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Hepatology Research 36 (2006) 294–300

HEPATOLOGY RESEARCH

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Identification of novel hepatitis C virus-specific cytotoxic T lymphocyte epiotpe in NS3 region

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Received 19 July 2006; received in revised form 16 August 2006; accepted 22 August 2006
Available online 22 September 2006

Abstract

Hepatitis C virus (HCV)-specific cytotoxic T lymphocytes (CTL) are thought to be effective in limiting viral spread and in clearing virus during infection. Therefore, we attempted to establish HCV-specific CTL and identify novel HCV-specific CTL epitopes in a patient with acute hepatitis C by a novel screening method using recombinant vaccinia viruses (rVV) and synthetic peptides. $CD8^+CD45RA^-$ T cells (memory T cells) were isolated from peripheral blood mononuclear cells (PBMC) of a patient with acute hepatitis C. HCV-specific CTL were cloned at limited dilutions and tested for HCV-specific CTL activity using a standard ^{51}Cr release assay. CTL assay was performed using rVV expressing regions of HCV-J, and overlapping and truncated synthetic peptides from HCV-J. CTL recognizing the NS3 region were isolated by ^{51}Cr release assay with rVV-HCV. Isolated CTL were restricted by HLA class I molecules B^*5603. We confirmed that isolated CTL recognized 8-mer amino acids in the NS3 region of HCV-J by ^{51}Cr release assay with overlapping and truncated synthetic peptides. In conclusion, we isolated HCV-specific CTL restricted by HLA-B^*5603 and identified a novel HCV-specific CTL epitope (IPFYGKAI, amino acids 1373–1380) in the NS3 region. The identified HCV-specific CTL epitope might be useful for HCV therapy.

Keywords: CTL; Epitope; HCV; NS3 region

1. Introduction

The hepatitis C virus (HCV) affects 200 million individuals worldwide [1]. HCV infection is a leading cause of chronic hepatitis and liver cirrhosis [2]. More than 50% of infected patients develop chronic hepatitis, and some of these will progress to hepatocellular carcinoma [3]. Current viral clearance treatment, peginterferon alfa-2a plus ribavirin, is only successful in about 60% of treated patients [4]. Therefore, development of new drugs and vaccines aiming to prevent and/or treat HCV infection is urgently needed.

Cytotoxic T lymphocytes (CTL) are thought to be a major host defense against viral infection [5] and have been implicated in the immunopathogenesis of viral infection [6]. HCV-specific CTL are also thought to be effective in limiting viral spread and in clearing virus during infection. HCV-specific CTL are particularly important in eradicating viruses in the acute phase. CTL interact through their polymorphic T cell receptors with HLA class I molecules containing endogenously synthesized peptides, such as peptides derived from viral proteins, of 8–11 residues (usually 9 or 10) on the surface of infected cells [7]. In previous studies, HCV-specific CTL responses have been detected in peripheral blood mononuclear cells (PBMC) [8] and liver-infiltrating lymphocytes in patients with chronic hepatitis [9].

CTL responses are less well characterized, in part of because of the technical difficulties involved in isolating and characterizing these cells [10]. Isolation of HCV-specific CTL is particularly difficult in the chronic phase; moreover,

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doi:10.1016/j.hepres.2006.08.011

identification of HCV-specific CTL epitopes is difficult using chronic-phase CTL. However, stronger CTL responses have been detected in PBMC in acute patients during hepatitis B virus (HBV) infection [11]. Previous methods to identify HCV-specific CTL epitopes have used screening of large numbers of overlapping synthetic peptides [8,9], recombinant vaccinia viruses (rVV) expressing HCV proteins [12] or testing synthesized peptides for HLA binding based on sequence motifs [13,14]. However, these methods do not allow the establishment of CTL recognizing all HCV regions and HLA molecules. Therefore, we attempted to establish HCV-specific CTL and identify novel HCV-specific CTL epitopes in a patient with acute hepatitis C using both rVV expressing HCV-J, and overlapping and truncated synthetic peptides from HCV-J.

The aim of this study was to isolate HCV-specific CTL and identify novel HCV-specific CTL epiotpes. Our methods and results are useful for investigating the CTL response in patients with HCV infection, and for the cell-mediated immune prevention of HCV infection.

2. Material and methods

2.1. Patients

An HLA-A*1101, A*2402, B*5201, B*5603, Cw*0102 and Cw*1202 positive 71-year-old patient with acute HCV infection with unknown etiology served as a subject. Diagnosis of acute hepatitis was based on high levels of serum alanine aminotransaminase (ALT), seropositive conversion of HCV RNA, seropositivity for HCV-specific antibody, seronegativity of other virus markers by enzyme immunoassay, recent onset of jaundice and other typical symptoms of acute hepatitis. The infected genotype of HCV was 1b. Sample was taken at about 4 months after ALT elevation. HCV was not eliminated spontaneously and persistently infected with normal range of ALT.

2.2. Isolation of $CD8^+$ memory T cells

PBMC were separated from heparinized peripheral blood by gradient centrifugation using Ficoll-Paque (Amersham Biosciences, Uppsata, Sweden). $CD8^+$ T cells were isolated from PBMC by positive selection using antibody-conjugated magnetic beads according to the manufacturer's instructions (Dynal, Oslo, Norway). Beads were detached from isolated cells using the DetatchaBead system (Dynal, Oslo, Norway). Furthermore, $CD45RA^+$ T cells (virgin T cells) were depleted by negative selection using antibody-conjugated magnetic beads according to the manufacturer's instructions (Dynal, Oslo, Norway). Isolated $CD8^+CD45RA^-$ T cells (memory T cells) were cultured in RPMI 1640 supplemented with L-glutamine (GIBCO, Grand Island, New York), penicillin-streptomycin (GIBCO, Grand Island, New York), 10% heat-inactivated fetal bovine serum (FBS) (BioWest, Logan, Utah), 40 U/ml rIL-2 (SIGMA, St. Louis, Missouri), 0.1 μg/ml anti-CD3 antibodies (eBioscience, San Diego, California) and 5 μg/ml phaseolus vulgaris agglutinin (PHA) (SIGMA, St. Louis, Missouri).

2.3. Target cell lines

Allogeneic and autologous Epstein–Barr virus-transformed B lymphoblastoid cells (B-LCL) were established from our own pool of patients and normal donors. B-LCL were maintained in RPMI 1640 supplemented with L-glutamine, penicillin-streptomycin and 10% heat-inactivated FBS.

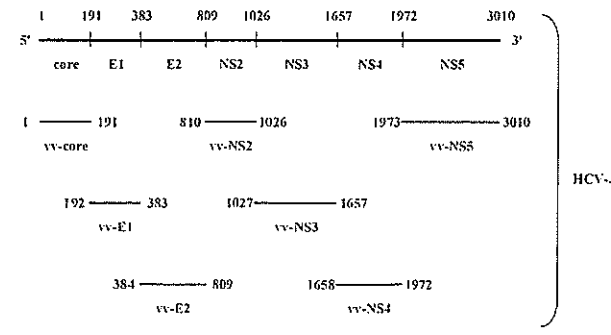
2.4. Construction of a recombinant HCV vaccinia virus

rVV expressing genotype 1b of HCV-J (accession no. D90208) were constructed (Table 1). The characteristics of the seven vaccinia constructs used in this study were as follows: vv-core (encoding amino acid (aa) 1–191), vv-E1 (encoding aa 192–383), vv-E2 (encoding aa 384–809), vv-NS2 (encoding aa 810–1026), vv-NS3 (encoding aa 1027–1657), vv-NS4 (encoding aa 1658–1972) and vv-NS5 (encoding aa 1973–3010). Wild-type (Western Reserve strain) vaccinia virus (vv-Wild) was used as a control. All vaccinia viruses were expanded with CV-1 cells, and titer was determined by standard plaque assay based on CV-1 cells. The HCV-J plasmid was provided by Prof. K, Shimotohno (Kyoto University) and the transfer plasmid vector, pAK10, was provided by Dr. A, Kojima (National Institute of Infectious Disease Diseases).

2.5. Synthetic peptides

Peptides were based on the amino acid sequence of genotype 1b of HCV-J (accession no. D90208). Peptides were synthesized by and purchased from Minotopes and were more than 80% pure according to high-performance liquid chromatography. Three types of peptide were synthesized: 10-mer overlapping 20-mer peptide, truncated 15-mer peptide and truncated 8-mer peptide.

Table 1
Schematic representation of vaccinia virus constructs used in this study



2.6. HLA typing

HLA genotypes of PBMC from patients and from normal donors were determined using the Pel-Freeze SSP UniTray Kit (DynalBiotech, Milwaukee, Wisconsin).

2.7. Generation of HCV-specific CTL clones

CTL lines were cloned at limited dilutions (0.3, 1, 3, 10 and 30 cells per well) and were then subcloned at 0.3 or 1 cells per well in 96-well microtiter plates. Cells were plated in the presence of RPMI 1640 supplemented with L-glutamine, penicillin-streptomycin, 20% heat-inactivated FBS, 40 U/ml rIL-2, 0.1 μg/ml anti-CD3 antibodies, 5 μg/ml PHA and irradiated (3500 rad) allogeneic PBMC (5×10^4 cells/well). HCV-specific CTL clones were expanded by restimulation in a 24-well plate, as described above.

2.8. CTL assay

Cytotoxic activity of CTL was assessed using standard 4 h sodium chromate (^{51}Cr) release assay. Briefly, B-LCL were suspended in RPMI 1640 supplemented with L-glutamine, penicillin-streptomycin and 10% heat-inactivated FBS, and were incubated overnight at 37 °C with either rVV-HCV at a multiplicity of infection of 5 plaque-forming units (pfu)/cell or with synthetic peptide. Infected or stimulated B-LCL were labeled with 100 μCi ^{51}Cr. After incubating the effector cells with target cells for 4 h at 37 °C in a humidified 5% CO_2 atmosphere, supernatants were collected and radioactivity was measured with a gamma counter.

2.9. Blocking of CTL response by antibodies

Each antibody used in this study was added to the 96-well plates of the CTL assay. Anti-CD4 (Pharmingen, San Diego, California), anti-CD8 (Pharmingen, San Diego, California), anti-HLA class I (serotec, Oxford, UK) and anti-HLA class II antibodies (Serotec, Oxford, UK) were used. Antibody reactions were performed at a final concentration of 10 μg/ml for anti-CD4 and anti-CD8, and at 50 μg/ml for anti-HLA-class I and anti-HLA class II.

3. Results

3.1. Screening of recombinant HCV vaccinia virus specific response

In order to isolate CTL, $CD8^+CD45RA^-$ T cells were separated from fresh PBMC. CTL assay was performed by using Target cells (B-LCL) infected with vv-core, vv-E1, vv-E2, vv-NS2, vv-NS3, vv-NS4 and vv-NS5. CTL recognizing the NS3 and NS5B region were isolated as the CTL line (data was not shown). We tried to identify CTL epitope in NS3 region, because NS3 region is important in the CTL target.

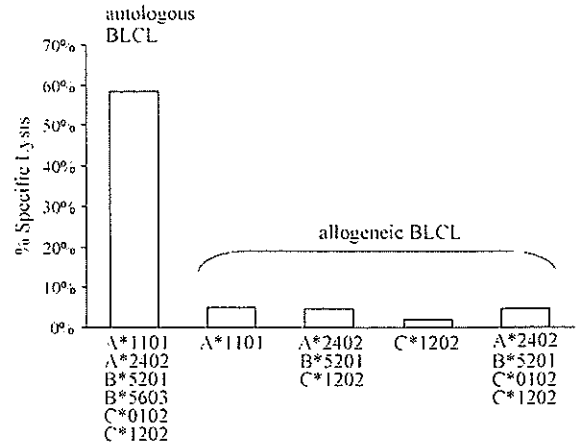


Fig. 1. HLA class I restriction analysis of CTL response to the NS3 region was performed. CTL were assayed for cytotoxicity against panels of autologous and allogeneic B-LCL infected with rVV-HCV-NS3, respectively, at an effector/target ratio of 3:1.

The CTL line was cloned at 0.3, 1, 3, 10 and 30 cells per well and was then subcloned at 0.3 or 1 cells per well in 96-well microtiter plates.

3.2. HLA restriction analysis

The HLA class I restriction of isolated CTL was confirmed by CTL assay (Fig. 1). This analysis was performed with a panel of autologous and allogeneic PBMC with known HLA haplotypes. CTL recognized autologous B-LCL (B^*5603 positive), but not allogeneic B-LCL (B^*5603 negative). Furthermore, CTL assay was performed against HLA-B^*07, HLA-B^*35, HLA-B^*51, HLA-B^*5601(data is not shown). However, we have no cytotoxicity. Therefore, we concluded that isolated CTL recognizing the NS3 region were restricted by HLA-B^*5603.

3.3. Identification of CTL epitope in NS3 region

In order to limit the amino acid residues in the NS3 region-recognizing CTL, we synthesized a 10-mer overlapping 20-mer peptide representing the NS3 region of HCV-J (peptide 1–63). When autologous B-LCL were pulsed with a mixture of synthetic peptides (5 peptide pool), CTL recognized autologous B-LCL pulsed with the pool containing peptides 31–35 (Fig. 2a). CTL assay was then performed using autologous B-LCL pulsed with individual synthetic peptides (31–35). CTL recognized autologous B-LCL pulsed with peptide 35 (Fig. 2b). To determine the epitopes of isolated CTL, based on the observation that the optimal length for peptides binding to MHC class I molecules is 8–15 amino acid residues, we synthesized truncated 15-mer peptides from the NS3 sequence (aa 1360–1393) of HCV-J. CTL assay was performed using autologous B-LCL pulsed with individual truncated synthetic peptides (Fig. 3; representative data is

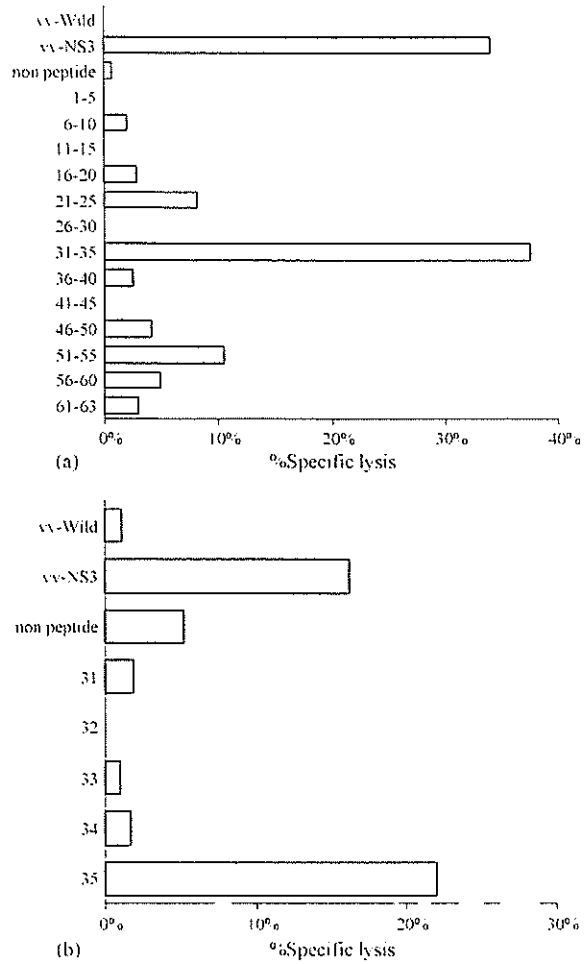


Fig. 2. (a) CTL were tested for recognition of B-LCL stimulated with synthetic 10-mer overlapping 20-mer peptide mixtures (5 peptide pool) from the NS3 sequence of the HCV-J. The peptide mixture comprising 31–35 were able to sensitize target cell for lysis. The effector/target ratio was 3:1 and the concentration of peptide used was 10 μM. (b) CTL were tested for their ability to recognize B-LCL stimulated with peptides 31–35. Only peptide 35 was able to sensitize target cells for lysis. The effector/target ratio was 3:1 and the concentration of peptide used was 10 μM.

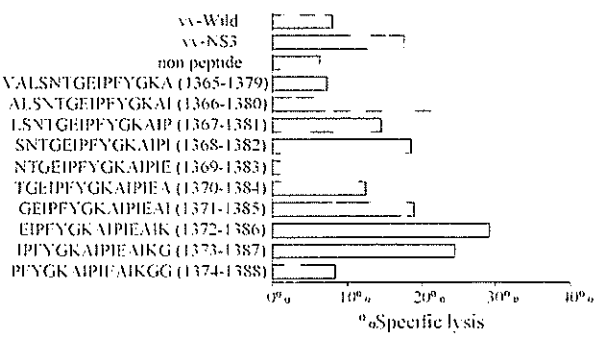


Fig. 3. CTL were tested for recognition of B-LCL stimulated with synthetic truncated 15-mer peptide from the NS3 sequence (aa 1365–1388) of HCV-J. CTL recognized an 8-mer peptide (IPFYGKAI; 1373–1380). The effector/target ratio was 3:1 and the concentration of peptide used was 10 μM.

Table 2
Truncated 8-mer peptide from NS3 region of HCV-J

Peptide	Sequence	Residues
II8	I P F Y G K A I	1373–1380

shown). We confirmed that CTL recognized an 8-mer peptide (IPFYGKAI; 1373–1380) in the NS3 region of HCV-J.

3.4. Minimal and optimal epitope

We confirmed the recognition of the 8-mer peptide as the minimal and optimal epitope by synthesizing truncated 8-mer peptide (Table 2). CTL recognized autologous B-LCL pulsed with the peptide (Fig. 4a). CTL activity with various effector/target ratios and concentrations of peptide II8 was assessed. The results showed that CTL is able to lyse the targets increased effector/target ratios (Fig. 4b) and in the presence of II8 in a dose-dependent manner (Fig. 4c).

3.5. Antibody blocking assay

In order to define the phenotype of cells responsible for II8 and restriction of HLA class I, CTL activity for II8 peptide was tested in the presence of anti-CD4, anti-CD8, anti-HLA class I and anti-HLA class II antibodies (Fig. 5). CTL activity was clearly blocked by anti-CD8 antibody, but not by anti-CD4 antibody. Moreover, CTL activity was clearly blocked by anti-HLA-class I antibody, but not by anti-HLA class II antibody. These results indicate that the cells responsible for II8-specific lysis are conventional $CD8^+CD4^-$ MHC class I restricted CTL.

4. Discussion

CTL are thought to play an important role in viral clearance. In HCV infection, recent reports have demonstrated the importance of CTL in the final outcome of infection [15], in the protection of individuals in contact with HCV [16] and in patients clearing HCV after IFN therapy [17]. However, a proportion of patients with chronic HCV infection remain resistant to antiviral therapy, including recently developed treatment modalities such as peginterferon alfa-2a plus ribavirin [4]. Such treatment failures may be a reflection of insufficient antiviral immune response. Thus, CTL response to HCV may not be sufficient to prevent disease progression of disease in chronic HCV infection [18].

However, CTL play an important role in eliminating HCV, particularly in acute HCV infection. Therefore, amplification of CTL response may be a useful therapeutic antiviral strategy. Augmentation of HCV-specific CTL response by therapeutic vaccines could enhance HCV elimination in IFN therapy, thus improving treatment outcome. Indeed, among patients with chronic HCV infection, the presence of HCV-specific CTL prior to treatment is associated with viral clear-

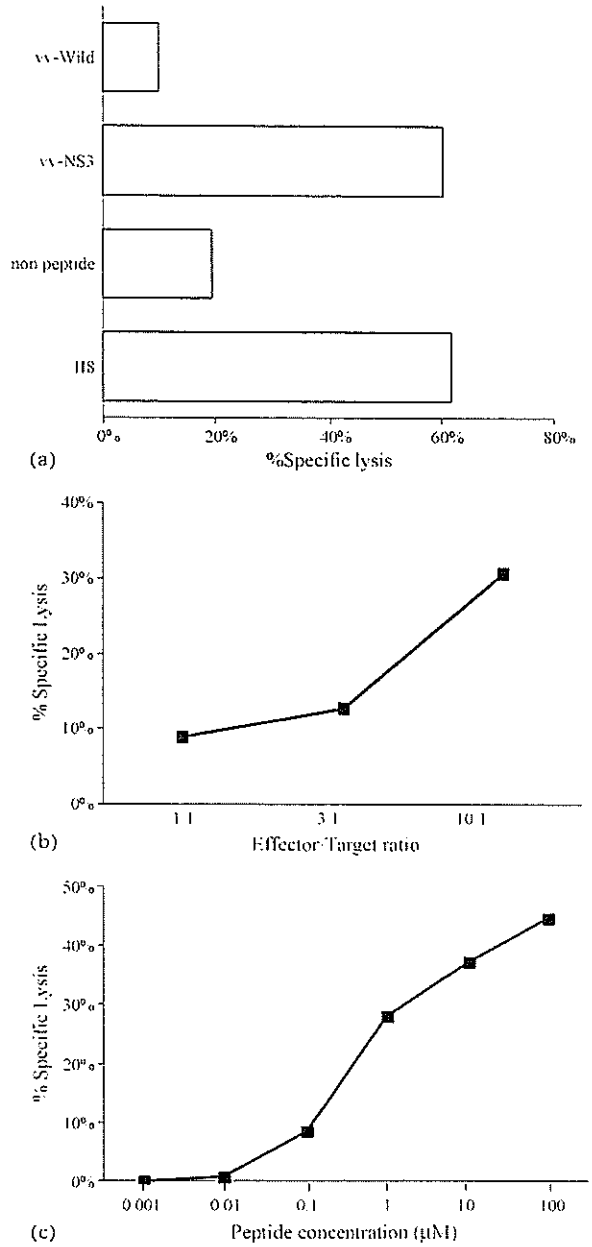


Fig. 4. (a) CTL were tested for recognition of B-LCL stimulated with synthetic truncated 8-mer peptide in order to confirm minimal and optimal epitope. The peptide was able to sensitize target cell for lysis. The effector/target ratio was 3:1 and the concentration of peptide used was 10 μM. (b) CTL were tested at varying effector/target ratios against autologous B-LCL stimulated with 10 μM peptide II8. (c) CTL were tested against autologous B-LCL stimulated with various concentrations of peptide II8 at an effector/target ratio of 3:1.

ance after a course of IFN plus ribavirin [19]. Therefore, development of a universally immunogenic vaccine requires the identification of as many CTL epitopes as possible.

While nonspecific stimulation of liver-infiltrating CD8 cells allows in vitro expansion and identification of immunodominant HCV-specific CTL populations present within

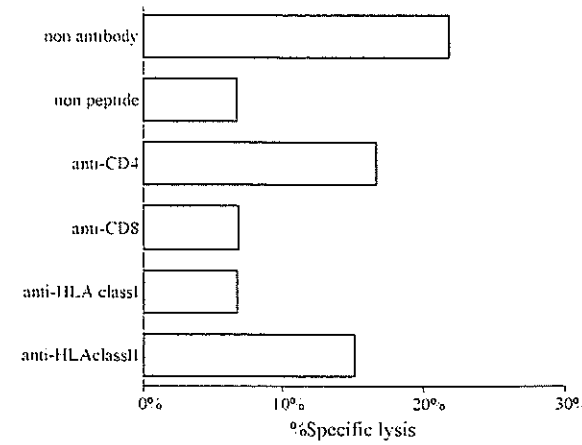


Fig. 5. Antibody blocking of CTL activity for the II8 peptide was performed. CTL were tested for cytotoxicity against autologous B-LCL in the presence of 10 μM II8 peptide with 10 μg of anti-CD4 and anti-CD8 antibody, or in the presence of 10 μM II8 peptide with 50 μg of anti-HLA class I and anti-HLA class II antibody. Antibodies were added to the assay before effector cells. Assays were performed at an effector/target ratio of 3:1.

chronically infected liver [20], the same method cannot be applied to study peripheral blood CTL response. This is because the frequency of HCV-specific CTL present in the circulation in most stages of HCV infection is too low [21]. Although HCV-specific CTL detected in peripheral blood are thought to be less frequent than those among liver-infiltrating lymphocytes in patients with chronic hepatitis C [22], circulating CTL can still be detected in the majority of individuals with chronic HCV infection and their epitope specificity is reportedly similar to that of liver infiltrating cells [12].

In the present study, we selected a patient with acute hepatitis C in order to establish HCV-specific CTL from PBMC, as the number of precursor HBV-specific CTL in PBMC from patients with acute hepatitis B is higher more than that from patients with chronic hepatitis B [11]. Indeed, in a previous study comparing acute-phase and chronic-phase HCV-specific CTL responses, HCV-specific CTL were detectable in chronic carriers but the percentage of HCV-specific CTL response was significantly higher in acute patients [23]. In addition, follow-up of acute HCV-infected patients during subsequent disease development showed a significant decrease in the intensity of HCV-specific CTL response [23]. In this study, we were able to isolate HCV-specific CTL from PBMC from a patient with acute hepatitis C and to identify a novel CTL epitope in these isolated CTL.

Previous studies to identify human CTL epitopes located in HCV proteins have used one of two approaches: screening using numerous overlapping synthetic peptides [8,9] or testing synthesized peptides based on binding motifs for HLA molecules [13,14]. However, it is difficult to isolate CTL recognizing all regions of HCV and to identify restriction by variable HLA molecules using these methods. On the other hand, the use of vaccinia vectors has facilitated the successful expansion of HCV-specific CTL from the periph-

eral blood of patients with HCV infection [12]. In this study, we isolated HCV-specific CTL recognizing the narrow region (NS3 region) using rVV-HCV expressing various regions of HCV-J. We then identified HCV-specific CTL epitopes using overlapping and truncated synthetic peptides representing the recognized NS3 region. The HCV-specific CTL epitope identified in this study was restricted by HLA-B*5603. Although HLA-B*5603 is a rare HLA class I molecule, we were able to isolate CTL and identify the CTL epitope.

The present method thus allows the identification of CTL epitopes in HCV and their restriction by HLA class I molecules. However, this method has potential problems in identifying HCV-specific CTL epitopes. Firstly, this method fully responds to HCV-J strain, but not to other HCV strains. Secondly, the HCV genome in each patient is highly variable for each epitope [24] and can change during the course of chronic hepatitis [25]. The use of PBMC during the acute phase, low mutation rates and high CTL activity were beneficial in establishing HCV-specific CTL in this study. Although these problems remain, this method is useful for isolation of HCV-specific CTL to identify epitopes within the entire HCV polyprotein and are recognized by various HLA class I molecules.

In recent studies, the NS3 region of HCV is a therapeutic target for HCV infection. This is because the NS3 region of HCV is indispensable for viral replication and includes multifunctional enzymes, such as serine protease and helicase. Indeed, internal cleavage of NS3 is associated with replication of HCV [26], and an NS3 serine protease inhibitor inhibits replication of HCV-RNA [27]. Moreover, mice immunized with a recombinant adenovirus expressing HCV NS3 protein were protected against infection with a recombinant vaccinia virus expressing HCV-polyprotein [28]. This protection was mediated by CD8-positive T cells.

In previous studies, HCV-specific CTL epitopes in the NS3 region were identified using various methods. However, the HCV-specific CTL epitope identified in this study was not reported previously, and thus it is a novel HCV-specific CTL epitope. CTL epitopes located in the NS3 region may be useful for therapeutic vaccines against HCV infection and the epitope identified in this study, IPFYGKAI, may also be useful for HCV therapy.

Because HCV is a highly heterogeneous virus and these epitopes are present within both conserved and variable regions of the genome, CTL-escaping variants seem to occur in acute and chronic HCV infection [29]. Therefore, precise definitions of the epitopes recognized by HCV-specific CTL may allow an understanding of effects of sequence variation on the immune response to HCV infection. Although we isolated HCV-specific CTL and identified HCV-specific CTL epitopes in this study, further analyses of the immune response to HCV infection and identification of additional HCV-specific CTL epitopes belonging to immunologically conserved regions are required.

In conclusion, we isolated HCV-specific CTL restricted by HLA-B*5603 and identified a novel HCV-specific CTL epitope in the NS3 region using rVV-HCV expressing various regions HCV-J, and overlapping and truncated synthetic peptide from HCV-J. The identified epitope, IPFYGKAI, may be useful in immunotherapy for HCV infection.

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Liver International 2006: 26: 1111–1118

Liver International
DOI: 10.1111/j.1478-3231.2006.01352.x

Clinical Studies

Zinc is a negative regulator of hepatitis C virus RNA replication

Yuasa K, Naganuma A, Sato K, Ikeda M, Kato N, Takagi H, Mori M. Zinc is a negative regulator of hepatitis C virus RNA replication.
Liver International 2006: 26: 1111–1118.

Abstract: *Background/Aims:* Hepatitis C virus (HCV) infection is a significant global public health problem. In clinical studies, zinc has been closely related to the pathogenesis of chronic hepatitis C. However, the role of zinc in both viral replication and the expression of viral proteins remains unclear. We aimed to clarify the effect of zinc on the replication of HCV *in vitro*. *Methods:* We incubated subgenomic HCV replicon cells (sO) and genome-length HCV RNA-replicating cells (O) treated with several chemicals including trace elements. Total RNAs were collected and subjected to real-time reverse-transcriptase polymerase chain reaction in order to examine the level of HCV RNA replication, and Western blotting was performed to confirm the expression of viral proteins. *Results:* Iron salts and interferon-α suppressed HCV RNA replication and protein expression in both sO and O cells. Zinc salts effectively reduced the viral replication in the genome-length HCV RNA replication system but not in the subgenomic HCV replicon system. *Conclusions.* We demonstrated that zinc may play an important role as a negative regulator of HCV replication in genome-length HCV RNA-replicating cells. Zinc supplementation thus appears to offer a novel approach to the development of future strategies for the treatment of intractable chronic hepatitis C.

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Key words: genome-length HCV RNA – hepatitis C virus – replication – subgenomic HCV replicon – zinc

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Received 5 December 2005,
accepted 18 June 2006

Hepatitis C virus (HCV) infection is a significant global public health problem. Persistent HCV infection eventually develops into liver cirrhosis or hepatocellular carcinoma (1). A sustained viral response (SVR) to anti-HCV therapy has been demonstrated to prevent the progression of liver disease and even to promote the regression of pathologic changes (2). Peginterferon plus oral ribavirin, currently the most powerful therapy for chronic hepatitis C, has successfully induced SVR in about half of treated patients of genotype 1b with high viral load (3, 4). However, there are still a number of non-responders to interferon (IFN)-based therapy. As a result, the treatment efficacy still needs to be improved.

HCV is a positive-polarity, single-stranded RNA virus, a member of the *Hepacivirus* genus of the Flaviviridae family (5). The HCV genome consists of an ~9.6 kb RNA molecule containing a large open reading frame flanked by structured 5′- and 3′-non-translated regions (NTR). Located within the 5′-NTR is an internal ribosome entry site (IRES) directing the translation of an approximately 3000-amino-acid polyprotein that is co- and posttranslationally cleaved by cellular and viral proteases into the following 10 products (listed from the N to the C termini): core, envelope protein 1 (E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B. The NS2–NS3 cleavage is performed by NS2, and the remaining processing of the NS3–NS4A–NS4B–NS5A–NS5B fragment depends on the NS3/NS4A protease, which is similar to chymotrypsin-like serine protease (6).

Zinc is an essential nutrient for a broad range of biological activities and for cell proliferation (7) and it also functions as an antioxidant (8). It also plays an important role in the function and maintenance of the crystal structures of such HCV proteins as NS2–NS3 (9–11) and NS5A (12, 13). The virus-encoded NS2–NS3 protease that is responsible for autocatalytic cleavage at the NS2–NS3 site is stimulated by $ZnCl_2$ (9, 10). The NS3 protease domain contains a zinc atom (11). These observations have led researchers to propose that zinc plays an important role in the NS2–NS3 protease activity. Several studies have examined the direct inhibitory effects of zinc on viruses, such as human immunodeficiency virus (14), rhinovirus (15), herpes simplex virus (16),

and respiratory syncytial virus (17) *in vitro*. However, the direct effect of zinc on the replication of HCV *in vitro* has never been previously reported.

Despite the clinical significance of HCV, molecular investigations of the virus have been hampered due to the lack of cell culture systems that efficiently support HCV replication, although a reproducible HCV proliferation system in cell culture has very recently been reported (18). In 1999, the situation changed for the better when a subgenomic HCV replicon cell culture system was introduced (19). The replicon RNA is composed of the HCV 5′-NTR containing an HCV IRES, a neomycin phosphotransferase (Neo) gene, and the HCV NS3 through NS5B under the control of an encephalomyocarditis virus (EMCV) IRES, followed by the HCV 3′-NTR. The Neo gene is expressed under the control of the HCV IRES, thereby inducing G-418 resistance to cells that contain replicon RNA. As the replicon RNA proliferates autonomously in cultured cells, this replicon system is thus considered to be a powerful tool for the analysis of molecular mechanisms underlying HCV replication and also for the screening of anti-HCV reagents (20). However, the subgenomic HCV replicon system may be insufficient because it lacks HCV structural proteins. A genome-length HCV RNA replication system may reflect the phenomenon that the HCV-infected human liver undergoes. To date, four genome-length HCV RNA replication systems, using N, Con-1, H77, and O strains, have so far been reported (21–24).

Clinical data suggest that the trace element metabolism is tightly linked to the pathogenesis of chronic hepatitis C (25, 26). We previously showed zinc supplementation to increase the therapeutic response of IFN-α for intractable chronic hepatitis C with genotype 1b (27, 28). However, it remains unclear as to whether or not zinc interferes with viral replication or the expression of viral proteins. We therefore examined the effect of zinc supplement on viral replication using HuH-7 cells harboring subgenomic HCV replicons (29) or genome-length HCV RNAs (24) derived from the HCV-O strain. We herein showed that zinc effectively suppressed the replication of genome-length HCV RNA but not that of the subgenomic HCV replicon.

Materials and methods

Cell culture systems

We incubated sO (previously described as 1B-2R1) cells (29) replicating the subgenomic HCV replicon and O cells (24) replicating the genome-length HCV RNA in a real-time reverse-transcriptase polymerase chain reaction (RT-PCR) analysis. The sequences of HCV RNA replicating in sO and O cells are derived from HCV RNA in non-neoplastic human hepatocytes PH5CH8 inoculated with HCV-O, and the basal replication levels of both O and sO cells were almost the same as those described previously (24, 29). In a luciferase reporter assay system, we incubated ORN/3-5B/KE cells supporting the subgenomic HCV replicon encoding the luciferase reporter gene, and ORN/C-5B/KE cells supporting genome-length HCV-RNA encoding the luciferase reporter gene (24). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Invitrogen Life Technology, Carlsbad, CA) supplemented with 10% fetal calf serum, penicillin, and streptomycin (complete DMEM) and maintained in the presence of G418 (300 μg/ml; Geneticin, Invitrogen). We passaged these cells twice a week at a 5:1 split ratio and used them within six to 10 passages for the experiments in this study.

Reagents

Iron sulfate ($FeSO_4$), iron chloride ($FeCl_3$), zinc sulfate ($ZnSO_4$), and zinc chloride ($ZnCl_2$) were purchased from Wako Pure Chemical Industries (Osaka, Japan). The purities of both reagents exceeded 99%. Purified human lymphoblast IFN-α (OIF) was kindly provided by the Otsuka Pharmaceutical Co. (Tokushima, Japan).

Cell viability

As it has been reported that the proliferation of the HCV subgenomic replicon is dependent on host-cell growth (30), we examined the cytotoxicities of $ZnSO_4$ and $ZnCl_2$ to sO or O cells. In brief, the cells were seeded at a density of 4×10^5 cells per dish onto dishes with a diameter of 95 mm. After a 24-h culture, the cells were treated with or without zinc salts at final concentrations of 50, 100, and 150 μM for 72 h in the absence of G418. Next, the number of viable cells was counted using an improved Neubauer-type hematocytometer after trypan blue dye (Invitrogen) treatment. The effect of zinc salts was calculated as a percentage of the number of control cells to which no reagent was added. All assays were conducted more than three times.

Quantification of HCV RNA by real-time RT-PCR

The subgenomic HCV replicon (29) and replicable genome-length HCV RNA (24) are both well known to be highly sensitive to IFN-α and

recently iron has been reported to suppress the subgenomic HCV replicon (31). To confirm that our subgenomic HCV replicon and genome-length HCV RNA replication system are useful for evaluating antiviral reagents, we examined the established inhibitory effects of IFN-α and iron on the replication of the subgenomic HCV replicon and genome-length HCV RNA using sO and O cells. Next, the effect of zinc salts on the replication of subgenomic HCV replicon and the genome-length HCV RNA was observed by real-time RT-PCR. In brief, sO or O cells seeded on six-well plates (1×10^5 cells per well) were treated with IFN-α, $FeSO_4$, $FeCl_3$, $ZnSO_4$, or $ZnCl_2$ at several concentrations. The total RNAs from cells were harvested at different time points using ISOGEN extraction kits (Nippon Gene Co., Tokyo, Japan) and subjected to a real-time RT-PCR analysis. The 5′-NTR of HCV genomic RNA was quantified using the ABI PRISM 7900 sequence detector (Applied Biosystems, Foster City, CA) as described previously (32), using the 5′-CGGGAG-AGCCATAGTGG-3′ (forward) and 5′-AGTACCACAAGGCCTTT CG-3′ (reverse) primers and the fluorogenic probe 5′-CTGCG-GAACCGGTGAGTACAC-3′. As an internal control, the level of human GAPDH mRNA was quantified using TaqMan hGAPDH reagents (Applied Biosystems). All experiments were conducted more than three times.

Western blot analysis

The cell lysates and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were prepared, and an immunoblotting analysis with a polyvinylidene difluoride membrane was performed as described previously (33). The antibodies used in this study were those against NS3 (Novocastra Laboratories, Newcastle, UK) and NS5B (a generous gift from M. Kohara, Tokyo Metropolitan Institute of Medical Science). Anti-β-actin antibody (Sigma-Aldrich, Tokyo, Japan) was also used to detect β-actin as the internal control. The immunocomplexes on the membranes were detected by an enhanced chemiluminescence assay (Amersham Co., Tokyo, Japan). Image scanning was analyzed using the Scion Image software program (Beta 4.0.2., Scion Corporation, NIH, Frederick, MD).

Luciferase reporter assay

To confirm the effect of zinc salts on the replication of HCV RNA by the different assay system, we performed the experiment while utilizing the luciferase reporter assay system using ORN/3-5B/KE cells and ORN/C-5B/KE cells with or without zinc salt. In brief, the cells were plated onto 24-well plates (1.5×10^4 cells per well) and cultured for 24 h. Next, the cells were treated with $ZnSO_4$ or $ZnCl_2$ at several concentrations for 24 h, and then the cells were subjected to the luciferase reporter assay using the *Renilla* luciferase assay system (Promega, Madison, MI) (24). Briefly, after removing the medium, the cells were washed twice with phosphate-buffered saline. The cells were extracted with 100 µl of *Renilla* lysis reagent, and the relative luciferase unit value in 10 µl of lysates was measured by adding 50 µl of *Renilla* luciferase assay reagent according to the manufacturer's protocol. Flash'n Glow LB 955 luminometer (EG&G Berthold, Bad Wildbad, Germany) was used to detect the luciferase activity.

Statistical analysis

All data were expressed as the mean ± standard deviation. The differences between groups were evaluated with Student's *t*-test or one-way analysis of variance $P<0.01$ was considered to be significant.

Results

Inhibitory effects of IFN-α on HCV RNA replication in sO and O cells

IFN-α efficiently inhibited the replication of the subgenomic HCV replicon and genome-length HCV RNA in a dose-dependent manner (Fig. 1). Based on the dose–response curve, the

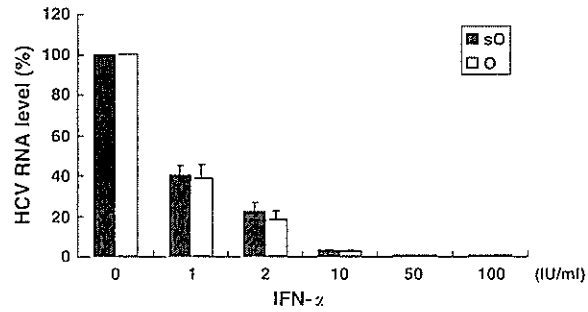

Fig. 1 Inhibition of hepatitis C virus (HCV) RNA replication in sO and O cells treated with interferon-α (IFN-α). IFN-α sensitivity of HCV RNA replication in sO (black bars) and O cells (white bars). Real-time reverse-transcriptase polymerase chain reaction was performed as described in the Materials and methods. sO and O cells were treated for 48 h with IFN-α (0, 1, 2, 10, 50, and 100 IU/ml). The replication level of HCV RNA of the respective non-treated cells was assigned as 100%. The replication level of HCV RNA was normalized to the respective GAPDH mRNA expression levels. The data indicate the mean ± SD of triplicates from three independent experiments.

concentrations of IFN-α required for a 50% reduction (IC_{50}) of the subgenomic HCV replicon and genome-length HCV RNA were calculated to be almost equal (0.7 IU/ml). These values were comparable to the previous findings obtained using another HCV-strain-derived subgenomic HCV replicon system (34) or an O-strain-derived HCV RNA replication system (35).

Inhibitory effects of iron salts on HCV RNA replication in sO and O cells

$FeSO_4$ or $FeCl_2$ significantly suppressed the replication of genome-length HCV RNA to the same extent as the subgenomic HCV replicon in a dose-dependent manner (Fig. 2). We demonstrated for the first time the inhibitory effect of iron via a genome-length HCV replication system. Both IFN-α and iron salts inhibited HCV RNA replication in sO and O cells in a dose-dependent manner, thus suggesting that our subgenomic HCV replicon and genome-length HCV RNA replication systems are useful for the evaluation of anti-HCV reagents.

Cytotoxicity of zinc salts to sO and O cells

Although 150 μM and higher of $ZnSO_4$ or $ZnCl_2$ was cytotoxic to sO and O cells, $ZnSO_4$ or $ZnCl_2$ at a concentration of 100 μM or lower had no significant cytotoxic effect on both cells in this assay (Fig. 3A and B). We therefore examined the inhibitory effects of zinc salts at a concentration of 100 μM or lower.

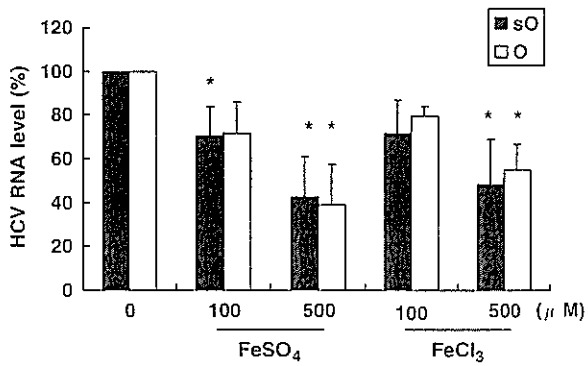

Fig. 2. Inhibition of hepatitis C virus (HCV) RNA replication in sO and O cells treated with iron. Iron inhibition of HCV RNA replication in sO (black bars) and O cells (white bars). sO and O cells were treated for 48 h with iron sulfate (100 and 500 μM) or iron chloride (100 and 500 μM). The control cells without iron salts (0 μM) were treated similarly. The quantification of HCV RNA was performed as described in Fig. 1. The data indicate the mean ± SD of triplicates from three independent experiments. The asterisk (*) indicates a significant inhibition of HCV RNA replication by iron sulfate or iron chloride ($P<0.01$).

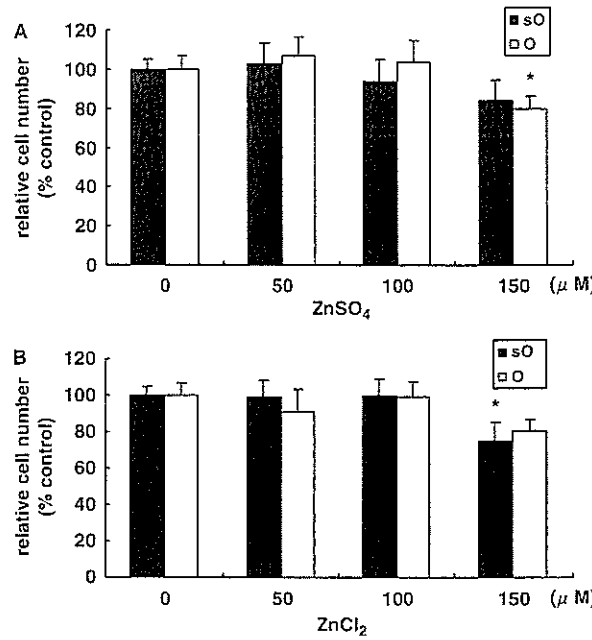

Fig. 3. Cytotoxicity of zinc salts to sO and O cells. (A) sO and O cells were cultured in the absence or presence of zinc sulfate (50, 100, and 150 μM each) for 72 h, and then the cell number was determined as described under the Materials and methods. The relative cell number (% control) calculated at each point, when the cell number of non-treated cells was assigned to be 100%, is presented herein. The data indicate the mean ± SD of three independent experiments. (B) sO and O cells were cultured in the absence or presence of zinc chloride as described in (A). The asterisk (*) indicates significant cytotoxicity by zinc sulfate or zinc chloride ($P<0.01$).

Different effects of zinc salts on the HCV RNA replication between sO and O cells

$ZnSO_4$ or $ZnCl_2$ significantly suppressed the genome-length HCV RNA replication in a dose-dependent manner. The IC_{50} values of $ZnSO_4$ and $ZnCl_2$ were calculated to be 76 and 89 μM, respectively. In contrast, only slight inhibitory effects on the subgenomic HCV replicon were observed in sO cells by 100 μM $ZnSO_4$ and $ZnCl_2$ (Fig. 4A and B). Zinc salts reduced the replication of the genome-length HCV RNA more markedly than that of the subgenomic HCV replicon. To determine whether the inhibitory effect of zinc on the genome-length HCV RNA replication is time dependent or not, O cells were incubated with 100 μM $ZnSO_4$ or $ZnCl_2$ and harvested at three different time points (24, 48, and 72 h) after treatment. The maximum inhibitory effect of zinc salts in O cells occurred at 48 h after treatment (Fig. 4C).

Effects of zinc salts on NS3 and NS5B protein expression

The expression levels of NS3 and NS5B proteins, which are the essential proteins for HCV RNA

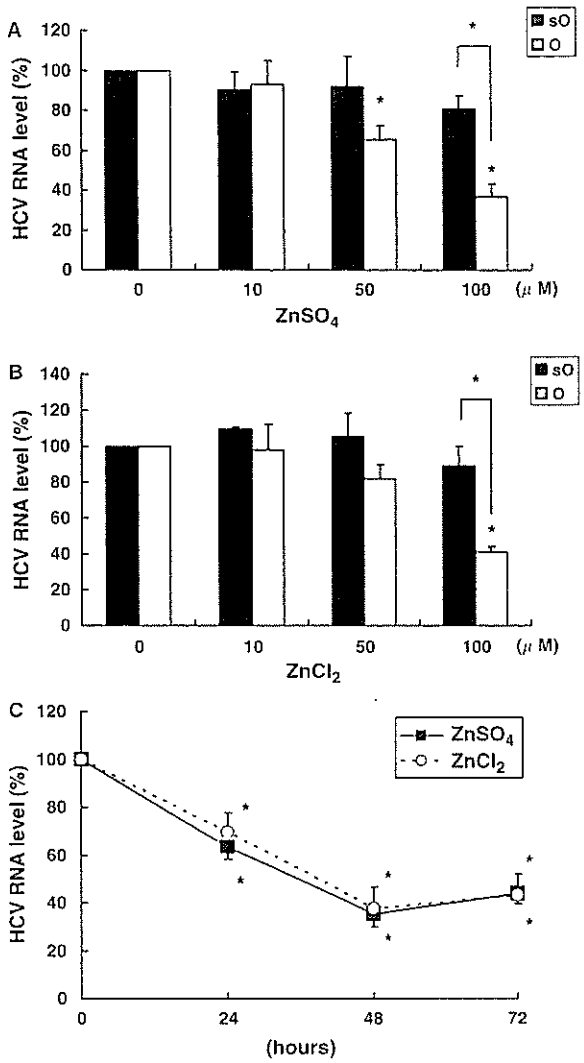

Fig. 4. Different effect of zinc salts between subgenomic hepatitis C virus (HCV) replicon and genome-length HCV RNA replication systems. (A) The sO and O cells were treated for 48 h with zinc sulfate (0, 10, 50, and 100 μM). The quantification of HCV RNA was performed as described in Fig. 1. (B) sO and O cells were treated for 48 h with zinc chloride (0, 10, 50, and 100 μM). The quantification of HCV RNA was performed as described in Fig. 1 (C) Time–response curve of zinc salts. O cells were treated with a fixed concentration (100 μM) of zinc sulfate or zinc chloride for 24, 48, and 72 h. Real-time reverse-transcriptase polymerase chain reaction was performed as described in the Materials and methods. We herein show the replication level of HCV RNA (%) calculated at each point, when the replication level of HCV RNA of the respective non-treated cells at 0 h was assigned as 100%. The replication level of HCV RNA was normalized by the level of GAPDH mRNA. The data indicate the mean ± SD of triplicates findings from three independent experiments. The asterisk (*) indicates a significant inhibition of HCV RNA replication by zinc sulfate or zinc chloride and a significant difference of the inhibitory effect of zinc salts between sO and O cells ($P<0.01$).

replication, did not decrease in the sO cells treated with $ZnSO_4$ or $ZnCl_2$ (100 μM), whereas the expression levels of NS3 and NS5B proteins were clearly decreased in the sO cells treated with $FeSO_4$, $FeCl_3$ (100 or 500 μM), or IFN-α (Fig. 5A and B). However, the expression levels of the NS3 and NS5B proteins both significantly decreased in the O cells treated with $ZnSO_4$ or $ZnCl_2$ (100 μM) as well as $FeSO_4$ or $FeCl_3$ (100 μM) (Fig. 5C and D). These results were consistent with those of the quantification analysis of HCV RNA as described above.

Anti-HCV activity of zinc salts on luciferase reporter assay system

Zinc salts significantly inhibited the *Renilla* luciferase activity in a dose-dependent manner but the extents of the suppressive effects were found to be rather weak depending on real-time RT-PCR (Fig. 6A and B). Zinc salts tend to reduce the replication of genome-length HCV RNA more markedly than that of the subgenomic HCV replicon even though the difference in chemical sensitivity to zinc salts was not significant.

Discussion

We demonstrated that zinc supplementation inhibited the replication of genome-length HCV RNA in O cells without causing cell toxicity, and the effects of zinc supplementation on HCV replication were significantly different between the genome-length HCV RNA replication system and the subgenomic HCV replicon system. On the other hand, IFN-α and iron supplementation suppressed the replication of HCV RNA almost equally between the subgenomic HCV replicon and genome-length HCV RNA replication system. However, other divalent cations, such as magnesium salts, did not suppress the replication of genome-length HCV RNA (data not shown). Therefore, the inhibition of the replication of HCV RNA is not an ubiquitous phenomenon caused by the divalent cations, but a specific phenomenon caused by certain divalent cations such as zinc and iron.

We showed the inhibitory effect of zinc salts in real-time RT-PCR and Western blotting on genome-length HCV RNA systems. In real-time RT-PCR, zinc inhibited the replication of HCV RNA as strongly as that of iron salts, whereas in Western blotting, the inhibitory effect of zinc salts was weaker than that of iron salts. There was a discrepancy in the inhibitory effects of zinc salts on RNA replication and protein expression in both systems. One possible reason is that zinc may affect the function of NS3 proteins of HCV through structural or NS2 proteins and consequently inhibit the replication of genome-length

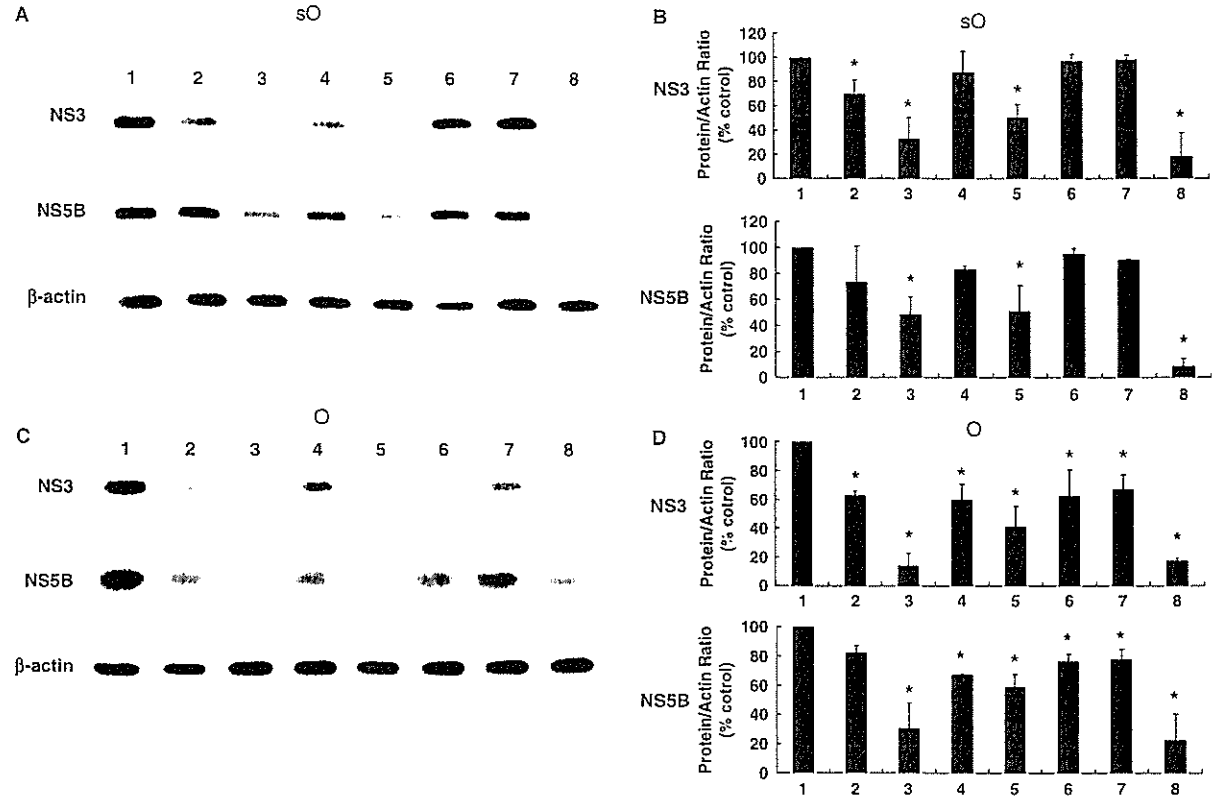

Fig. 5. Inhibitory effect of the expression of hepatitis C virus (HCV) proteins. (A) The sO cells were treated for 48 h without reagents (0 μM) as control wells (lane 1), or treated with 100 and 500 μM of iron sulfate (lanes 2 and 3), 100 and 500 μM of iron chloride (lanes 4 and 5), 100 μM zinc sulfate (lane 6), 100 μM zinc chloride (lane 7), or 20 IU/ml interferon-α (IFN-α) (lane 8). The production of NS3 and NS5B protein in sO cells was analyzed by immunoblotting using anti-NS3 and anti-NS5B antibodies, respectively. β-actin was used as an internal control for the amount of protein loaded per lane. (B) The quantification of NS3 and NS5B production by densitometry using Scion Image software in sO normalized for the respective β-actin and the density of non-treated cells was assigned as 100%. These results were based on three separate experiments performed using three separate sets of cells and expressed as the mean ± SD for Western blotting. The asterisk (*) indicates a significant inhibition of NS3 and NS5B production by reagents ($P<0.01$). (C) The treatment of O cells with reagents and a Western blot analysis for NS3 and NS5B production were performed as described in (A). (D) The quantification of NS3 and NS5B in O cells was performed as described in (B).

HCV RNA, because sO cells replicate the subgenomic HCV replicon RNA lacking the HCV core to the NS2 region. This hypothesis is supported by the findings that the expression levels of NS3 proteins seemed to decrease more than that of NS5B expression in the O cells-treated zinc salts as shown in Fig. 5C and D.

We showed that iron supplementation inhibited the HCV RNA replication in both systems almost equally. It has recently been reported that iron directly inactivates the RNA-dependent RNA polymerase activity of HCV, which is mediated by the viral NS5B, thus impairing the HCV replication using the subgenomic HCV replicon system (31). The iron compound-induced inhibitory effect of HCV RNA replication on genome-length HCV RNA system may be caused by NS5B, which is a common structure in both systems.

We could not confirm the inhibitory effect of zinc on other genome-length HCV RNA replication systems because we could not obtain any other cell lines. However, the previous reports that describe iron and not zinc to inhibit significantly the HCV RNA replication in another subgenomic HCV replicon system is consistent with the result of our subgenomic HCV replicon (31), and it is also consistent with our results. In the luciferase reporter system, we confirmed that the inhibitory effect of zinc salts in the genome-length HCV replication system was also observed in a dose-dependent manner. However, 100 μM zinc salts significantly inhibited the luciferase activity in the subgenomic HCV replicon, but less than that in the genome-length HCV RNA replication systems, in contrast to the results of real-time RT-PCR. The luciferase reporter assay system showed reproducible results but the extent of the inhibitory effect between the replication system of subgenomic HCV and that of genome-length HCV RNA was slightly different. In our results of real-time RT-PCR as shown in Fig. 4A

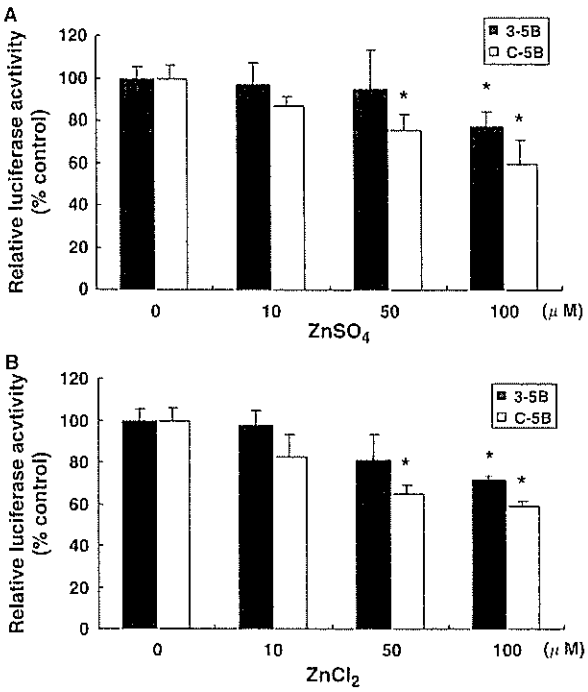

Fig. 6. Inhibitory effect of zinc of hepatitis C virus (HCV) RNA replication in ORN/3-5B/KE and ORN/C-5B/KE cells treated with zinc salts on a luciferase reporter assay system. (A) Inhibitory effect of zinc sulfate against HCV RNA replication in ORN/3-5B/KE (black bars) and ORN/C-5B/KE cells (white bars). A *Renilla* Luciferase reporter assay was performed as described in Materials and methods. ORN/3-5B/KE and ORN/C-5B/KE cells were treated for 24 h with zinc sulfate (0, 10, 50, and 100 μM), and the relative luciferase activity was calculated. The relative luciferase activity of respective non-treated cells was assigned to be 100%. The data indicate the mean ± SD of triplicate findings from three independent experiments. (B) The inhibitory effect of zinc chloride against HCV RNA replication in ORN/3-5B/KE (black bars) and ORN/C-5B/KE cells (white bars). A *Renilla* Luciferase reporter assay was performed as described in (A). The asterisk (*) indicates a significant inhibition of the *Renilla* luciferase activity by zinc sulfate or zinc chloride ($P < 0.01$).

and B, the HCV replication level in 100 μM zinc salts tended to be slightly lower than in either the control or 50 μM zinc salts. Therefore, this is probably due to a difference in the detection sensitivity of the real-time RT-PCR and luciferase reporter assay systems.

The subgenomic HCV replicon and the replicable genome-length HCV RNA in this study were highly sensitive to IFN-α as described previously (33, 34). Moreover, clinically, zinc supplementation increased the therapeutic response of IFN-α for intractable chronic hepatitis C (27, 28). However, zinc supplementation did not show the additional or synergistic inhibitory effect of IFN-α in a genome-length HCV RNA replication system (data not shown). The inhibitory effect of zinc on the replication of both replicon cells may be masked with that of IFN-α, because the inhibitory effect of IFN-α on the replication of both replicon cells is much more effective than that of zinc.

To date, four genome-length HCV RNA replication systems, using N, Con-1, H77, and O strains, have been reported (21–24). Genome-length HCV RNA replication, including the structural region of HCV RNA, closely mimics the *in vivo* situation within an HCV-infected hepatocyte. In this study, different degrees of chemical sensitivity were observed between the subgenomic HCV replicon system and genome-length HCV RNA replication system. This significant difference suggests that a useful investigation may have been overlooked in the subgenomic HCV replicon systems. Previous studies using subgenomic HCV replicon systems should therefore be re-examined using genome-length HCV RNA replication systems.

In conclusion, our study suggests that zinc may play an important role as a negative regulator of HCV replication in genome-length HCV RNA-replicating cells. Zinc supplementation appears to be a novel approach in the development of future strategies for the treatment of chronic hepatitis C. The mechanisms underlying the inhibitory effect of zinc on virus replication are presently being investigated in our laboratory.

Acknowledgements

This study was supported by a grant from the 21st century Center of Excellence (COE) in Biomedical Research Using Accelerator Technology, Gunma, Japan.

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Different Anti-HCV Profiles of Statins and Their Potential for Combination Therapy With Interferon

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We recently developed a genome-length hepatitis C virus (HCV) RNA replication system (OR6) with luciferase as a reporter. The OR6 assay system has enabled prompt and precise quantification of HCV RNA replication. Pegylated interferon (IFN) and ribavirin combination therapy is the world standard for chronic hepatitis C, but its effectiveness is limited to about 55% of patients. Newer therapeutic approaches are needed. In the present study, we used the OR6 assay system to evaluate the anti-HCV activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, called statins, and their effects in combination with IFN-α. Five types of statins (atorvastatin, fluvastatin, lovastatin, pravastatin, and simvastatin) were examined for their anti-HCV activities. Fluvastatin exhibited the strongest anti-HCV activity (IC_{50}: 0.9 μmol/L), whereas atorvastatin and simvastatin showed moderate inhibitory effects. However, lovastatin, reported recently as an inhibitor of HCV replication, was shown to exhibit the weakest anti-HCV activity. The anti-HCV activities of statins were reversed by the addition of mevalonate or geranylgeraniol. Surprisingly, however, pravastatin exhibited no anti-HCV activity, although it worked as an inhibitor for HMG-CoA reductase. The combination of IFN and the statins (except for pravastatin) exhibited strong inhibitory effects on HCV RNA replication. In combination with IFN, fluvastatin also exhibited a synergistic inhibitory effect. In conclusion, statins, especially fluvastatin, could be potentially useful as new anti-HCV reagents in combination with IFN. (HEPATOLOGY 2006;44:117-125.)

Persistent hepatitis C virus (HCV) infection causes liver fibrosis and hepatocellular carcinoma. Approximately 170 million people worldwide are infected with HCV. The combination of pegylated interferon (IFN) with ribavirin is the current standard therapy for chronic hepatitis C (CHC) and yields a sustained virological response (SVR) rate of about 55%.[1] This means that about 45% of patients with CH C are still threatened by the progression of the disease to cirrhosis and hepatocellular carcinoma. Until 1999, when Lohmann et al. developed the subgenomic replicon of HCV, it was difficult to screen anti-HCV reagents.[2] Many improvements followed that breakthrough, such as a genome-length HCV RNA replication system[3,4] and a subgenomic replicon with a reporter assay system[5]; more recently, Wakita et al. used a genotype 2a strain, JFH1, to produce the infectious virus in cell culture.[6-8]

Genotype 1 is the major genotype of HCV found in Japan, the United States, and many other countries. Unfortunately, the SVR rate after combination therapy of pegylated IFN with ribavirin is less than 50% for this genotype. To find a more effective therapy especially for CHC patients with genotype 1, we recently developed a genome-length HCV RNA (strain O of genotype 1b) replication reporter system (OR6), which has been an effective screening tool.[9,10]

Statins, which are 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, are in wide use for the treatment of hypercholesterolemia. Recently, it

Abbreviations: HCV, hepatitis C virus; IFN, interferon; CHC, chronic hepatitis C; SVR, sustained virological response; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; 2'-5'-OAS1, 2'-5'-oligoadenylate synthetase 1; LOV, lovastatin; ATV, atorvastatin; PRV, pravastatin; SMV, simvastatin; FLV, fluvastatin; RL, renilla luciferase; LST-1, human liver-specific organic anion transporter-1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; EMCV, encephalomyocarditis virus; IRES, internal ribosome entry site; AMPH-B, amphotericin B; RT-PCR, reverse-transcription polymerase chain reaction.

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Received November 17, 2005; accepted April 17, 2006.

Supported by a grant-in-aid for the third-term comprehensive 10-year strategy for cancer control and by a grant-in-aid for research on hepatitis, both from the Ministry of Health, Labor, and Welfare of Japan, and by the Program for the Promotion of Fundamental Studies in Health Science of the Pharmaceutical and Medical Device Agency (PMAD), Japan.

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Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.21232

Potential conflict of interest: Nothing to report.

was reported that lovastatin (LOV) inhibited HCV RNA replication.[11,12] These reports suggested the anti-HCV activity of LOV resulted from inhibition of the geranylgeranylation of cellular proteins rather than the inhibition of cholesterol synthesis.[11,12] More recently, FBL2 has been reported to be a host target protein for geranylgeranylation, which is responsible for HCV replication.[13] Although several types of statins are used clinically to lower cholesterol, thus far only LOV has been tested for anti-HCV activity. In the present study, we used the OR6 assay system to test the anti-HCV activity of five statins: atorvastatin (ATV), fluvastatin (FLV), pravastatin (PRV), simvastatin (SMV), and LOV. We found that ATV, FLV, and SMV exhibited stronger anti-HCV activity than that previously reported for LOV and that PRV exhibited no anti-HCV activity, although it worked as an inhibitor for HMG-CoA reductase. Because FLV showed the strongest anti-HCV activity, we also examined the effect of the combination of IFN-α and FLV on HCV RNA replication and found a synergistic inhibitory effect of IFN-α and FLV on HCV RNA replication.

Materials and Methods

Cell Cultures. OR6 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin, streptomycin, and G418 (300 μg/mL; Geneticin, Invitrogen) and passaged twice a week at a 5:1 split ratio. OR6c cells are cured OR6 cells from which genome-length HCV RNA was eliminated by IFN-α treatment (500 IU/mL for 2 weeks) without G418, as previously described.[9] HepG2 and PH5CH8 (human immortalized hepatocytes) cells were cultured as previously described.[14,15]

Luciferase Reporter Assay. For the renilla luciferase (RL) assay, 2×10^4 OR6 cells were plated in 24-well plates at least in triplicate for each assay and were cultured for 24 hours. The cells were treated with statins for 72 hours and were harvested with Renilla lysis reagent (Promega) and subjected to the RL assay according to the manufacturer's protocol.

Reagents. FLV, LOV, and PRV were purchased from Calbiochem. ATV and SMV were purchased from Astellas Pharma Inc. and Banyu Pharmaceutical Co. Ltd., respectively. Mevalonate, geranylgeraniol, and geranylgeranyl pyrophosphate were purchased from Sigma.

Reverse-Transcriptase Polymerase Chain Reaction. Total RNA from the cultured cells was extracted with the RNeasy Mini Kit (Qiagen). Reverse-transcriptase polymerase chain reaction (RT-PCR) for HMG-CoA reductase, human liver-specific organic anion transporter (LST-1), 2′-5′-oligoadenylate synthetase 1 (2′-5′-OAS1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed by a method described previously.[16] Briefly, using cellular total RNA (2 μg), cDNA was synthesized using Superscript II with oligo dT primer. One-tenth of the synthesized cDNA was subjected to polymerase chain reaction (PCR) with the following primer pairs: HMG-CoA reductase, 5′-ATGCCATCCCTGTTGGAGTG-3′ and 5′-TGTTCATCCCCATGGCATCCC-3′; LST-1, 5′-TGGCACACGTGGGTCATGTAGG-3′ and 5′-CACTATCTGCCCCAGCAGAAGG-3′, 2′-5′-OAS1, 5′-AGTACCTGAGAAGGCAGCTCACGA-3′ and 5′-ACTGGCATTCAGAGGATGGTGCAG-3′; and GAPDH, 5′-GACTCATGACCACAGTCCATGC-3′, and 5′-GAGGAGACCACCTGGTGCTCAG-3′.

Western Blot Analysis. Preparation of the cell lysate, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and immunoblotting were performed as previously described.[17] The antibodies used in this study were those against Core (Institute of Immunology, Tokyo), NS3 (Novocastra Laboratories, Newcastle upon Tyne, UK), NS5B (a generous gift from Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science), β-actin (Sigma, St. Louis, MO), STAT1 (BD Transduction Laboratories, San Diego, CA), and phospho-STAT1 (Y701; Cell Signaling Technology, Beverly, MA). Immunocomplexes on the membranes were detected by the enhanced chemiluminescence assay (Renaissance; Perkin Elmer Life Science, Wellesley, MA).

Plasmid Construction. The plasmids pORN/C-5B/KE (Fig. 1A) and pHCV-O were described previously.[9] To construct the pEMCV-RL, two fragments, the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) and the *RL* gene, were amplified by PCR from pORN/C-5B/KE using the following primer pairs: EMCV IRES, 5′-CGGGATCCGCGGGACTCGGGGGTTCG-3′ and 5′-CCGCTCGAGGGTATTATCGTGTTTTTCAAAGG-3′; and RL, 5′-CCGCTCGAGATGGCTTCCAAGGTGTACGACC-3′, and 5′-GCTCTAGACTAGACGTTGATCCTGGCGC-3′. The two fragments were ligated into the *Bam*HI and *Xba*I sites in pcDNA 3.1/Zeo (Invitrogen).

Statistical Analysis. Differences in anti-HCV activity between statins were tested using the Student t test. P values $< .05$ were considered statistically significant.

Results

Inhibition of HCV RNA Replication by Statins. We recently developed a genome-length HCV RNA (strain O of genotype 1b) replication system (OR6) with luciferase as a reporter.[9,10] This OR6 reporter assay system has enabled prompt and precise quantification of HCV

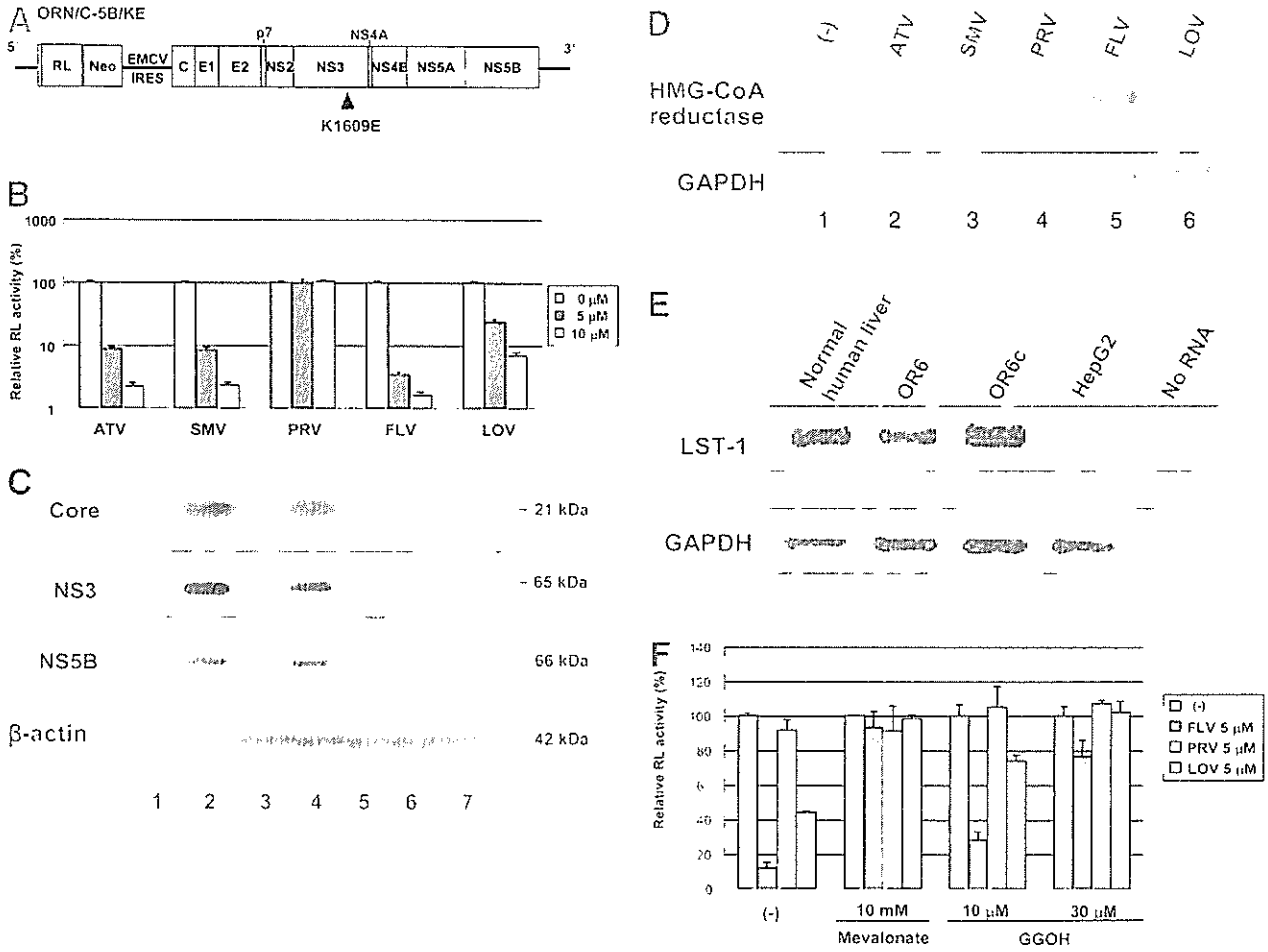

Fig. 1. Different inhibitory effects of various statins on HCV RNA replication in OR6 cells. (A) Schematic gene organization of genome-length HCV RNA encoding the *RL* gene, ORN/C-5B/KE, replicating in OR6 cells. RL is expressed as a fusion protein with Neo. The position of an adaptive mutation, K1609E, is indicated by a triangle. (B) Reporter assay on statin sensitivity of HCV RNA replication. OR6 cells were treated with ATV, SMV, PRV, FLV, and LOV (0, 5, and 10 μmol/L each). After 72 hours of treatment, the RL assay was performed as described in the Materials and Methods section. Shown here is the relative RL activity (%) calculated when the RL activity of untreated cells was assigned as 100%. The data indicate means ± SDs of triplicates from three independent experiments. (C) Western blot analysis of statin sensitivity of HCV RNA replication: lane 1, cured OR6 cells (OR6c) used as a negative control; lane 2, untreated OR6 cells; lane 3, OR6 cells treated with IFN-α (10 IU/mL); lane 4, OR6 cells treated with PRV (5 μmol/L); lane 5, OR6 cells treated with LOV (5 μmol/L); lane 6, OR6 cells treated with FLV (5 μmol/L); lane 7, OR6 cells treated with FLV (10 μmol/L). After 96 hours of treatment, the production of Core, NS3, and NS5B was analyzed by immunoblotting using anti-Core, anti-NS3, and anti-NS5B antibodies, respectively. β-actin was used as a control for the amount of protein loaded per lane. (D) Feedback induction of HMG-CoA reductase after treatment of statins: lane 1, untreated OR6c cells; lane 2, OR6c cells treated with ATV (10 μmol/L); lane 3, OR6c cells treated with SMV (10 μmol/L); lane 4, OR6c cells treated with PRV (10 μmol/L); lane 5, OR6c cells treated with FLV (10 μmol/L); lane 6, OR6c cells treated with LOV (10 μmol/L). After 24 hours of treatment, RT-PCR for HMG-CoA reductase was performed as described in the Materials and Methods section. RT-PCR for GAPDH was performed as an internal control. RT-PCR products (376 bp for HMG-CoA reductase and 334 bp for GAPDH) were detected by staining with ethidium bromide after 3% agarose gel electrophoresis. (E) RT-PCR analysis of LST-1 mRNA. Total RNA was extracted from OR6, OR6c, and HepG2 cells and subjected to RT-PCR analysis with primer sets for LST-1 (241 bp) and GAPDH (334 bp) as described in (D). Normal human liver total RNA (Clontech) was used as a positive control for LST-1. (F) Mevalonate and geranylgeraniol restore HCV RNA replication in statin-treated cells. OR6 cells were treated with FLV, PRV, and LOV (5 μmol/L each) alone or in the presence of mevalonate (10 mmol/L) or geranylgeraniol (GGOH, 10 and 30 μmol/L). After 72 hours of treatment, the RL assay was performed as described in (B).

RNA replication. Therefore, in the present study, we examined whether several types of statins currently used in clinical therapy exhibit anti-HCV activity, as has already been reported for LOV.[11,12] This time, the antiviral activities of five statins—ATV, FLV, LOV, PRV, and SMV—were tested using the OR6 assay system (Fig. 1A). The results revealed that ATV, FLV, and SMV exhibited stronger anti-HCV activity than did LOV and that FLV exhibited the strongest anti-HCV activity among the statins tested (Fig. 1B). Surprisingly, however, PRV had no inhibitory effect on HCV RNA replication (Fig. 1B). Similar results were obtained from the analysis of the ex-

pression levels of HCV proteins (Fig. 1C). The anti-HCV activity of 5 μmol/L FLV was compatible with that of 10 IU/mL IFN-α (Fig. 1C).

To exclude the possibility that only PRV was unable to inhibit HMG-CoA reductase, we examined the expression of HMG-CoA reductase in statin-treated OR6c cells by RT-PCR, because HMG-CoA reductase was known to show positive feedback when statins were active in the cells.[18] OR6c cells were treated with statins in the same way as were the OR6 cells used to measure the anti-HCV activity of statins, except that sampling for RT-PCR was performed after 24 hours of statin treatment. All statins, including PRV, enhanced expression of the HMG-CoA reductase gene (Fig. 1D). Although PRV is hydrophilic and does not cross cellular membranes passively, it has been reported that a human liver–specific organic anion transporter, LST-1, mediates the uptake of PRV in human hepatocytes but not in HepG2 cells, which showed very low PRV uptake.[19,20] Therefore, we examined the expression levels of LST-1 in OR6 and OR6c cells using an RT-PCR method. OR6 and OR6c cells expressed LST-1 at levels equivalent to that in normal human liver, confirming that LST-1 was not expressed in the HepG2 cells (Fig. 1E). These findings suggest that PRV is taken up actively in OR6 and OR6c cells. In summary, these results indicate all statins tested inhibit HMG-CoA reductase and suggest the anti-HCV action of statins is not a result of direct inhibition of HMG-CoA reductase.

Regarding the mechanism underlying the anti-HCV action of statins, it has thus far been reported that the inhibitory effect of LOV can be overcome by the addition of mevalonate (the product of the HMG-CoA reductase reaction) or geranylgeraniol (a donor of prenyl groups for protein geranylgeranyl transferase reaction).[11,12] These observations suggest that some geranylgeranylated proteins are required for HCV RNA replication and that LOV blocks HCV RNA replication by depleting endogenous geranylgeranyl pyrophosphate (the mevalonate-derived donor of protein geranylgeranylation). To evaluate this mechanism, we examined the effects of mevalonate and geranylgeraniol on the anti-HCV activities of the statins used in this study. OR6 cells were treated with 5 μmol/L FLV, PRV, or LOV alone or in the presence of mevalonate (10 mmol/L) or geranylgeraniol (10 or 30 μmol/L). Mevalonate and geranylgeraniol restored HCV RNA replication in the statin-treated cells, although 10 μmol/L geranylgeraniol exhibited partial restoration (Fig. 1F). In addition, we observed that the anti-HCV activities of the statins could be blocked by the addition of geranylgeranyl pyrophosphate (20 μmol/L) in the OR6 cells (data not shown), indicating geranylgeranyl pyrophosphate is also taken up in OR6 cells. These findings support two previous reports[11,12] that found the inhibition of HCV RNA replication by statins was not correlated with their cholesterol-lowering activities, although the reason for the lack of anti-HCV activity by PRV remains vague.

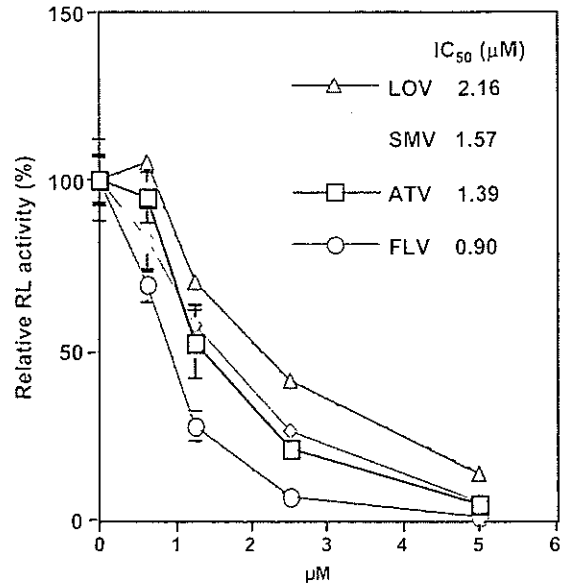

Fig. 2. Dose-dependent inhibition of HCV RNA replication by statins. OR6 cells were treated with LOV, SMV, ATV, and FLV at concentrations of 0.625, 1.25, 2.5, and 5 μmol/L. After 72 hours of treatment, the RL assay was performed, and RL activity was calculated as shown in Fig. 1B.

Anti-HCV Activity of FLV Significantly Stronger Than Those of Other Statins. From the dose-response curves after 72 hours of treatment with the statins, the concentrations of FLV, ATV, SMV, and LOV required for a 50% reduction in RL activity (IC_{50}) were calculated to be 0.90, 1.39, 1.57, and 2.16 μmol/L, respectively (Fig. 2). Consistent with the results shown in Fig. 1, the anti-HCV activity of FLV ($P < .01$ at 0.625-5 μmol/L), ATV ($P < .05$ at 1.25 μmol/L; $P < .01$ at 2.5 and 5 μmol/L), and SMV ($P < .05$ at 0.625 and 1.25 μmol/L; $P < .01$ at 2.5 and 5 μmol/L) was significantly stronger than that previously reported for LOV. In addition, the anti-HCV activity of FLV was significantly stronger than those of SMV ($P < .01$ at 1.25-5 μmol/L) and ATV ($P < .05$ at 1.25 μmol/L; $P < .01$ at 2.5 and 5 μmol/L).

Anti-HCV Activity of Statins Not Due to Their Cytotoxicity. Since it has been reported that the proliferation of the HCV replicon is dependent on host-cell growth,[21] it remained to be clarified whether the inhibitory effects of statins on HCV RNA replication were caused by their cytotoxicity. To examine this possibility, we investigated the cytotoxic effects of statins on OR6 cells. A comparison of cell viability in the untreated cells with that in the cells treated with each statin (5 μmol/L each) showed no significant decrease in the number of

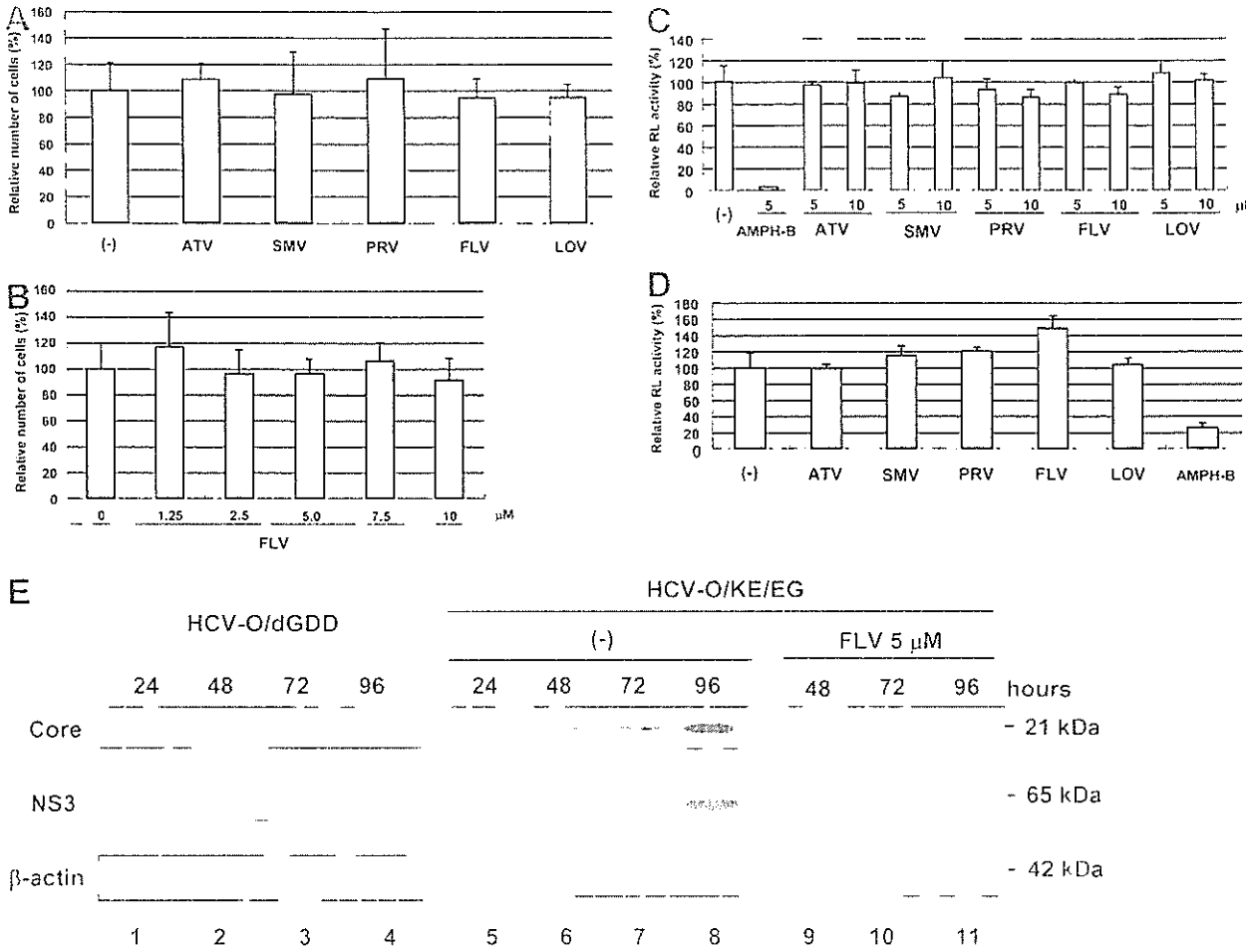

Fig. 3. Anti-HCV activity of the statins was not a result of inhibition of cell growth, RL activity, or EMCV IRES activity. (A) Cell viability after treatment with the statins. OR6 cells were cultured in the absence and in the presence of ATV, SMV, PRV, FLV, and LOV (5 μmol/L each), and then the number of viable cells was counted after trypan blue dye treatment. Shown here is the relative cell number (%) calculated when the cell number of untreated cells was assigned as 100%. The data indicate means ± SDs of triplicates from two independent experiments. (B) Cell viability after treatment with FLV. OR6 cells were cultured in the absence and in the presence of FLV (1.25, 2.5, 5, 7.5, and 10 μmol/L) for 72 hours, and then the number of viable cells was counted as described in (A). (C) No inhibition of RL activity by the statins. ATV, SMV, PRV, FLV, and LOV (5 or 10 μmol/L each) were directly added to the cell lysates from OR6 cells, and then the RL assay was performed. The relative RL activity was calculated as shown in Fig. 1B. AMPH-B (5 μmol/L) was used as a control reagent, which directly affected RL activity. (D) No inhibition of EMCV IRES activity by the statins. After pEMCV-RL was introduced into the OR6c cells, the cells were treated with ATV, SMV, PRV, FLV, and LOV (5 μmol/L each). After 72 hours of treatment, the RL assay was performed. Relative RL activity was calculated as shown in Fig. 1B. AMPH-B (2.5 μmol/L) was used as a control reagent, which directly affected RL activity. (E) Replication of authentic HCV RNA prevented by the statins. Authentic HCV-O RNA, HCV-O/KE/EG RNA, was introduced into the OR6c cells by electroporation as described previously.[3] After 24 (lane 5), 48 (lane 6), 72 (lane 7), and 96 (lane 8) hours of electroporation, production of Core and NS3 was analyzed by immunoblotting using anti-Core and anti-NS3 antibodies. Production of Core and NS3 after 24 (lane 1), 48 (lane 2), 72 (lane 3), and 96 (lane 4) hours of electroporation of HCV-O/dGDD RNA (negative control) was also analyzed. After 24 hours of electroporation of HCV-O/KE/EG RNA, the cells were treated with FLV (5 μmol/L), and then production of Core and NS3 was analyzed after 48 (lane 9), 72 (lane 10), and 96 (lane 11) hours of electroporation. β-actin was used as a control for the amount of protein loaded per lane.

cells following treatment with statins (Fig. 3A). However, it was recently reported that statins inhibited the proliferation of hepatocellular carcinoma cell lines (HuH-7 and HepG2) by inducing apoptosis and G1/S cell-cycle arrest.[22] Because that study found the IC_{50} of FLV in HuH-7 cells to be 10 ± 3 μmol/L, we further examined the effects of FLV on the proliferation of OR6 cells by varying the concentration (up to 10 μmol/L) of FLV. FLV (at least at concentrations ≤ 10 μmol/L) did not inhibit the proliferation of OR6 cells (Fig. 3B), suggesting that FLV does not induce apoptosis or G1/S cell-cycle arrest in OR6 cells, although the OR6 cell line is a HuH-7-derived cell line.[10] In summary, these results indicate that none of the statins showed any cytotoxicity to the OR6 cells at the concentrations used in our assay system. This suggests the statins possess the