, however, our study provides the first evidence that the viral protease plays a role in protecting the virus from the complement defense system via proteolytic processing of the complement component.

In particular, C4 is involved in the classical and mannose-binding lectin pathways of the complement system, and it is responsible for the major activity of complement components. Upon antibody binding to an antigen, C4 is cleaved into C4a and C4b by the C1q-C1r-C1s complex, and C4b then binds to C2a (C4b2a) on the cell membrane to cleave C3 into C3a and C3b. Subsequently, C3b binds to C4b2a to cleave C5, and finally C5b and C6-C9 form the membrane attack complex to cause lysis of the cell membrane [7]. The erythrocyte hemolysis assay used in this study reproduces this cascade and revealed that HCV NS3/4A protease cleaves C4 and decreases the activity of the classical pathway. The specific assay was constructed to evaluate the function of C4 in the classical pathway by allowing HCV NS3/4A protease to act on C4 alone, without influence from other complement components. Therefore, further work is needed to determine whether HCV NS3/4A protease affects other components.

Several studies have demonstrated that HCV proteins influence complement systems and may be involved in evading antiviral immune responses of the host, as follows. Amet et al. reported that CD59, which may inhibit formation of the membrane attack complex, is incorporated into cultured cells and plasma primary HCV virions and inhibited activation of complement components, whereas administration of a CD59 inhibitor increases the sensitivity of component activation against endogenous HCV viral particles [25]. Banerjee et al. found that the HCV core protein reduces the expression of upstream stimulatory factor (USF)-1, a transcription factor important for basal C4 expression, and that expression of interferon regulatory factor (IRF)-1, which is important for IFN-γ–induced C4 expression, is inhibited by hepatocytes expressing HCV NS5A [26]. Mazumdar et al. showed that NS5A strongly downregulates C3 promoter activity in the presence of IL-1β, acting as an inducer [27]. HCV core inhibits T-cell proliferative responses *in vitro*, and this effect can be reversed by addition of anti–C1q receptor antibody to a T-cell proliferation assay [28]. Here, we identified C4γ as a novel cellular substrate of the HCV NS3/4A protease.

The results of this study suggest that C4γ cleavage by NS3/4A decreased the activity of the classical complement pathway, and might thereby attenuate activation of the complement system. An understanding of the viral protease–mediated inhibition of the complement system should provide new insights into the roles played by immune evasion in persistent HCV infection.

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Author Contributions

Conceived and designed the experiments: SM HU YS MS HT. Performed the experiments: SM KN TS SK KK KO KT TT AM MO. Analyzed the data: SM HU AI KN TS YS. Contributed reagents/materials/analysis tools: HU IS HT. Wrote the manuscript: SM HU TS HT.

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Original article

Retinoids and rexinoids inhibit hepatitis C virus independently of retinoid receptor signaling

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Abstract

Using a high-throughput screening system involving HCV JFH-1-Huh 7.5.1 cells, we determined that the ligands of class II nuclear receptors, retinoids and rexinoids inhibit HCV infection. Retinoids, ligands of retinoic acid receptor (RAR), and rexinoids, ligands of retinoid X receptor (RXR), reduced extracellular HCV RNA of HCV infected cells in a dose-dependent manner. The 50% effective concentrations were below 10 nM, and the 50% cytotoxic concentrations were over 10 μM. Both agonists and antagonists demonstrated inhibition, which indicates that the effect is not dependent on retinoic acid signaling. These chemicals reduced HCV RNA and NS5A protein levels in cells harboring the sub-genomic HCV replicon RNA, which suggests that the chemicals affect HCV RNA replication. These compounds were also effective against persistently infected cells, although the reduction in the intracellular HCV RNA was smaller than that of the extracellular HCV RNA, suggesting that viral post-replication step is also inhibited. In combination with interferon (IFN), retinoid exhibited a synergistic effect. Retinoids did not enhance expression of the IFN effector molecule PKR. These series of compounds warrant further investigation as new class of HCV drugs, for the clinical translation of our observation may lead to increased anti-HCV efficacy.
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Keywords: Hepatitis C virus; HCV; Retinoid; Retinoic acid; Rexinoid

1. Introduction

Hepatitis C virus (HCV) is a leading cause of chronic liver diseases such as hepatitis, cirrhosis, and hepatocellular carcinoma (HCC). HCC usually occurs after establishment of liver cirrhosis in HCV-infected individuals. Although the prevalence of HCV infection in HCC differs noticeably with geographical regions, two-thirds of hepatocellular carcinoma patients are chronically infected with HCV in Japan [1]. Because of limited efficacy and high cost of preexisting drugs, HCV infection has not yet been eradicated from the world especially Asia. The current standard therapy, a combination of interferon (IFN) and ribavirin, is not effective for all the patients, in addition to having serious side effects. Because of the urgent need for novel HCV therapies, many studies on HCV drugs have been conducted. In addition to *in vitro* screenings targeting specific HCV viral enzymes and screenings using HCV genome-harboring replicon cells, the HCV JFH-1-Huh 7.5 cell infection system has been recently developed [2] and is now used for screening. This system is applicable to easy screening assays [3,4] and is capable of identifying and analyzing inhibitors that have effects on any stages of HCV life cycle: viral attachment, entry, replication, and post-replication. This system targets not only viral components but also the host components involved in HCV infection.

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We screened chemical libraries using an easy high-throughput screening with the JFH-1-Huh 7.5 cell system [4], and discovered that several ligands of class II nuclear receptors inhibited HCV infection. These ligands had a notable effect on HCV infection; the 50% effective concentrations (EC_{50}) were below 10 nM. The nuclear receptors are classified into two classes: receptors for steroid hormones (class I) and receptors for non-steroid ligands (class II). The class II nuclear receptors include retinoic acid receptor (RAR), retinoid X receptor (RXR), peroxisome proliferator-activated receptors (PPARs), vitamin D receptor (VDR), thyroid hormone receptor (TR), and liver X receptor (LXR). The common biological characteristic of the class II receptors is that they work as a dimer with the RXR [5–7]. The RAR consists of three subtypes, α, β and γ, encoded by separate genes. All-*trans*-retinoic acid (ATRA) is a retinol (vitamin A) metabolite and considered as a natural ligand of RARs. ATRA and some synthetic analogs bind RARs and are referred to as retinoids [5,6]. ATRA is used as an effective anticancer drug for the treatment of acute promyelocytic leukemia (APL). Am80 is a synthetic retinoid with specific activation of RARα, which is also clinically used for APL as tamibarotene. RXR also consists of three subtypes, α, β, and γ. In addition, 9-*cis*-retinoic acid (9CRA) and some synthetic ligands of RXRs are called rexinoids [7]. RXR is unique in that it forms a homodimer, whereas all the other class II receptors exclusively form heterodimers with the RXR. 9CRA is also a metabolite of retinol and is believed to be a natural ligand of the RXR, but is capable of binding the RAR. Bexarotene is a synthetic selective agonist of RXR prescribed as Targretin and used for cutaneous T cell lymphomas in USA and some other countries.

There are some reports regarding the *in vitro* inhibitory effect on HCV of ATRA and other retinoids [8,9]. Furthermore, Böcher et al. reported a preclinical use of ATRA for the treatment of hepatitis C patients and demonstrated its therapeutic potential [10]. Nevertheless, the mechanism of the retinoid inhibitory effect on HCV has not been examined and remains unclear. Retinoids have been already approved for treatments of other diseases. That is, their pharmacological properties had been already investigated and safety is verified, suggesting benefit for HCV treatment. Therefore, in this study, we attempted to elucidate how retinoids and related chemicals inhibit HCV infection and obtain some clues allowing the understanding of the mechanism of action.

2. Materials and methods

2.1. Cells and virus

Huh 7.5.1 cells and 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma–Aldrich Co. St. Louis, MO, USA) with 10% fetal bovine serum (FBS). HCV JFH-1 (genotype 2a) (HCVcc) was generated and stocked as described previously [3]. Subgenomic replicon cells, clone #4-1 and clone #5-15, which are derived from Huh 7 cells, harbor the JFH-1 genome (genotype 2a) [11] and the genotype 1b HCV genome [12], respectively. A persistently infected cell line was prepared as described below. Huh 7 cells were inoculated with HCVcc at a multiplicity of infection (MOI) of 1. The cells were passaged every 3–5 days with a cell density at more than 5×10^5 cells/10 cm-dish. HCVcc inoculated cells were monitored via intracellular and extracellular HCV core protein levels as determined by ELISA (HCV ELISA Test System, Ortho-Clinical Diagnostic K. K., Tokyo, Japan) and immunostaining and immunoblot using a specific antibody (anti-HCV core protein antibody, #40015B, Anogen, Mississauga, Canada). After HCVcc inoculation of Huh 7 cells, the intracellular HCV RNA and extracellular infectivity were detected for over ten passages. These cells were also cultured in DMEM with FBS.

2.2. Chemicals

All-*trans*-retinoic acid (ATRA) was purchased from Calbiochem-Merck KGaA (Darmstadt, Germany) and 9-*cis*-retinoic acid (9CRA) was from LKT (St. Paul, MN, USA). Am80, adapalene, TTNTB, and GW3965 were purchased from Tocris Bioscience (Bristol, UK). LE135, bexarotene, CD3254, UVI3003, SR11237, and TO901317 were purchased from Sigma–Aldrich Co. Ro41-5253, GW1929, and vitamin D3 were purchased from Enzo Life Science Inc. (Farmingdale, NY, USA). Clofibrate was purchased from Cayman Chemicals (Ann Arbor, MI, USA), and thyroxin was from Santa Cruz Biotech Inc. (Santa Cruz, CA, USA). Human interferon (IFN) α was purchased from PeproTech Inc. (Princeton, NJ, USA).

2.3. Quantification of viral titer in the supernatant and cells

To detect the reduction of HCV RNA in the supernatant, we used an easy quantitative real-time RT-PCR, the tube-capture-RT-PCR described before [3]. Briefly, the test compounds were added to Huh 7.5.1 cells seeded in 96-well plates as was HCVcc (MOI = 0.01), and after 5 days, the culture supernatant was taken and subjected to quantitative real-time RT-PCR using the SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit™ (Invitrogen Co., Carlsbad, CA, USA) by the Agilent Technologies Mx3000P (Santa Clara, CA, USA) [3]. For measuring the copy number of HCV RNA, 5×10^4 cells were seeded in 24-well plate per well, infected, and cultured for the indicated time. RNA was extracted with the QIAamp viral RNA Mini Kit™ (Qiagen GmbH, Hilden, Germany) from the supernatant or with the RNeasy Mini Kit ™ (Qiagen) from cells for quantitative real-time RT-PCR. The primers for HCV RNA were 5′-GAGT GTCGTACAGCCTCCAG-3′ (nucleotides 97–116) and 5′-AGGCCTTTCGCAACCCA-3′ (nucleotides 280–264). The standard JFH-1 RNA protocol for measurement of copy number has been previously described [3]. As an internal control, glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) mRNA was measured with the primers 5′-CCACCCATGGCAAATTCC-3′ and 5′-TGGGATTTCCATTGAT-3′. To evaluate the expression of protein kinase R (PKR) and 2′, 5′-oligoadenylate synthetase 1 (OAS1), we used 5′-TGGCCGCTAAACTTGCATATC-3′ and 5′-GCGAGTGTGCTGGTCACTAAAG-3′ as primers for PKR

and 5′-TGACGCTGACCTGGTTGTCTT-3′ and 5′-GAAC TCTCCCCGGCGATT-3′ as primers for OAS1.

Cell growth was monitored using an MTT assay, as described previously [13].

2.4. Western blotting

Western blotting was performed as previously described [3]. Briefly, cell lysates containing equal amounts of protein separated by SDS-PAGE were transferred onto PVDF membranes, and probed with antibodies against core (2H9), NS5 (Austral Biologicals, San Ramon, CA, USA), and GAPDH (Santa Cruz Biotech. Inc.).

2.5. Production and infection of pseudo-particles

The methods of production and infection of pseudo-particles have been previously described [14]. Briefly, HCV pseudo-particles (HCVpp) were generated with 3 plasmids: a Gag-Pol packaging construct, a transfer vector construct, and a HCV envelope glycoprotein-expressing construct (E1E2 of JFH-1), according to the method of Bartosch et al. [15] To produce pseudo-particles of vesicular stomatitis virus (VSV) (VSVpp), a plasmid coding VSV envelope, pCAG-VSV (kindly given by Dr. Y. Matsuura, Osaka University, Japan) was used instead of the plasmid expressing HCV envelope protein. The 3 plasmids were simultaneously transfected into 293T cells, and the supernatant was used as the pseudo-particle solution. For infection assay, the pseudo-particle solution with or without drug was inoculated onto Huh 7.5.1 cells. After 2-h incubation, the supernatants were changed to new complete medium, followed by 3-day incubation. Luciferase activities were assayed with the One-Glo™ luciferase assay system (Promega Co. Madison, WI, USA).

3. Results

3.1. Ligands of RAR and RXR inhibited HCV infection at nanomolar concentrations

Using an easy high-throughput screening for HCV inhibitors of the HCV JFH-1-Huh 7.5.1 cell infection system [4], we determined that some class II receptor agonists inhibit HCV infection. Therefore, we initiated further intensive study of the effect of various RAR and RXR agonists and antagonists. Huh 7.5.1 cells were infected with HCVcc at a MOI of 0.01 in the presence or absence of the chemicals. Five days after infection, the HCV RNA levels in the supernatant were measured by the tube-capture-RT-PCR. As shown in Fig. 1a, ATRA and all the synthetic retinoids we tested produced a marked reduction of HCV RNA in a dose-dependent manner, whereas the dose–response curves gradually lowered. The 50% effective concentrations (EC_{50}) were below 10 nM (Table 1). Notably, an antagonist of RARα, Ro41-5253 and an antagonist of RARβ, LE135 also reduced HCV RNA in a similar manner (Fig. 1a). We also examined the effects of various RXR ligands such as 9CRA, which is believed to be a natural agonist of the RXR and also an agonist of the RAR, synthetic RXR agonists (bexarotene, SR11237, and CD3254), and an RXR antagonist, UVI3003. All the RXR ligands we examined inhibited HCV RNA with a similar dose-dependent curve and the EC_{50} values were also below 10 nM (Fig. 1b and Table 1). The results demonstrated that although RXR ligands appeared to have a higher EC_{50}, both the agonists and antagonists of these receptors exhibited inhibitory activity on the production of extracellular HCV RNA. Given that the 50% cytotoxic concentrations (TC_{50}) of these compounds were over 10 μM, the specific indexes were over 1000 (Fig. 1a and b, and Table 1).

As the ligands of the two members of the class II nuclear receptors presented inhibitory effects, we attempted to examine some ligands of other class II receptors, such as PPAR, LXR, VR, and TR. These receptors form a heterodimer with RXR but do not form the respective homodimer. A PPARα agonist clofibrate did not exhibit any inhibition, and although a PPARγ agonist GW1929 showed some inhibition, there was no concentration dependence (Fig. 1c). LXR agonists GW3965 and TO901317 displayed inhibition in a dose-dependent manner, although the EC_{50} of these drugs were higher, in that they were between 10 nM and 1 μM (Fig. 1c). Thyroxin and vitamin D3 did not inhibit HCV infection at these concentrations (Fig. 1c). These results demonstrated that not all the class II receptor ligands have an inhibitory effect on HCV infection. The RAR and RXR ligands exhibited a robust inhibitory effect, whereas LXR agonists displayed a weak dose-dependent inhibition.

Next, we examined the intracellular and extracellular HCV RNA amount in the presence of Am80 for 5 days. The Huh 7.5.1 cells were infected with HCVcc at a MOI of 0.01 in the presence of 10 μM Am80 and incubated, and the RNA was extracted from the supernatant and the cells every day for 5 days. The viral RNA copy number was increased, but inhibition was observed in the presence of 10 μM Am80 (Fig. 1d). Am80 gradually attenuated the increase of HCV RNA both in the supernatant and in the cells with the passage of days. On day 2 post-infection, the relative amount of extracellular HCV RNA to the control was 54% (Fig. 1e). However, the relative amount of extracellular HCV RNA fell to 7% of the control level on day 5 post-infection, although the intracellular HCV RNA level decreased to 23% of the control level (Fig. 1e). It took 5 days until full repression of HCV infection was observed, and the viral release from the cells appeared to be inhibited during this period.

We also examined the production of HCV core protein in cells using western blotting. As expected, the tested chemicals inhibited the core protein expression in the cells (Fig. 1f).

3.2. Retinoids and rexinoids inhibited HCV RNA replication but not entry

To know the mechanism of inhibitory action we first examined the effect on viral entry of these compounds using pseudo-particles. HCVpp or VSVpp solution was mixed with the drug and inoculated into Huh 7.5.1 cells for 2 h. Then the cells were washed and supplied fresh medium without drug, and we performed a luciferase assay after a 3-day culture. The

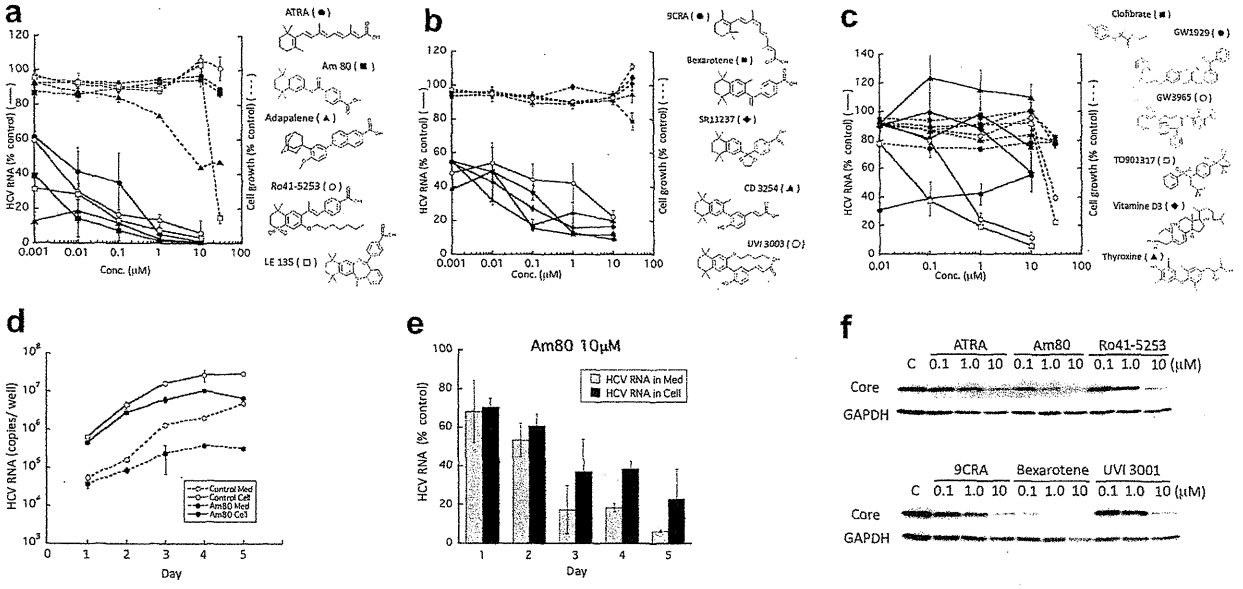

Fig. 1. Effects of agonists and antagonists of RAR and RXR on JFH-1 HCV infection. Huh 7.5.1 cells infected with HCVcc (MOI = 0.01) in the presence of drugs were incubated for 5 days. HCV RNA in the supernatant was measured by tube-capture RT-PCR previously described [3]. Parallel cultures of cells without virus were analyzed using an MTT assay to detect the inhibition of cell growth due to drug exposure. The percentages to control HCV RNA and control cell growth are indicated by solid lines and dotted lines, respectively. (a) Effects of RAR agonists and antagonists. ATRA (all-*trans*-retinoic acid, closed circles), Am80 (closed rectangles) and adapalene (closed triangles) are agonists of RAR. ATRA has no subtype specificity. Am80 and adapalene binds specific to RARα and RARβ, respectively. Ro41-5253 (open circles) and LE135 (open rectangles) are antagonists of RARβ and RARα, respectively. (b) Effects of RXR agonists and antagonists. 9CRA (9-*cis*-retinoic acid, closed circles) is an agonist of both RAR and RXR. Bexarotene (closed rectangles), SR11237 (closed rhombus), and CD3254 (closed triangles) are RXR agonists, whereas UVI3003 (open circles) is an RXR antagonist. (c) Effects of other class II nuclear receptor ligands. Clofibrate (closed rectangles) and GW1929 (closed circles) are PPARα and PPARγ agonists, respectively. GW3965 (open circles) and TO901317 (open rectangles) are LXR agonists. Vitamin D3 and thyroxin are indicated by closed rhombus and closed triangles, respectively. The values are the averages of triplicates, and the error bars represent the standard deviation. (d) Time-dependent expression profile of the HCV RNA in the supernatant and in the cells. Huh 7.5.1 cells were infected with HCVcc (MOI = 0.01) in the presence or absence of 10 μM of Am80 and incubated for 5 days. HCV RNA was extracted from the supernatant and from cells every day and subjected to quantitative real-time RT-PCR. The values indicate the copy numbers/well of 24-well plate. (e) Time-dependent expression profile represented by percentages of those in untreated cells based on (d). The results are represented by percentages of the levels of untreated cells. The values are averages of triplicates and the error bars represent the standard deviation. (f) Huh 7.5.1 cells were infected (MOI = 0.01) in the presence of retinoids and incubated for 5 days. The cell lysates were blotted with anti-core antibody and anti-GAPDH antibody.

luciferase activity of the cells infected with HCVpp was elevated and was not inhibited by retinoids or rexinoids (Fig. 2a). The luciferase activity of VSVpp was also not reduced at these concentrations (Fig. 2a). These results indicate that these drugs did not affect the viral entry.

Next, to examine whether these drugs inhibit the viral replication step, we conducted experiments using subgenomic replicon cells. The subgenomic replicon is a special cell system in which HCV RNA replicates autonomously and expresses viral proteins without viral entry or release. We treated subgenomic replicon cells derived from Huh 7 cells that is harboring the JFH-1 genome (#4-1, genotype 2a) with retinoids and rexinoids for 6 days at the indicated concentrations and measured the amount of cellular replicon RNA. Although the effective concentration was considerably high (EC_{50} of Am80 was 6 μM), the HCV RNA was reduced by the treatment with the compounds in a dose-dependent manner compared with GAPDH mRNA expression (Fig. 2b). Furthermore, we examined the effects of the drugs on HCV NS5A protein expression in the replicon cells by western blotting. These drugs, except ATRA, reduced the NS5A expression of the cells at a concentration of 30 μM but not the GAPDH protein (Fig. 2c, upper figure). We also examined the expression of NS5A protein in another subgenomic replicon cell line (#5-15) that harbors a genotype 1b HCV replicon. Treatment for 5 days with 30 μM of the retinoid and rexinoid compounds, except for Am80, resulted in a decrease in NS5A protein expression without affecting the amount of cellular GAPDH. However, in addition to Am80, 10 μM ATRA and bexarotene appeared to increase NS5A expression (Fig. 2c, lower figure). Although the protein level is influenced by metabolism or subcellular modification,

Table 1
EC_{50} and TC_{50} of retinoids and rexinoids.

	EC_{50} (nM)[a]	TC_{50} (μM)	TC_{50}/EC_{50}
ATRA	4.45	30<	6000<
Am80	0.00647	30<	4,000,000<
Adapalene	0.001>	20	20,000,000<
Ro41-5253	0.943	30<	30,000<
LE135	0.00477	20	4,260,000
9CRA	4.24	30<	7000<
Bexarotene	0.190	30<	100,000<
SR11237	1.45	30<	20,000<
CD3254	0.260	30<	100,000<
UVI3003	5.68	30<	5000<

[a] EC_{50} were calculated by equations of logarithmic fitting curves based on the data in Fig. 1a and b.

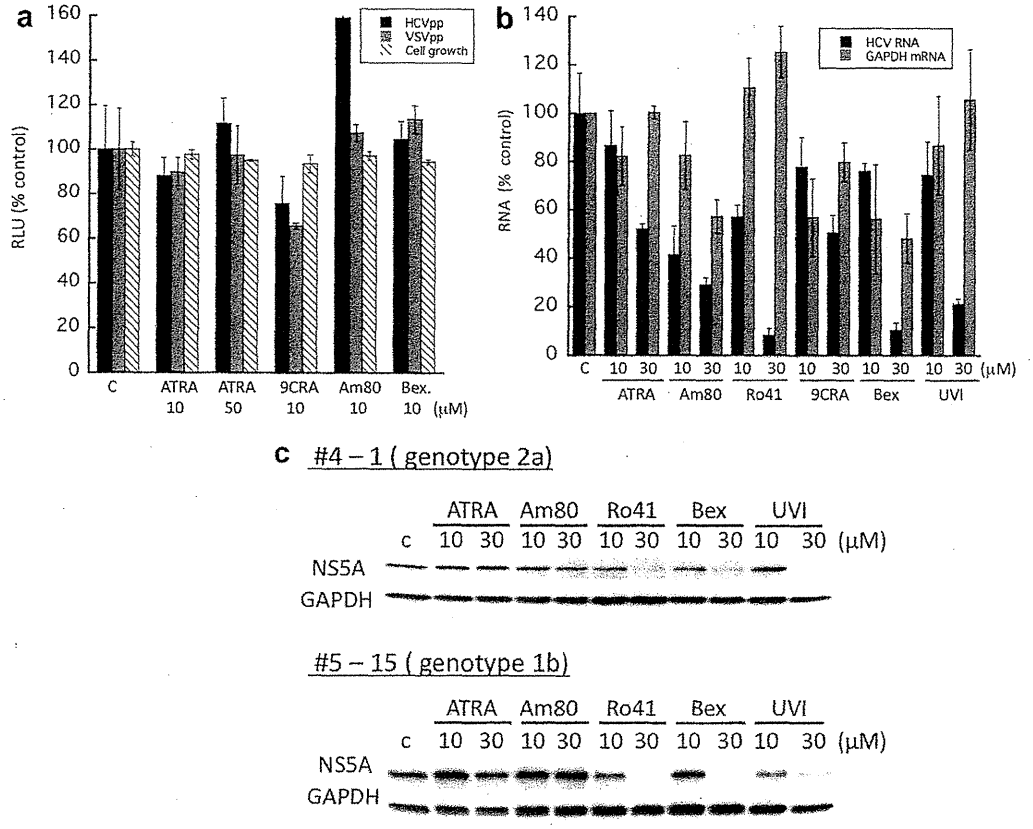

Fig. 2. Effects of retinoids and rexinoids on infection of HCV pseudo-particles and subgenomic replicon cells. (a) Huh 7.5.1 cells received HCVpp mixed with drug, and the medium was replaced after 2 h, followed by culturing for 3 days. Parallel infection of VSVpp with drugs was performed to compare the effects. Another parallel culture without pseudo-particles was evaluated by MTT assay for detecting effects on cell growth. The values are the averages of triplicates, and the error bars represent the standard deviation. (b) Subgenomic replicon cell harboring genotype 2a (JFH-1), #4-1 was treated with retinoids for 6 days. Total RNA was extracted from the cells and applied to quantitative real-time RT-PCR using primers for HCV or GAPGH. (c) Subgenomic replicon cell #4-1 (JFH-1, genotype 2a) or #5-15 (genotype 1b) were treated with the drugs for 7 days or 5 days, respectively. The cell lysates were applied to western blotting with anti-NS5A antibody or anti-GAPDH antibody. Ro41, Ro41-5253; Bex, bexarotene; UVI, UVI3003.

NS5A protein expression depends on the viral replication in the replicon cells. Therefore, the reduction in NS5A protein suggested that these compounds inhibited molecule(s) involved in HCV replication. The cell growth of the replicon cells was not suppressed at 30 μM or less concentrations of all the compounds (data not shown), indicating that the reduction in the viral protein level does not result from cell toxicity. In conclusion, although the effective concentrations were markedly higher compared with those in the JFH-1-Huh 7.5.1 cell system, the tested compounds inhibited HCV RNA replication. In addition, the compounds were effective against not only HCV genotype 2a but also genotype 1b.

3.3. Effect of retinoids on persistently infected cells

To investigate the possibility that retinoids are effective on chronic hepatitis C, we used HCV-persistently infected cells. We had established the cell line as described in the 'Materials and methods'. The cell is derived from Huh 7 cells, and constitutively infected with HCV JFH-1, and the control intracellular HCV RNA and extracellular HCV RNA were 4.4×10^6 copies/well and 3.5×10^5 copies/well of 24-well plate, respectively, in the present experiment. Although the effective concentrations were higher (3–30 μM) than those in the JFH-1-Huh 7.5.1 infection experiment, the amount of HCV RNA was reduced in both the supernatant and the cell by retinoid treatment (Fig. 3). These results suggest that retinoids may be useful in the treatment of chronically infected hepatitis C. Smaller reduction in the intracellular HCV RNA compared with extracellular HCV RNA, which corresponds to the results of the JFH-1-Huh 7.5.1 cell system in Fig. 1e, suggests that retinoids also inhibited the viral post-replication step. In contrast, IFNα (10 unit/ml), which is believed to inhibit the viral replication, exhibited reduction in extracellular HCV RNA equivalent to those in intracellular HCV RNA (Fig. 3).

3.4. Effect of drug combinations on HCV infection

When considering the application to clinical use of retinoids and rexinoids, we attempted to access the effect of combination treatment on the JFH-1-Huh 7.5.1 cell system. First, we treated Huh 7.5.1 cells with the indicated concentrations of Am80 and bexarotene together, followed by viral inoculation (MOI = 0.01) and incubation for 5 days. Then, we

lysed the cells to evaluate the intracellular core protein expression using western blotting. As shown in Fig. 4a, Am80 and bexarotene singly inhibited the expression of HCV core protein; however, a combined treatment produced a more marked reduction, that is, an additive or synergistic effect.

Although the antagonists were solely effective against HCV infection, we next attempted to examine the effect of the combination of RAR agonists and antagonists. An RAR agonist, TTNTB, in combination with an antagonist, Ro41-5253, also displayed an additive or synergistic inhibition of HCV core protein expression (Fig. 4b). This result supports that the inhibitory effect on HCV infection by retinoids is independent of the RARE (retinoic acid response element) signal mediated by RAR-RXR. Such combination treatments might be useful for HCV therapy because the antagonist would counteract other unfavorable RARE-dependent effects.

Furthermore, as IFNα is used as a standard medicine for the treatment of hepatitis C, it would be useful if retinoids exert an additive or synergistic effect on IFNα treatment. When we performed western blotting with IFNα and Am80 under similar conditions, the HCV core protein in the IFNα (1.0 unit/ml)-treated cells also displayed a marked reduction in the existence of 0.1 μM Am80 (Fig. 4c). To determine whether the combined administration of retinoid and IFNα is additive or synergistic inhibition, we examined the effect on HCV RNA in the presence of the two drugs. We added the various concentrations of IFNα in combination with Am80 into Huh 7.5.1 cells, followed by viral inoculation (MOI = 0.01). After 5 days, HCV RNA was extracted from the supernatant, and the amount was measured using real-time RT-PCR. The result revealed that IFNα and Am80 singly reduced HCV RNA to 90% of the levels observed in the control at a concentration of 14.5 unit/ml and 0.192 μM, respectively (Fig. 4d). Combinations of 5.0 unit/ml IFNα with 0.0001 μM Am80, and 2.5 unit/ml IFNα with 0.001 μM Am80 demonstrated 90% inhibition of HCV RNA. The isobologram analysis indicated that the combined administration of IFNα and Am80 exhibited a synergistic effect (Fig. 4d). These results demonstrate the possibility that a combination treatment of retinoids and IFNα is useful for HCV treatment.

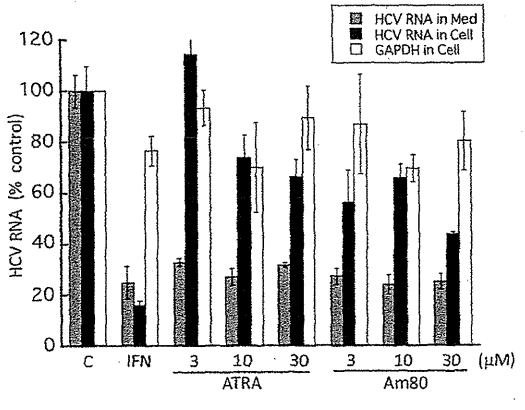

Fig. 3. Effects of retinoids on persistently HCV-infected cells. Persistently HCV-infected cells were treated with indicated concentrations of retinoids or IFN (10 unit/ml) for 5 days. HCV RNA was extracted from supernatant or cells. Quantitative real-time RT-PCR was performed with primers for HCV or GAPDH. The values are the averages of triplicates and the error bars represent the standard deviation.

3.5. Retinoids did not activate the IFN signaling effector molecule PKR

Retinoic acid reportedly activates the expression of some molecules, which play central roles in IFN signaling [16,17]. We attempted to investigate if retinoids inhibit HCV replication through IFN signaling. As there are multiple pathways involved in IFN signaling, we examined the expression of the IFN signaling downstream antiviral effector molecules, OAS1 and PKR. These molecules are present ubiquitously at constitutive levels but are increased and activated by IFN and play critical roles in the degradation of HCV RNA [18,19]. We treated Huh 7.5.1 cells with 10 unit/ml IFNα for 48 h and the cellular RNA was extracted. The level of PKR mRNA in the IFNα-treated cells was approximately 4-fold higher than that of the untreated cells, whereas that of OAS1 was 1.7-fold higher (Fig. 5). These results suggest the possibility that PKR considerably contributes to the antiviral effect of IFNα in the examined cells. We treated the cell with 10 μM of Am80, bexarotene, or Ro41-5253 for 48 h, extracted RNA from the cell, and estimated the RNA expression levels of OAS1, PKR and GAPDH using quantitative real-time RT-PCR. The relative RNA levels compared with the control were calibrated by a standard curve of RNA extracted from IFNα (10 unit/ml)-treated cells. The three chemicals at the concentration of 10 μM, produced no enhancement of PKR expression and a slight enhancement of OAS1 expression (1.2–1.3-fold) (Fig. 5). GAPDH expression of the cells was slightly enhanced by the three compounds and IFNα. Although there were some increases in OAS1 expression, it is unlikely that these compounds inhibited through enhancement of OAS1. These results, when taken together, suggest that retinoids and rexinoids presumably inhibit HCV infection through some other modes of action than the activation of IFN signaling.

4. Discussion

This study revealed that RAR and RXR ligands inhibited HCV infection at significantly low concentrations (EC_{50} <10 nM) in the JFH-1-Huh 7.5.1 cell-culture system (Fig. 1 and Table 1). Until now, there have been a few reports concerning the *in vitro* or *in vivo* effect of retinoids on HCV infection. Regarding *in vitro* inhibition, the RAR agonists, TTNPB and 13-*cis*-retinoic acid, were determined to have an inhibitory effect in screening studies [8,9], whereas a slight activation by TTNPB in a HCV replicon system was also reported [20]. On the other hand, there had been some reports of retinoids being used for preclinical application against hepatitis C. Böcher et al. reportedly treated HCV patients who were resistant to IFNα with ATRA and obtained good results, especially in combination with IFNα [10]. Kohge et al. reported a pilot study involving retinol in combination with

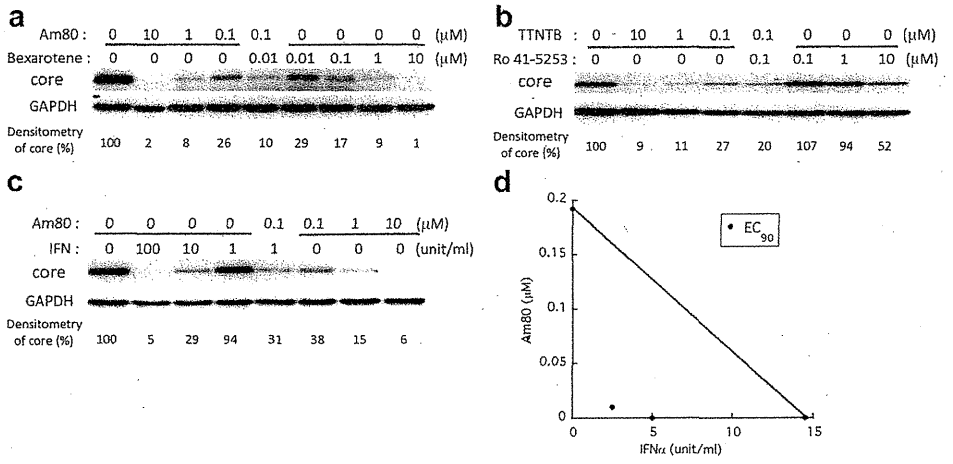

Fig. 4. Effects of combined application on HCV infection. with Huh 7.5.1 cells were infected with HCVcc at a MOI of 0.01 in the presence of (a) Am80 and bexarotene, (b) TTNTB and Ro41-5253, and (c), (d) Am80 and IFNα. After 5 days of incubation, the cells were lysed and subjected to western blotting with anti-core antibody and anti-GAPDH antibody (a, b, and c), or RNA was extracted from the supernatant, applied to real-time RT-PCR, and the EC_{90} were plotted in an isobologram (d). The relative amounts of core protein to the untreated cells (control) were determined using ImageJ and described under the figures (a–c).

IFNα-ribavirin to chronic hepatitis C, and they obtained some positive results from the combined treatment [21].

Nevertheless, the inhibitory mechanism of HCV replication by retinoid has been scarcely studied. In this study, we performed some experiments to clarify the mechanism or target molecule(s) of retinoids and rexinoids. Although we were not able to provide an explicit mechanism of action, this study identified some suggestions that implicate the mechanism. First, these compounds affect some of the molecules involved in HCV viral replication and post-replication steps (Figs. 1e, 2, and 3). Second, the mechanism is not related to the RARE-dependent signaling mediated by RAR/RXR because the antagonists also demonstrated inhibition of HCV infection and further additive/synergistic effects (Fig. 1a, b, and 4b). Third, these compounds appeared to inhibit HCV via a mechanism other than IFN signaling because they could not enhance the expression of the IFN effector molecule PKR (Fig. 5).

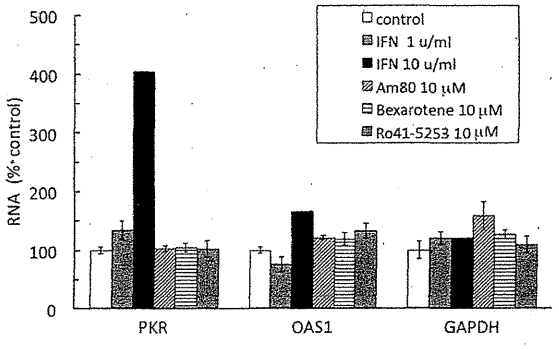

Fig. 5. Effects of retinoids and rexinoids on the expression of PKR, OAS1, and GAPDH. Huh 7.5.1 cells were treated with IFN, Am80, bexarotene, or Ro41-5253 for 48 h. RNA was extracted from the cells and subjected to real-time RT-PCR. The relative amount of PKR, OAS1, and GAPDH mRNA are represented by percentages relative to the values observed with untreated cells. The values are the averages of triplicates and the error bars represent the standard deviation.

As shown in Fig. 1, dose-dependent inhibition was observed in the cells treated with retinoid, rexinoid, and LXR ligands but not in the cells treated with PPAR ligands, vitamin D3, or thyroxin. Although RXR is common partner of these class II receptors, not all of the class II ligands inhibited HCV infection, suggesting that the RXR is not the main target of the HCV inhibitory effect. The finding that vitamin D3 or thyroxin did not inhibit HCV infection might be explained by the absence of the expression of VDR or TR in the tested liver cells. However, as PPARα is generally expressed in liver cells, it was deduced that RXR is not the target molecule of retinoids in regards to HCV inhibition. Furthermore, the fact that antagonists have inhibitory activity as well as agonists suggested that the effect is not mediated via RAR-RXR signaling.

After the report of Böcher et al., Kast believed that the inhibitory effect of retinoids would be explained by the enhancement of IFN signal via RIG-I (retinoic acid inducible gene I) [22]. RIG-I is an intracellular sensor molecule that recognizes HCV dsRNA to activate IFN signaling [23]. However, there is a retinoid-binding site in the promoter domain of RIG-I gene, and RIG-I expression requires RARE-dependent signaling [24]. In addition, the Huh 7.5.1 cells reportedly are defective for RIG-I signaling by a point mutation in this gene [25]. Retinoids also modulate expression of STAT1 (signal transducer and activator of transcription 1) and IRF-1 (interferon regulatory factor-1) [26–28], which also play critical roles in the IFN system during HCV infection [29,30]. The STAT1 gene has a RARE motif in the promoter, and the enhancement is dependent on RAR-RXR signaling [26], whereas IRF-1 expression was not mediated through the RARE motif [31].

As the IFN system is highly complicated, we examined the possibility that the IFN system is involved in the retinoid activity by examining the expression of the effector molecules PKR and OAS1. Since PKR expression was enhanced 4-fold by 10 unit/ml IFNα, the main effector molecule appeared to be PKR in the cells. However, 10 μM of retinoids, with

antiviral activity corresponding to 10 unit/ml IFNα, produced no enhancement of PKR (Fig. 5). These findings suggest that these compounds inhibited HCV infection mainly via a mechanism other than the IFN signaling.

This study suggested that there are two target steps of viral lifecycle affected by retinoids and rexinoids: the HCV replication step and the post-replication step. Given that the compounds reduced the HCV RNA of the subgenomic replicon cells, the HCV viral replication step appears to be inhibited (Fig. 2). Based on the observation that the intracellular HCV RNA was less reduced compared with extracellular HCV RNA (Figs. 1e and 3), the post-replication step also appears to be inhibited. On the other hand, higher concentrations of ligands were required to affect the subgenomic replicon cells (Fig. 2b and c). This suggests that the post-replication step might be the major target rather than the replication step. If so, some molecules critical for viral maturation or release may be disturbed. Some common structures of these compounds might interact with such a target molecule. However, a higher concentration of retinoids is also required for the effect on the persistently infected cells (Fig. 3). Given that both the subgenomic replicon cells and the persistently infected cells are derived from Huh 7 cells, the lower sensitivity of these cells might be the result of some property of Huh 7 cells (e.g., the abundance of or accessibility to the target molecule involved in HCV replication).

Although the mechanism of the inhibitory effect is not clear, the results in this study revealed the usefulness of the combination of retinoids. The combination treatment of agonists and antagonists is useful because the antagonists can counteract the unfavorable effects other than the anti-HCV activity of retinoids. Treatment with retinoids in combination with IFNα also has further potential. Given that Am80 and bexarotene are already used clinically to treat other diseases, and many agonists and antagonists of nuclear receptors II are being developed [5,7,32], further studies of retinoids and rexinoids for practical application against HCV are warranted.

Acknowledgments

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Signal Peptidase Complex Subunit 1 Participates in the Assembly of Hepatitis C Virus through an Interaction with E2 and NS2

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Abstract

Hepatitis C virus (HCV) nonstructural protein 2 (NS2) is a hydrophobic, transmembrane protein that is required not only for NS2-NS3 cleavage, but also for infectious virus production. To identify cellular factors that interact with NS2 and are important for HCV propagation, we screened a human liver cDNA library by split-ubiquitin membrane yeast two-hybrid assay using full-length NS2 as a bait, and identified signal peptidase complex subunit 1 (SPCS1), which is a component of the microsomal signal peptidase complex. Silencing of endogenous SPCS1 resulted in markedly reduced production of infectious HCV, whereas neither processing of structural proteins, cell entry, RNA replication, nor release of virus from the cells was impaired. Propagation of Japanese encephalitis virus was not affected by knockdown of SPCS1, suggesting that SPCS1 does not widely modulate the viral lifecycles of the *Flaviviridae* family. SPCS1 was found to interact with both NS2 and E2. A complex of NS2, E2, and SPCS1 was formed in cells as demonstrated by co-immunoprecipitation assays. Knockdown of SPCS1 impaired interaction of NS2 with E2. Our findings suggest that SPCS1 plays a key role in the formation of the membrane-associated NS2-E2 complex via its interaction with NS2 and E2, which leads to a coordinating interaction between the structural and non-structural proteins and facilitates the early step of assembly of infectious particles.

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Introduction

Over 170 million people worldwide are chronically-infected with hepatitis C virus (HCV), and are at risk of developing chronic hepatitis, cirrhosis, and hepatocellular carcinoma [1]. HCV is an enveloped virus of the family *Flaviviridae*, and its genome is an uncapped 9.6-kb positive-strand RNA consisting of the 5′ untranslated region (UTR), an open reading frame encoding viral proteins, and the 3′ UTR [2]. A precursor polyprotein is further processed into structural proteins (Core, E1, and E2), followed by p7 and nonstructural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B), by cellular and viral proteases. The structural proteins (Core to E2) and p7 reside in the N-terminal region, and are processed by signal peptidase from the polyprotein. NS2, NS3, and NS4A are prerequisites for proteolytic processing of the NS proteins. NS3 to NS5B are considered to assemble into a membrane-associated HCV RNA replicase complex. NS3 also possesses activities of helicase and nucleotide triphosphatase. NS4 is a cofactor that activates the NS3 protease. NS4B induces vesicular membrane alteration. NS5A is considered to play an important but undefined role in viral RNA replication. NS5B is the RNA-dependent RNA polymerase. It is now accepted that NS proteins, such as NS2, NS3, and NS5A, contribute to the assembly or release of infectious HCV [3–9].

NS2 protein is a transmembrane protein of 21–23 kDa, with highly hydrophobic N-terminal residues forming transmembrane helices that insert into the endoplasmic reticulum (ER) membrane [5,10]. The C-terminal part of NS2 resides in the cytoplasm, enabling zinc-stimulated NS2/3 autoprotease activity together with the N-terminal domain of NS3. The crystal structure of the C-terminal region of NS2 reveals a dimeric cysteine protease containing two composite active sites [11]. Prior work showed that NS2 is not essential for RNA replication of subgenomic replicons [12]; however, the protein is required for virus assembly independently of protease activity [5,6]. Several adaptive mutations in NS2 that increase virus production have been reported [13–17]. In addition, there is increasing evidence for genetic and biochemical interaction of NS2 with other HCV proteins, including E1, E2, p7, NS3-4A, and NS5A [10,18–25]. Thus, NS2 is now suggested to act as a scaffold to coordinate interactions between the structural and NS proteins for viral assembly. However, the molecular mechanism by which NS2 is involved in virus assembly remains unclear.

In this study, we identified signal peptidase complex subunit 1 (SPCS1) as a host factor that interacts with NS2 by yeast two-hybrid screening with a split-ubiquitin system. SPCS1 is a component of the microsomal signal peptidase complex which is

Author Summary

Viruses hijack host cells and utilize host-derived proteins for viral propagation. In the case of hepatitis C virus (HCV), many host factors have been identified that are required for genome replication; however, only a little is known about cellular proteins that interact with HCV proteins and are important for the viral assembly process. The C-terminal half of nonstructural protein 2 (NS2), and the N-terminal third of NS3, form the NS2-3 protease that cleaves the NS2/3 junction. NS2 also plays a key role in the viral assembly process independently of the protease activity. We performed split-ubiquitin yeast two-hybrid screening and identified signal peptidase complex subunit 1 (SPCS1), which is a subunit of the microsomal signal peptidase complex. In this study, we provide evidence that SPCS1 interacts with both NS2 and E2, resulting in E2-SPCS1-NS2 complex formation, and has a critical role in the assembly of infectious HCV particles. To our knowledge, SPCS1 is the first NS2-interacting cellular factor that is involved in regulation of the HCV lifecycle.

responsible for the cleavage of signal peptides of many secreted or membrane-associated proteins. We show that SPCS1 is a novel host factor that participates in the assembly process of HCV through an interaction with NS2 and E2.

Results

SPCS1 is a novel host protein that interacts with HCV NS2 protein

To gain a better understanding of the functional role of NS2 in the HCV lifecycle, we screened a human liver cDNA library by employing a split-ubiquitin membrane yeast two-hybrid system with the use of NS2 as a bait. It is known that the split ubiquitin-based two-hybrid system makes it possible to study protein-protein interactions between integral membrane proteins at the natural sites of interactions in cells [26]. From the screening, several positive clones were identified from the 13 million transformants, and the nucleotide sequences of the clones were determined. A BLAST search revealed that one of the positive clones encodes a full-length coding region of signal peptidase complex subunit 1 (SPCS1). SPCS1 is a component of the microsomal signal peptidase complex which consists of five different subunit proteins in mammalian cells [27]. Although catalytic activity for SPCS1 has not been indicated to date, a yeast homolog of this subunit is involved in efficient membrane protein processing as a component of the signal peptidase complex [28].

To determine the specific interaction of NS2 with SPCS1 in mammalian cells, FLAG-tagged NS2 (FLAG-NS2; Fig. 1A) was co-expressed in 293T cells with myc-tagged SPCS1 (SPCS1-myc; Fig. 1A), followed by co-immunoprecipitation and immunoblotting. SPCS1 was shown to be co-immunoprecipitated with NS2 (Fig. 1B). Co-immunoprecipitation of SPCS1-myc with NS2 was also observed in the lysate of Huh-7 cells infected with cell culture-produced HCV (HCVcc) derived from JFH-1 isolate [29] (Fig. 1C). To determine the region of SPCS1 responsible for the interaction with NS2, deletion mutants of myc-tagged SPCS1 were constructed (Fig. 1A) and co-expressed with FLAG-tagged NS2. Since the expression of C-terminal deletion mutants, d3 and d4, was difficult to detect (Fig. 1D), N-terminal deletions (d1 and d2) as well as wild-type SPCS1 were subjected to immunoprecipitation analysis. SPCS1-myc, -d1, and -d2 were co-immunoprecipitated with NS2 (Fig. 1E), suggesting that the SPCS1 region spanning amino acids (aa) 43 to 102 is involved in its interaction with NS2. Next, to identify the NS2 region responsible for its interaction with SPCS1, deletion mutants for FLAG-NS2 (Fig. 1A) were co-expressed with SPCS1-myc-d2 in cells, followed by being immunoprecipitated with anti-myc antibody. SPCS1 was co-immunoprecipitated with the NS2 deletions, except for a mutant lacking transmembrane (TM) 2 and TM3 (dTM23) domains (Fig. 1F). These finding suggests that the TM3 region of NS2 is involved in the interaction with SPCS1.

To investigate SPCS1-NS2 interaction *in situ*, the proximity ligation assay (PLA) [30], which is based on antibodies tagged with circular DNA probes, was used. Only when the antibodies are in close proximity, the probes can be ligated together and subsequently be amplified with a polymerase. We were able to detect PLA signal predominantly in the cytoplasm of the cells expressing FLAG-NS2 and SPCS1-myc-d2 tagged with V5 at N-terminus (Fig. 1G). By contrast, the PLA signal was not observed in the context of NS2-Core co-expression. We further analyzed the SPCS1-NS2 interaction by the monomeric Kusabira-Green (mKG) system [31], which is based on fusion proteins with complementary fragments (mKG-N and mKG-C) of the monomeric coral fluorescent reporter protein. When the mKG fragments are in close proximity due to the protein-protein interaction, the mKG fragments form a beta-barrel structure and emit green fluorescence. Co-expression of SPCS1-mKG-N and NS2-mKG-C fusion proteins in cells reconstituted green cellular fluorescence as shown in Fig. 1H. Thus, these results represented structures with SPCS1 and NS2 in close proximity, and strongly suggest their physical interaction in cells.

SPCS1 participates in the propagation of infectious HCV particles

To investigate the role(s) of endogenous SPCS1 in the propagation of HCV, four small interfering RNAs (siRNAs) for SPCS1 with different target sequences or scrambled control siRNA were transfected into Huh7.5.1 cells, followed by infection with HCVcc. Among the four SPCS1-siRNAs, the highest knockdown level was observed by siRNA #2. siRNAs #3 and #4 showed moderate reductions of SPCS1 expression, and only a marginal effect was obtained from siRNA #1 (Fig. 2A). As indicated in Fig. 2B, the infectious viral titer in the culture supernatant was significantly reduced by the knockdown of SPCS1. It should be noted that the infectious titers correlated well with the expression levels of endogenous SPCS1. siRNA #2 reduced the HCV titer to ~5% of the control level in Huh7.5.1 cells. To rule out the possibility of off-target effect of SPCS1-siRNA on HCV propagation, we also used "C911" mismatch control siRNAs in which bases 9 through 11 of siRNAs are replaced with their complements but other parts of antisense- and sense-strand sequences are kept intact. These mismatch designed-control siRNAs have been shown to reduce the down-regulation of the targeted mRNA, but maintains the off-target effects of the original siRNA [32]. The C911 controls against SPCS1-siRNA #2, #3, and #4 (C911-#2, -#3, and -#4) showed little effect on knockdown of SPCS1 as well as propagation of HCV (Fig. S1A and B).

We further determined the loss- and gain-of-function of SPCS1 on HCV propagation in an SPCS1-knockdown cell line. To this end, Huh-7 cells were transfected with a plasmid encoding a short hairpin RNA (shRNA) targeted to SPCS1 and were selected with hygromycin B, resulting in clone KD#31 where little or no expression of SPCS1 was detectable (Fig. 2C). KD#31 cells and parental Huh-7 cells were transfected with an RNA polymerase I (pol)-driven full-genome HCV plasmid [33] in the presence or

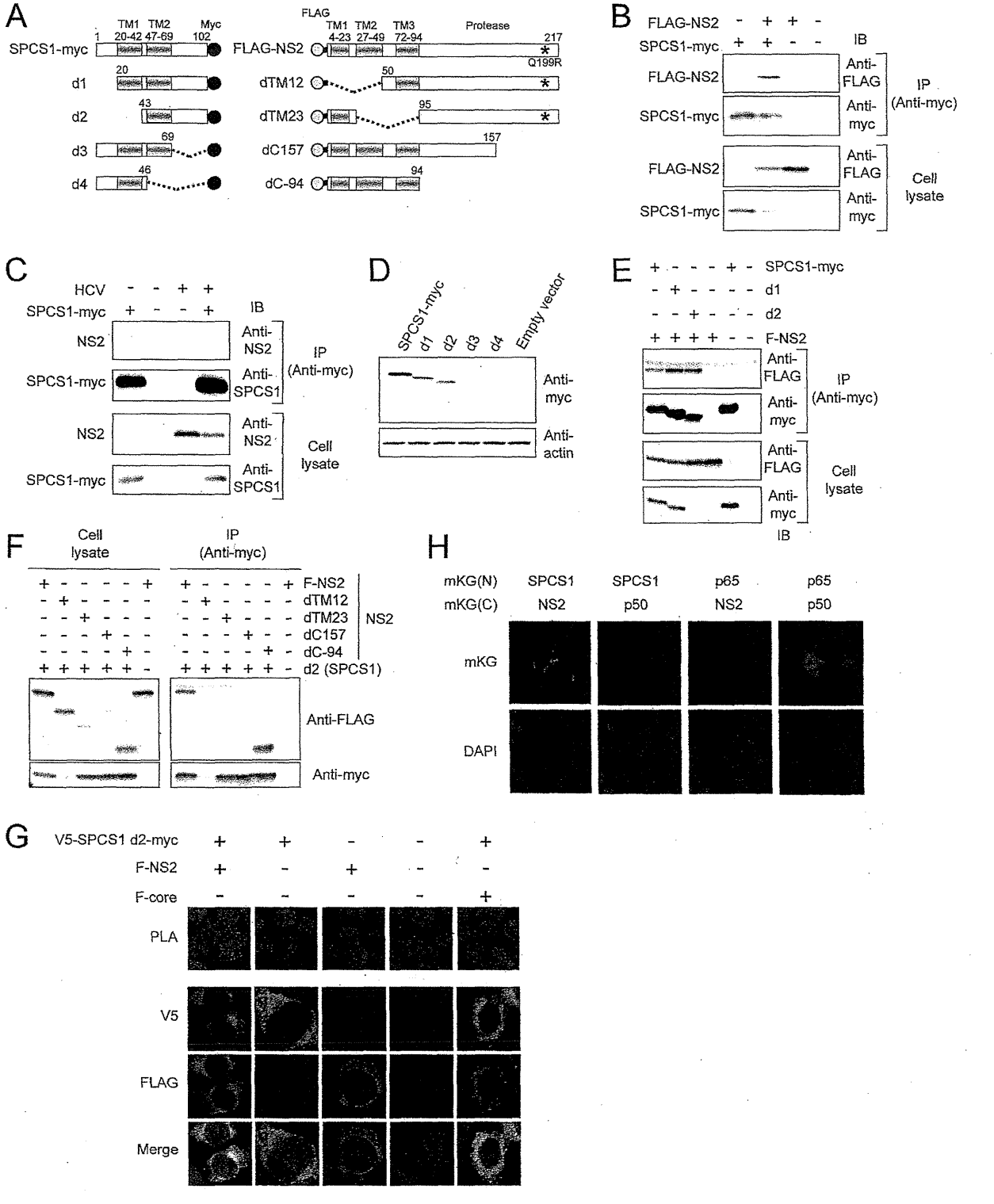

Figure 1. Interaction of HCV NS2 protein with SPCS1 in mammalian cells. (A) Expression constructs of SPCS1-myc and FLAG-NS2 used in this study. TM regions are represented as gray. Myc-tag regions are depicted by the black circles. Gray circles and bold lines indicated FLAG-tag and spacer (GGGGS) sequences, respectively. Adaptive mutations are indicated as asterisks. Positions of the aa resides are indicated above the boxes. (B) 293T cells were co-transfected with a FLAG-tagged NS2 expression plasmid in the presence of a SPCS1-myc expression plasmid. Cell lysates of the transfected cells were immunoprecipitated with anti-myc antibody. The resulting precipitates and whole cell lysates used in immunoprecipitation (IP) were examined by immunoblotting using anti-FLAG- or anti-myc antibody. An empty plasmid was used as a negative control. (C) HCVcc infected