Saeed,M., Gondeau,C., Hmwe,S., Yokokawa,H., Date,T., Suzuki,T., Kato,T., Maurel,P., and Wakita,T.	Replication of hepatitis C virus genotype 3a in cultured cells.	Gastroenterology	144 (1)	56-58	2013
Sakamoto N	NX-PVKA assay, a conventional but refined prognostic biomarker for Hepatocellular carcinoma.	J Gastroenterol Hepatol			2013 in press
Takayama K., Kawabata K., Nagamoto Y., Kishimoto K., Tashiro K., Sakurai F., Tachibana M., Kanda K.,Hayakawa T., Furue MK., Mizuguchi H.	3D spheroid culture of hESC/hiPSC-derived hepatocyte-like cells for drug toxicity testing.	Biomaterials	34	1781-1789	2013

Ⅳ．研究成果の刊行物・別冊

Liver INTERNATIONAL
OFFICIAL JOURNAL OF THE INTERNATIONAL ASSOCIATION FOR THE STUDY OF THE LIVER

Liver International ISSN 1478-3223

BASIC STUDIES

Amino acid substitutions of hepatitis C virus core protein are not associated with intracellular antiviral response to interferon-α *in vitro*

Fusao Ikeda[1,2,3], Hiromichi Dansako[1], Go Nishimura[1], Kyoko Mori[1], Yoshinari Kawai[1,2], Yasuo Ariumi[1], Yasuhiro Miyake[2,3], Akinobu Takaki[2], Kazuhiro Nouso[2,3], Yoshiaki Iwasaki[2], Masanori Ikeda[1], Nobuyuki Kato[1] and Kazuhide Yamamoto[2,3]

1 Department of Tumor Virology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan
2 Department of Gastroenterology and Hepatology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan
3 Department of Molecular Hepatology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

Keywords
antiviral activity – HCV core – hepatitis C virus – interferon

Correspondence
Fusao Ikeda, MD, Department of Gastroenterology and Hepatology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, 2-5-1, Shikata-cho, Okayama 700-8558, Japan
Tel: +81 86 235 7219
Fax: +81 86 225 5991
e-mail: fikeda@md.okayama-u.ac.jp

Received 12 March 2010
Accepted 23 May 2010

DOI:10.1111/j.1478-3231.2010.02299.x

Abstract

Background: Studies on patients with hepatitis C virus (HCV) of genotype 1b have suggested that amino acids (aa) 70 and/or 91 of the HCV core protein affect the outcome of interferon (IFN)-α and ribavirin (RBV) therapy, although there are no clear supporting data *in vitro*. *Aims:* This study was designed to determine the differences among the antiviral activities of HCV core proteins with various substitutions at aa70 and/or aa91. *Methods:* The retroviral vectors expressing the HCV core proteins with substitutions of arginine/leucine, arginine/methionine, glutamine/leucine or glutamine/methionine at aa70/aa91 were transiently transfected or stably transducted into an immortalized hepatocyte line (PH5CH8), hepatoma cell lines and an HCV-RNA replicating cell line (sOR) to evaluate antiviral responses to IFN-α or IFN-α/RBV. Sequence analysis was performed using genome-length HCV-RNA replicating cells (OR6 and AH1) to evaluate HCV core mutations during IFN-α treatment. *Results:* The promoter activity levels of IFN-stimulated genes in the transiently transfected cells or the mRNA levels of 2′-5′-oligoadenylate synthetase in the stably transducted PH5CH8 cells were not associated with the HCV core aa70 and/or aa91 substitutions during IFN-α treatment. Antiviral responses to IFN-α or IFN-α/RBV treatment were enhanced in sOR cells stably transducted with the HCV core, although there were no differences in antiviral responses among the cells expressing different core types. Sequence analysis showed no aa mutations after IFN-α treatment. *Conclusions:* Antiviral activities were enhanced by HCV core transduction, but they were not associated with the HCV core aa70 and/or aa91 substitutions by *in vitro* analysis.

Hepatitis C virus (HCV) infection causes chronic hepatitis, and may progress to cirrhosis and hepatocellular carcinoma. More than 170 million people worldwide are infected with HCV, creating a serious global health problem (1, 2). Interferon (IFN)-α is widely used in the treatment of patients with chronic hepatitis C, and the current combination treatment with pegylated IFN-α and ribavirin (RBV) has improved the sustained virological response, and has a success rate of more than 50% (3). Despite this therapeutic success rate, however, there are still non-viral responders (NVR) to IFN-α treatment. High viral load and genotype 1 of HCV are major viral causes of IFN-α resistance. For patients with HCV genotype 1, variations in the amino acid (aa) sequence of the IFN sensitivity-determining region (ISDR) (4) and IFN/RBV resistance-determining region (IRRDR) (5) in the non-structural 5A region have also been reported as important predictors of therapeutic outcomes.

Recent studies on the virological features of HCV patients that are most predictive of NVR to IFN-α/RBV therapy (6, 7) proposed that HCV core protein aa70 and/or aa91 substitutions were independent and significant factors for therapeutic outcomes. In particular, substitutions of arginine by glutamine at aa70 and/or of leucine by methionine at aa91 were common in NVR. Patients with the HCV core aa70 substitutions often had a slow or no decrease in HCV-RNA levels during the early phase of IFN-α treatment (6–9). A previous report evaluating HCV dynamics during IFN-α therapy described a biphasic kinetic pattern of HCV-RNA decline, and the viral

© 2010 John Wiley & Sons A/S

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 2010, p. 3179–3186 Vol. 54, No. 8
0066-4804/10/$12.00 doi:10.1128/AAC.00113-10
Copyright © 2010, American Society for Microbiology. All Rights Reserved.

Inhibition of Hepatitis C Virus Replication by a Specific Inhibitor of Serine-Arginine-Rich Protein Kinase▽

Yuko Karakama,[1]§ Naoya Sakamoto,[1,2]§* Yasuhiro Itsui,[1,5] Mina Nakagawa,[1,2] Megumi Tasaka-Fujita,[1] Yuki Nishimura-Sakurai,[1] Sei Kakinuma,[1,2] Masaya Oooka,[3] Seishin Azuma,[1] Kiichiro Tsuchiya,[1] Hiroshi Onogi,[4] Masatoshi Hagiwara,[4] and Mamoru Watanabe[1]

Department of Gastroenterology and Hepatology[1] and Department for Hepatitis Control,[2] Tokyo Medical and Dental University, Tokyo, Japan; Department of General Medicine, Tokyo Medical and Dental University, Tokyo, Japan[3]; Department of Functional Genomics, Medical Research Institute, Tokyo Medical and Dental University, Tokyo, Japan[4]; and Department of Internal Medicine, Soka Municipal Hospital, Saitama, Japan[5]

Received 26 January 2010/Returned for modification 9 March 2010/Accepted 11 May 2010

Splicing of messenger RNAs is regulated by site-specific binding of members of the serine-arginine-rich (SR) protein family, and SR protein kinases (SRPK) 1 and 2 regulate overall activity of the SR proteins by phosphorylation of their RS domains. We have reported that specifically designed SRPK inhibitors suppressed effectively several DNA and RNA viruses *in vitro* and *in vivo*. Here, we show that an SRPK inhibitor, SRPIN340, suppressed in a dose-dependent fashion expression of a hepatitis C virus (HCV) subgenomic replicon and replication of the HCV-JFH1 clone *in vitro*. The inhibitory effects were not associated with antiproliferative or nonspecific cytotoxic effects on the host cells. Overexpression of SRPK1 or SRPK2 resulted in augmentation of HCV replication, while small interfering RNA (siRNA) knockdown of the SRPKs suppressed HCV replication significantly. Immunocytochemistry showed that SRPKs and the HCV core and NS5A proteins colocalized to some extent in the perinuclear area. Our results demonstrate that SRPKs are host factors essential for HCV replication and that functional inhibitors of these kinases may constitute a new class of antiviral agents against HCV infection.

Hepatitis C virus (HCV) infects up to 170 million people worldwide, and these infections frequently are characterized by chronic liver inflammation, leading to decompensated liver cirrhosis and hepatocellular cancers (1). Alpha and beta interferons are the mainstay of HCV therapeutics. However, the most effective pegylated interferon plus ribavirin combination therapies can eliminate HCV from around half of the patients only (6). These difficulties in eradicating HCV are compounded by the limited treatment options. For this reason, the development of safe and effective therapeutic agents against HCV has been a strong motivation in academia and industry (23).

Serine-arginine-rich (SR) proteins are a family of non-small nuclear ribonucleoprotein particle (non-snRNP) splicing factors that are highly conserved throughout the eukaryotes. They harbor one or two RNA recognition motifs and an RS domain at the amino and carboxyl termini, respectively (29). RS domains consist of multiple consecutive Arg-Ser/Ser-Arg dipeptide repeats, in which the Ser residues are extensively phosphorylated by several kinases, including SR protein kinases (SRPKs). SRPK1 was the first SR protein kinase to be cloned, on the basis of its ability to phosphorylate SR proteins *in vitro* (8, 9), and two other structurally related kinases, SRPK2 and SRPK3, also have been shown to phosphorylate SR proteins (16, 31). Although the precise physiological role of this phosphorylation remains unknown, it is expected that phosphorylation of SR proteins affects their protein-protein and protein-RNA interactions, intracellular localization and trafficking, and alternative splicing of pre-mRNA (21).

As SRPK-dependent herpes simplex virus (HSV) splicing and SRPK-mediated phosphorylation of hepatitis B virus (HBV) core protein have been reported (4, 25, 33), it is reasonable to expect that SR proteins and SRPK might be suitable targets for therapeutic modulation of various viral infections. Actually, we found that increased activity of SRPK2 upregulated human immunodeficiency virus (HIV) expression and that an isonicotinamide compound, SRPIN340, which preferentially inhibited SRPK1 and SRPK2, suppressed propagation of Sindbis virus, HIV, and cytomegalovirus (7). In this study, we investigated the effects of SRPIN340 on HCV replication using the HCV subgenomic replicon system (27, 32) and HCV-JFH1 virus cell culture (30, 34). Here, we demonstrate that cellular SRPK is required for HCV replication and suggest that the inhibitor of SRPK could be used therapeutically.

MATERIALS AND METHODS

SRPK inhibitor. SRPIN340, *N*-[2-(1-piperidinyl)-5-(trifluoromethyl)phenyl] isonicotinamide, inhibits SRPK1 and SRPK2 kinase activities potently (7). SRPIN340 does not inhibit other classes of SRPKs significantly, including Clk1 and Clk and other classes of SR kinases. SRPIN614, *N*-methyl-*N*-[2-(1-piperidinyl)-5-(trifluoromethyl)phenyl]isonicotinamide, is a negative-control compound that has no suppressive effects on SRPK1 or SRPK2. SRPIN340 and SRPIN614 were synthesized in-house (7).

***In vitro* kinase assay.** Kinase activities of SRPKs were assayed as described previously (18). Briefly, His_6-tagged recombinant SRPK1 or SRPK2 was expressed in *Escherichia coli* and purified by Ni-nitrilotriacetic acid (NTA) affinity chromatography. The purified SRPK1 or SRPK2 was incubated in the presence of ATP, [γ-^{32}P]ATP, and a synthetic peptide of the SF2/ASF RS domain (NH_2-RSPSYGRSRSRSRSRSRSRSNSRSRSY-OH) at pH 7.5 and 30°C for 10 min. The reaction mixtures were spotted onto phosphocellulose membranes (What-

* Corresponding author. Mailing address: Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. Phone: 81 3-5803-5877. Fax: 81 3-5803-0268. E-mail: nsakamoto.gast@tmd.ac.jp.
§ Y.K. and N.S. contributed equally to this work.
▽ Published ahead of print on 24 May 2010.

MAJOR ARTICLE

Hepatitis B Virus Replication Could Enhance Regulatory T Cell Activity by Producing Soluble Heat Shock Protein 60 From Hepatocytes

Yasuteru Kondo,[1] Yoshiyuki Ueno,[1] Koju Kobayashi,[2] Eiji Kakazu,[1] Masaaki Shiina,[1] Jun Inoue,[1] Keiichi Tamai,[1] Yuta Wakui,[1] Yasuhito Tanaka,[5] Masashi Ninomiya,[1] Noriyuki Obara,[1] Koji Fukushima,[1] Motoyasu Ishii,[3] Tomoo Kobayashi,[4] Hirofumi Niitsuma,[1] Satonori Kon,[2] and Tooru Shimosegawa[1]

[1]Division of Gastroenterology, Tohoku University Hospital, [2]School of Health Science, Tohoku University, [3]Department of Gastroenterology, Miyagi Social Insurance Hospital, and [4]Department of Gastroenterology, Tohoku Rosai Hospital, Sendai, and [5]Clinical Molecular Informative Medicine, Nagoya City University, Nagoya, Japan

Background. HBcAg-specific regulatory T (T_{reg}) cells play an important role in the pathogenesis of chronic hepatitis B. Soluble heat shock proteins, especially soluble heat shock protein 60 (sHSP60), could affect the function of T_{reg} cells via Toll-like receptor.

Methods. We analyzed the relationship between soluble heat shock protein production and hepatitis B virus (HBV) replication with both clinical samples from HBeAg-positive patients with chronic hepatitis B ($n = 24$) and HBeAb-positive patients with chronic hepatitis B ($n = 24$) and in vitro HBV-replicating hepatocytes. Thereafter, we examined the biological effects of sHSP60 with isolated T_{reg} cells.

Results. The serum levels of sHSP60 in patients with chronic hepatitis B were statistically significantly higher than those in patients with chronic hepatitis C ($P < .01$), and the levels of sHSP60 were correlated with the HBV DNA levels ($R = 0.532$; $P < .001$) but not with the alanine aminotransferase levels. Moreover, the levels of sHSP60 in HBV-replicating HepG2 cells were statistically significantly higher than those in control HepG2 cells. Preincubation of $CD4^+$ $CD25^+$ cells with recombinant HSP60 (1 ng/mL) statistically significantly increased the frequency of HBcAg-specific interleukin 10–secreting T_{reg} cells. The frequency of $IL7R^-CD4^+CD25^+$ cells, the expression of Toll-like receptor 2, and the suppressive function of T_{reg} cells had declined during entecavir treatment.

Conclusion. The function of HBcAg-specific T_{reg} cells was enhanced by sHSP60 produced from HBV-infected hepatocytes. Entecavir treatment suppressed the frequency and function of T_{reg} cells; this might contribute to the persistence of HBV infection.

Hepatitis B virus (HBV) is a noncytopathic DNA virus that causes chronic hepatitis and hepatocellular carcinoma as well as acute hepatitis and fulminant hepatitis [1]. HBV now affects more than 400 million people worldwide [2], and persistent infection develops in ~5% of adults and 95% of neonates who become infected with HBV.

It has been shown that the cellular immune system, including cytotoxic T lymphocytes, $CD4^+$ T helper 1 cells, and $CD4^+C25^+FoxP3^+$ regulatory T (T_{reg}) cells, plays a central role in the control of viral infection [3–6]. The hyporesponsiveness of HBV-specific T helper 1 cells and the excessive regulatory function of T_{reg} cells in peripheral blood in patients with chronic hepatitis B has been shown elsewhere [7–10]. Lamivudine treatment of chronic hepatitis B has been reported to restore both $CD4^+$ T cells and cytotoxic T lymphocyte hyporesponsiveness following the decrease of serum levels of HBV DNA and HBV-derived Ag [8, 11–13]. In our previous study, we observed that HBcAg-specific interleukin 10 (IL-10)–secreting T_{reg} cells could play an important role in the immunopathogenesis of chronic hepatitis B [9].

Received 14 July 2009; accepted 3 February 2010; electronically published 9 June 2010.

Potential conflicts of interest: none reported.

Financial support: Ministry of Health, Labor, and Welfare of Japan (Health and Labor Sciences Research Grants for the Research on Measures for Intractable Diseases); Ministry of Education, Culture, Sports, Science, and Technology of Japan (grant 21790642 to Y.K.).

Reprints or correspondence: Yoshiyuki Ueno, Division of Gastroenterology, Tohoku University Graduate School of Medicine, Seiryo 1-1, Aobaku, Sendai, 980-8574, Japan (yueno@mail.tains.tohoku.ac.jp).

The Journal of Infectious Diseases 2010; 202(2):202–213
© 2010 by the Infectious Diseases Society of America. All rights reserved.
0022-1899/2010/20202-0004$15.00
DOI: 10.1086/653496

JOURNAL OF VIROLOGY, Sept. 2010, p. 9118–9127 Vol. 84, No. 18
0022-538X/10/$12.00 doi:10.1128/JVI.00402-10
Copyright © 2010, American Society for Microbiology. All Rights Reserved.

A Disulfide-Bonded Dimer of the Core Protein of Hepatitis C Virus Is Important for Virus-Like Particle Production▽†

Yukihiro Kushima,[1,2] Takaji Wakita,[3] and Makoto Hijikata[1,2]*

Department of Viral Oncology, Institute for Virus Research, Kyoto University, Kyoto 606-8507, Japan[1]; Graduate School of Biostudies, Kyoto University, Kyoto 606-8507, Japan[2]; and Department of Virology II, National Institute of Infectious Diseases, Tokyo 162-8640, Japan[3]

Received 24 February 2010/Accepted 20 June 2010

Hepatitis C virus (HCV) core protein forms the nucleocapsid of the HCV particle. Although many functions of core protein have been reported, how the HCV particle is assembled is not well understood. Here we show that the nucleocapsid-like particle of HCV is composed of a disulfide-bonded core protein complex (dbc-complex). We also found that the disulfide-bonded dimer of the core protein (dbd-core) is formed at the endoplasmic reticulum (ER), where the core protein is initially produced and processed. Mutational analysis revealed that the cysteine residue at amino acid position 128 (Cys128) of the core protein, a highly conserved residue among almost all reported isolates, is responsible for dbd-core formation and virus-like particle production but has no effect on the replication of the HCV RNA genome or the several known functions of the core protein, including RNA binding ability and localization to the lipid droplet. The Cys128 mutant core protein showed a dominant negative effect in terms of HCV-like particle production. These results suggest that this disulfide bond is critical for the HCV virion. We also obtained the results that the dbc-complex in the nucleocapsid-like structure was sensitive to proteinase K but not trypsin digestion, suggesting that the capsid is built up of a tightly packed structure of the core protein, with its amino (N)-terminal arginine-rich region being concealed inside.

Hepatitis C virus (HCV) infection is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma, affecting approximately 200 million people worldwide (13, 29, 44). Current treatment strategies, including interferon coupled with ribavirin, are not effective for all patients infected with HCV. An error-prone replication strategy allows HCV to undergo rapid mutational evolution in response to immune pressure and thus evade adaptive immune responses (10). New approaches to HCV therapy include the development of specifically targeted antiviral therapies for hepatitis C (STAT-Cs) which target such HCV proteins as the nonstructural 3/4A (NS3/4A), serine protease, and RNA-dependent RNA polymerase NS5B proteins (3). Despite the potent antiviral activities of some of these approaches, many resistant HCV strains have been reported after treatment with existing STAT-Cs (23, 48, 51). Therefore, identification of new targets that are common to all HCV strains and that are associated with low mutation rates is an area of active research.

HCV has a 9.6-kb, plus-strand RNA genome composed of a 5′ untranslated region (UTR), an open reading frame that encodes a single polyprotein of about 3,000 amino acids, and a 3′ UTR. The polyprotein is processed by host and viral proteases to produce three structural proteins (the core, envelope 1 [E1], and E2 proteins) and seven nonstructural proteins (the p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B proteins) (14, 16, 17, 22, 49). The HCV core protein is produced cotranslationally via carboxyl (C)-terminal cleavage to generate an immature core protein, 191 amino acids in length, on the endoplasmic reticulum (ER) (16). This protein consists of three predicted domains: the N-terminal hydrophilic domain (D1), the C-terminal hydrophobic domain (D2), and the tail domain (33), which serves as a signal peptide for the E1 protein. D1 includes a number of positively charged amino acids responsible for viral RNA binding (amino acids 1 to 75) (43) and the region involved in multimerization of the core protein via homotypic interactions (amino acids 36 to 91 and 82 to 102) (32, 40) (see Fig. S1 in the supplemental material). Hydrophobic D2 includes the region responsible for core protein association with lipid droplets (LDs; amino acids 125 to 144) (7, 18, 37), which accumulate in response to core protein production (1, 6).

Many functions of the core protein have been reported (13, 38, 50), yet because infectious HCV particles cannot be appropriately produced in currently available experimental systems, HCV particle assembly has not been elucidated to date. A cell culture system that reproduces the complete life cycle of HCV *in vitro* was developed by Wakita et al. using a cloned HCV genome (JFH1) (53). Using this system, the assembly of infectious HCV particles was found to occur near LDs and ER-derived LD-associated membranes (36, 47). Neither the structures nor the functions of the virus proteins involved in virus particle assembly are known, however. To elucidate this point, we have analyzed the biochemical characteristics of the proteins within the fraction containing the HCV particle and found a disulfide-bonded core protein complex (dbc-complex). We revealed that the disulfide-bonded dimer of core protein (dbd-core) was formed by a single cysteine residue at amino

* Corresponding author. Mailing address: Department of Viral Oncology, Institute for Virus Research, Kyoto University, 53 Kawaharacho Shougoin, Kyoto 606-8507, Japan. Phone: 81-75-751-4046. Fax: 81-75-751-3998. E-mail: mhijikat@virus.kyoto-u.ac.jp.

† Supplemental material for this article may be found at http://jvi.asm.org/.

▽ Published ahead of print on 30 June 2010.

Involvement of PA28γ in the Propagation of Hepatitis C Virus

Kohji Moriishi,[1] Ikuo Shoji,[2] Yoshio Mori,[1] Ryosuke Suzuki,[3] Tetsuro Suzuki,[3] Chikako Kataoka,[1] and Yoshiharu Matsuura[1]

We have reported previously that the proteasome activator PA28γ participates not only in degradation of hepatitis C virus (HCV) core protein in the nucleus but also in the pathogenesis in transgenic mice expressing HCV core protein. However, the biological significance of PA28γ in the propagation of HCV has not been clarified. PA28γ is an activator of proteasome responsible for ubiquitin-independent degradation of substrates in the nucleus. In the present study, knockdown of PA28γ in cells preinfection or postinfection with the JFH-1 strain of HCV impaired viral particle production but exhibited no effect on viral RNA replication. The particle production of HCV in PA28γ knockdown cells was restored by the expression of an small interfering RNA (siRNA)-resistant PA28γ. Although viral proteins were detected in the cytoplasm of cells infected with HCV, suppression of PA28γ expression induced accumulation of HCV core protein in the nucleus. HCV core protein was also degraded in the cytoplasm after ubiquitination by an E3 ubiquitin ligase, E6AP. Knockdown of PA28γ enhanced ubiquitination of core protein and impaired virus production, whereas that of E6AP reduced ubiquitination of core protein and enhanced virus production. Furthermore, virus production in the PA28γ knockdown cells was restored through knockdown of E6AP or expression of the siRNA-resistant wild-type but not mutant PA28γ incapable of activating proteasome activity. *Conclusion:* Our results suggest that PA28γ participates not only in the pathogenesis but also in the propagation of HCV by regulating the degradation of the core protein in both a ubiquitin-dependent and ubiquitin-independent manner. (HEPATOLOGY 2010;52:411-420)

Over 170 million individuals worldwide are infected with hepatitis C virus (HCV), which is a major etiological agent of liver diseases, including hepatic steatosis, cirrhosis, and hepatocellular carcinoma (HCC).[1] HCV is classified into the genus Hepacivirus of the *Flaviviridae* family and has a positive, single-strand RNA genome that encodes a single polyprotein consisting of about 3,000 amino acids.[2] The N-terminal one-third of the polyprotein is occupied by the structural proteins, and the remaining portion consists of nonstructural proteins involved in viral replication and assembly. Host and viral proteases cleave the appropriate sites of the polyprotein, resulting in generation of at least 10 viral proteins. The capsid (core), E1 and E2 proteins, and p7 are cleaved off by signal peptidase from the polyprotein. Furthermore, the C-terminal signal sequence of the core protein is processed by signal peptide peptidase.[3] Our recent data indicate that signal peptide peptidase cleaves the polyprotein between Phe[177] and Leu[178] in the signal sequence, and this processing is required for HCV propagation.[4] The mature core proteins make nucleocapsid with viral RNA, and HCV particles bud into the lumen of the endoplasmic reticulum bearing E1 and E2 glycoproteins on the host lipid components, and are released from the host cells.

Several reports suggest that HCV core protein plays an important role in the development of various outcomes of liver failure, including steatosis and HCC.[5,6]

Abbreviations: HA, hemagglutinin; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; JEV, Japanese encephalitis virus; moi, multiplicity of infection; shRNA, short hairpin RNA; siRNA, small interfering RNA.

From the [1]Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan; the [2]Division of Microbiology, Kobe University Graduate School of Medicine, Hyogo, Japan; and the [3]Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan.

Received February 3, 2010; accepted March 13, 2010.

Supported in part by grants-in-aid from the Ministry of Health, Labor, and Welfare; the Ministry of Education, Culture, Sports, Science, and Technology; the Osaka University Global Center of Excellence Program; and the Foundation for Biomedical Research and Innovation.

Potential conflict of interest: Nothing to report.

Address reprint requests to: Yoshiharu Matsuura, D.V.M., Ph.D.,Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamada-oka, Suita, Osaka 565-0871, Japan. E-mail: matsuura@biken.osaka-u.ac.jp; fax: (81)-6-6879-8269.

Copyright © 2010 by the American Association for the Study of Liver Diseases.

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

DOI 10.1002/hep.23680

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

Hepatology Research

Hepatology Research 2010; **40**: 1248–1253 doi: 10.1111/j.1872-034X.2010.00730.x

Short Communication

Gene expression profile of Li23, a new human hepatoma cell line that enables robust hepatitis C virus replication: Comparison with HuH-7 and other hepatic cell lines

Kyoko Mori,* Masanori Ikeda, Yasuo Ariumi and Nobuyuki Kato*

Department of Tumor Virology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

Aim: Human hepatoma cell line HuH-7-derived cells are currently the only cell culture system used for robust hepatitis C virus (HCV) replication. We recently found a new human hepatoma cell line, Li23, that enables robust HCV replication. Although both cell lines had similar liver-specific expression profiles, the overall profile of Li23 seemed to differ considerably from that of HuH-7. To understand this difference, the expression profile of Li23 cells was further characterized by a comparison with that of HuH-7 cells.

Methods: cDNA microarray analysis using Li23 and HuH-7 cells was performed. Li23-derived ORL8c cells and HuH-7-derived RSc cells, in which HCV could infect and efficiently replicate, were also used for the microarray analysis. For the comparative analysis by reverse transcription polymerase chain reaction (RT–PCR), human hepatoma cell lines (HuH-6, HepG2, HLE, HLF and PLC/PRF/5) and immortalized hepatocyte cell line (PH5CH8) were also used.

Results: Microarray analysis of Li23 versus HuH-7 cells selected 80 probes to represent highly expressed genes that have ratios of more than 30 (Li23/HuH-7) or 20 (HuH-7/Li23). Among them, 17 known genes were picked up for further analysis. The expression levels of most of these genes in Li23 and HuH-7 cells were retained in ORL8c and RSc cells, respectively. Comparative analysis by RT–PCR using several other hepatic cell lines resulted in the classification of 17 genes into three types, and identified three genes showing Li23-specific expression profiles.

Conclusion: Li23 is a new hepatoma cell line whose expression profile is distinct from those of frequently used hepatic cell lines.

Key words: hepatitis C virus, hepatoma cell line, HuH-7, Li23, microarray

INTRODUCTION

HUH-7, A HUMAN hepatoma cell line,[1] is frequently used in the research of hepatitis C virus (HCV), since an HCV replicon system enabling HCV subgenomic RNA replication was developed using HuH-7 cells.[2] Even with the use of an efficient HCV production system developed in 2005,[3] HuH-7-derived cells are still used as the only cell line for persistent HCV production systems.

Correspondence: Professor Nobuyuki Kato, Department of Tumor Virology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama 700-8558, Japan. Email: nkato@md.okayama-u.ac.jp

*These authors contributed equally to this work.

Received 21 July 2010; revision 16 August 2010; accepted 17 August 2010.

We previously developed HCV replicon systems[4,5] and an HCV production system[6] using HuH-7-derived cells. Furthermore, we recently found a new human hepatoma cell line, Li23, that enables robust HCV RNA replication and persistent HCV production.[7] In that study, using microarray analysis, we excluded the possibility that the obtained Li23-derived cells were derived from contamination of HuH-7-derived cells used for HCV replication.[7] In addition, we noticed that the gene expression profile of Li23 cells seemed considerably different from that of HuH-7 cells. Therefore, we assumed that the Li23 cell line possesses a unique expression profile among widely used human hepatoma cell lines. To evaluate this assumption, we further characterized the expression profile of Li23 cells by comparing it with those of other human hepatoma cell lines, including HuH-7,[1] HuH-6,[8] HepG2,[9] HLE,[10] HLF[10] and PLC/PRF/5.[11] Human immortalized hepatocyte cell line

© 2010 The Japan Society of Hepatology

J Gastroenterol
DOI 10.1007/s00535-009-0195-7

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Mutations in the interferon sensitivity determining region and virological response to combination therapy with pegylated-interferon alpha 2b plus ribavirin in patients with chronic hepatitis C-1b infection

Mina Nakagawa · Naoya Sakamoto · Mayumi Ueyama · Kaoru Mogushi · Satoshi Nagaie · Yasuhiro Itsui · Seishin Azuma · Sei Kakinuma · Hiroshi Tanaka · Nobuyuki Enomoto · Mamoru Watanabe

Received: 1 June 2009 / Accepted: 11 December 2009
© Springer 2010

Abstract
Background Pegylated-interferon-alpha 2b (PEG-IFN) plus ribavirin (RBV) therapy is currently the de-facto standard treatment for hepatitis C virus (HCV) infection. The aims of this study were to analyze the clinical and virological factors associated with a higher rate of response in patients with HCV genotype 1b infection treated with combination therapy.
Methods We analyzed, retrospectively, 239 patients with chronic hepatitis C-1b infection who received 48 weeks of combination therapy. We assessed clinical and laboratory parameters, including age, gender, pretreatment hemoglobin, platelet counts, HCV RNA titer, liver histology, the number of interferon sensitivity determining region (ISDR) mutations and substitutions of the core amino acids 70 and 91. Drug adherence was monitored in each patient. We carried out univariate and multivariate statistical analyses of these parameters and clinical responses.
Results On an intention-to-treat (ITT) analysis, 98 of the 239 patients (41%) had sustained virological responses (SVRs). Patients with more than two mutations in the ISDR had significantly higher SVR rates ($P < 0.01$). Univariate analyses showed that stage of fibrosis, hemoglobin, platelet counts, ISDR mutations, serum HCV RNA level, and adherence to PEG-IFN plus RBV were significantly correlated with SVR rates. Multivariate analysis in subjects with good drug adherence extracted the number of ISDR mutations (two or more: odds ratio [OR] 5.181).
Conclusions The number of mutations in the ISDR sequence of HCV-1b (≥ 2) is the most effective parameter predicting a favorable clinical outcome of 48-week PEG-IFN plus RBV therapy in patients with HCV genotype 1b infection.

Keywords Hepatitis C virus (HCV) · Chronic hepatitis C · PEG-IFN plus RBV therapy · Combination therapy · Interferon sensitivity determining region (ISDR)

Abbreviations

HCV	Hepatitis C virus
IFN	Interferon
PEG	Polyethylene glycol
PEG-IFN	Pegylated-interferon-alpha 2b
RBV	Ribavirin
ISDR	Interferon sensitivity determining region
BMI	Body mass index

M. Nakagawa and N. Sakamoto contributed equally to this work.

M. Nakagawa · N. Sakamoto (✉) · M. Ueyama · Y. Itsui · S. Azuma · S. Kakinuma · M. Watanabe
Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan
e-mail: nsakamoto.gast@tmd.ac.jp

M. Nakagawa · N. Sakamoto · S. Kakinuma
Department for Hepatitis Control, Tokyo Medical and Dental University, Tokyo, Japan

K. Mogushi · S. Nagaie · H. Tanaka
Information Center for Medical Science, Tokyo Medical and Dental University, Tokyo, Japan

Y. Itsui
Department of Internal Medicine, Soka Municipal Hospital, Saitama, Japan

N. Enomoto
First Department of Internal Medicine, University of Yamanashi, Yamanashi, Japan

Published online: 30 January 2010

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i2.184

World J Gastroenterol 2010 January 14; 16(2): 184-192
ISSN 1007-9327 (print)
© 2010 Baishideng. All rights reserved.

ORIGINAL ARTICLE

An antioxidant resveratrol significantly enhanced replication of hepatitis C virus

Mitsuyasu Nakamura, Hidetsugu Saito, Masanori Ikeda, Ryota Hokari, Nobuyuki Kato, Toshifumi Hibi, Soichiro Miura

Mitsuyasu Nakamura, Ryota Hokari, Soichiro Miura, Department of Internal Medicine 2, National Defense Medical Collage, 3-2 Namiki, Tokorozawa, Saitama 3598513, Japan
Mitsuyasu Nakamura, Hidetsugu Saito, Toshifumi Hibi, Department of Internal Medicine, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 1608582, Japan
Masanori Ikeda, Nobuyuki Kato, Department of Molecular Biology, Okayama University Graduate School of Medicine and Dentistry, 2-5-1 Shikada-cho, Okayama 7008558, Japan
Author contributions: Nakamura M performed the majority of experiments; Ikeda M and Kato N provided reagents and were involved in editing the manuscript; Hokari R, Hibi T and Miura S provided financial support for this work; Saito H designed the study, provided financial support and wrote the manuscript.
Supported by The grant from the Japanese Ministry of Education and Science
Correspondence to: Hidetsugu Saito, MD, PhD, Department of Internal Medicine, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 1608582, Japan. hsaito@sc.itc.keio.ac.jp
Telephone: +81-3-33531211 Fax: +81-3-33518705
Received: September 25, 2009 Revised: November 5, 2009
Accepted: November 12, 2009
Published online: January 14, 2010

Abstract

AIM: To elucidate the effect of antioxidants, resveratrol (RVT) and astaxanthin (AXN), on hepatitis C virus (HCV) replication.

METHODS: We investigated the effect of recent popular antioxidant supplements on replication of the HCV replicon system OR6. RVT is a strong antioxidant and a kind of polyphenol that inhibits replication of various viruses. AXN is also a strong antioxidant. The replication of HCV RNA was assessed by the luciferase reporter assay. An additive effect of antioxidants on antiviral effects of interferon (IFN) and ribavirin (RBV) was investigated.

RESULTS: This is the first report to investigate the effect of RVT and AXN on HCV replication. In contrast to other reported viruses, RVT significantly enhanced HCV RNA replication. Vitamin E also enhanced HCV RNA replication as reported previously, although AXN didnot affect replication. IFN and RBV significantly reduced HCV RNA replication, but these effects were dose-dependently hampered and attenuated by the addition of RVT. AXN didnot affect antiviral effects of IFN or RBV.

CONCLUSION: These results suggested that RVT is not suitable as an antioxidant therapy for chronic hepatitis C.

© 2010 Baishideng. All rights reserved.

Key words: Replicon system; Luciferase assay; Ribavirin; Interferon; Polyphenol; Astaxanthin

Peer reviewer: Yutaka Yata, MD, PhD, Director, Department of Gastroenterology, Saiseikai Maebashi Hospital, 564-1 Kamishinden-machi, Maebashi-city, Gunma 371-0821, Japan

Nakamura M, Saito H, Ikeda M, Hokari R, Kato N, Hibi T, Miura S. An antioxidant resveratrol significantly enhanced replication of hepatitis C virus. *World J Gastroenterol* 2010; 16(2): 184-192 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i2/184.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i2.184

INTRODUCTION

Chronic liver disease develops in over 70% of those infected with hepatitis C virus (HCV), and HCV is now the most common cause of liver cirrhosis and also hepatocellular carcinoma (HCC), especially in Japan. It has been said that the median time for progression to cirrhosis is 30-40 years, but other factors such as male gender, the age at infection, co-infection with hepatitis

Arch Virol (2010) 155:601–605
DOI 10.1007/s00705-010-0624-1

BRIEF REPORT

Hydroxyurea as an inhibitor of hepatitis C virus RNA replication

Akito Nozaki · Manabu Morimoto · Masaaki Kondo · Takashi Oshima · Kazushi Numata · Shin Fujisawa · Takeshi Kaneko · Eiji Miyajima · Satoshi Morita · Kyoko Mori · Masanori Ikeda · Nobuyuki Kato · Katsuaki Tanaka

Received: 13 October 2009 / Accepted: 29 December 2009 / Published online: 5 March 2010
© Springer-Verlag 2010

Abstract Hepatitis C virus (HCV) is the main causative agent of chronic liver disease, which may develop into liver cirrhosis and hepatocellular carcinoma. By using a recently developed reporter assay system in which genome-length HCV RNA replicates efficiently, we found that hydroxyurea (HU), a DNA synthesis inhibitor, inhibited HCV RNA replication. Moreover, we demonstrated that the anti-HCV activity of the combination of IFN-alpha and HU was higher than that of IFN-alpha alone. These results suggest that HU may be an effective anti-HCV reagent that can be used not only singly but also in combination with IFN-alpha to treat chronic hepatitis C.

Introduction

Hepatitis C virus (HCV) is the main causative agent of chronic hepatitis C (CHC), which can develop into liver cirrhosis and hepatocellular carcinoma [4, 12, 19]. HCV infection is a global health problem, with over 170 million people being infected with the virus [24]. HCV genotype 1 is the major genotype found in Japan, the United States, and many other countries. Unfortunately, less than 50% of the patients infected with HCV of this genotype respond to the standard combination therapy of pegylated interferon (IFN) and ribavirin [6, 15]. In order to develop a more effective therapy especially for these patients, 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, 17]. By using this

A. Nozaki (✉) · M. Morimoto · M. Kondo · T. Oshima · K. Numata · K. Tanaka
Gastroenterological Center, Yokohama City University Medical Center, 4-57, Urafune-cho, Minami-ku, Yokohama 232-0024, Japan
e-mail: akino@yokohama-cu.ac.jp

A. Nozaki
Department of Transfusion Medicine, Yokohama City University Medical Center, 4-57, Urafune-cho, Minami-ku, Yokohama 232-0024, Japan

S. Fujisawa
Department of Hematology, Yokohama City University Medical Center, 4-57, Urafune-cho, Minami-ku, Yokohama 232-0024, Japan

T. Kaneko
Respiratory Disease Center, Yokohama City University Medical Center, 4-57, Urafune-cho, Minami-ku, Yokohama 232-0024, Japan

E. Miyajima
Department of Laboratory Medicine and Clinical Investigation, Yokohama City University Medical Center, 4-57, Urafune-cho, Minami-ku, Yokohama 232-0024, Japan

S. Morita
Department of Biostatistics and Epidemiology, Yokohama City University Medical Center, 4-57, Urafune-cho, Minami-ku, Yokohama 232-0024, Japan

K. Mori · M. Ikeda · N. Kato
Department of Tumor Virology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama 700-8558, Japan

J Gastroenterol (2010) 45:523–536
DOI 10.1007/s00535-009-0162-3

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Comparison of HCV-associated gene expression and cell signaling pathways in cells with or without HCV replicon and in replicon-cured cells

Yuki Nishimura-Sakurai · Naoya Sakamoto · Kaoru Mogushi · Satoshi Nagaie · Mina Nakagawa · Yasuhiro Itsui · Megumi Tasaka-Fujita · Yuko Onuki-Karakama · Goki Suda · Kako Mishima · Machi Yamamoto · Mayumi Ueyama · Yusuke Funaoka · Takako Watanabe · Seishin Azuma · Yuko Sekine-Osajima · Sei Kakinuma · Kiichiro Tsuchiya · Nobuyuki Enomoto · Hiroshi Tanaka · Mamoru Watanabe

Received: 2 September 2009 / Accepted: 2 November 2009 / Published online: 12 December 2009
© Springer 2009

Abstract

Background Hepatitis C virus (HCV) replication is affected by several host factors. Here, we screened host genes and molecular pathways that are involved in HCV replication by comprehensive analyses using two genotypes of HCV replicon-expressing cells, their *cured* cells and naïve Huh7 cells.

Methods Huh7 cell lines that stably expressed HCV genotype 1b or 2a replicon were used. The *cured* cells were established by treating HCV replicon cells with interferon-alpha. Expression of 54,675 cellular genes was analyzed by GeneChip DNA microarray. The data were analyzed by using the KEGG Pathway database.

Results Hierarchical clustering analysis showed that the gene-expression profiles of each cell group constituted clear clusters of naïve, HCV replicon-expressed, and cured cell lines. The pathway process analysis between the replicon-expressing and the *cured* cell lines identified significantly altered pathways, including MAPK, steroid biosynthesis and TGF-beta signaling pathways, suggesting that these pathways were affected directly by HCV replication. Comparison of *cured* and naïve Huh7 cells identified pathways, including steroid biosynthesis and sphingolipid metabolism, suggesting that these pathways were required for efficient HCV replication. Cytoplasmic lipid droplets were obviously increased in replicon-expressing and *cured* cells as compared to naïve cells. HCV replication was significantly suppressed by peroxisome proliferator-activated receptor (PPAR)-alpha agonists but augmented by PPAR-gamma agonists.

Conclusion Comprehensive gene expression and pathway analyses show that lipid biosynthesis pathways are crucial to support proficient virus replication. These metabolic pathways could constitute novel antiviral targets against HCV.

Keywords DNA microarray · KEGG database · HCV replicon · Lipid metabolism

Y. Nishimura-Sakurai and N. Sakamoto contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s00535-009-0162-3) contains supplementary material, which is available to authorized users.

Y. Nishimura-Sakurai · N. Sakamoto (✉) · M. Nakagawa · Y. Itsui · M. Tasaka-Fujita · Y. Onuki-Karakama · G. Suda · K. Mishima · M. Yamamoto · M. Ueyama · Y. Funaoka · T. Watanabe · S. Azuma · Y. Sekine-Osajima · S. Kakinuma · K. Tsuchiya · M. Watanabe
Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan
e-mail: nsakamoto.gast@tmd.ac.jp

N. Sakamoto · M. Nakagawa · S. Kakinuma
Department for Hepatitis Control, Tokyo Medical and Dental University, Tokyo, Japan

K. Mogushi · S. Nagaie · H. Tanaka
Information Center for Medical Science, Tokyo Medical and Dental University, Tokyo, Japan

Y. Itsui
Department of Internal Medicine, Soka Municipal Hospital, Saitama, Japan

N. Enomoto
First Department of Internal Medicine, University of Yamanashi, Yamanashi, Japan

Abbreviations

HCV	Hepatitis C virus
TLR	Toll-like receptor
BMP	Bone morphogenetic protein

Hepatology Research

Hepatology Research 2010; 40: 1063–1071 doi: 10.1111/j.1872-034X.2010.00741.x

Original Article

ITPA gene variant protects against anemia induced by pegylated interferon-α and ribavirin therapy for Japanese patients with chronic hepatitis C

Naoya Sakamoto,[1,2]* Yasuhito Tanaka,[3]* Mina Nakagawa,[1,2] Hiroshi Yatsuhashi,[4] Shuhei Nishiguchi,[5] Nobuyuki Enomoto,[6] Seishin Azuma,[1] Yuki Nishimura-Sakurai,[1] Sei Kakinuma,[1,2] Nao Nishida,[7] Katsushi Tokunaga,[7] Masao Honda,[8] Kiyoaki Ito,[9] Masashi Mizokami[9] and Mamoru Watanabe[1]

[1]*Department of Gastroenterology and Hepatology,* [2]*Department for Hepatitis Control, Tokyo Medical and Dental University, Tokyo,* [3]*Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Nagoya,* [4]*Clinical Research Center, National Nagasaki Medical Center, Nagasaki,* [5]*Department of Internal Medicine, Hyogo College of Medicine,* [6]*First Department of Internal Medicine, University of Yamanashi, Yamanashi,* [7]*Department of Human Genetics, Graduate School of Medicine, The University of Tokyo, Tokyo,* [8]*Department of Gastroenterology, Kanazawa University Graduate School of Medicine, Kanazawa,* [9]*Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Ichikawa, Japan*

Aim: Host genetic variants leading to inosine triphosphatase (ITPA) deficiency, a condition not thought to be clinically important, protect against hemolytic anemia in chronic hepatitis C patients receiving ribavirin. In this study, we evaluated the clinical significance of *ITPA* variants in Japanese hepatitis C patients who were treated with pegylated interferon plus ribavirin.

Methods: In this multicenter retrospective cross-sectional study, 474 hepatitis C patients were enrolled who were treated with pegylated interferon plus ribavirin in four geographically different hospitals in Japan. Patients were grouped according to hemoglobin decline of more than 3 g/dL at week 4. Two single nucleotide polymorphisms (SNP) within or adjacent to the *ITPA* gene (rs6051702, rs1127354) were genotyped.

Results: A functional SNP, rs1127354, within the *ITPA* exon was strongly associated with protection against anemia with only one (0.8%) in 129 patients with the *ITPA* minor variant A developing severe anemia ($P = 5.9 \times 10^{-20}$). For rs6051702, which had significant association in European-Americans, significant but weak association with severe hemoglobin reduction was found in Japanese ($P = 0.009$). In patients excluding genotype 1b and high viral load, those with the *ITPA* minor variant A achieved significantly higher sustained viral response rate than those with the major variant (CC) (96% vs 70%, respectively, $P = 0.0066$).

Conclusion: *ITPA* SNP, rs1127354, is confirmed to be a useful predictor of ribavirin-induced anemia in Japanese patients. Patients with the *ITPA* minor variant A (~27%) have an advantage in pegylated interferon plus ribavirin-based therapies, due to expected adherence of ribavirin doses, resulting in a higher viral clearance rate.

Key words: *c20orf194*, hemolytic anemia, hepatitis C virus, *ITPA* (inosine triphosphatase), pegylated interferon plus ribavirin therapy

INTRODUCTION

APPROXIMATELY 3% OF the worldwide population is infected with the hepatitis C virus (HCV), which represents 170 million people, with 3–4 million individuals newly infected each year. Chronic hepatitis C (CHC) has a variable course; although 20–25% of CHC patients maintain persistently normal serum aminotransferases and experience relatively slow histological progression, other patients present a more active biochemical course.[1–3] Overall, 30% of the CHC patients progress to cirrhosis in their lifetime,[3] and 3–8% of cirrhosis patients develop hepatocellular carcinoma (HCC) every year.[4–6] Among various factors, older age and hepatic steatosis are significant factors accelerating the rate of progression in CHC.[3,7–9]

Correspondence: Professor Masashi Mizokami, Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, 1-7-1 Konodai, Ichikawa 272-8516, Japan. Email: mmizokami@hospk.ncgm.go.jp

*These authors contributed equally to this study.

Received 28 June 2010; revision 26 August 2010; accepted 20 September 2010.

© 2010 The Japan Society of Hepatology

Virology 407 (2010) 80–90

Contents lists available at ScienceDirect

Virology

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

IL-6-mediated intersubgenotypic variation of interferon sensitivity in hepatitis C virus genotype 2a/2b chimeric clones

Goki Suda [a,1], Naoya Sakamoto [a,b,*,1], Yasuhiro Itsui [a,d], Mina Nakagawa [a,b], Megumi Tasaka-Fujita [a], Yusuke Funaoka [a], Takako Watanabe [a], Sayuri Nitta [a], Kei Kiyohashi [a], Seishin Azuma [a], Sei Kakinuma [a], Kiichiro Tsuchiya [a], Michio Imamura [c], Nobuhiko Hiraga [c], Kazuaki Chayama [c], Mamoru Watanabe [a]

[a] Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo, Japan
[b] Department for Hepatitis Control, Tokyo Medical and Dental University, Tokyo, Japan
[c] Department of Medicine and Molecular science, Division of Frontier Medical Sciences, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan
[d] Department of Internal Medicine, Soka Municipal Hospital, Saitama, Japan

ARTICLE INFO

Article history:
Received 25 May 2010
Returned to author for revision 19 June 2010
Accepted 26 July 2010
Available online 25 August 2010

Keywords:
HCV-JFH1
Inter-genotypic chimeric virus
SOCS3
Interleukin-6

ABSTRACT

Mechanisms of difference in interferon sensitivity between hepatitis C virus (HCV) strains have yet to be clarified. Here, we constructed an infectious genotype2b clone and analyzed differences in interferon-alpha sensitivity between HCV-2b and 2a-JFH1 clones using intergenotypic homologous recombination. The HCV-2b/JFH1 chimeric virus able to infect Huh7.5.1 cells and was significantly more sensitive to IFN than JFH1. IFN-induced expression of MxA and 25-OAS was significantly lower in JFH1 than in 2b/JFH1-infected cells. In JFH1-infected cells, expression of SOCS3 and its inducer, IL-6, was significantly higher than in 2b/JFH1-infected cells. The IFN-resistance of JFH1 cells was negated by siRNA-knock down of SOCS3 expression and by pretreatment with anti-IL6 antibody. In conclusion, intergenotypic differences of IFN sensitivity of HCV may be attributable to the sequences of HCV structural proteins and can be determined by SOCS3 and IL-6 expression levels.
© 2010 Elsevier Inc. All rights reserved.

Introduction

Hepatitis C virus (HCV) is one of the most important pathogens causing liver-related morbidity and mortality (Alter, 1997). There is no therapeutic or prophylactic vaccine available for HCV and type I interferons have been the mainstay of HCV therapeutics (Hoofnagle and di Bisceglie, 1997). Antiviral therapeutic options against HCV are limited and yield unsatisfactory responses (Fried et al., 2002). Given these situations, gaining a detailed understanding of the molecular mechanisms of interferon resistance has been a high priority in academia and industry.

Molecular studies of HCV have been hampered by the lack of efficient *in vitro* and *in vivo* models of infection, which has been partly overcome by the development of HCV subgenomic replicons (Blight et al., 2000; Kato et al., 2003; Lohmann et al., 1999) and the HCV-JFH1 cell culture system (Wakita et al., 2005). HCV-JFH1 is an isolate of HCV genotype 2a that was obtained from a patient with fulminant hepatitis C. The full-length JFH1 genome has been shown to produce infectious particles in cell culture. Simultaneously, a robustly replicating intragenotypic chimera has been reported, which consists of the structural region of a genotype 2a, J6-clone and nonstructural region of JFH-1 (Lindenbach et al., 2005).

HCV isolates are classified into seven major genotypes and multiple subtypes (Gottwein et al., 2009). In infected individuals, HCV exists as quasispecies of closely related genomes (Bukh et al., 1995). A number of studies have suggested that the outcome of HCV infection, as well as the response to interferon treatment, depends on the genotype or quasispecies with which the patient is infected. However, it is not clear how these subtle genetic differences of HCV affect viral replication, infectivity and host responses. Thus, it is important to establish multiple cell culture-permissive strains of different genotypes and isolates of the same genotype for their potential value for characterizing the virus life cycle, drug sensitivity and virus-related cell signaling.

Our present work describes the generation of chimeric viruses with their structural regions from genotype 2b and non-structural genes from the HCV-JFH1 strain. The intergenotypic 2b/JFH1 viruses were compared in terms of intracellular replication, infectious virus production and sensitivity to interferon-alpha. Here we show that the differences in sensitivity to interferon are attributable to upregulated expression of the cellular interferon signal attenuator, SOCS3, and that this upregulation is caused by overexpression of interleukin-6 (IL-6).

Abbreviations: HCV, hepatitis C virus; TLR, toll-like receptor; FBS, fetal bovine serum; ISG, interferon-stimulated gene; IFN, interferon; SOCS, suppressor of cytokine signaling; IL, interleukin; ALT, alanine aminotransferase; UTR, untranslated region; CLEIA, chemiluminescence enzyme immunoassay; PVDF, polyvinylidene fluoride; STAT, signal transducer and activator of transcription; IFNAR, interferon alpha/beta receptor.

* Corresponding author. Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. Fax: +81 3 5803 0268.
E-mail address: nsakamoto.gast@tmd.ac.jp (N. Sakamoto).
[1] G.S. and N.S. contributed equally to this work.

0042-6822/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2010.07.041

Journal of Bioscience and Bioengineering
VOL. 110 No. 3, 374–376, 2010

ELSEVIER

www.elsevier.com/locate/jbiosc

TECHNICAL NOTE

Generation of single-chain Fvs against detergent-solubilized recombinant antigens with a simple coating procedure

Torahiko Tanaka,[1,*] Yuichiro Hasegawa,[2,3] Makoto Saito,[2,3] Masanori Ikeda,[4] and Nobuyuki Kato[4]

Department of Advanced Medical Science, Nihon University School of Medicine, 30-1 Oyaguchi-kamimachi, Itabashi, Tokyo 173-8610, Japan[1]
Department of Obstetrics and Gynecology, Nihon University School of Medicine, 30-1 Oyaguchi-kamimachi, Itabashi, Tokyo 173-8610, Japan[2]
Open Research Center for Genome and Infectious Disease Control, Nihon University School of Medicine, 30-1 Oyaguchi-kamimachi, Itabashi, Tokyo 173-8610, Japan[3] *and Department of Tumor Virology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama 700-8558, Japan*[4]

Received 5 February 2010; accepted 29 March 2010

Antigen coating on polystyrene is prevented by detergent. We present here a simple procedure to coat detergent-solubilized antigen for subsequent panning selection of single-chain Fv (scFv), the target antigen of which was the hepatitis C virus (HCV) non-structural protein (NS) 4B, an integral membrane protein.

© 2010, The Society for Biotechnology, Japan. All rights reserved.

[**Key words:** Single-chain Fv; Detergent; Critical micelle concentration; Hepatitis C virus; NS4B]

The single-chain Fv (scFv)-phage display (1) is a useful technology to obtain antibodies against a wide category of antigens, including non-protein antigens, autoantigens, and antigens that are difficult to generate in animals. To obtain specific scFvs, panning selection has been performed on antigen-coated polystyrene using an scFv-phage display library (2). Antigen coating is achieved by the simple incubation of a soluble antigen solution in polystyrene tubes and wells. However, when an antigen is detergent-solubilized, detergent severely disturbs antigen coating on polystyrene (3,4) and this becomes an obstacle to the panning selection of scFv. To overcome the problem, we developed a simple procedure to coat an antigen by lowering the detergent concentration in an antigen solution with no additional material or time-consuming work. The target antigen was the hepatitis C virus (HCV) non-structural protein (NS) 4B, an integral membrane protein. HCV has a positive-stranded RNA genome encoding at least 10 viral proteins, namely, a core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B (5). The 5' untranslated region has a functional internal ribosome entry site, and the 3' untranslated region contains a highly conserved 98 nucleotide structure, the 3' X (6), which is indispensable for the viral genome replication. The NS proteins are thought to form complexes to replicate the viral genome. Little is known about the role of NS4B which harbors at least four transmembrane domains. Anti-NS4B scFvs to various epitopes are a useful tool for analyzing the roles of NS4B in virus replication.

We prepared the N-terminal hexahistidine (His)-tagged NS4B (NS4BHis) as an antigen based on the sequence of strain O (subtype 1b HCV) (7) using the pET expression system (Novagen, USA). The NS4B fragment was amplified by PCR using the restriction-site-tagged primers 5'-TTACATATGCATCACCACCATCACCATGGTGCCTCGCACC-TCCCTTAC-3' (with the *Nde*I site as underlined) and 5'-TTAGGATCCT-TAGCATGGCGTGGAGCAGTC-3' (with the *Bam*HI site as underlined) with a plasmid pON/C-5B/KE (7) as a template. The expression construct was created by ligating the *Nde*I–*Bam*HI-digested fragment of NS4B into the *Nde*I–*Bam*HI-digested pET3a vector. Similarly, the N-terminal Myc (EQKLISEEDL)-His-tagged NS4B (NS4BMycHis) construct was created by PCR using the primers 5'-TTACATATGGAACA-GAAACTGATTAGCGAAGAAGATCTGCATCACCACCATCACCATG-3' (with the *Nde*I site as underlined) and 5'-TTAGGATCCTTAGCATGGCGTGGAG-CAGTC-3' (with the *Bam*HI site as underlined) with the NS4BHis construct as the PCR template. NS4B proteins were expressed in *Escherichia coli* strain KRX (Promega, USA) in the presence of 0.1% rhamnose at 25 °C. The cells were suspended in a buffer containing 10 mM Tris–HCl, pH 7.4, 5 mM EDTA, and a Complete™ protease inhibitor cocktail (Roche, Germany), sonicated three times with 5 s bursts, and centrifuged at 5000*g* for 3 min. Because NS4BHis was recovered in the pellet, the solubilization conditions were examined. NS4BHis was efficiently solubilized in the presence of 0.5 M NaCl with 1% n-dodecyl β-D-maltoside (DDM) or Triton X-100 but not with Tween-20 and n-octyl β-D-glucoside (OG). After solubilization with DDM, NS4BHis was affinity-purified using Ni NTA agarose (Qiagen, USA) to near-homogeneity according to the manufacturer's protocol.

In the usual panning selection of antigen-specific scFv, the antigen is coated on polystyrene by simple incubation in an aqueous buffer. In the present work, the purified NS4BHis preparation contains 1% DDM, and, as described above, detergents are known to severely disturb antigen coating on polystyrene. Upon a preliminary experiment, we failed to efficiently coat NS4BHis with 50-fold simple dilution (final

* Corresponding author. Present address: Division of Biochemistry, Department of Biomedical Sciences, Nihon University School of Medicine, 30-1 Oyaguchi-kamimachi, Itabashi, Tokyo 173-8610, Japan. Tel.: +81 3 3972 8111x2241; fax: +81 3 3972 8199.
E-mail address: tanakat@med.nihon-u.ac.jp (T. Tanaka).

1389-1723/$ - see front matter © 2010, The Society for Biotechnology, Japan. All rights reserved.
doi:10.1016/j.jbiosc.2010.03.014

J Gastroenterol (2010) 45:195–203
DOI 10.1007/s00535-009-0132-9

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Inhibition of hepatitis C virus replication by chloroquine targeting virus-associated autophagy

Tomokazu Mizui · Shunhei Yamashina · Isei Tanida · Yoshiyuki Takei · Takashi Ueno · Naoya Sakamoto · Kenichi Ikejima · Tsuneo Kitamura · Nobuyuki Enomoto · Tatsuo Sakai · Eiki Kominami · Sumio Watanabe

Received: 28 May 2009 / Accepted: 22 August 2009 / Published online: 17 September 2009
© Springer 2009

Abstract
Background Autophagy has been reported to play a pivotal role on the replication of various RNA viruses. In this study, we investigated the role of autophagy on hepatitis C virus (HCV) RNA replication and demonstrated anti-HCV effects of an autophagic proteolysis inhibitor, chloroquine.
Methods Induction of autophagy was evaluated following the transfection of HCV replicon to Huh-7 cells. Next, we investigated the replication of HCV subgenomic replicon in response to treatment with lysosomal protease inhibitors or pharmacological autophagy inhibitor. The effect on HCV replication was analyzed after transfection with siRNA of ATG5, ATG7 and light-chain (LC)-3 to replicon cells. The antiviral effect of chloroquine and/or interferon-α (IFNα) was evaluated.
Results The transfection of HCV replicon increased the number of autophagosomes to about twofold over untransfected cells. Pharmacological inhibition of autophagic proteolysis significantly suppressed expression level of HCV replicon. Silencing of autophagy-related genes by siRNA transfection significantly blunted the replication of HCV replicon. Treatment of replicon cells with chloroquine

T. Mizui (✉) · S. Yamashina · K. Ikejima · T. Kitamura · S. Watanabe
Department of Gastroenterology, Juntendo University, School of Medicine, Hongo 2-1-1, Bunkyo-ku, Tokyo 113-8421, Japan
e-mail: don-chip@aqua.email.ne.jp

S. Yamashina
e-mail: ryou0607jp@ybb.ne.jp

K. Ikejima
e-mail: ikejima@juntendo.ac.jp

T. Kitamura
e-mail: kitamura@juntendo.ac.jp

S. Watanabe
e-mail: sumio@juntendo.ac.jp

I. Tanida
Department of Biochemistry and Cell Biology, Laboratory of Biomembranes, National Institute of Infectious Disease, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan
e-mail: tanida@nih.go.jp

Y. Takei
Department of Gastroenterology, Mie University, Kurimamachiya-cho 1577, Tsu, Mie 514-8507, Japan
e-mail: ytakei@clin.medic.mie-u.ac.jp

T. Ueno · E. Kominami
Department of Biochemistry, Juntendo University School of Medicine, Hongo 2-1-1, Bunkyo-ku, Tokyo 113-8421, Japan
e-mail: upfield@juntendo.ac.jp

E. Kominami
e-mail: kominami@juntendo.ac.jp

N. Sakamoto
Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Yushima 1-5-45, Bunkyo-ku, Tokyo 113-8510, Japan
e-mail: nsakamoto.gast@tmd.ac.jp

N. Enomoto
First Department of Internal Medicine, University of Yamanashi, Kakedo 4-3-11, Kofu-shi, Yamanashi 400-8511, Japan
e-mail: enomoto@yamanashi.ac.jp

T. Sakai
Department of Anatomy, Juntendo University School of Medicine, Hongo 2-1-1, Bunkyo-ku, Tokyo 113-8421, Japan
e-mail: tatsuo@juntendo.ac.jp

Virology 405 (2010) 361–369

Contents lists available at ScienceDirect

Virology

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

Cell culture and in vivo analyses of cytopathic hepatitis C virus mutants☆,☆☆

Kako Mishima [a,1], Naoya Sakamoto [a,b,*,1], Yuko Sekine-Osajima [a], Mina Nakagawa [a,b], Yasuhiro Itsui [a,c], Seishin Azuma [a], Sei Kakinuma [a,b], Kei Kiyohashi [a], Akiko Kitazume [a], Kiichiro Tsuchiya [a], Michio Imamura [d], Nobuhiko Hiraga [d], Kazuaki Chayama [d], Takaji Wakita [e], Mamoru Watanabe [a]

[a] Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan
[b] Department for Hepatitis Control, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan
[c] Department of Internal Medicine, Soka Municipal Hospital, 2-21-1 Soka, Soka City, Saitama 340-8560, Japan
[d] Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima City, Hiroshima 734-8551, Japan
[e] Department of Virology II, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan

ARTICLE INFO

Article history:
Received 11 March 2010
Returned to author for revision 7 April 2010
Accepted 7 June 2010
Available online 6 July 2010

Keywords:
HCV-JFH1 cell culture
Plaque assay
Cytopathic effect
Adaptive mutations
Human hepatocyte chimeric mice

ABSTRACT

HCV-JFH1 yields subclones that develop cytopathic plaques (Sekine-Osajima Y, et al., Virology 2008; 371:71). Here, we investigated viral amino acid substitutions in cytopathic mutant HCV-JFH1 clones and their characteristics in vitro and in vivo. The mutant viruses with individual C2441S, P2938S or R2985P signature substitutions, and with all three substitutions, showed significantly higher intracellular replication efficiencies and greater cytopathic effects than the parental JFH1 in vitro. The mutant HCV-inoculated mice showed significantly higher serum HCV RNA and higher level of expression of ER stress-related proteins in early period of infection. At 8 weeks post inoculation, these signature mutations had reverted to the wild type sequences. HCV-induced cytopathogenicity is associated with the level of intracellular viral replication and is determined by certain amino acid substitutions in HCV-NS5A and NS5B regions. The cytopathic HCV clones exhibit high replication competence in vivo but may be eliminated during the early stages of infection.
© 2010 Elsevier Inc. All rights reserved.

Introduction

Hepatitis C virus (HCV) is one of the most important pathogens causing liver-related morbidity and mortality (Alter, 1997). Antiviral therapeutic options against HCV have been limited to type I interferons and ribavirin and have yielded unsatisfactory responses (Fried et al., 2002). Given this situation, a precise understanding of the molecular mechanisms of interferon resistance has been a high priority of research in academia and industry.

Molecular analyses of the HCV life cycle, virus–host interactions, and mechanisms of liver cell damage by the virus are not understood completely, mainly because of the lack of cell culture systems. These problems have been overcome to some extent by the development of the HCV subgenomic replicon (Lohmann et al., 1999) and HCV cell culture systems (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). The HCV-JFH1 strain, which is a genotype 2a clone derived from a Japanese fulminant hepatitis patient and can replicate efficiently in Huh7 cells (Kato, 2001; Kato et al., 2003), has contributed to the establishment of the HCV cell culture system. Furthermore, the Huh7-derived cell lines, Huh-7.5 and Huh-7.5.1 cells, allow production of higher viral titers and have a greater permissivity for HCV (Koutsoudakis et al., 2007; Lindenbach et al., 2005; Zhong et al., 2005). The HCV-JFH1 cell culture system now allows us to study the complete HCV life cycle: virus–cell entry, translation, protein processing, RNA replication, virion assembly and virus release.

HCV belongs to the family *Flaviviridae*. One of the characteristics of the *Flaviviridae* is that they cause cytopathic effects (CPE). The viruses have positive strand RNA genomes of ~10 kilo-bases that encode polyproteins of ~3000 amino acids. These proteins are processed post-translationally by cellular and viral proteases into at least 10 mature proteins (Sakamoto and Watanabe, 2009). The viral non-structural proteins accumulate in the ER and direct genomic replication and viral protein synthesis (Bartenschlager and Lohmann, 2000; Jordan et al., 2002; Mottola et al., 2002). It has been recently

Abbreviations: HCV, hepatitis C virus; CPE, cytopathic effect; ER, endoplasmic reticulum; RdRp, RNA dependent RNA polymerase.

☆ The authors, K.M., N.S., Y.S., M.N., Y.I., S.A., S.K., K.K., A.K., K.T., M.I., N.H., K.C., T.W. and M.W. declare that there is no conflict of interest.

☆☆This study was supported by grants from Ministry of Education, Culture, Sports, Science and Technology-Japan, the Japan Society for the Promotion of Science, Ministry of Health, Labour and Welfare-Japan, Japan Health Sciences Foundation, and National Institute of Biomedical Innovation.

* Corresponding author. Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. Fax: +81 3 5803 0268.
E-mail address: nsakamoto.gast@tmd.ac.jp (N. Sakamoto).

[1] K. M. and N. S. contributed equally to this work.

0042-6822/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2010.06.020

FEBS Letters xxx (2009) xxx–xxx

ELSEVIER

FEBS Letters

journal homepage: www.FEBSLetters.org

Curcumin inhibits hepatitis C virus replication via suppressing the Akt-SREBP-1 pathway

KyeongJin Kim [a], Kook Hwan Kim [a], Hye Young Kim [a], Hyun Kook Cho [a], Naoya Sakamoto [b], JaeHun Cheong [a,*]

[a] Department of Molecular Biology, College of Natural Sciences, Pusan National University, Busan 609-735, Republic of Korea
[b] Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo 113-8519, Japan

ARTICLE INFO

Article history:
Received 20 October 2009
Revised 3 December 2009
Accepted 12 December 2009
Available online xxxx

Edited by Lukas Huber

Keywords:
Akt
Curcumin
HCV
NS5A
SREBP-1

ABSTRACT

A polyphenolic compound from the curry spice turmeric, curcumin, is known to show anti-viral activity against the influenza virus, adenovirus, coxsackievirus, and the human immunodeficiency virus. However, it remains to be determined whether curcumin can inhibit the replication of hepatitis C virus (HCV). In this study, we showed that curcumin decreases HCV gene expression via suppression of the Akt-SREBP-1 activation, not by NF-κB pathway. The combination of curcumin and IFNα exerted profound inhibitory effects on HCV replication. Collectively, our results indicate that curcumin can suppress HCV replication in vitro and may be potentially useful as novel anti-HCV reagents.

© 2009 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.

1. Introduction

The hepatitis C virus (HCV), a member of the *Flaviviridae* family, is an enveloped virus with a single-stranded 9.6 kb RNA genome. HCV infection is characterized by a high rate of progression to fibrosis and chronic hepatitis, resulting in cirrhosis, and ultimately in hepatocellular carcinoma [1]. The best anti-viral therapy presently known involves the combination of pegylated interferon (IFN) alpha and ribavirin, but almost half of all patients manifest no response to exogenous IFNα [2]. Therefore, the development of novel drugs for the safer and more efficient treatment of HCV is urgently required.

Many bioactive polyphenolic compounds have been shown to perform candidate agent functions in chemoprevention and in cancer chemotherapy [3]. Among this class, curcumin (diferuloylmethane) is one of the most widely studied compounds. Curcumin is the major component of the curry spice tumeric (*Curcuma longa* Linn) and can affect the metabolism of cells and organisms in a number of ways, including anti-inflammatory, anti-oxidant, and anti-proliferative properties via the modulation of multiple cellular mechanisms [4,5]. Furthermore, some recent reports have shown that these compounds show anti-viral activity against the influenza virus, adenovirus, coxsackievirus, and the human immunodeficiency virus [6–9]. Also, curcumin has been shown to suppress transcription activation by the host protein AP-1, leading to diminished HTLV-1 and HPV-mediated cellular transformation [10]. However, it remains to be determined whether curcumin can inhibit the gene expression of HCV.

On the basis of our previous knowledge of the regulation of HCV replication and the biological properties of curcumin, we evaluated the effects of curcumin on the intracellular replication of the HCV genome in vitro, using an HCV replicon system. We showed that curcumin at concentrations that do not affect cell viability reduced HCV RNA replication in vitro to a significant degree. Curcumin inhibited a lipogenic transcription factor, sterol regulatory element binding protein-1 (SREBP-1)-induced HCV replication via the PI3K/Akt pathway. Finally, the combination of curcumin and IFNα showed cooperative inhibitory effects on HCV RNA replication. Our results indicate that curcumin may potentially prove useful as a treatment for HCV infection.

2. Materials and methods

2.1. Plasmid constructs

pEMCV/IRES-Rluc was utilized as a control for the analysis of translation efficiency mediated by an encephalomyocarditis virus internal ribosome entry site (EMCV-IRES) which mediates the translation of the HCV non-structure gene of replicon constructs, Huh7/Rep-Feo [11]. pCIneo-Rluc-IRES-Fluc was constructed in or-

* Corresponding author. Fax: +82 51 513 9258.
E-mail addresses: molecule85@pusan.ac.kr, jhcheong2@lycos.co.kr (J. Cheong).

0014-5793/$36.00 © 2009 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2009.12.019

Please cite this article in press as: Kim, K., et al. Curcumin inhibits hepatitis C virus replication via suppressing the Akt-SREBP-1 pathway. FEBS Lett. (2009), doi:10.1016/j.febslet.2009.12.019

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 2010, p. 3179–3186 Vol. 54, No. 8
0066-4804/10/$12.00 doi:10.1128/AAC.00113-10
Copyright © 2010, American Society for Microbiology. All Rights Reserved.

DOWNLOADED FROM aac.ASM.ORG - at TOKYO IKASHIKA DAIGAKU July 15, 2010

Inhibition of Hepatitis C Virus Replication by a Specific Inhibitor of Serine-Arginine-Rich Protein Kinase▽

Yuko Karakama,[1]§ Naoya Sakamoto,[1,2]§* Yasuhiro Itsui,[1,5] Mina Nakagawa,[1,2] Megumi Tasaka-Fujita,[1] Yuki Nishimura-Sakurai,[1] Sei Kakinuma,[1,2] Masaya Oooka,[3] Seishin Azuma,[1] Kiichiro Tsuchiya,[1] Hiroshi Onogi,[4] Masatoshi Hagiwara,[4] and Mamoru Watanabe[1]

Department of Gastroenterology and Hepatology[1] and Department for Hepatitis Control,[2] Tokyo Medical and Dental University, Tokyo, Japan; Department of General Medicine, Tokyo Medical and Dental University, Tokyo, Japan[3]; Department of Functional Genomics, Medical Research Institute, Tokyo Medical and Dental University, Tokyo, Japan[4]; and Department of Internal Medicine, Soka Municipal Hospital, Saitama, Japan[5]

Received 26 January 2010/Returned for modification 9 March 2010/Accepted 11 May 2010

Splicing of messenger RNAs is regulated by site-specific binding of members of the serine-arginine-rich (SR) protein family, and SR protein kinases (SRPK) 1 and 2 regulate overall activity of the SR proteins by phosphorylation of their RS domains. We have reported that specifically designed SRPK inhibitors suppressed effectively several DNA and RNA viruses *in vitro* and *in vivo*. Here, we show that an SRPK inhibitor, SRPIN340, suppressed in a dose-dependent fashion expression of a hepatitis C virus (HCV) subgenomic replicon and replication of the HCV-JFH1 clone *in vitro*. The inhibitory effects were not associated with antiproliferative or nonspecific cytotoxic effects on the host cells. Overexpression of SRPK1 or SRPK2 resulted in augmentation of HCV replication, while small interfering RNA (siRNA) knockdown of the SRPKs suppressed HCV replication significantly. Immunocytochemistry showed that SRPKs and the HCV core and NS5A proteins colocalized to some extent in the perinuclear area. Our results demonstrate that SRPKs are host factors essential for HCV replication and that functional inhibitors of these kinases may constitute a new class of antiviral agents against HCV infection.

Hepatitis C virus (HCV) infects up to 170 million people worldwide, and these infections frequently are characterized by chronic liver inflammation, leading to decompensated liver cirrhosis and hepatocellular cancers (1). Alpha and beta interferons are the mainstay of HCV therapeutics. However, the most effective pegylated interferon plus ribavirin combination therapies can eliminate HCV from around half of the patients only (6). These difficulties in eradicating HCV are compounded by the limited treatment options. For this reason, the development of safe and effective therapeutic agents against HCV has been a strong motivation in academia and industry (23).

Serine-arginine-rich (SR) proteins are a family of non-small nuclear ribonucleoprotein particle (non-snRNP) splicing factors that are highly conserved throughout the eukaryotes. They harbor one or two RNA recognition motifs and an RS domain at the amino and carboxyl termini, respectively (29). RS domains consist of multiple consecutive Arg-Ser/Ser-Arg dipeptide repeats, in which the Ser residues are extensively phosphorylated by several kinases, including SR protein kinases (SRPKs). SRPK1 was the first SR protein kinase to be cloned, on the basis of its ability to phosphorylate SR proteins *in vitro* (8, 9), and two other structurally related kinases, SRPK2 and SRPK3, also have been shown to phosphorylate SR proteins (16, 31). Although the precise physiological role of this phosphorylation remains unknown, it is expected that phosphorylation of SR proteins affects their protein-protein and protein-RNA interactions, intracellular localization and trafficking, and alternative splicing of pre-mRNA (21).

As SRPK-dependent herpes simplex virus (HSV) splicing and SRPK-mediated phosphorylation of hepatitis B virus (HBV) core protein have been reported (4, 25, 33), it is reasonable to expect that SR proteins and SRPK might be suitable targets for therapeutic modulation of various viral infections. Actually, we found that increased activity of SRPK2 upregulated human immunodeficiency virus (HIV) expression and that an isonicotinamide compound, SRPIN340, which preferentially inhibited SRPK1 and SRPK2, suppressed propagation of Sindbis virus, HIV, and cytomegalovirus (7). In this study, we investigated the effects of SRPIN340 on HCV replication using the HCV subgenomic replicon system (27, 32) and HCV-JFH1 virus cell culture (30, 34). Here, we demonstrate that cellular SRPK is required for HCV replication and suggest that the inhibitor of SRPK could be used therapeutically.

MATERIALS AND METHODS

SRPK inhibitor. SRPIN340, *N*-[2-(1-piperidinyl)-5-(trifluoromethyl)phenyl] isonicotinamide, inhibits SRPK1 and SRPK2 kinase activities potently (7). SRPIN340 does not inhibit other classes of SRPKs significantly, including Clk1 and Clk and other classes of SR kinases. SRPIN614, *N*-methyl-*N*-[2-(1-piperidinyl)-5-(trifluoromethyl)phenyl]isonicotinamide, is a negative-control compound that has no suppressive effects on SRPK1 or SRPK2. SRPIN340 and SRPIN614 were synthesized in-house (7).

***In vitro* kinase assay.** Kinase activities of SRPKs were assayed as described previously (18). Briefly, His_6-tagged recombinant SRPK1 or SRPK2 was expressed in *Escherichia coli* and purified by Ni-nitrilotriacetic acid (NTA) affinity chromatography. The purified SRPK1 or SRPK2 was incubated in the presence of ATP, [γ-^{32}P]ATP, and a synthetic peptide of the SF2/ASF RS domain (NH_2-R SPSYGRSRSRSRSRSRSRSNSRSRSY-OH) at pH 7.5 and 30°C for 10 min. The reaction mixtures were spotted onto phosphocellulose membranes (What-

* Corresponding author. Mailing address: Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. Phone: 81 3-5803-5877. Fax: 81 3-5803-0268. E-mail: nsakamoto.gast@tmd.ac.jp.
§ Y.K. and N.S. contributed equally to this work.
▽ Published ahead of print on 24 May 2010.

Strain-promoted double-click reaction for chemical modification of azido-biomolecules†

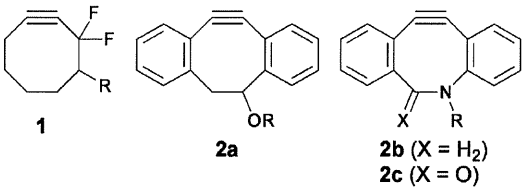
Isao Kii,*[a] Akira Shiraishi,[a] Toshiyuki Hiramatsu,[a,b] Takeshi Matsushita,[c] Hidehiro Uekusa,[d] Suguru Yoshida,[b] Makoto Yamamoto,[e] Akira Kudo,[a] Masatoshi Hagiwara[e] and Takamitsu Hosoya*[a,b]

Received 15th April 2010, Accepted 8th July 2010
First published as an Advance Article on the web 26th July 2010
DOI: 10.1039/c0ob00003e

Published on 26 July 2010 on http://pubs.rsc.org | doi:10.1039/C0OB00003E
Downloaded by Kyoto Daigaku on 15 February 2013

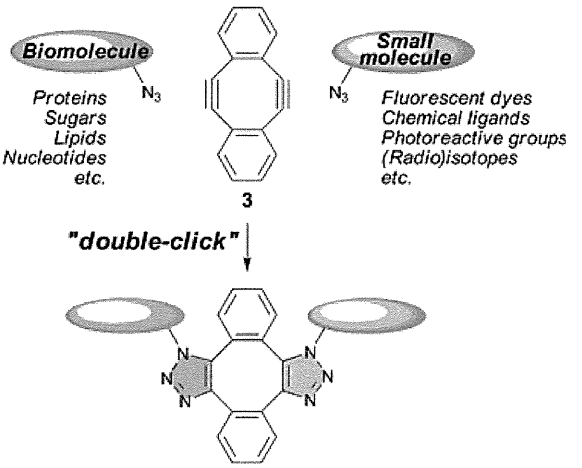
The strain-promoted "double-click" (SPDC) reaction using Sondheimer diyne, a novel convergent method conjugating three molecules spontaneously, has enabled us to readily modify an azido-biomolecule with a small reporter azido-molecule.

"Click reaction," copper(I)-catalyzed azide–alkyne [3+2] cycloaddition (CuAAC), is an emerging method for conjugating molecules in the fields of chemistry and biology.[1–3] With the progress in preparation techniques of biomolecules incorporated with bioorthogonal groups, various chemically-modified biomolecules have become available.[4] However, cytotoxicity by the copper catalyst and the slow rate of the reaction have restricted its application. To overcome these limitations, Bertozzi has introduced a copper-free click reaction, the strain-promoted azide–alkyne [3+2] cycloaddition (SPAAC),[5] exploiting the spontaneous reactivity of cyclooctynes toward an azide by its ring strain.[6] Furthermore, a rapid SPAAC reaction has been achieved using difluorinated cyclooctyne (DIFO, **1**),[7] dibenzocyclooctynol (DIBO, **2a**)[8] and aza-dibenzocyclooctyne (DIBAC, **2b**;[9] BARAC, **2c**[7f]) derivatives. Fluorescence-labeled or biotinylated derivatives of these cyclooctynes have enabled us to visualize the distribution of azido-glycoconjugates in cultured cells and in living animals.[7,8]



1 **2a** **2b** (X = H_2) **2c** (X = O)

To expand the versatility of the SPAAC reaction, we have conceived the idea of connecting two azides by a bisreactive molecule, thereby avoiding the on-demand preparation of cyclooctyne derivatives. We envisaged that *sym*-dibenzo-1,5-cyclooctadiene-3,7-diyne (**3**), reported by Sondheimer[10] and now easily available,[11] is an ideal compound because it has two highly strained alkyne bonds[12] ready to react spontaneously.[13] Herein, we show the catalyst-free dual annulation of diyne **3** with two different azido-molecules, the strain-promoted "double-click" (SPDC) reaction (Scheme 1), and demonstrate the chemical modification of an azido-biomolecule with a reporter azido-molecule in both *in vitro* and living cells.



Scheme 1 Chemical modification of azido-biomolecules with small azido-molecules by the strain-promoted double-click (SPDC) reaction.

In an initial study, diyne **3** in methanol (8 mM) was treated with an excess amount of benzyl azide (**4a**, 2.4 equiv.) at room temperature (Scheme 2, R = benzyl). After 70 min, diyne **3** completely reacted to give two regioisomeric bis-cycloadducts **6a** (*trans*) and **7a** (*cis*) in 60% and 38% yields, respectively.[14] X-Ray crystallographic analysis showed their unique saddle-shaped structures (Fig. 1).[15] The mono-cycloadduct **5a**, the presumed monoyne intermediate, was neither isolated nor detected even when an equimolar amount of **4a** was used,[14] indicating that the monoyne intermediate is more reactive toward azides than the starting diyne **3**.

The broad substrate scope in the SPDC reaction was shown from the reactions of diyne **3** with various azides, including ethyl azidoacetate (**4b**), phenyl azide (**4c**), 4-(azidomethyl)benzyl alcohol (**4d**) and methyl 4-(azidomethyl)benzoate (**4e**) (Scheme 2).[14–16] Not surprisingly, the reaction of diyne **3** with an equimolar mixture

[a]*Department of Biological Information, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku, Yokohama 226-8501, Japan. E-mail: ikii@bio.titech.ac.jp*
[b]*Laboratory of Chemical Biology, Graduate School of Biomedical Science, Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University and SORST, Japan Science and Technology Agency (JST), 2-3-10 Kanda-Surugadai, Chiyoda-ku, Tokyo 101-0062, Japan. E-mail: thosoya.cb@tmd.ac.jp; Fax: +81-3-5280-8117*
[c]*Goi Research Center, Chisso Petrochemical Corporation, 5-1 Goikaigan, Ichihara, Chiba 290-8551, Japan*
[d]*Department of Chemistry and Materials Science, Graduate School of Science and Engineering, Tokyo Institute of Technology, 2-12-1 Ookayama, Meguro-ku, Tokyo, 152-8551, Japan*
[e]*Department of Functional Genomics, Medical Research Institute, Graduate School of Biomedical Science, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan*

† Electronic supplementary information (ESI) available: Experimental details for syntheses and characterization of new compounds, kinetic study, computational studies and biological experiments. See DOI: 10.1039/c0ob00003e