Journal of Viral Hepatitis, 2009 doi:10.1111/j.1365-2893.2009.01102.x

Inhibition of intracellular hepatitis C virus replication by nelfinavir and synergistic effect with interferon-α

S. Toma,[1] T. Yamashiro,[2] S. Arakaki,[1] J. Shiroma,[1] T. Maeshiro,[1] K. Hibiya,[1] N. Sakamoto,[3] F. Kinjo,[4] M. Tateyama[1] and J. Fujita[1] [1]*First Department of Internal Medicine, School of Medicine, University of the Ryukyus, Okinawa, Japan;* [2]*Department of Blood Transfusion Medicine, Ryukyu University Hospital, Okinawa, Japan;* [3]*Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo, Japan; and* [4]*Department of Endoscopy, Ryukyu University Hospital, Okinawa, Japan*

Received September 2008; accepted for publication December 2008

SUMMARY. Liver diseases associated with hepatitis C virus (HCV) infection have become the major cause of mortality in patients with human immunodeficiency virus (HIV) infection since the introduction of highly active anti-retroviral therapy. HCV-related liver disease is more severe in HIV-infected patients than in non-HIV-infected patients, but the standard therapies used to treat chronic hepatitis C in HCV/HIV coinfected patients are the same as those for patients infected with HCV alone. HIV protease inhibitors might have potential to down-regulate HCV load of HCV/HIV coinfected patients. In this study, we evaluated the effects of nelfinavir on intracellular HCV replication using the HCV replicon system. We constructed an HCV replicon expressing a neomycin-selectable chimeric firefly luciferase reporter protein. Cytotoxicity and apoptosis induced by nelfinavir were assessed and synergism between nelfinavir and interferon (IFN) was calculated using CalcuSyn analysis. Nelfinavir dose-dependently repressed HCV replication at low concentrations (IC_{50}, 9.88 μmol/L). Nelfinavir failed to induce cytotoxicity or apoptosis at concentrations that inhibited HCV replication. Clinical concentrations of nelfinavir (5 μmol/L) combined with IFN showed synergistic inhibition of HCV replication in our replicon model. Our results suggest that the direct effects of nelfinavir on the HCV subgenome and its synergism with IFN could improve clinical responses to IFN therapy in HCV/HIV coinfected patients.

Keywords: hepatitis C virus, human immunodeficiency virus, nelfinavir.

INTRODUCTION

Patients with human immunodeficiency virus (HIV) infection are frequently coinfected with hepatitis C virus (HCV), because these viruses have similar routes of transmission, including blood transfusion, intravenous drug use and sexual contact [1,2]. The optimal therapy for HIV infection is highly active anti-retroviral therapy (HAART), which combines HIV reverse transcriptase inhibitors, often with HIV protease inhibitors. Since the introduction of HAART, the morbidity and mortality associated with HIV infection have declined. This reduction in mortality due to opportunistic infections has made HCV-associated liver disease the leading causes of mortality [3].

Several studies have reported that HCV-related liver disease is more severe in HIV-infected patients than in non-HIV-infected patients [4,5]. The severity of liver disease increases as the immunodeficiency progresses and HIV seropositivity accelerates the progression of liver fibrosis related to chronic hepatitis C [6,7]. In addition, many studies have documented that HIV/HCV coinfected patients have higher HCV loads than do HCV monoinfected controls [8–10]. However, the standard therapies used to treat chronic hepatitis C in HCV/HIV coinfected patients are the same as those for patients infected with HCV alone [11].

HAART has been reported to reduce serum HCV RNA levels accompanied by immune improvement [12], but the decrease in HIV viral load was associated with a persistent and significant increase in HCV viral load [13]. There is no consistent evidence that HAART results in suppression of HCV viraemia, suggesting that multiple factors may be affecting viral load in coinfected patients [14,15]. However,

Abbreviations: CI, combination index; HAART, highly active anti-retroviral therapy; HCV, hepatitis C virus; HIV, human immunodeficiency virus; IFN, interferon; LDH, lactate dehydrogenase; MTS, 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazoly)-3-(4-sulfophenyl) tetrazolium inner salt; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end-labelling.

Correspondence: Tsuyoshi Yamashiro, MD, PhD, Department of Blood Transfusion Medicine, Ryukyu University Hospital, 207 Uehara, Nishihara-cho, Okinawa 903-0215, Japan.
E-mail: tsuyo-y@med.u-ryukyu.ac.jp

Trimoulet *et al.* showed that patients treated with HAART that included protease inhibitors had significantly lower intrahepatic HCV loads than those treated with HAART without protease inhibitors [16].

Liver injury has been reported to be a potential side-effect of HAART [17] and potential hepatotoxicity of HIV protease inhibitors had been well realized before these drugs were licensed for the first time [18]. Concomitant hepatic damage prior to the start of HAART is an important risk factor, which can intensify hepatotoxic side-effects of HAART. The presence of chronic hepatitis C has been reported to increase the risk of HAART-associated hepatotoxicity (relative risk, 2.46; 95% confidence interval, 1.43–4.24) [19]. The mechanisms underlying the association of HCV and hepatotoxicity remain unclear, but in some patients liver enzyme elevations may be a manifestation of immune reconstitution that follows antiretroviral therapy. After immune recovery, CD4+ cell counts rise and the ability of T cells to identify and lyse HCV-infected hepatocytes may be increased [20]. The differences in the potential for hepatotoxicity have been reported to exist among the commercially available protease inhibitors and nelfinavir was associated with the low rate of severe hepatotoxicity among patients coinfected with hepatitis viruses [21].

HIV protease is a small, dimeric aspartyl protease that specifically cleaves the polyprotein precursors encoding the structural proteins and enzymes of the virus. This proteolytic activity is absolutely required for the production of mature, infectious virions. HIV protease inhibitors block HIV maturation and show remarkable antiviral potency [22]. It has recently been reported that HIV protease inhibitors also have nonviral effects on the host cells, beyond their effect of blocking HIV protease enzymatic activity [23]. NF-κB is central to the overall immune response through its ability to activate genes coding for regulators of apoptosis and cell proliferation [24]. The HIV protease inhibitor nelfinavir has been shown to regulate NF-κB activation [25].

In the present study, we investigated the action of nelfinavir alone, or in combination with interferon (IFN), on HCV replication using the replicon system.

MATERIALS AND METHODS

Cell culture

The human hepatoma cell line, Huh7, was maintained in Dulbecco's modified minimal essential medium (Sigma, St Louis, MO, USA) supplemented with 2 mM L-glutamine and 10% fetal calf serum at 37 °C under 5% CO_2. Huh7 cells expressing the HCV replicon were cultured in medium containing 500 μg/mL G418 (Nakalai Tesque, Kyoto, Japan).

HCV replicon constructs and transfected cell lines

An HCV subgenomic replicon plasmid, pHCVIbneo-delS (designated pRep-N), was derived from an infectious HCV clone, HCV-N, genotype Ib [26]. The replicon, pRep-N was reconstructed by substituting the neomycin phosphotransferase gene with a fusion gene comprising firefly luciferase and neomycin phosphotransferase (pRep-Feo) [27–29]. RNA was synthesized from pRep-Feo using T7-polymerase (Promega, Madison, WI, USA) and transfected into Huh7 cells. After culture in the presence of G418, cell lines stably expressing the replicon were established. We have previously reported that firefly luciferase activities of Feo replicon-expressing cells correlated well with HCV NS3, NS4A and NS5A protein expression levels and with replicon RNA expression levels [27].

Treatment with IFN and nelfinavir

Recombinant human IFN-α-2b (Schering-Plough, Kenilworth, NJ, USA) and purified nelfinavir (Japan Tobacco Inc., Tokyo, Japan) were used. IFN and nelfinavir treatment schedules were as described in the results.

Luciferase assays

Luciferase activity was quantified using a luminometer (Lumat LB9501; Promega) and the Bright-Glo Luciferase Assay System (Promega). Typically, 5×10^3 cells/well, plated onto 24-well plates and cultured for 48 h, were lysed with 100 μL 1× Glo luciferase Buffer (Promega), and the luciferase activity in 100 μL of the lysate was measured by adding an equal volume of Bright-Glo Luciferase Assay Reagent (Promega). Assays were performed in triplicate, and the results were expressed as mean ± SD relative light units.

Western blot analysis

Cells were lysed in buffer containing 62.5 μM Tris–HCl (pH 6.8), 2% sodium dodecyl sulfate, 10% glycerol, 6% 2-mercaptoethanol and 0.01% bromophenol blue. Equal amounts of protein (10 μg) were subjected to electrophoresis on sodium dodecyl sulfate-polyacrylamide gels (Invitrogen, Carlsbad, CA, USA), followed by transfer to a polyvinylidene difluoride membrane (Roche, Basle, Switzerland) and sequential probing with a monoclonal anti-NS5A antibody (Virogen, Watertown, MA, USA) and β-actin antibody (Thermo Fisher Scientific, Fremont, CA, USA), respectively. The bands were visualized using an enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ, USA).

Cytotoxicity assay

Lactate dehydrogenase (LDH) tests and 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazoly)-3-(4-sulfophenyl)tetrazolium inner salt (MTS) reduction assays were performed to investigate cytotoxicity and cell viability. LDH levels were measured in the supernatants using the LDH-Cytotoxic Test (Wako Pure Chemical Industries, Osaka, Japan), according

to the manufacturer's instructions. The level of specific cytotoxicity was calculated using the following formula: % of specific LDH release = ([experimental LDH release – the mean of negative control release]/[the mean of positive control release – the mean of negative control release]) ×100. LDH release from cells treated with 0.2% Tween 20 was used as a positive control, while LDH release from nontreated cells was used as a negative control. Viable cell growth was determined by MTS assay using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega), according to the manufacturer's instructions.

TUNEL method

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end-labelling (TUNEL) was used to detect DNA fragmentation of nuclei. Using 24-well plates, 5×10^3 cells/well were plated with 5.0 μM nelfinavir. After incubation for 2 days, the glass coverslips were harvested, fixed with 4% paraformaldehyde and washed with phosphate-buffered saline. The cells were permeabilized with 0.5% Tween 20 and treated with MEBSTAIN Apoptosis Kit Direct (Medical and Biological Laboratories Co., Nagoya, Japan). Cells were then treated with RNase and propidium iodide. The nick end-labelling was analysed using a confocal laser scanning microscope (Fluorview; Olympus, Tokyo, Japan).

Analysis of drug synergism

The effects of treatment of Huh7/Rep-Feo cells with nelfinavir and IFN, alone and in combination, were analysed using isobologram analysis. Dose-inhibition curves were drawn for IFN and nelfinavir, used alone or in combination. For each drug combination, the 50% inhibitory concentration (IC_{50}) values were plotted against the fractional concentration of IFN and nelfinavir on the x axis and y axis, respectively. IC_{50}, IC_{20} and IC_{80} values ware determined using the Calcusyn™ software package (Biosoft, Cambridge, UK), which performs single and multiple drug dose-effect calculations and determines the presence of antagonism, additivity or synergism. Using the median effect equation, we used this program to plot dose-effect curves for each drug and combination of drugs. The x intercept of the median effect equation gives the ID_{50} for each drug. The median effect plot also gives information on the slope of the dose-effect curve. This information can then be used to calculate the combination index (CI). CI >1 denotes antagonism, CI = 1 denotes additivity, and CI < 1 denotes synergism.

Statistical analysis

Statistical analysis was performed using the Student's t-test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Effect of nelfinavir on HCV replication

To assess the effects of nelfinavir on intracellular replication of the HCV genome, Huh7/Rep-Feo cells were cultured with various concentrations of nelfinavir. The dose-effect correlation and time course of replicon expression were measured using the luciferase assay 48 h after treatment. Culture of Huh7/Rep-Feo cells with nelfinavir, at concentrations ranging from 0 to 10 μmol/L, showed dose-dependent repression of internal luciferase activity (Fig. 1a). The inhibition of HCV-RNA replication was detectable at concentrations of nelfinavir as low as 2.5 μmol/mL. Western blot hybridization also demonstrated a reduction of the replicon protein levels after nelfinavir treatment (Fig. 1b). To determine the cytotoxic effect of nelfinavir in Huh7/Rep-Feo cells, LDH levels in the supernatants were measured. No significant change in LDH levels was detectable after 48 h incubation (Fig. 1c). MTS assays of the cells cultured with nelfinavir indicated no significant effects on cell viability (Fig. 1d). Nuclear DNA fragmentation in Huh7/Rep-Feo cells, a possible mechanism of nelfinavir induced cytotoxicity, was evaluated by TUNEL staining. No fragmentation of nuclear DNA was observed in Huh7/Rep-Feo cells treated with 5.0 μM nelfinavir (Fig. 1e).

Inhibition of HCV RNA replication by IFN alone and in combination with nelfinavir

Huh7/Rep-Feo cells were cultured with various concentrations of IFN, and the dose-effect correlation and time courses of replicon expression were measured by luciferase assay. IFN caused a marked dose-dependent inhibition of HCV RNA replication (Fig. 2a). The inhibition of HCV RNA replication was detectable at concentrations of IFN as low as 0.01 U/mL. In contrast, measurement of LDH levels and the results of the MTS assay suggested that IFN had little effect on cell viability and replication (data not shown). A dose-effect curve for the effects of nelfinavir and IFN on the replicon was generated by treating Huh7/Rep-Feo cells with various concentrations of IFN (1.0, 0.1, 0.01, 0.001 and 0 U/mL) and nelfinavir (5, 10 and 0 μmol/L). The luciferase activities were plotted against the drug concentrations after 48 h incubation. The inhibition curves were shifted to the left with increasing concentrations of nelfinavir (Fig. 2b), demonstrating synergy between the two drugs against the HCV replicon. There were no significant differences in MTS reduction values at the different drug concentrations (data not shown).

Synergistic inhibitory effects of nelfinavir and IFN on the replicon

We investigated a possible synergistic anti-HCV effect between nelfinavir and IFN, using the isobologram method

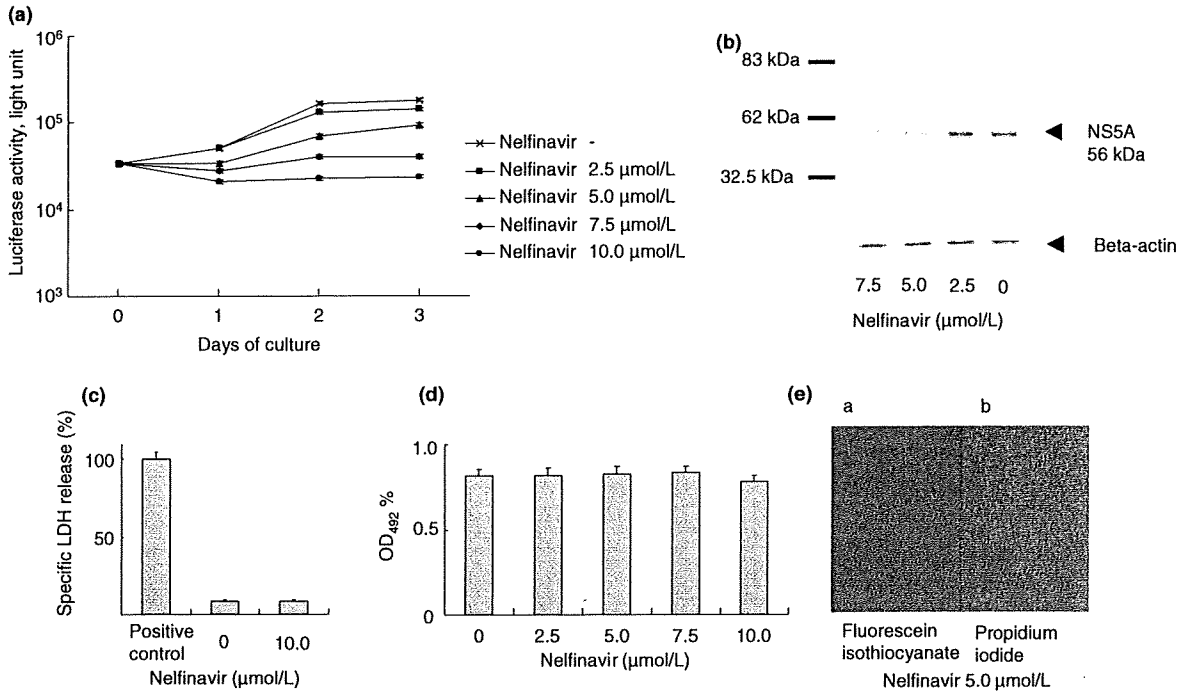

Fig. 1 Dose-dependent inhibition of hepatitis C virus (HCV) RNA replication by nelfinavir. (a) Huh7/Rep-Feo cells were cultured with concentrations of nelfinavir as indicated. (b) Western blotting. The cells were cultured in the presence of nelfinavir, as indicated and were harvested after 48 h exposure. (c) Cytotoxicity assay. Lactate dehydrogenase (LDH) assay of Huh7/Rep-Feo cells cultured with the concentrations of nelfinavir indicated. (d) 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazoly)-3-(4-sulfophenyl) tetrazolium inner salt (MTS) assay of Huh7/Rep-Feo cells cultured with the concentrations of nelfinavir indicated. (e) Nuclear DNA fragmentation in Huh7/Rep-Feo cells detected by the TUNEL method. Cells were observed using a confocal laser scanning microscopy (all 200×). Nuclear DNA fragmentation is shown in green (a: fluorescein isothiocyanate staining), and Huh7/Rep-Feo cell nuclei in red (b: propidium iodide staining).

and Calcusyn software, as described in Material and methods. A log dose-effect curve and median effect plot were made for both drugs. Both drugs showed linear regression of effect on the logarithms of doses (R^2 = 0.94 for nelfinavir; R^2 = 0.99 for IFN). The IC_{50} values were 9.88 ± 0.43 μmol/L for nelfinavir and 0.099 ± 0.14 U/mL for IFN (Fig. 3a,b).

In order to determine if nelfinavir and IFN had a synergistic inhibitory effect on the replicon, Huh7/Rep-Feo cells were treated with combinations of IFN and nelfinavir at various concentrations. Isobolograms were generated based on the two drugs administered in combination at fixed ratios, adjusted for the IC_{50} of each drug (FIC ratio): 1:0, 4:1, 1:1, 1:4 and 0:1. Using the Calcusyn software, each 90% inhibition of HCV replication (Fa = 0.90), 75% inhibition of HCV replication (Fa = 0.75), and 50% inhibition of HCV replication were plotted on the x and y axes (Fig. 3c). The ED_{90}, ED_{75} and ED_{50} plots for each drug ratio fell below the line representing additivity, indicating synergistic effects of the drug combination on intracellular HCV-RNA replication. The CI at an Fa value of 0.5 was 0.58, generated from Fig. 3c using CalcuSyn. There was no significant difference in MTS reduction at different drug concentrations (data not shown), suggesting that the synergistic action of nelfinavir and IFN on HCV replication was through their pharmacological effects, and not due to augmentation of cytotoxicity.

DISCUSSION

The results of this study suggest that nelfinavir inhibits HCV replication at concentrations that show no cytotoxicity, and that nelfinavir and IFN act synergistically against HCV.

Nelfinavir inhibited HCV replication in a concentration-dependent manner and its effects could be observed at concentrations as low as <3.0 μmol/L. In clinical use, the plasma concentration of nelfinavir ranges from 3.3 to 6.0 μmol/L. These results support those of Trimoulet *et al.*, who found a reduction in HCV loads in patients treated with HAART including nelfinavir [16]. García-Samaniego *et al.* reported that indinavir, another HIV protease inhibitor, failed to reduce HCV viral titres [4]. In a preliminary study using the replicon system, we tested the ability of several unpurified HIV protease inhibitors to inhibit HCV replication: nelfinavir, ritonavir and saquinavir reduced HCV-replication, but indinavir and fosamprenavir had no effect (data not

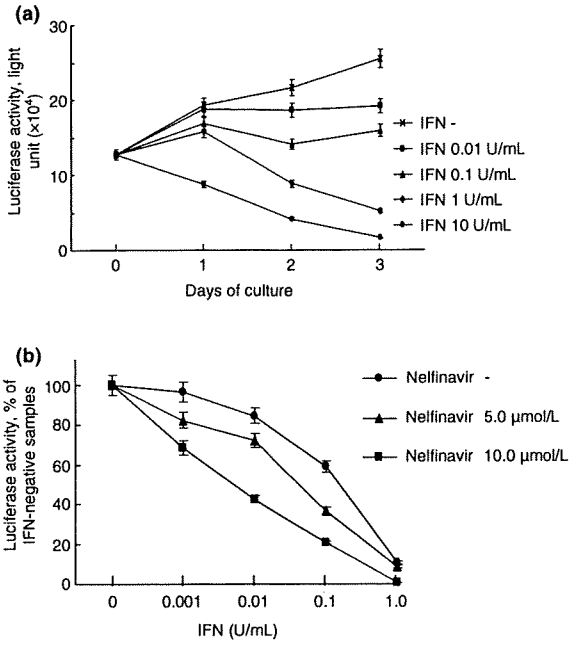

Fig. 2 Dose-dependent inhibition of hepatitis C virus (HCV) RNA replication by IFN. (a) Huh7/Rep-Feo cells were cultured with concentrations of IFN as indicated. (b) Dose-inhibition curves for IFN combined with the concentrations of nelfinavir indicated. Luciferase activities are displayed as percentages of the IFN-negative samples.

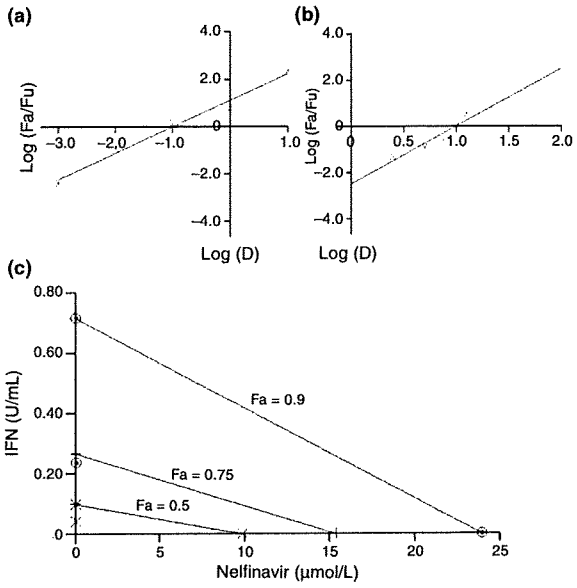

Fig. 3 CalcuSyn analysis of the interferon (IFN)/nelfinavir combination effects on intracellular hepatitis C virus (HCV) replication. Huh7/Rep-Feo cells were cultured with various concentrations of nelfinavir and IFN. Luciferase activities of the cell lysates were measured after 48 h exposure. The CalcuSyn median-effect plot was generated from three separate experiments in triplicate with SD <20% (Fa: affected fraction, Fu: unaffected fraction, D: concentration of drug used). (a) Log dose-effect curve and median effect plot for nelfinavir. Median effect plot has the form of a straight line, $y = 2.47x + 0.43$. (b) Log dose-effect curve and median effect plot for IFN. Median effect plot has the form of a straight line, $y = 1.12x - 0.14$. (c) Isobologram analysis of the combination of IFN and nelfinavir in Huh7/Rep-Feo cells. The individual doses of IFN and nelfinavir required to achieve 90% inhibition of HCV-replication (Fa = 0.90), 75% inhibition of HCV-replication (Fa = 0.75), 50% inhibition of HCV-replication were plotted on the x and y axes. Combination index (CI) values calculated using the Calcusyn software are represented by points above (indicate antagonism between drugs) or below the lines (indicate synergy). (X symbol) ED50, (plus sign) ED75 and (open dotted circle) ED90.

shown). These discrepancies in the effects of different protease inhibitors could explain the different results found in clinical studies.

Combination therapy using ribavirin and IFN is a standard therapy for patients with chronic HCV infection, including HCV/HIV coinfected patients [11]. We previously reported that the use of a clinically achievable concentration of ribavirin, in combination with IFN, showed strong synergistic inhibitory effects on HCV replication using the replicon system [29]. In this study, nelfinavir showed similar strong synergy with IFN. These results suggest that nelfinavir could improve the antiviral effects of IFN in HCV/HIV coinfected patients.

HIV protease inhibitors have a strong affinity for the active site of the HIV viral aspartyl protease, and irreversibly inhibit the catalytic activity of the enzyme. However, HIV protease inhibitors are thought not to inhibit HCV viral serine protease. The above-mentioned findings which demonstrate that different HIV protease inhibitors have different effects on HCV replication support this idea because if HIV protease inhibitors inhibited HCV serine protease, then all HIV protease inhibitors should inhibit HCV replication. The mechanism by which nelfinavir inhibits HCV replication is uncertain: Several studies have shown that HCV infection alters NF-κB promoter activity, possibly contributing to the persistence of HCV infection [30–32]. Equils *et al.* reported that nelfinavir blocked TLR2-, TLR4- and TNF-α-induced NF-κB activation [25]. Nelfinavir may play an important role in the regulation of the cellular inflammatory and immune responses through NF-κB, but further studies are needed to investigate the role of NF-κB promoter activity in nelfinavir-induced HCV replication inhibition.

The decreased clearance of antiretroviral drugs is suspected to be a possible cause of increased susceptibility for HAART-associated liver toxicity in HIV/HCV coinfected patients, because the metabolism of the HIV protease inhibitors depends on the amount of functional cytochrome

P450, which is reduced in severe liver disease [33]. Indeed, increased toxic drug concentrations have been reported in patients with hepatic disease, receiving standard dose of nelfinavir [34]. Meanwhile, Bruno *et al.* have reported, in a clinical study, that nelfinavir was associated with the lowest rates of severe hepatotoxicity in patients coinfected with hepatitis viruses among the available HIV protease inhibitors [21]. In our study, nelfinavir failed to induce cytotoxicity or apoptosis at concentrations that inhibited HCV replication.

The implications of our results for understanding the effect of nelfinavir on HCV replication are limited because this study used an *in vitro* HCV subgenomic replicon system, which only expresses viral nonstructural, and not structural, proteins. To elucidate the effects of nelfinavir on full-length, infectious hepatitis C virions, further studies using full genomic replicons are needed. In addition, the influence of immune reconstitution induced by HAART, including nelfinavir, on HCV replication needs to be investigated before clinical application of our data to therapy for HCV/HIV coinfected patients.

Because end-stage chronic liver disease resulting from co-infection with HCV is now the major cause of death in individuals infected with HIV, our results suggest a potentially promising approach for improving the standard therapies for chronic hepatitis C in HCV/HIV coinfected patients.

CONFLICT OF INTEREST

The authors report no conflict of interest.

REFERENCES

1 Hayashi PH, Flynn N, McCurdy SA, Kuramoto IK, Holland PV, Zeldis JB. Prevalence of hepatitis C virus antibodies among patients infected with human immunodeficiency virus. *J Med Virol* 1991; 33(3): 177–180.

2 Sherman KE, Rouster SD, Chung RT, Rajicic N. Hepatitis C Virus prevalence among patients infected with human immunodeficiency virus: a cross-sectional analysis of the US adult AIDS Clinical Trials Group. *Clin Infect Dis* 2002; 34(6): 831–837.

3 Salmon-Ceron D, Lewden C, Morlat P *et al.* Liver disease as a major cause of death among HIV infected patients: role of hepatitis C and B viruses and alcohol. *J Hepatol* 2005; 42(6): 799–805.

4 Soriano V, Martin-Carbonero L, Garcia-Samaniego J, Puoti M. Mortality due to chronic viral liver disease among patients infected with human immunodeficiency virus. *Clin Infect Dis* 2001; 33(10): 1793–1795.

5 Valle Tovo C, Alves de Mattos A, Ribeiro de Souza A *et al.* Impact of human immunodeficiency virus infection in patients infected with the hepatitis C virus. *Liver Int* 2007; 27(1): 40–46.

6 Benhamou Y, Bochet M, Di Martino V *et al.* Liver fibrosis progression in human immunodeficiency virus and hepatitis C virus coinfected patients. The Multivirc Group. *Hepatology (Baltimore, MD)* 1999; 30(4): 1054–1058.

7 Puoti M, Bonacini M, Spinetti A *et al.* Liver fibrosis progression is related to CD4 cell depletion in patients coinfected with hepatitis C virus and human immunodeficiency virus. *J Infect Dis* 2001; 183(1): 134–137.

8 Daar ES, Lynn H, Donfield S *et al.* Relation between HIV-1 and hepatitis C viral load in patients with hemophilia. *J Acquir Immune Defic Syndr* 2001; 26(5): 466–472.

9 Goedert JJ, Hatzakis A, Sherman KE, Eyster ME. Lack of association of hepatitis C virus load and genotype with risk of end-stage liver disease in patients with human immunodeficiency virus coinfection. *J Infect Dis* 2001; 184(9): 1202–1205.

10 Sherman KE, O'Brien J, Gutierrez AG *et al.* Quantitative evaluation of hepatitis C virus RNA in patients with concurrent human immunodeficiency virus infections. *J Clin Microbiol* 1993; 31(10): 2679–2682.

11 Strader DB, Wright T, Thomas DL, Seeff LB. Diagnosis, management, and treatment of hepatitis C. *Hepatology (Baltimore, MD)* 2004; 39(4): 1147–1171.

12 Yokozaki S, Takamatsu J, Nakano I *et al.* Immunologic dynamics in hemophiliac patients infected with hepatitis C virus and human immunodeficiency virus: influence of antiretroviral therapy. *Blood* 2000; 96(13): 4293–4299.

13 Ragni MV, Bontempo FA. Increase in hepatitis C virus load in hemophiliacs during treatment with highly active antiretroviral therapy. *J Infect Dis* 1999; 180(6): 2027–2029.

14 Babik JM, Holodniy M. Impact of highly active antiretroviral therapy and immunologic status on hepatitis C virus quasispecies diversity in human immunodeficiency virus/hepatitis C virus-coinfected patients. *J Virol* 2003; 77(3): 1940–1950.

15 Sulkowski MS, Benhamou Y. Therapeutic issues in HIV/HCV-coinfected patients. *J Viral Hepat* 2007; 14(6): 371–386.

16 Trimoulet P, Neau D, Le Bail B *et al.* Intrahepatic HCV RNA loads in 37 HIV-HCV co-infected patients with controlled HIV infection. *J Med Virol* 2002; 67(2): 143–151.

17 Wit FW, Weverling GJ, Weel J, Jurriaans S, Lange JM. Incidence of and risk factors for severe hepatotoxicity associated with antiretroviral combination therapy. *J Infect Dis* 2002; 186(1): 23–31.

18 Danner SA, Carr A, Leonard JM *et al.* A short-term study of the safety, pharmacokinetics, and efficacy of ritonavir, an inhibitor of HIV-1 protease. European-Australian Collaborative Ritonavir Study Group. *N Engl J Med* 1995; 333(23): 1528–1533.

19 den Brinker M, Wit FW, Wertheim-van Dillen PM *et al.* Hepatitis B and C virus co-infection and the risk for hepatotoxicity of highly active antiretroviral therapy in HIV-1 infection. *AIDS (London, England)* 2000; 14(18): 2895–2902.

20 John M, Flexman J, French MA. Hepatitis C virus-associated hepatitis following treatment of HIV-infected patients with HIV protease inhibitors: an immune restoration disease? *AIDS (London, England)* 1998; 12(17): 2289–2293.

21 Bruno R, Sacchi P, Maiocchi L, Zocchetti C, Filice G. Hepatotoxicity and nelfinavir: a meta-analysis. *Clin Gastroenterol Hepatol* 2005; 3(5): 482–488.

22 Mitsuya H, Yarchoan R, Kageyama S, Broder S. Targeted therapy of human immunodeficiency virus-related disease. *FASEB J* 1991; 5(10): 2369–2381.

23 Vlahakis SR, Bennett SA, Whitehead SN, Badley AD. HIV protease inhibitors modulate apoptosis signaling in vitro and in vivo. *Apoptosis* 2007; 12(5): 969–977.
24 Ghosh S, Karin M. Missing pieces in the NF-kappaB puzzle. *Cell* 2002; 109(Suppl): S81–S96.
25 Equils O, Shapiro A, Madak Z, Liu C, Lu D. Human immunodeficiency virus type 1 protease inhibitors block toll-like receptor 2 (TLR2)- and TLR4-Induced NF-kappaB activation. *Antimicrob Agents Chemother* 2004; 48(10): 3905–3911.
26 Guo JT, Bichko VV, Seeger C. Effect of alpha interferon on the hepatitis C virus replicon. *J Virol* 2001; 75(18): 8516–8523.
27 Yokota T, Sakamoto N, Enomoto N *et al.* Inhibition of intracellular hepatitis C virus replication by synthetic and vector-derived small interfering RNAs. *EMBO Rep* 2003; 4(6): 602–608.
28 Yamashiro T, Sakamoto N, Kurosaki M *et al.* Negative regulation of intracellular hepatitis C virus replication by interferon regulatory factor 3. *J Gastroenterol* 2006; 41(8): 750–757.
29 Tanabe Y, Sakamoto N, Enomoto N *et al.* Synergistic inhibition of intracellular hepatitis C virus replication by combination of ribavirin and interferon-alpha. *J Infect Dis* 2004; 189(7): 1129–1139.
30 Chung YM, Park KJ, Choi SY, Hwang SB, Lee SY. Hepatitis C virus core protein potentiates TNF-alpha-induced NF-kappaB activation through TRAF2-IKKbeta-dependent pathway. *Biochem Biophys Res Commun* 2001; 284(1): 15–19.
31 Joo M, Hahn YS, Kwon M, Sadikot RT, Blackwell TS, Christman JW. Hepatitis C virus core protein suppresses NF-kappaB activation and cyclooxygenase-2 expression by direct interaction with IkappaB kinase beta. *J Virol* 2005; 79(12): 7648–7657.
32 Wagoner J, Austin M, Green J *et al.* Regulation of CXCL-8 (interleukin-8) induction by double-stranded RNA signaling pathways during hepatitis C virus infection. *J Virol* 2007; 81(1): 309–318.
33 Nakai K, Tanaka H, Hanada K *et al.* Decreased expression of cytochromes P450 1A2, 2E1, and 3A4 and drug transporters Na+-taurocholate-cotransporting polypeptide, organic cation transporter 1, and organic anion-transporting peptide-C correlates with the progression of liver fibrosis in chronic hepatitis C patients. *Drug Metab Dispos Biol Fate Chem* 2008; 36(9): 1786–1793.
34 Maserati R, Villani P, Seminari E, Pan A, Lo Caputo S, Regazzi MB. High plasma levels of nelfinavir and efavirenz in two HIV-positive patients with hepatic disease. *AIDS (London, England).* 1999; 13(7): 870–871.

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)

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.

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

B virus or human immunodeficiency virus (HIV), and alcohol consumption accelerate progression of this chronic disease. Oxidative stress has been postulated to be one of the deleterious factors of chronic hepatitis, and it was reported that antioxidant levels were significantly reduced in chronic hepatitis and cirrhosis[1]. Moreover, it was also found that HCV proteins themselves generate oxidative stress, and the additive effect of oxidative stress caused by the inflammatory process in hepatitis and that induced by HCV proteins may further advance the disease stage of chronic hepatitis C[2]. Therefore, it is thought that antioxidant therapy has a role in slowing disease progression to cirrhosis and subsequent HCC. In fact, there are studies suggesting a beneficial effect of antioxidant therapy for patients who didnot respond to interferon (IFN) therapy, and that a combination of antiviral and antioxidant therapies may enhance the overall response rate of patients with chronic hepatitis C[3,4].

Current recommended therapy for previously untreated and relapsed patients is a combination of pegylated interferon (Peg-IFN) and ribavirin (RBV), resulting in a sustained virological response in around 50% of genotype 1 patients with high viral load[5]. Recent studies have shown that protease and polymerase inhibitors possess a strong additive effect on antiviral therapy of Peg-IFN/RBV and seem to be promising[6]. However, these regimens are expensive and adverse effects are sometimes severe and frequent, meaning that large numbers of patients give up treatment for a variety of reasons[7]. These conditions have lead patients with chronic hepatitis C to use complementary and alternative medicine (CAM) including various supplements, and a previous survey found that about 40% of patients of liver disease outpatient clinics in the US used CAM at least once during the preceding month[8]. Moreover, a large number of supplements are used by patients universally to maintain their health condition or improve quality of life even if they are cared by medical doctors and they always do not tell doctors whether they used CAM and/or supplements. The most frequent CAM or supplements taken by patients with chronic hepatitis C were antioxidants, which may be beneficial for this disease as described above.

Resveratrol (RVT) was discovered to be a strong activator of *sirtuin*, a gene for longevity[9], and has been implicated as the most important polyphenol responsible for the beneficial effects of red wine consumption, which has been called as the "French Paradox"[10]. Polyphenols contained in red wine have shown a strong antioxidative effect on cardioprotection, anti-atherosclerosis and relaxation of vascular endothelium through nitric oxide release. *Sirtuin* is activated when a person undergoes calorie restriction, and RVT is thought to be a surrogate for calorie restriction, which induces stabilization of DNA and also increases longevity by 70%. RVT is also known to improve liver lesions such as acetaminophen-induced hepatic injury and liver fibrosis in the mouse. When RVT was administered to mice fed a high-fat diet, fatty liver induced by this high-calorie diet was significantly improved[11]. In addition to these favorable reactions, it has been reported that RVT inhibited viral replication of several major viruses, such as cytomegalovirus (CMV), varicella-zoster, influenza A, and herpes simplex virus (HSV)[12-15]. This supplement is popular and is thought to be one of candidates for the supplemental treatment of chronic hepatitis C.

Another candidate is astaxanthin (AXN: 3,3'-dihydroxy-b, b-carotene-4,4'-dione), which also showed a strong antioxidative effect[16]. AXN is the carotenoid responsible for the pink pigmentation in the flesh of salmon, lobster, krill and other aquatic animals and plants. Recent studies have indicated that AXN is more powerful than its carotenoid cousin, β carotene, at neutralizing singlet oxygen[17]. This supplement is known to improve the condition of so-called metabolic syndrome[18,19], and is therefore popular. The antiviral effect of this supplement has not been examined so far.

These reports suggested that RVT and AXN might be good candidates for an antioxidative as well as an anti-HCV agent. However, we have no information whether these antioxidants affect HCV replication or not and if they are suitable for patients with chronic hepatitis C. In this study, we tried to assess the effect of these antioxidants on HCV replication using the HCV replicon system as an *in vitro* tool[20]. This is the first report to investigate the effect of RVT and AXN on HCV replication *in vitro*.

MATERIALS AND METHODS

Cells and virus

OR6 cells, a cell line cloned from ORN/C-5B/KE cells[21] supporting genome-length HCV RNA (strain O of genotype 1b) encoding the luciferase reporter gene, were used. This cell line was originally derived from a hepatoma cell line, HuH-7, as described elsewhere[21]. The schematic organization of the *ORN/C-5B/KE* gene is shown in Figure 1A. This cell line was cultured and maintained as previously reported[22]. Another cell line used was sKAH-5R[23], which was established from a patient with acute hepatitis C, having subgenomic HCV RNA encoding the luciferase reporter gene (Figure 1B). The latter cell line was cultured under the same conditions as the OR6 cells, which includes the gene without a structural region of HCV RNA from the *ORN/C-5B/KE* gene.

Chemicals

We evaluated RVT and AXN as new supplements, and vitamin E (VE) was used as a control because its effect on the HCV replicon system was already reported elsewhere[24]. RVT (3,5,4'-trihydroxystilbene), RBV (1-b-D-ribofuranosyl-1H-1,2,4-triazole-3-carboxamide), AXN (3,3'-dihydroxy-b,b-carotene-4,4'-dione), VE and IFN-2b were purchased from Sigma-Aldrich Japan (Tokyo, Japan). AXN, RVT and VE were prepared as 10-20 mg/mL stock solutions in dimethylsulfoxide (DMSO) and stored at -80℃ until used. This stock solution was diluted with culture medium. The final

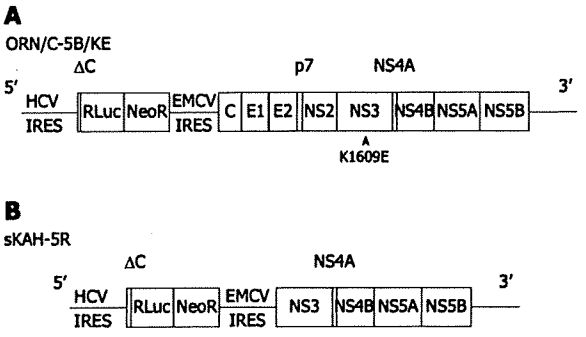

Figure 1 Organization of hepatitis C virus (HCV) RNA used in the replicon systems. A: Genome-length HCV RNA generated in OR6 cells; B: Subgenomic HCV RNA used in sKAH-5R and s1B-4R cells. Structural region of HCV RNA was deficient in the replicated RNA of sKAH-5R and s1B-4R cells.

concentration of DMSO was 0.2%, which didnot interfere with viral replication, the highest concentration of RVT used in this study was 100 μmol/L, that of AXN was 50 μmol/L, and that of VE was 15 μmol/L. To monitor the anti-HCV effects of IFN and RBV on replication, OR6 cells (1.5×10^4 /well) were plated onto 24-well plates at least in triplicate for each assay and cultured for 4 h. Then the cells were treated with IFN at a final concentration of 1, 2, 4, 10, or 20 U/mL or RBV at a final concentration of 10 or 25 μmol/L for 72 h, harvested with renilla lysis reagent (Promega, Madison, WI), and assayed for luciferase activity according to the manufacturer's protocol. The same protocol was applied for sKAH-5R cells. The additive effect of RVB, RVT and AXN on the antiviral effect of IFN (1 U) was studied and compared using luciferase activity.

Luciferase reporter assay

Approximately 1 to 4.5×10^4 cells were plated onto 6-well plates and cultured for 24 h. Cells were treated with each agent for 72 h. The cells were then harvested with Renilla lysis reagent and subjected to the Renilla luciferase (RL) assay according to the manufacturer's protocol.

Cell viability

We tested the toxic effect of RBV as described elsewhere[22]. The effect of RVT (5-100 μmol/L) and AXN (1-50 μmol/L) on cell viability was investigated. To examine the cytotoxic effect of RVT and AXN on cells with replicon RNA, the cells were seeded at a density of 2×10^5 cells per dish onto 6-well plates. After 24 h culture, the cells were treated with RVT at final concentrations of 25 and 50 μmol/L in the absence of G418. After incubation for 72 h, the number of viable cells was counted in an improved Neubauer-type hemocytometer after trypan blue dye (Invitrogen, Carlsbad, CA) treatment.

Statistical analysis

The difference in relative luciferase activity was tested using the Student's *t*-test and the Mann-Whitney *U*-test as appropriate. *P*-values < 0.05 were considered statistically significant. Every experiment was performed in triplicate and two independent experiments were done.

RESULTS

RVT dose-dependently enhanced HCV RNA replication but AXN inhibited replication

The effect of RVT and AXN was examined in comparison to that of VE, using the OR6 assay system, in which genomic length HCV RNA replication is represented by RL fluorescence activity.

After treatment of OR6 cells with various concentrations of RVT for 72 h, the luciferase activity was dose-dependently increased up to 20 mol/L (Figure 2A). The activity gradually decreased at higher concentrations, but at 100 mol/L it was still higher than that without RVT. Since it has been reported that the proliferation of the HCV subgenomic replicon is dependent on host-cell growth, we examined the effect of RVT on cell number and viability of OR6 cells by the trypan blue dye exclusion test. As shown in Figure 2B, RVT didnot increase OR6 cells until 15 μmol/L, and the cell viability decreased at higher concentrations than 20 μmol/L of RVT. This decrease seen in higher concentrations paralleled the luciferase activity shown in Figure 2A, and it seemed that RVT further enhanced HCV RNA replication at concentrations higher than 20 μmol/L when estimated by the number of viable cells. Different from the effect of RVT, AXN didnot enhance luciferase activity (Figure 2C). The luciferase activity decreased at a concentration more than 10 μmol/L, and this decrease seemed to be due to decrease in cell viability (Figure 2D).

VE dose-dependently increased luciferase activity up to a concentration of 15 μmol/L (Figure 3). This result indicated that VE also upregulates HCV RNA replication in OR6 cells. The cytotoxic effect of VE was not observed in the cell viability test. This result was compatible to the data already reported elsewhere[24].

Proliferative effect of RVT was also observed in the subgenomic replicon

Next, we further investigated the effect of RVT on other clones of HCV RNA. We tested the effect of RVT on the subgenomic HCV RNA-replicating cell, sKAH-5R. RVT also enhanced the replication of sKAH-5R subgenomic HCV RNA at 2-10 μmol/L (Figure 4A). This concentration is not toxic to the sKAH-5R cells, but more than 20 μmol/L of RVT was toxic to sKAH-5R cells (Figure 4B). Thus, subgenomic replicons showed the same results observed in the full-genome length replicon.

Anti-viral effects of IFN and RBV

The effects of IFN-2b and RBV were independently applied to these cells to demonstrate that anti-viral agents reduce HCV RNA replication in this cell line. Figure 5A shows the time course of luciferase activity after administration of IFN. Luciferase activity was

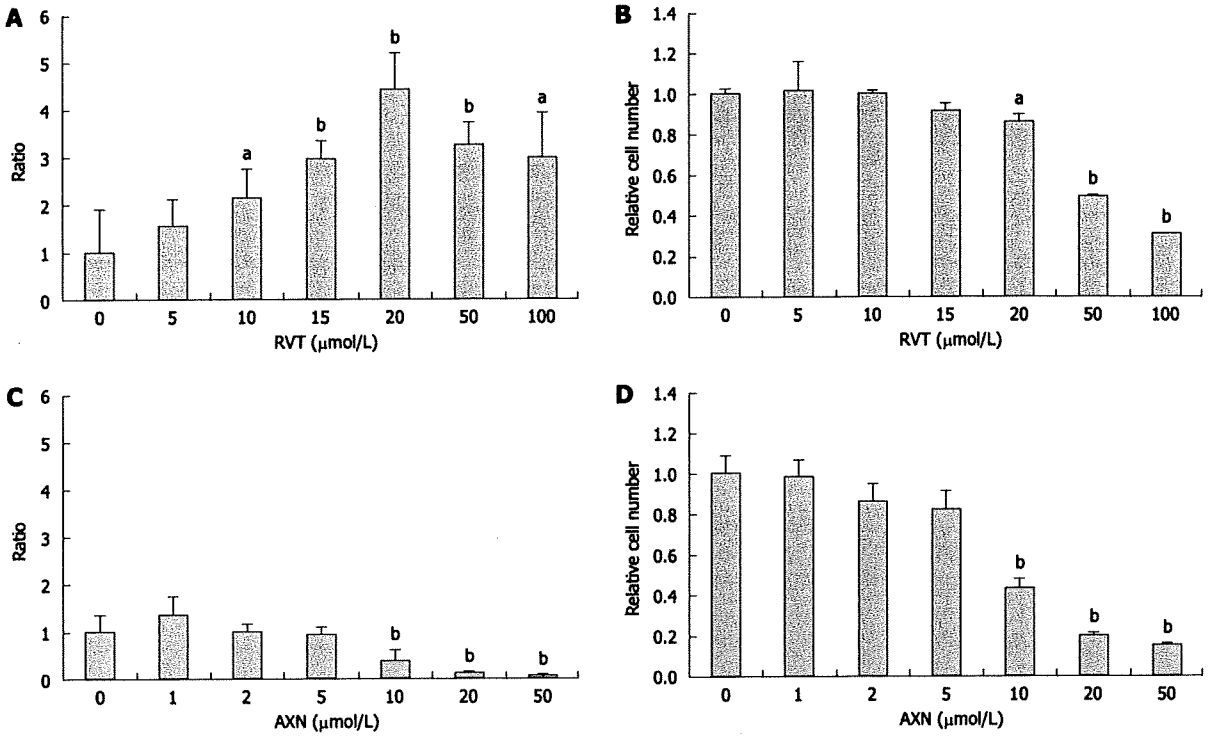

Figure 2 Luciferase activity and cell viability of OR6 cells after addition of resveratrol (RVT) and astaxanthin (AXN) for 72 h. The indicated concentrations of RVT or AXN were added to the culture medium of OR6 cells, and after 72 h of culture, cells were harvested with Runilla lysis buffer and the lysate was subjected to the luciferase assay. Cell viability was evaluated by a trypan blue dye exclusion assay. A: Luciferase activity after addition of RVT; B: Cell viability after addition of RVT; C: Luciferase activity after addition of AXN; D: Cell viability after addition of AXN. $^{a}P < 0.05$, $^{b}P < 0.01$.

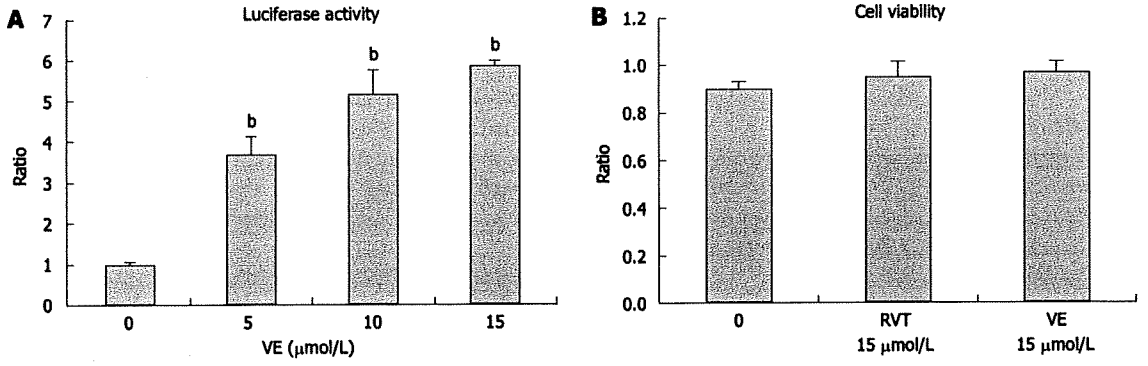

Figure 3 The effect of vitamin E (VE) on luciferase activity and cell viability of OR6 cells (72 h). Cells were treated with the indicated concentrations of VE for 72 h. A: Luciferase activity after addition of VE; B: Cell viability after addition of RVT and VE. $^{b}P < 0.01$.

dose-dependently inhibited by IFN. Figure 5B shows the effect of RBV at concentrations of 10 and 25 μmol/L on luciferase activity of OR6 cells 72 h after addition of RBV in comparison to that of IFN (1 U/mL). RBV also reduced luciferase activity of OR6 cells dose-dependently, but the effect was smaller than that of IFN. Anti-viral effects of IFN and RBV were thus confirmed in this cell line.

RVT reversed anti-viral activity of IFN and RBV, but AXN didnot affect it

We then investigated whether RVT reverses the anti-viral effects of RBV and IFN. Luciferase activity of OR6 cells 72 h after treatment with RBV or IFN was compared to treatment with RBV plus RVT or IFN plus RVT (Figure 6A). The effect of treatment with 10 μmol/L of RVT alone on OR6 cells showed a 2.1-fold increase of luciferase activity. The addition of RVT (10 μmol/L) to RBV- or IFN-treated cells reversed the anti-proliferative effect of RBV on HCV RNA even when the cells were treated with 25 μmol/L of RBV plus 1 U/mL of IFN, which was normally enough to reduce HCV RNA to 1/5 (Figure 5B). Ten μmol/L RVT upregulated luciferase activity in RBV- or IFN-treated OR6 cells around 2-fold

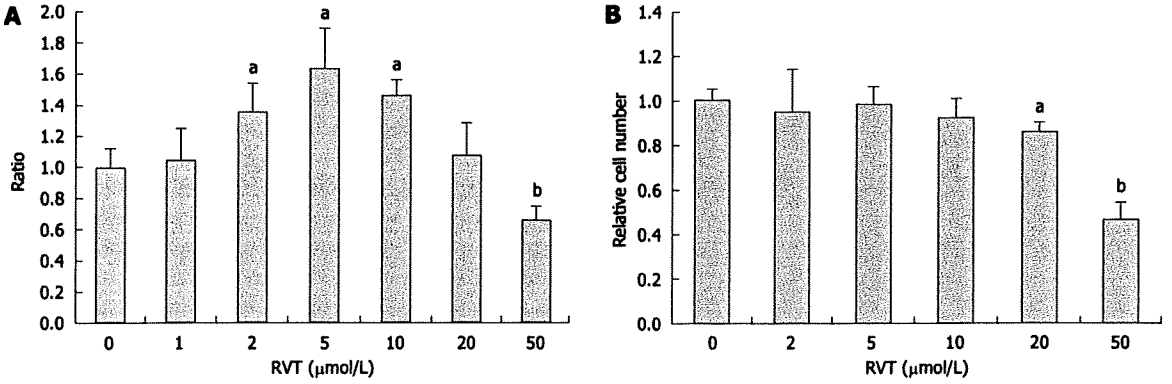

Figure 4 The effect of resveratrol on subgenomic replicon cells, sKAH-5R. RVT was added to the cells for 72 h and luciferase activity was assayed as was indicated in Figure 2. A: Luciferase activity after addition of RVT; B: Cell viability after addition of RVT. $^{a}P < 0.05$, $^{b}P < 0.01$.

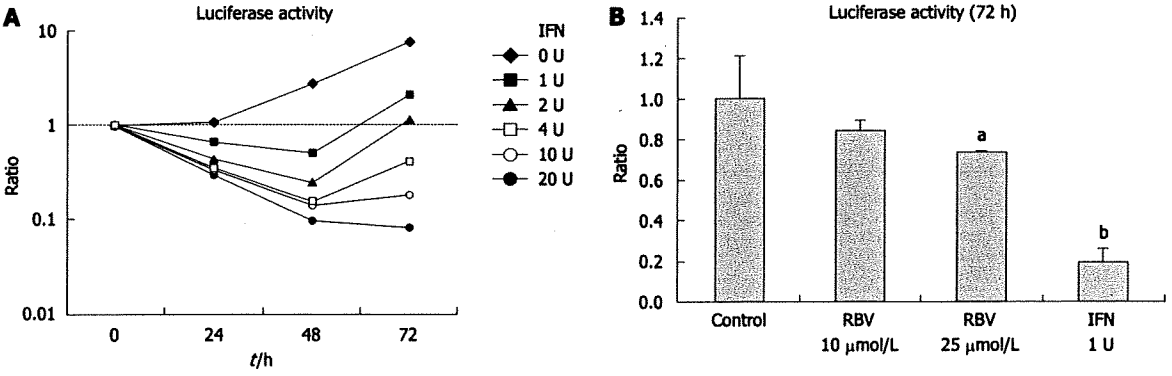

Figure 5 Sequential change of luciferase activity of OS6 cells after addition of interferon (IFN). A: IFN was added for the indicated duration and the ratio to the luciferase activity at time 0 was shown. Statistical significance ($^{a}P < 0.05$, $^{b}P < 0.01$) is shown below: at 24 h 0 *vs* 1^{a}, 2^{b}, 4^{b}, 10^{b} and 20^{b}; at 48 h, 0 *vs* 1^{b}, 2^{b}, 4^{b}, 10^{b} and 20^{b}; 1 *vs* 2^{b}, 4^{b}, 10^{b} and 20^{b}; 20 *vs* 2^{b}, 4^{b} and 10^{b}; at 72 h, 0 *vs* 1^{b}, 2^{b}, 4^{b}, 10^{b} and 20^{b}; 1 *vs* 2^{a}; 1 *vs* 4^{b}, 10^{b} and 20^{b}; 2 *vs* 4^{b}, 10^{b} and 20^{b}; 4 *vs* 10^{b} and 20^{b}; 10 *vs* 20^{b}; B: Luciferase activity after addition of RVB and IFN for 72 h was shown as the ratio to that without antiviral agents. $^{a}P < 0.05$, $^{b}P < 0.01$.

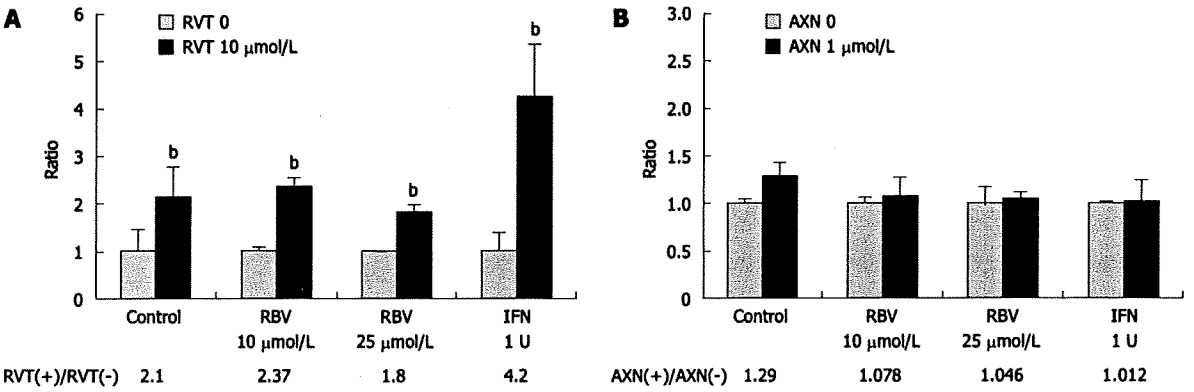

Figure 6 The enhancing effect of RVT on replication of HCV RNA, even when treated with ribavirin (RBV) and IFN. RVT was simultaneously added to RBV or IFN. A: The ratio was calculated as the ratio of luciferase activity with RVT to that without RVT. $^{b}P < 0.01$ *vs* RVT 0; B: The same procedure was applied in AXN.

and 4.2-fold, respectively (Figure 6A). On the other hand, AXN didnot affect the effect of RBV and IFN (Figure 6B), indicating that AXN has no disadvantageous effect on antiviral activity of IFN and RBV.

This proliferative effect of RVT was further emphasized by comparison to the additive effects of RBV with IFN. We compared the dose-dependent effect of RBV, RVT and AXN on the anti-proliferative effect of IFN (Figure 7). RBV (A), RVT (B) and AXN (C) were added to OS6 cells at concentrations of 0, 1, 2, 5, 10 or 15 μmol/L with 1 U/mL of IFN for 48 h. The ratio between luciferase activity of RBV-, RVT- or AXN-treated

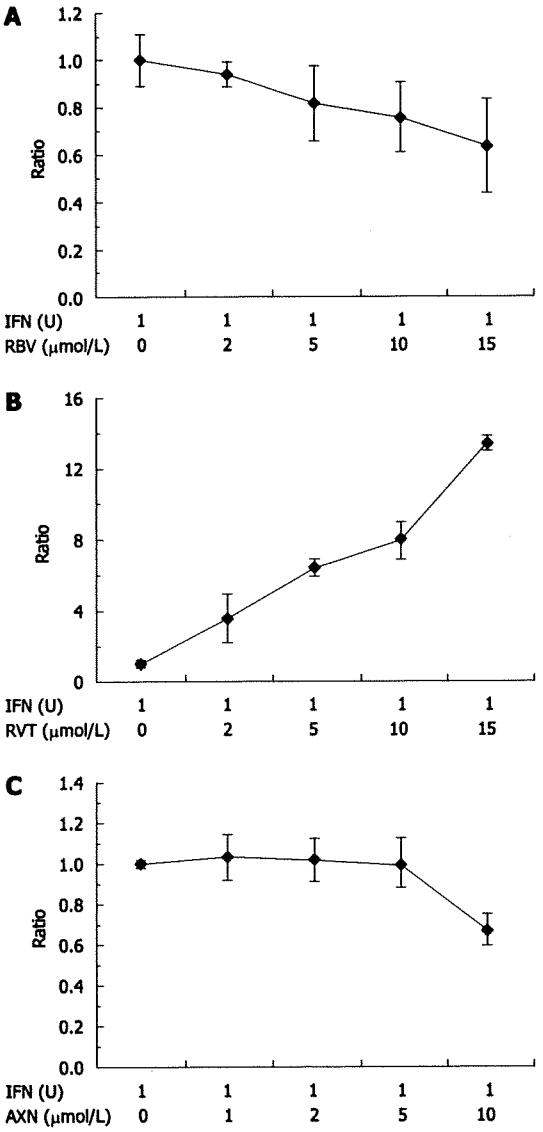

Figure 7 The comparative effect of RBV (A), RVT (B) and AXN (C) on the luciferase activity of IFN-treated cells. Cells were simultaneously treated with 1 U/mL of IFN and the indicated concentrations of RBV, RVT and AXN were added with IFN.

cells and that without co-treatment is shown. RBV further reduced the IFN-induced decrease in luciferase activity (Figure 7A), while RVT reversed this decrease, and further increased luciferase activity (Figure 7B). This effect was dose-dependent and it is noted that the enhanced ratio was strikingly large. On the other hand, AXN didnot affect antiviral effect of IFN (Figure 7C).

DISCUSSION

We have shown that RVT, a natural polyphenol contained in red wine and peanuts, enhanced *in vitro* replication of HCV RNA without producing significant proliferation of host cells. This is the first report demonstrating a proliferative effect of RVT on HCV. RVT inhibited replication of HSV-1, HSV-2[25-27] human CMV[13], Influenza A and orthomyxo virus[15]. Moreover, it was reported that RVT inhibited replication of HIV-1 synergistically with nucleoside analogues[28]. These reports suggest that RVT has a broad spectrum of anti-viral activities, and that RVT may selectively target the host, rather than the virus, as a mode of action for inhibiting viral replication. In spite of these inhibitory effects on viral replication, the mechanism of enhancing replication of HCV RNA is unclear. These results suggested that RVT is not suitable for antioxidant therapy of chronic hepatitis C. We also examined the effect of VE on replication of HCV RNA in OS6 cells, and VE enhanced its replication as effectively as RVT. On the other hand, AXN didnot enhance replication of HCV RNA and had no effect on antiviral activity of IFN and RBV. These results indicated that we could recommend patients with chronic hepatitis C do not take RVT, especially when they receive antiviral therapy.

RVT is a non-flavonoid polyphenol and exerts anti-oxidative, anti-neoplastic and anti-inflammatory properties[11]. Moreover, RVT has received much attention as an agent for prolongation of lifespan by activating silent information regulator 2 proteins, or sirtuins[25], which are implicated in influencing aging and regulating transcription, apoptosis and stress resistance[29]. These are causes for the popularity of this supplementation. Therapeutic intervention in liver injury with RVT has been suggested in various liver diseases[30], such as alcohol-induced liver disease[31], drug-induced liver injury[32], ischemia-reperfusion injury[33], and fatty liver diseases[11,34]. Furthermore, RVT has been implicated to be favorable for prevention of hepatic fibrosis[35,36]. These observations in combination with anti-viral effects indicated that RVT might be therapeutically beneficial or suitable for chronic hepatitis C. However, the direct effect of RVT on HCV RNA replication has not been studied thus far. In spite of our expectation, RVT didnot inhibit replication of HCV, and on the contrary, it enhanced replication. Moreover, RVT hampered the anti-viral effect of IFN or RBV, and HCV RNA replication was enhanced even when enough concentration of IFN or RBV was administered to OR6 cells to reduce HCV replication. This condition was quite different from that observed in HIV-1 replication, in which the effect of RVT was synergistic with anti-viral effect of nucleotide analogues. Unlike RVT, AXN didnot affect HCV replication and IFN-based antiviral activity, while it possesses strong antioxidant power.

An immunological response against virus-infected cells is an important pathogenic mechanism of chronic viral hepatitis. Reactive oxygen species (ROS) produced by activated macrophages and a consequent rise of lipid peroxidation cause direct activation of hepatic stellate (Ito) cells, leading to hepatic fibrosis and cirrhosis[37]. Moreover, HCV core protein directly increases ROS as well as lipid peroxidation products and antioxidant gene expression[38]. HCV infection is also associated with liver

iron accumulation[39], which further produces ROS in the liver. These observations suggested that anti-oxidant therapy has an important role in slowing disease progression to cirrhosis in chronic hepatitis C. In consequence of this theory, the use of CAM is common in patients with chronic liver disease[8]. Liu *et al*[40] reviewed medical herbs for HCV infection and concluded that some agents may have an effect on liver enzymes, but there is no firm evidence supporting efficacy of CAM. However, few studies have investigated the effect of antioxidants on HCV itself. Yano *et al*[24] investigated the effect of ordinary nutrients on HCV RNA replication using the replicon system, and found that some antioxidants such as β-carotene, vitamin D_2 and linoleic acid inhibited replication. They also showed an effect of VE on HCV RNA replication that was the same as in our study. In our study, AXN didnot affect replication of HCV RNA. Thus, there is a group of antioxidants which inhibit replication of HCV, while there is another group of antioxidants which enhance its replication. The precise mechanism of this difference has not been clarified, but the investigation of this mechanism may provide new insights into anti-viral mechanisms. Recently it has been demonstrated that anti-HCV nutrients induce activation of the MEK-ERK1/2 signaling pathway through phosphorylation of ERK1/2[41]. Study of this phenomenon may provide clues for a new therapeutic strategy in anti-viral treatment of HCV.

RVT has been shown to have a large number of regulatory biological functions, and Docherty *et al*[25-27] extensively studied the mechanism by which RVT inhibits the replication of HSV. However, even though it has been extensively studied, the molecular mechanism of RVT's action is not clear. Our results were quite different from those of Docherty's. In our study, not all antioxidants but 2 of 3 antioxidants increased the replication of HCV suggesting that the molecular mechanism of each agent is likely variable depending on viruses when we speculate in combination with studies of Docherty *et al*[25-27] and Yano *et al*[24]. On the other hand, reports suggesting a correlation between HCV replication and lipid metabolism have accumulated recently. It has been demonstrated that the cellular lipid droplet is an important structure for replication or assembly of viral components of HCV, especially HCV core protein[42]. The inhibitory effect of 3-hydroxyl-3-methylglutaryl coenzyme A reductase inhibitors on HCV have also been reported[43]. Moreover, the success of peg-IFN plus RBV combination therapy, that resulted in the disappearance of HCV, affected lipid metabolism thereafter *in vivo*[44]. Thus, it is conceivable that HCV genomic structure as well as the intracellular lipid is indispensable for viral replication of HCV. It is thought that RVT and VE affect intracellular lipid metabolism because they are lipid-soluble antioxidants. It is also interesting that HCV itself produces ROS, and that antioxidants affect the replication of HCV.

Bechmann *et al*[45] recently demonstrated that RVT in response to free fatty acid administration deteriorates fibrogenic activation of human hepatic stellate cells. They showed that RVT upregulated the expression of key mRNAs associated with activated, fibrogenic stellate cells, and also demonstrated that the combined presence of free fatty acids and RVT significantly reduced the hepatic stellate cells' susceptibility to apoptosis. This report was controversial since previous reports[35,36] demonstrated favorable effects of RVT on prevention of fibrosis progression. Bechmann *et al*[45] pointed out that the concentration of RVT was different from the previous study, and species' differences (employing rat *vs* human hepatic stellate cells) might be significant. Thus, RVT may have different therapeutic effects at various concentrations, and further investigation is needed to clarify a role of RVT in chronic liver diseases. Their result also suggested that patients with chronic hepatitis C should not take RVT as an additive nutrient, especially when they receive IFN-based antiviral therapy. Further investigations focusing on the enhancing mechanism of RVT on HCV RNA and different responses between RVT and AXN is necessary, and these approaches may develop a new strategy of anti-HCV agents.

In conclusion, we recommend patients with chronic hepatitis C who receive IFN-based antiviral therapy not to take RVT as an antioxidant supplement, although AXN may not affect anti-viral therapy.

COMMENTS

Background

Antiviral therapy for chronic hepatitis C has been developing, but the current standard therapy with pegylated interferon (Peg-IFN) and ribavirin combination therapy for 12 mo has achieved around 50% of patients who are infected with genotype 1 hepatitis C virus (HCV). Patients who have not attained viral clearance tend to take several supplementations for this chronic disease with an expectation for retardation of disease progression. A previous survey found that about 40% of patients of liver disease outpatient clinics in the US used complementary and alternative medicine (CAM) at least once during the preceding month. Among CAM, antioxidants have been popularly used by patients with chronic hepatitis C because it is said that oxidative stress deteriorates chronic hepatitis. However, the information about the use of supplementations for chronic hepatitis C was insufficient.

Research frontiers

Resveratrol (RVT) was discovered to be a strong activator of *sirtuin*, a gene for longevity, and the most important polyphenol responsible for the beneficial effects of red wine, which has been called the "French Paradox". RVT showed a strong antioxidative effect on cardioprotection, anti-atherosclerosis and relaxation of vascular endothelium through nitric oxide release. This information resulted in the popularity of this supplementation for people who suffered from chronic diseases. Since RVT inhibits the replication of other viruses, it is thought that RVT also inhibits HCV replication. However they revealed RVT enhanced HCV replication.

Innovations and breakthroughs

The investigation on HCV replication has been enabled by using the replicon system, in which HCV RNA replicates but unfortunately viral particles were not produced. Recently, the cell culture system in which HCV particles are produced was developed by Dr. Wakita T, and many new insights of HCV virology have been discovered. This study focused on the effect of taking daily supplementations on viral replication and antiviral therapy of HCV.

Applications

The authors recommend patients with chronic hepatitis C who receive IFN-

based antiviral therapy not to take RVT as an antioxidant supplement, although astaxanthin (AXN) may not affect anti-viral therapy.

Peer review

In this study, Nakamura *et al* tried to show the efficacy of antioxidants, RVT and AXN, on HCV replication. Since RVT inhibits the replication of other viruses, it was thought that RVT also inhibited HCV replication. However they revealed RVT enhanced HCV replication. These results are very interesting.

REFERENCES

1 **Jain SK**, Pemberton PW, Smith A, McMahon RF, Burrows PC, Aboutwerat A, Warnes TW. Oxidative stress in chronic hepatitis C: not just a feature of late stage disease. *J Hepatol* 2002; **36**: 805-811

2 **Koike K**, Miyoshi H. Oxidative stress and hepatitis C viral infection. *Hepatol Res* 2006; **34**: 65-73

3 **Melhem A**, Stern M, Shibolet O, Israeli E, Ackerman Z, Pappo O, Hemed N, Rowe M, Ohana H, Zabrecky G, Cohen R, Ilan Y. Treatment of chronic hepatitis C virus infection via antioxidants: results of a phase I clinical trial. *J Clin Gastroenterol* 2005; **39**: 737-742

4 **Gabbay E**, Zigmond E, Pappo O, Hemed N, Rowe M, Zabrecky G, Cohen R, Ilan Y. Antioxidant therapy for chronic hepatitis C after failure of interferon: results of phase II randomized, double-blind placebo controlled clinical trial. *World J Gastroenterol* 2007; **13**: 5317-5323

5 **Manns MP**, Foster GR, Rockstroh JK, Zeuzem S, Zoulim F, Houghton M. The way forward in HCV treatment--finding the right path. *Nat Rev Drug Discov* 2007; **6**: 991-1000

6 **Kieffer TL**, Sarrazin C, Miller JS, Welker MW, Forestier N, Reesink HW, Kwong AD, Zeuzem S. Telaprevir and pegylated interferon-alpha-2a inhibit wild-type and resistant genotype 1 hepatitis C virus replication in patients. *Hepatology* 2007; **46**: 631-639

7 **Coon JT**, Ernst E. Complementary and alternative therapies in the treatment of chronic hepatitis C: a systematic review. *J Hepatol* 2004; **40**: 491-500

8 **Seeff LB**, Lindsay KL, Bacon BR, Kresina TF, Hoofnagle JH. Complementary and alternative medicine in chronic liver disease. *Hepatology* 2001; **34**: 595-603

9 **Brooks CL**, Gu W. How does SIRT1 affect metabolism, senescence and cancer? *Nat Rev Cancer* 2009; **9**: 123-128

10 **Sun AY**, Simonyi A, Sun GY. The "French Paradox" and beyond: neuroprotective effects of polyphenols. *Free Radic Biol Med* 2002; **32**: 314-318

11 **Baur JA**, Pearson KJ, Price NL, Jamieson HA, Lerin C, Kalra A, Prabhu VV, Allard JS, Lopez-Lluch G, Lewis K, Pistell PJ, Poosala S, Becker KG, Boss O, Gwinn D, Wang M, Ramaswamy S, Fishbein KW, Spencer RG, Lakatta EG, Le Couteur D, Shaw RJ, Navas P, Puigserver P, Ingram DK, de Cabo R, Sinclair DA. Resveratrol improves health and survival of mice on a high-calorie diet. *Nature* 2006; **444**: 337-342

12 **Docherty JJ**, Sweet TJ, Bailey E, Faith SA, Booth T. Resveratrol inhibition of varicella-zoster virus replication in vitro. *Antiviral Res* 2006; **72**: 171-177

13 **Evers DL**, Wang X, Huong SM, Huang DY, Huang ES. 3,4',5-Trihydroxy-trans-stilbene (resveratrol) inhibits human cytomegalovirus replication and virus-induced cellular signaling. *Antiviral Res* 2004; **63**: 85-95

14 **Faith SA**, Sweet TJ, Bailey E, Booth T, Docherty JJ. Resveratrol suppresses nuclear factor-kappaB in herpes simplex virus infected cells. *Antiviral Res* 2006; **72**: 242-251

15 **Palamara AT**, Nencioni L, Aquilano K, De Chiara G, Hernandez L, Cozzolino F, Ciriolo MR, Garaci E. Inhibition of influenza A virus replication by resveratrol. *J Infect Dis* 2005; **191**: 1719-1729

16 **Barros MP**, Pinto E, Colepicolo P, Pedersén M. Astaxanthin and peridinin inhibit oxidative damage in Fe(2+)-loaded liposomes: scavenging oxyradicals or changing membrane permeability? *Biochem Biophys Res Commun* 2001; **288**: 225-232

17 **Shen H**, Kuo CC, Chou J, Delvolve A, Jackson SN, Post J, Woods AS, Hoffer BJ, Wang Y, Harvey BK. Astaxanthin reduces ischemic brain injury in adult rats. *FASEB J* 2009; **23**: 1958-1968

18 **Aoi W**, Naito Y, Takanami Y, Ishii T, Kawai Y, Akagiri S, Kato Y, Osawa T, Yoshikawa T. Astaxanthin improves muscle lipid metabolism in exercise via inhibitory effect of oxidative CPT I modification. *Biochem Biophys Res Commun* 2008; **366**: 892-897

19 **Hussein G**, Nakagawa T, Goto H, Shimada Y, Matsumoto K, Sankawa U, Watanabe H. Astaxanthin ameliorates features of metabolic syndrome in SHR/NDmcr-cp. *Life Sci* 2007; **80**: 522-529

20 **Nakamura M**, Saito H, Hibi T. Advances in genomic research on hepatitis C virus with a useful tool, replicon system. *Keio J Med* 2008; **57**: 75-83

21 **Ikeda M**, Abe K, Dansako H, Nakamura T, Naka K, Kato N. Efficient replication of a full-length hepatitis C virus genome, strain O, in cell culture, and development of a luciferase reporter system. *Biochem Biophys Res Commun* 2005; **329**: 1350-1359

22 **Nakamura M**, Saito H, Ikeda M, Tada S, Kumagai N, Kato N, Shimotohno K, Hibi T. Possible molecular mechanism of the relationship between NS5B polymorphisms and early clearance of hepatitis C virus during interferon plus ribavirin treatment. *J Med Virol* 2008; **80**: 632-639

23 **Ikeda M**, Kato N, Mizutani T, Sugiyama K, Tanaka K, Shimotohno K. Analysis of the cell tropism of HCV by using in vitro HCV-infected human lymphocytes and hepatocytes. *J Hepatol* 1997; **27**: 445-454

24 **Yano M**, Ikeda M, Abe K, Dansako H, Ohkoshi S, Aoyagi Y, Kato N. Comprehensive analysis of the effects of ordinary nutrients on hepatitis C virus RNA replication in cell culture. *Antimicrob Agents Chemother* 2007; **51**: 2016-2027

25 **Docherty JJ**, Fu MM, Hah JM, Sweet TJ, Faith SA, Booth T. Effect of resveratrol on herpes simplex virus vaginal infection in the mouse. *Antiviral Res* 2005; **67**: 155-162

26 **Docherty JJ**, Fu MM, Stiffler BS, Limperos RJ, Pokabla CM, DeLucia AL. Resveratrol inhibition of herpes simplex virus replication. *Antiviral Res* 1999; **43**: 145-155

27 **Docherty JJ**, Smith JS, Fu MM, Stoner T, Booth T. Effect of topically applied resveratrol on cutaneous herpes simplex virus infections in hairless mice. *Antiviral Res* 2004; **61**: 19-26

28 **Heredia A**, Davis C, Redfield R. Synergistic inhibition of HIV-1 in activated and resting peripheral blood mononuclear cells, monocyte-derived macrophages, and selected drug-resistant isolates with nucleoside analogues combined with a natural product, resveratrol. *J Acquir Immune Defic Syndr* 2000; **25**: 246-255

29 **Guarente L**. Sirtuins as potential targets for metabolic syndrome. *Nature* 2006; **444**: 868-874

30 **Muriel P**, Rivera-Espinoza Y. Beneficial drugs for liver diseases. *J Appl Toxicol* 2008; **28**: 93-103

31 **Bujanda L**, García-Barcina M, Gutiérrez-de Juan V, Bidaurrazaga J, de Luco MF, Gutiérrez-Stampa M, Larzabal M, Hijona E, Sarasqueta C, Echenique-Elizondo M, Arenas JI. Effect of resveratrol on alcohol-induced mortality and liver lesions in mice. *BMC Gastroenterol* 2006; **6**: 35

32 **Sener G**, Toklu HZ, Sehirli AO, Velioğlu-Oğünç A, Cetinel S, Gedik N. Protective effects of resveratrol against acetaminophen-induced toxicity in mice. *Hepatol Res* 2006; **35**: 62-68

33 **Hassan-Khabbar S**, Cottart CH, Wendum D, Vibert F, Clot JP, Savouret JF, Conti M, Nivet-Antoine V. Postischemic treatment by trans-resveratrol in rat liver ischemia-reperfusion: a possible strategy in liver surgery. *Liver Transpl* 2008; **14**: 451-459

34 **Ahn J**, Cho I, Kim S, Kwon D, Ha T. Dietary resveratrol

alters lipid metabolism-related gene expression of mice on an atherogenic diet. *J Hepatol* 2008; **49**: 1019-1028
35 **Kawada N**, Seki S, Inoue M, Kuroki T. Effect of antioxidants, resveratrol, quercetin, and N-acetylcysteine, on the functions of cultured rat hepatic stellate cells and Kupffer cells. *Hepatology* 1998; **27**: 1265-1274
36 **Souza IC**, Martins LA, Coelho BP, Grivicich I, Guaragna RM, Gottfried C, Borojevic R, Guma FC. Resveratrol inhibits cell growth by inducing cell cycle arrest in activated hepatic stellate cells. *Mol Cell Biochem* 2008; **315**: 1-7
37 **Svegliati Baroni G**, D'Ambrosio L, Ferretti G, Casini A, Di Sario A, Salzano R, Ridolfi F, Saccomanno S, Jezequel AM, Benedetti A. Fibrogenic effect of oxidative stress on rat hepatic stellate cells. *Hepatology* 1998; **27**: 720-726
38 **Okuda M**, Li K, Beard MR, Showalter LA, Scholle F, Lemon SM, Weinman SA. Mitochondrial injury, oxidative stress, and antioxidant gene expression are induced by hepatitis C virus core protein. *Gastroenterology* 2002; **122**: 366-375
39 **Hézode C**, Cazeneuve C, Coué O, Roudot-Thoraval F, Lonjon I, Bastie A, Duvoux C, Pawlotsky JM, Zafrani ES, Amselem S, Dhumeaux D. Liver iron accumulation in patients with chronic active hepatitis C: prevalence and role of hemochromatosis gene mutations and relationship with hepatic histological lesions. *J Hepatol* 1999; **31**: 979-984
40 **Liu JP**, Manheimer E, Tsutani K, Gluud C. Medicinal herbs for hepatitis C virus infection. *Cochrane Database Syst Rev* 2001; CD003183
41 **Yano M**, Ikeda M, Abe K, Kawai Y, Kuroki M, Mori K, Dansako H, Ariumi Y, Ohkoshi S, Aoyagi Y, Kato N. Oxidative stress induces anti-hepatitis C virus status via the activation of extracellular signal-regulated kinase. *Hepatology* 2009; **50**: 678-688
42 **Miyanari Y**, Atsuzawa K, Usuda N, Watashi K, Hishiki T, Zayas M, Bartenschlager R, Wakita T, Hijikata M, Shimotohno K. The lipid droplet is an important organelle for hepatitis C virus production. *Nat Cell Biol* 2007; **9**: 1089-1097
43 **Delang L**, Paeshuyse J, Vliegen I, Leyssen P, Obeid S, Durantel D, Zoulim F, Op de Beeck A, Neyts J. Statins potentiate the in vitro anti-hepatitis C virus activity of selective hepatitis C virus inhibitors and delay or prevent resistance development. *Hepatology* 2009; **50**: 6-16
44 **Tada S**, Saito H, Ebinuma H, Ojiro K, Yamagishi Y, Kumagai N, Inagaki Y, Masuda T, Nishida J, Takahashi M, Nagata H, Hibi T. Treatment of hepatitis C virus with peg-interferon and ribavirin combination therapy significantly affects lipid metabolism. *Hepatol Res* 2009; **39**: 195-199
45 **Bechmann LP**, Zahn D, Gieseler RK, Fingas CD, Marquitan G, Jochum C, Gerken G, Friedman SL, Canbay A. Resveratrol amplifies profibrogenic effects of free fatty acids on human hepatic stellate cells. *Hepatol Res* 2009; **39**: 601-608

S- Editor Wang YR **L- Editor** O'Neill M **E- Editor** Zheng XM

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 285, NO. 2, pp. 845–854, January 8, 2010

Ethanol Enhances Hepatitis C Virus Replication through Lipid Metabolism and Elevated NADH/NAD$^+$*

Received for publication, July 17, 2009, and in revised form, October 30, 2009 Published, JBC Papers in Press, November 12, 2009, DOI 10.1074/jbc.M109.045740

Scott Seronello[‡], Chieri Ito[‡], Takaji Wakita[§], and Jinah Choi[‡1]

From the [‡]School of Natural Sciences, University of California, Merced, Atwater, California 95343 and the [§]National Institute of Infectious Diseases, Tokyo 162-8640, Japan

Ethanol has been suggested to elevate HCV titer in patients and to increase HCV RNA in replicon cells, suggesting that HCV replication is increased in the presence and absence of the complete viral replication cycle, but the mechanisms remain unclear. In this study, we use Huh7 human hepatoma cells that naturally express comparable levels of CYP2E1 as human liver to demonstrate that ethanol, at subtoxic and physiologically relevant concentrations, enhances complete HCV replication. The viral RNA genome replication is affected for both genotypes 2a and 1b. Acetaldehyde, a major product of ethanol metabolism, likewise enhances HCV replication at physiological concentrations. The potentiation of HCV replication by ethanol is suppressed by inhibiting CYP2E1 or aldehyde dehydrogenase and requires an elevated NADH/NAD$^+$ ratio. In addition, acetate, isopropyl alcohol, and concentrations of acetone that occur in diabetics enhance HCV replication with corresponding increases in the NADH/NAD$^+$. Furthermore, inhibiting the host mevalonate pathway with lovastatin or fluvastatin and fatty acid synthesis with 5-(tetradecyloxy)-2-furoic acid or cerulenin significantly attenuates the enhancement of HCV replication by ethanol, acetaldehyde, acetone, as well as acetate, whereas inhibiting β-oxidation with β-mercaptopropionic acid increases HCV replication. Ethanol, acetaldehyde, acetone, and acetate increase the total intracellular cholesterol content, which is attenuated with lovastatin. In contrast, both endogenous and exogenous ROS suppress the replication of HCV genotype 2a, as previously shown with genotype 1b. Conclusion: Therefore, lipid metabolism and alteration of cellular NADH/NAD$^+$ ratio are likely to play a critical role in the potentiation of HCV replication by ethanol rather than oxidative stress.

Ethanol consumption is a well-known risk factor for chronic liver diseases. Ethanol is also a key cofactor in the pathogenesis induced by hepatitis C virus (HCV),[2] and it decreases the efficacy of anti-HCV treatments (1, 2). Likewise, HCV infection exacerbates liver damage caused by prolonged alcohol abuse (2). It has also been reported that patients with a history of alcohol abuse are more likely to be infected with HCV than the rest of the population (1).

In addition, ethanol has been suggested to exacerbate HCV-induced liver diseases in part by affecting the viral titer (2–5). Hepatitis C patients who drink alcohol typically show a pattern of hepatic injury that is more characteristic of chronic viral hepatitis than alcohol-induced injury, suggesting that alcohol enhances the pathogenic effects of HCV rather than exerting its independent effects on liver (6). Several clinical studies have correlated increased serum and intrahepatic HCV titer with the amount of alcohol consumed (2, 4, 5). Abstinence or moderation of alcohol consumption could reduce the HCV titer in some patients (2, 5). Furthermore, *in vitro* studies suggest that ethanol increases HCV RNA levels in Huh7 human hepatoma replicon cell lines that continuously support the HCV RNA replication without virus production (3, 7, 8). These studies suggest that ethanol enhances HCV replication both in the presence and absence of the complete viral replication cycle. HCV replicon systems and more recent virus-producing cell culture models have increased our understanding of HCV and provide us with tools for studying potential interactions between HCV and pathological cofactors, such as ethanol (9).

Nevertheless, whether ethanol directly enhances HCV production in the context of the complete viral replication cycle has not been demonstrated. Furthermore, the mechanism by which ethanol modulates HCV RNA replication remains controversial as reactive oxygen species (ROS) and lipid peroxidation products, which can be generated during ethanol metabolism, can suppress, rather than increase, HCV RNA replication in cells, suggesting the involvement of other metabolites of ethanol (10–14). Oxidative hepatic ethanol metabolism is a multistep process (4). Alcohol dehydrogenase, the predominant ethanol-metabolizing enzyme, is found in the cytosol and produces acetaldehyde and NADH. Ethanol-inducible cytochrome P450 (CYP2E1), which is induced during extended ethanol exposure, is another major ethanol-metabolizing enzyme located in the endoplasmic reticulum and generates NADP$^+$ and ROS in addition to acetaldehyde. Catalase, which is found in peroxisomes, is thought to not contribute significantly to ethanol metabolism under normal conditions. Once ethanol is metabolized into acetaldehyde, it is rapidly converted into acetate and NADH by aldehyde dehydrogenase. Acetaldehyde and other products of ethanol metabolism have been implicated in many pathogenic effects of ethanol. Whether these metabolites also

* This work was supported by start-up funds from the University of California, Merced (to J. C.).

[1] To whom correspondence should be addressed: School of Natural Sciences, University of California, Merced, 5200 N. Lake Rd, Merced, CA 95343. Tel.: 209-228-4386; Fax: 209-228-4060; E-mail: jchoi@ucmerced.edu.

[2] The abbreviations used are: HCV, hepatitis C virus; ROS, reactive oxygen species; nt, nucleotides; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction; siRNA, small interfering RNA; IRES, internal ribosomal entry site; DADS, diallyl disulfide; BSO, L-buthionine *S,R*-sulfoximine; GSH, glutathione; GO, glucose oxidase; NAC, *N*-acetylcysteine; 4MP, 4-methylpyrazole; TOFA, 5-(tetradecyloxy)-2-furoic acid.

participate in the modulation of HCV replication by ethanol, however, has not yet been tested.

Therefore, the goal of this study was to determine the effects of ethanol exposure on HCV replication in the context of the complete HCV replication cycle and the mechanisms, comparing the effects of other metabolites of ethanol with those of ROS. Our data show that ethanol and acetaldehyde, at subtoxic and physiologically relevant concentrations, elevate complete HCV replication, as opposed to the suppression caused by endogenous and exogenous ROS. Our data further suggest that elevation of the ratio of NADH/NAD^+ and modulation of lipid metabolism are likely to play critical roles in the modulation of HCV replication by ethanol. Possible implications on *in vivo* HCV replication, patient education, and disease management are also discussed.

EXPERIMENTAL PROCEDURES

HCV Constructs—The genotype 2a HCV constructs, pJFH1 (produces infectious virus particles), replicative-null pJFH1-GND, and subgenomic pSgJFH1-Luc (contains a luciferase reporter gene), are described elsewhere (15, 16). Huh7 cell clones (SgPC2 and Clone B) supporting continuous replication of a subgenomic HCV replicon of genotype 1b (Con1 sequence) were also used (11, 17). The subgenomic replicons support HCV RNA replication but no virus is formed.

HCV RNA Transfection, Infection, and Cell Culture—The *in vitro* transcription, transfection of HCV RNA, and Huh7 human hepatoma cell culture were performed as described (10, 11). For the *in vitro* infectivity assays, 2 ml of the extracellular medium from JFH1 RNA-transfected cells were used to inoculate naïve Huh7 or Huh7.5 cells with 3 ml of fresh medium, as described (16, 18). Treatments were initiated 24 h after infection, and the cells were harvested after another 24 or 48 h.

Northern Blot Analysis—Intracellular RNA extraction and Northern blots were carried out, as described (10, 11). DNA probes were prepared from nucleotides (nt) 4128–8273 or 358–2816 of JFH1, generated with ScaI and ApaL I, respectively, or 3669 to 6016 of the Con1 subgenomic replicons. Images were quantified by densitometry, using Optiquant Cyclone 4.00 (Perkin Elmer), and data were normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA content.

Quantitative Real-time Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)—The total intracellular RNA was obtained from cells using TRIzol (Invitrogen). To obtain extracellular HCV RNA, cell culture medium samples were first treated with RNase A (100 μg/ml) for 30 min at room temperature, then RNA was extracted using TRIzol LS, and glycogen as a carrier. HCV RNA was quantified by qRT-PCR as described (11, 16). The primer sequences for JFH1 were 5′-TCTGCGGAACCGGTGAGTA-3′ (nt 146–164; forward), and 5′-TCAGGCAGTACCACAAGGC-3′ (nt 277–295; reverse), and the sequence of the fluorogenic probe, labeled with 6-FAM and TAMRA (Biosearch Technologies, Inc.), was 5′-CCAGTCTTCCCGGCAATTCCG-3′ (nt 168–188). Standard curves were generated using *in vitro* transcribed HCV RNA. Intracellular HCV RNA levels were normalized by 18 S rRNA or GAPDH mRNA.

Western Blot Analysis—Cells were sonicated in Laemmli buffer, and proteins were analyzed for NS5A and β-actin by Western blot, as described (10). Loading was normalized by protein assay of acetone-precipitated proteins determined with the bicinchoninic acid assay kit (Pierce). Quantification of Western blots was performed by densitometry using the Kodak IS2000R software.

Luciferase Assays—After various treatments, SgJFH1Luc RNA-transfected cells were lysed with 1× Reporter Lysis Buffer, and the luciferase activity was determined using Luciferase Reporter Assay kit (Promega) (15). Luciferase activities were normalized by total protein content, determined with bicinchoninic acid assay kit (Pierce).

In Vitro HCV Replication Assay—*In vitro* replication assay was carried out, as previously described (10, 11). Briefly, cytoplasmic lysates were prepared, and the replication was allowed to proceed for 1 h at 30 °C in the presence of [α-^{32}P]CTP and actinomycin D. Then, RNA products were analyzed on a 1% formaldehyde-agarose gel, which was subsequently analyzed, using Optiquant Cyclone 4.00 (PerkinElmer Life Sciences).

CYP2E1 Small Interfering RNA (siRNA)—Huh7 and SgPC2 cells were transfected with 50 nM CYP2E1 (Santa Cruz Biotechnology) or non-targeting control (Dharmacon) siRNAs, using RNAiMax (Invitrogen) per the manufacturer's recommendations.

CYP2E1 Activity Assay—Cells were treated with and without 0.2% ethanol for 48 h and lysed. CYP2E1 activity was determined by measuring hydroxylation of *p*-nitrophenol as described, except NADPH, instead of the NADPH-generating system, was used (19). The specificity was demonstrated by inhibiting the reaction with 100 μM CYP2E1 antibodies, and portion of the activity that is inhibited by CYP2E1 antibodies was then calculated and reported.

NADH/NAD^+, Cholesterol, and ATP Assays—NADH and NAD^+ levels were determined by enzymatic NADH recycling assay, using the NAD^+/NADH Quantification kit from Biovision, per the manufacturer's recommendations. After various treatments, cells were collected in 400 μl of NADH/NAD^+ extraction buffer. Samples were immediately subjected to two freeze/thaw cycles and filtered using Microcon YM-10 (Millipore). Then, the samples were split into two sets, one of which was used to carry out the thermal decomposition of NAD^+ followed by the cycling assay for the determination of NADH content of the cell. The other set was used to measure the total NADH plus NAD^+ content by performing the cycling assay without the thermal decomposition. Then, the NADH/NAD^+ ratio was calculated. Total intracellular cholesterol was measured using the Cholesterol/Cholesteryl Ester Quantitation kit (Biovision) per the manufacturer's instructions. Briefly, cells were homogenized in chloroform/isopropyl alcohol/Triton X-100 (7:11:0.1). The lipids were extracted, and all traces of organic solvents were evaporated prior to resuspending the lipids in the reaction buffer and performing the assay. Total ATP content was measured using Somatic Cell ATP Assay kit (Sigma-Aldrich). The data were normalized by total protein content, determined with the bicinchoninic acid assay kit (Pierce).

Statistics—Data were analyzed using Student's *t* test or one-way analysis of variance, using SigmaStat 3.1 (Jandel Scientific).

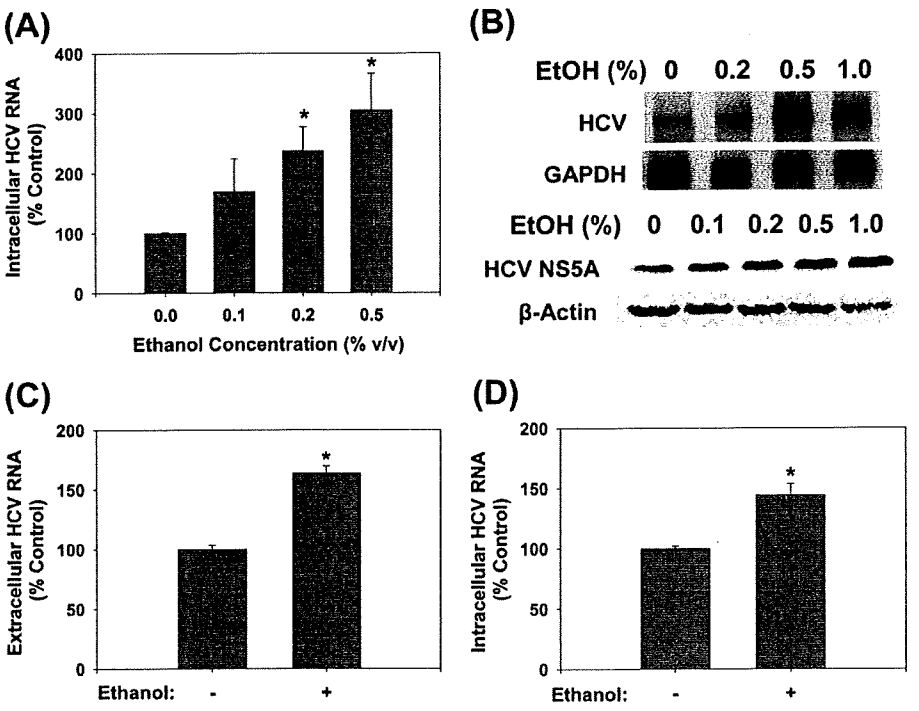

FIGURE 1. **Ethanol increases JFH1 replication.** Huh7 cells transfected with JFH1 RNA were analyzed for intracellular HCV RNA by (*A*) qRT-PCR ($n = 6$) or (*B*) Northern blots ($n = 4$) and for HCV NS5A protein content by Western blot ($n = 3$) (*B, bottom panel*) after 48 h of ethanol treatments. *C*, extracellular HCV RNA levels were analyzed by qRT-PCR for 0.2% ethanol treatments ($n = 4$). *D*, naïve Huh7 cells were inoculated with virus-containing medium and analyzed for HCV RNA after 48 h of 0.2% ethanol treatment ($n = 4$). *, indicates statistically significant difference for indicated sample sizes ($p < 0.05$).

A p value ≤ 0.05 was considered significant. All experiments were repeated three to six times, and either the means ± S.E. of several independent experiments or the representative Northern or Western blot images are shown.

RESULTS

Ethanol Increases the Complete Replication of HCV at Physiological Concentrations—To examine whether ethanol increased the complete replication of HCV, positive-sense genomic JFH1 RNA was produced by *in vitro* transcription using T7 RNA polymerase and transfected into Huh7 human hepatoma cells. Then, the transfected cells were exposed to 0–1.0% (v/v, 0–172 mM) ethanol once daily for 48 h. Then, the cells and the cell culture medium were harvested and analyzed for intracellular and RNase A-resistant extracellular HCV RNAs by a combination of Northern blots and qRT-PCR. Ethanol significantly increased the intracellular JFH1 HCV RNA levels to 237 ± 40 and 305 ± 61% of untreated controls at 0.2 and 0.5% concentrations, respectively ($p < 0.05$) (Fig. 1, *A* and *B, top panel*). HCV NS5A protein level similarly increased with ethanol treatments (Fig. 1*B, bottom panel*). Extracellular HCV RNA was also significantly elevated with the 0.2% ethanol treatment, indicating increased virus secretion (Fig. 1*C*). Next, we examined whether virus-infected cells responded similarly to ethanol treatment with elevated HCV RNA. We found that 0.2% ethanol also increased HCV RNA in Huh7 cells infected with cell culture-generated JFH1 virions (Fig. 1*D*). JFH1 GND mutant, which harbors a critical mutation (GDD:GND) in NS5B, the viral polymerase, did not replicate or generate infectious virus particles, as expected (data not shown). These concentrations of ethanol did not induce any cytotoxicity, as assessed by cell morphology and measuring the ATP content (data not shown). The 0.2% ethanol, equivalent to blood alcohol concentration of 34.4 mM, that significantly enhanced HCV replication, is approximately twice the legal limit for driving under the influence in many countries, including the United States. The 0.5% ethanol lies in the toxic range but can also be achieved physiologically, particularly in chronic alcohol users. In addition, ethanol is volatile, and the amount that remains would be significantly less than what was added to cell culture medium (20). These data, therefore, suggest that ethanol can enhance complete HCV replication, at physiologically attainable concentrations.

Ethanol Enhances HCV RNA Replication of Genotypes 2a and 1b—Previously, ethanol was shown to elevate HCV RNA content in Huh7 cells that supported subgenomic HCV RNA replication without virus production (3, 7, 8). To test whether the JFH1 RNA replication was also affected by ethanol, we transfected Huh7 cells with SgJFH1-Luc RNA and exposed the cells to ethanol for 48 h. Then, HCV replication was monitored by measuring the firefly luciferase activity (15). Ethanol increased the luciferase activity in these cells, suggesting that the JFH1 RNA genome replication was affected (Fig. 2*A*).

Genotype 2a HCV infection is found globally, with the prevalence ranging from less than 2 to about 30% depending on the geographical region (21, 22). However, as the most prevalent HCV genotype is genotype 1, we also repeated these experiments, using Con1 subgenomic replicon (SgPC2) cells (11, 17). Again, significant increases in the genotype 1b HCV RNA could be demonstrated with 0.1–1% ethanol (Fig. 2*B, top panel*). Similar increases in the HCV NS5A protein content was demonstrated by Western blots (Fig. 2*B, bottom panel*).

To confirm that the rate of the HCV RNA genome replication is accelerated by ethanol, we measured the activity of the HCV RNA replication complex. JFH1-transfected cells were exposed to ethanol for 5 h and then, the cytoplasmic lysates, containing the HCV replication complex, were isolated. Then, the *in vitro* RNA replication assay was performed in the presence of α-^{32}P-labeled CTP and actinomycin D, as previously described (11). JFH1 cell extracts produced a single band that corresponded to the expected size of the HCV RNA, indicating active viral RNA replication, whereas the JFH1 GND extracts did not (Fig. 2*C*). Ethanol significantly increased the rate of

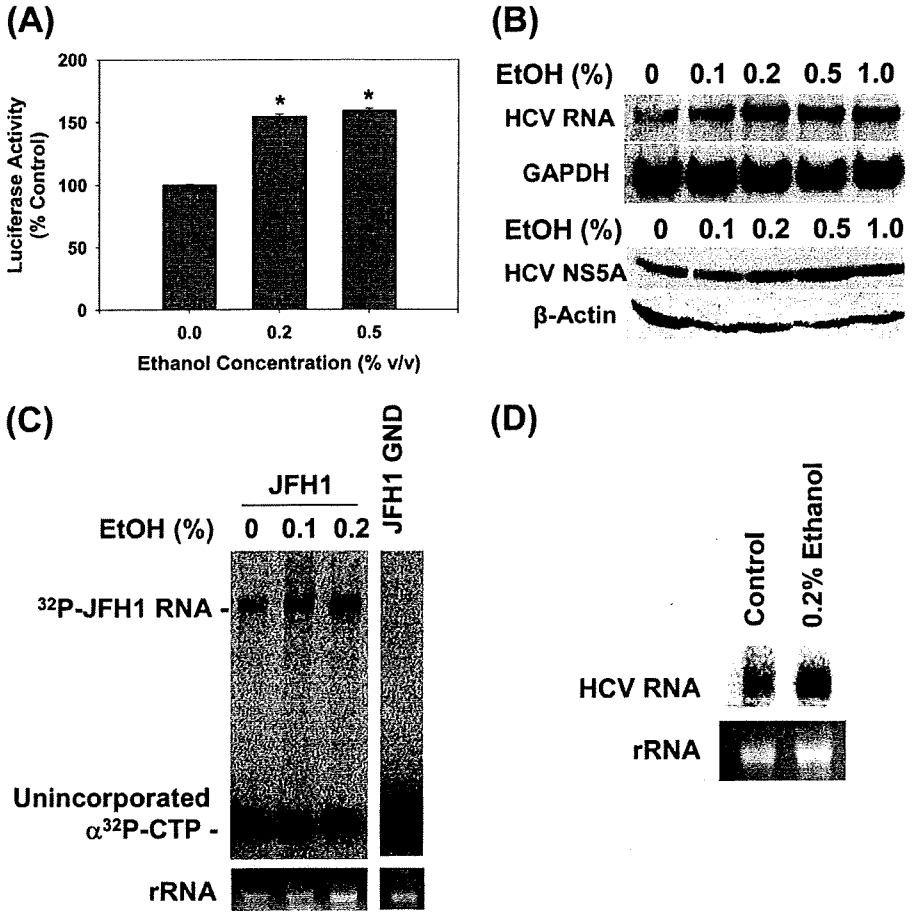

FIGURE 2. **Ethanol increases the replication of subgenomic JFH1 and Con1 replicon RNAs.** *A*, Huh7 cells transfected with SgJFH1-Luc RNA were analyzed for luciferase activity after 48-h ethanol treatments ($n = 3$). *B*, stable Huh7 clones expressing SgCon1-Neo (SgPC2) were incubated with ethanol for 24 h and analyzed for HCV RNA, GAPDH mRNA, and NS5A and β-actin proteins ($n = 3$) by Northern and Western blots, respectively ($n = 3$). *C* and *D*, cytosolic lysates were prepared from (*C*) JFH1 and JFH1-GND RNA-transfected cells and (*D*) SgPC2 cells, after 5 h of ethanol treatment, these lysates were used to carry out *in vitro* replication assays ($n = 3$). *Bottom panels* show ethidium bromide staining of rRNA as the loading control. *, indicates statistically significant difference for indicated sample sizes ($p < 0.05$).

HCV RNA replication (Fig. 2*C*). Ethanol also accelerated the *in vitro* replication rate of Con1 strain (Fig. 2*D*). On the other hand, ethanol did not increase the HCV internal ribosomal entry site (IRES) activity, as assessed by the HCV IRES activity assay, using pRL-HL (data not shown) (23). The data suggest that ethanol increases the rate of HCV RNA replication without directly enhancing its translation rate, at least when these processes are evaluated separately. Therefore, increases in the NS5A protein content with ethanol (Fig. 2*B*) are likely to have resulted from increased levels of the viral RNA template available for translation.

CYP2E1 Is Present in Huh7 Cells at Significant Levels as in Human Liver—Next, we started examining the mechanism by which ethanol increased HCV replication, first, by identifying key steps of ethanol metabolism that mediated this effect (Fig. 3*A*). Alcohol dehydrogenase I was not detected in significant levels in our Huh7 cells (data not shown). To confirm that ethanol metabolism is occurring in our cells, we then analyzed our Huh7 cells for the expression of CYP2E1. Our Huh7 cells expressed significant levels of CYP2E1 protein, which was about 2.2 ± 0.5-fold less than human liver (Fig. 3*B*). CYP2E1 expression could also be enhanced by 1.5 ± 0.2-fold with daily treatment with 0.2% (v/v) ethanol for 48 h (Fig. 3*C*). This enhanced expression of CYP2E1 could be maintained for at least 2 weeks. The CYP2E1 activities (Fig. 3*D*) were within the expected range for human liver, which is 0.25–3.3 nmol/min/mg protein, and paralleled the CYP2E1 expression levels (Fig. 3*C*) (19). CYP2E1 activity of human liver shown in Fig. 3*B* was 1.83 ± 0.01 nmol/min/mg. Con1 SgPC2 cells had similar expression and activity levels of CYP2E1 as Huh7 cells (0.81 ± 0.02 nmol/min/mg without ethanol; 1.01 ± 0.06 nmol/min/mg with 0.2% ethanol).

The ethanol-induced potentiation of HCV replication could be abrogated with 25 μM diallyl disulfide (DADS), an inhibitor of CYP2E1 (Fig. 3*E*). In addition, CYP2E1 siRNA, which decreased CYP2E1 protein level to 35 ± 9% ($p < 0.05$) of the controls transfected with non-targeting control siRNA, also significantly blunted the potentiation of HCV replication by ethanol (Fig. 3*E*). These data suggest that ethanol is being metabolized by these cells, and that CYP2E1 activity is critical for the potentiation of HCV replication by ethanol in our system.

ROS Suppresses JFH1 Replication—Hepatic ethanol, particularly CYP2E1-mediated, metabolism generates ROS in addition to acetaldehyde (24) (Fig. 3*A*) and previously, we showed that ROS could suppress subgenomic Con1 and H77c/Con1 hybrid HCV RNA replication in these cells (10, 11). To resolve these seemingly conflicting observations, we continued to examine how ROS affected JFH1. To examine the effects of endogenously generated ROS, we first used L-buthionine *S,R*-sulfoximine (BSO). BSO depletes glutathione (GSH), a major endogenous antioxidant, by inhibiting its *de novo* synthesis. Therefore, BSO would amplify the effects of endogenous ROS, generated during normal cellular metabolism and in response to HCV (4). BSO decreased intracellular GSH content by ~80 ± 12% in Huh7 cells ($p < 0.05$). In addition, BSO decreased both intracellular and extracellular JFH1 RNA levels (Fig. 4, *A* and *B*). To confirm that BSO was acting specifically by decreasing GSH, cells were treated with BSO and GSH ethyl ester, which enters cells and is cleaved by cellular esterases to restore GSH inside cells, bypassing the inhibition of GSH biosynthesis by BSO. GSH ethyl ester partially restored both intracellular and extracellular HCV RNA (Fig. 4, *A* and *B*).