[10] M. Ikeda, K. Abe, H. Dansako, T. Nakamura, K. Naka, N. Kato, 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. 329 (2005) 1350-1359.
[11] N. Kato, K. Sugiyama, K. Namba, H. Dansako, T. Nakamura, M. Takami, K. Naka, A. Nozaki, K. Shimotohno, Establishment of a hepatitis C virus subgenomic replicon derived from human hepatocytes infected in vitro, Biochem. Biophys. Res. Commun. 306 (2003) 756-766.
[12] T. Wakita, T. Pietschmann, T. Kato, T. Date, M. Miyamoto, Z. Zhao, K. Murthy, A. Habermann, H.G. Krausslich, M. Mizokami, R. Bartenschlager, T.J. Liang, Production of infectious hepatitis C virus in tissue culture from a cloned viral genome, Nat. Med. 11 (2005) 791–796.
[13] N. Kato, H. Sekiya, Y. Ootsuyama, T. Nakazawa, M. Hijikata, S. Ohkoshi, K. Shimotohno, Humoral immune response to hypervariable region 1 of the putative envelope glycoprotein (gp70) of hepatitis C virus, J. Virol. 67 (1993) 3923–3930.
[14] H. Dansako, A. Naganuma, T. Nakamura, F. Ikeda, A. Nozaki, N. Kato, Differential activation of interferon-inducible genes by hepatitis C virus core protein mediated by the interferon stimulated response element, Virus Res. 97 (2003) 17–30.
[15] V. Lohmann, S. Hoffmann, U. Herian, F. Penin, R. Bartenschlager, Viral and cellular determinants of hepatitis C virus RNA replication in cell culture, J. Virol. 77 (2003) 3007–3019.
[16] K. Abe, M. Ikeda, H. Dansako, K. Naka, N. Kato, Cell culture-adaptive NS3 mutations required for the robust replication of genome-length hepatitis C virus RNA, Virus Res. 125 (2007) 88-97.
[17] N. Ishii, K. Watashi, T. Hishiki, K. Goto, D. Inoue, M. Hijikata, T. Wakita, N. Kato, K. Shimotohno, Diverse effects of cyclosporine on hepatitis C virus strain replication, J. Virol. 80 (2006) 4510–4520.
[18] MP. Windisch, M. Frese, A. Kaul, M. Trippler, V. Lohmann, R. Bartenschlager, Dissecting the interferon-induced inhibition of hepatitis C virus replication by using a novel host cell line, J. Virol. 79 (2005) 13778–13793.

LIVER TRANSPLANTATION 14:292–298, 2008

ORIGINAL ARTICLE

# Differential Effects of Calcineurin Inhibitors, Tacrolimus and Cyclosporin A, on Interferon-Induced Antiviral Protein in Human Hepatocyte Cells

**Kumi Hirano,[1]* Tatsuki Ichikawa,[2]* Kazuhiko Nakao,[2] Azusa Matsumoto,[1] Hisamitsu Miyaaki,[2] Hidetaka Shibata,[2] Susumu Eguchi,[3] Mitsuhisa Takatsuki,[3] Masanori Ikeda,[5] Hironori Yamasaki,[4] Nobuyuki Kato,[5] Takashi Kanematsu,[3] Nobuko Ishii,[4] and Katsumi Eguchi[1]**

*[1]Department of Clinical Pharmaceutics, [2]First Department of Internal Medicine, [3]Department of Transplantation and Digestive Surgery, and [4]Health Research Center, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan; and [5]Department of Molecular Biology, Graduate School of Medicine and Dentistry, Okayama University, Okayama, Japan*

The premise of our study is that selective inhibition of interferon (IFN) by calcineurin inhibitors contribute to the increased severity of hepatitis C virus (HCV) posttransplantation. Therefore, we examined the influence of calcineurin inhibitors in the human hepatocyte cell line on IFN-$\alpha$-induced phosphorylation of Janus kinase (Jak) and signal transducers and activators of transcription (STAT), nuclear translocation of IFN-stimulated gene factor 3 (ISGF-3), IFN-stimulated regulatory element (ISRE)-contained promoter activity, and the expressions of antiviral proteins. Tacrolimus (Tac), but not cyclosporin A (CyA), had an inhibitory effect on IFN-$\alpha$-induced double-stranded ribonucleic acid (RNA)-dependent protein kinase (PKR) in a dose-dependent manner. STAT-1 also acted in a similar fashion to PKR. IFN-$\alpha$ combined with Tac attenuated the ISRE-containing promoter gene activity as compared with IFN-$\alpha$ alone. In contrast, its expression in pretreated CyA was slightly attenuated. In pretreated Tac, but not CyA, the levels of IFN-$\alpha$-induced tyrosine phosphorylated STAT-1 and -2 were clearly lower than those induced by IFN-$\alpha$ alone. Tac and CyA did not decrease the IFN-$\alpha$-induced JAK-1 phosphorylation. The nuclear translocation rate of tyrosine phosphorylated STAT-1 was inhibited by pretreatment of both Tac and CyA by western blotting and immunohistochemistry. In an HCV replicon system, pretreated Tac diminished the replication inhibitory effect of IFN-$\alpha$. In this study, we show that calcineurin inhibitors, especially Tac, are the negative regulators of IFN signaling in the hepatocyte; the greatest cause of such inhibition is the phosphorylation disturbance of STAT-1, next to inhibition of the nuclear translocation of STAT-1. In conclusion, disturbance of tyrosine phosphorylation of STAT-1 resulted in diminished ISRE-containing promoter activity and a decline in antiviral protein expression. Moreover, the replication of HCV was activated. This phenomenon is detrimental to IFN therapy after liver transplantation, and the selection of calcineurin inhibitors may warrant further discussion depending on the transplant situation. *Liver Transpl 14:292–298, 2008.* © 2008 AASLD.

Received March 8, 2007; accepted August 17, 2007.

See Editorial on Page 265

Hepatitis C virus (HCV) infection is widespread worldwide. A major problem of chronic HCV infection is hepatocellular carcinoma. Currently, liver transplantation for HCV-related liver disease is an option worldwide.[1] Recently, it has been demonstrated that the prognosis for liver transplantation patients with HCV-related disease deteriorates with time,[2] resulting in a poorer out-

**Abbreviations:** CyA, cyclosporin A; HCV, hepatitis C virus; IFN, interferon; ISGF-3, IFN-stimulated gene factor 3; ISRE, IFN-stimulated regulatory element; Jak, Janus kinase; NF-AT, nuclear factor of activated T cells; PKR, double-stranded RNA-dependent protein kinase; RNA, ribonucleic acid; STAT, signal transducers and activators of transcription; Tac, tacrolimus.

*These authors contributed equally to this study.
Address reprint requests to Tatsuki Ichikawa, First Department of Internal Medicine, Graduate School of Biomedical Sciences, Nagasaki University, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan. Telephone: 81-95-849-7260; FAX: 81-95-849-7270; E-mail: ichikawa@net.nagasaki-u.ac.jp

DOI 10.1002/lt.21358
Published online in Wiley InterScience (www.interscience.wiley.com).

© 2008 American Association for the Study of Liver Diseases.

come than in the non-HCV course.[3,4] The transplanted liver in HCV-related disease undergoes a rapid progression of fibrosis and worsens to cirrhosis and graft failure.[5] The factors for a worsening outcome were speculated to be increased donor age,[3-5] stronger immunosuppression,[3] and high levels of HCV-ribonucleic acid (RNA) at transplantation.[4] These factors have no small effect on the reinfection and reactivation of HCV in the graft liver.

Reinfection of HCV in the graft liver is rapid after transplantation, and the virus immediately proliferates in the graft. In the natural course of reinfection, approximately 10 to 25% of recipients will develop cirrhosis, and a strategy for the prevention of reinfection has not been developed.[6] At present, treatment of HCV after transplantation is inadequate, and does not result in a cure.[7] Recently, pegylated interferon (IFN) and ribavirin combination therapy has been effective in the treatment of HCV genotype 1a chronic hepatitis, with a sustained viral response rate of 45%.[8] However, reinfection after transplantation is the norm despite combined therapy.[9,10] Meanwhile, the patients with a sustained viral response after transplantation show no progression or reversal of liver fibrosis.[11,12] The refractory nature of pegylated IFN and ribavirin combination therapy for liver transplantation patients contributes to a worsening outcome in HCV-related transplantation.

We speculated that posttransplantation immunosuppression is part of the reason for IFN resistance to HCV reinfection of the graft liver. Methylprednisolone pulse therapy is a risk factor for severe outcome after transplantation, and the treatment of acute cellular rejection using heavy immunosuppressive agents is also a risk factor.[3,4,6] Previous reports described the fact that glucocorticoid inhibits the expression of signal transducers and activators of transcription (STAT)-1, as a signal transduction factor of IFN, and diminishes the signaling of IFN.[13] However, the effects on HCV reinfection and IFN therapy by calcineurin inhibitors, the most frequently used immunosuppressants, have not been fully evaluated, until now. Therefore, we have attempted to evaluate the influences of calcineurin inhibitors on IFN signaling in the hepatocytes.

IFN-α and β, after binding to their receptors, stimulate the intracellular IFN-signaling cascade including the Janus kinase (Jak)-STAT tyrosine kinases, the phosphorylation of STAT-1 and -2, and the formation of IFN-stimulated gene factor 3 (ISGF-3), which consists of STAT-1, STAT-2, and p48.[14] ISGF-3 translocates into the nucleus and binds to the IFN-stimulated regulatory element (ISRE) in the promoter sequences of a variety of IFN-inducible genes, including antiviral proteins such as double-stranded RNA-dependent protein kinase (PKR).[15] Several negative regulation systems of Jak-STAT signaling, including the suppressor of cytokines signaling family, the protein inhibitor of activated STAT family, and the SH2-containing protein tyrosine phosphatase family, are notorious contributors to a state of inflammation and carcinogenesis in the hepatocyte.[16,17] In addition, the nucleus-cytoplasm transport of ISGF-3 was regulated by translocated specific proteins along with the phosphorylation of STAT.[18] We examined the influence of calcineurin inhibitors on IFN-induced phosphorylation of Jak and STAT, nuclear translocation of ISGF-3, ISRE contained promoter activity, and the expressions of antiviral proteins.

## MATERIALS AND METHODS

### Reagents and Cell Culture

Recombinant human IFN-α2b, tacrolimus (Tac), and cyclosporine A (CyA) were generous gifts from Schering-Plough KK (Tokyo, Japan), Astellas Co. (Tokyo, Japan), and Novartis Pharma Co. (Basel, Switzerland), respectively. Hc human hepatocyte cells (Applied Cell Biology Research Institute, Kirkland, WA) and HuH-7 human hepatoma cells (American Type Culture Collection, Rockville, MD) were maintained in a chemically-defined medium, CS-C completed (Cell Systems, Kirkland, WA) and RPMI (Invitrogen, Grand Island, NY), respectively, supplemented with 5% fetal bovine serum. In the pretreatment of calcineurin inhibitors, the cells were cultured in 5% RPMI containing varying concentrations of Tac and CyA, and then the medium was exchanged and the cells were treated with IFN 100 IU/mL at the indicated time.

### HCV Replicon System

OR6 cells stably harboring the full-length genotype 1 replicon, ORN/C-5B/KE[19] were used to examine the influence on the anti-HCV effect of IFN of calcineurin inhibitors. The cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL; Invitrogen) supplemented with 10% fetal bovine serum, penicillin, and streptomycin and maintained in the presence of G418 (300 mg/L; Geneticin; Invitrogen). This replicon was derived from the 1B-2 strain (strain HCV-o, genotype 1b), in which the *Renilla* luciferase gene is introduced as a fusion protein with neomycin to facilitate the monitoring of HCV replication. After the treatment, the cells were harvested with *Renilla* lysis reagent (Promega, Madison, WI) and subjected to luciferase assay according to the manufacturer's protocol.

### Western Blotting and Antibody

Western blotting with anti-PKR, anti-STAT-1, anti-STAT-2 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-tyrosine-701 phosphorylated STAT-1, anti-tyrosine-689 STAT-2, anti-JAK-1 or anti-tyrosine 1022/1023 JAK-1 (New England Biolabs, Beverly, MA) was performed as described previously.[20] Briefly, Hc cells were lysed by the addition of lysis buffer (50 mmol/L Tris-HCl, pH 7.4, 1% Np40, 0.25% sodium deoxycholate, 0.02% sodium azide, 0.1% sodium dodecyl sulfate buffer, 150 mmol/L NaCl, 1 mmol/L ethylene diamine tetraacetic acid, 1 mmol/L phenylmethanesulfonylfluoride, 1 μg/mL each of aprotinin, leupeptin, and pepstatin, 1 mmol/L sodium o-vanadate, and 1 mmol/L NaF). Extraction of nucleus and cytoplasm were performed using the NE-PER Nuclear and Cyto-

plasmic Extraction kit (Pierce, France). Samples were analyzed by electrophoresis on 8 to 12% sodium dodecyl sulfate buffer polyacrylamide gel and electrotransferred to nitrocellulose membranes, and then blotted with each antibody. The membranes were incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G or anti-mouse immunoglobulin G, and the immunoreactive bands were visualized by the ECL chemiluminescence system (Amersham Life Science, Buckinghamshire, England). The density of each band was quantified using the National Institutes of Health image analysis software program.

### Reporter Gene Assay

pISRE-Luc containing 5 copies of the ISRE sequence and firefly luciferase gene and pRL-SV40 containing SV40 early enhancer/promoter and *Renilla* luciferase gene were obtained from Clontech (San Diego, CA) and Promega, respectively. The HuH-7 cells were grown in 24-well multiplates and transfected with 1 μg of pISRE-Luc and 10 ng of pRL-SV40 as a standard by the lipofection method. One day later, the cells were incubated in the absence or presence of varying concentrations of Tac, CyA, and IFN-α, and the luciferase activities in the cells were determined using a dual-luciferase reporter assay system and a TD-20/20 luminometer (Promega). The data were expressed as the relative ISRE-luciferase activity.

### Fluorescence Immunohistochemistry

The Hc cells were seeded onto 11-mm glass coverslips in 24-well plates at 240,000 cells/well. The next day, the medium was replaced with serum-free medium, and the cells were pretreated with 10 μmol/L of Tac, 100 μmol/L of CyA, or vehicle, for 16 hours and then stimulated with 100 IU/mL of IFN-α for 10 minutes. Fluorescence immunohistochemistry was performed as described previously.[21] The cells were incubated with anti-tyrosine-701 phosphorylated STAT-1 antibody for 1 hour at room temperature, washed 3 times in phosphate buffered saline, incubated with rhodamine-conjugated donkey anti-rabbit immunoglobulin G (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 hour, washed in phosphate buffered saline, and mounted in Vectashield Mounting Medium (Vector Laboratories Inc., Burlingame, CA). Nuclear staining was performed using Hoechst 33258 (Invitrogen Japan K.K., Tokyo, Japan). Immunofluorescence analysis was done by an Olympus BX50 microscope (Tokyo, Japan) and the image was captured by a Nikon DXM 1200 digital camera (Tokyo, Japan).

## RESULTS

### Differential Effects of Tac and CyA on IFN-induced Antiviral Protein Expression

To elucidate how calcineurin inhibitors exert influence on IFN-induced antiviral protein, the Hc cells were incubated in the absence or presence of IFN-α after the

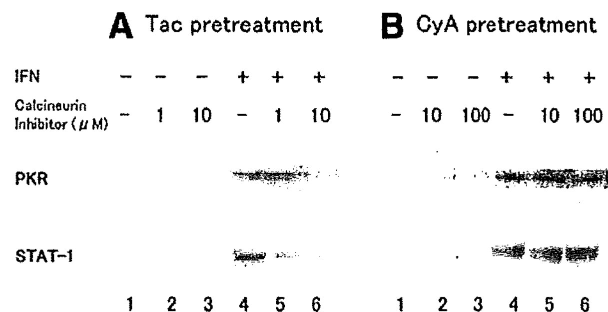


**Figure 1. Effect of (A) Tac and (B) CyA on IFN-α-induced PKR and STAT-1. Hc cells were treated with 100 IU/mL of IFN-α in the absence (lane 4) or presence of pretreatment (lanes 5 and 6). Indicated concentration of calcineurin inhibitor alone was lanes 2 and 3, lane 1 was not treated with IFN-α and calcineurin inhibitors. One day later, PKR and STAT-1 were determined by western blotting.**

presence or absence of pretreatment of Tac (Fig. 1A) or CyA (Fig. 1B) for 16 hours, and then were harvested for the western blot analysis. Pretreated Tac had an inhibitory effect on IFN-α-induced PKR expression, antiviral protein as messenger RNA translation inhibitor activated by double-stranded RNA dependent, in a dose-dependent manner, but no inhibitory effect of pretreatment CyA for PKR expression was recognized in our experiment. STAT-1 is an essential signal transmitter substance of IFN and IFN-inducible proteins.

The expression of IFN-inducible STAT-1 also decreased in a dose-dependent manner after the administration of Tac, but not after the administration of CyA.

### Alterations of IFN-α-Stimulated Reporter Gene Expression by Tac and CyA

Because the formation of IFN stimulating gene factor (ISGF) 3 by IFN-α leads to transactivation of the ISRE in the promoter regions of the IFN-α-inducible genes, we performed the reporter gene transfection assay using plasmids containing ISRE in their promoter sequence. Because there were not enough Hc cells for reporter gene transfection, we used HuH-7 cells in the transfection assay. HuH-7 cells were transfected with pISRE-Luc containing 5 repeats of ISRE sequence and pRV-SV40 as a standard and then were treated with IFN-α after 16 hours in the presence or absence of pretreated Tac or CyA (Fig. 2). Tac and CyA alone did not influence the ISRE-luciferase activities. IFN-α combined with Tac and attenuated its expression compared with IFN-α alone. In contrast, there was a slight attenuation effect of its expression in 100 μmol/L of pretreated CyA.

### Inhibitory Effect of Tac on IFN-α-Induced Tyrosine Phosphorylation of STATs

The activation of STAT-1 and -2 by phosphorylation of tyrosine-701 and 689 residues, respectively, is essential for the relay of IFN-α signal with the formation of ISGF-3. Therefore, we examined the effect of Tac and CyA on the IFN-α-induced tyrosine phosphorylation of

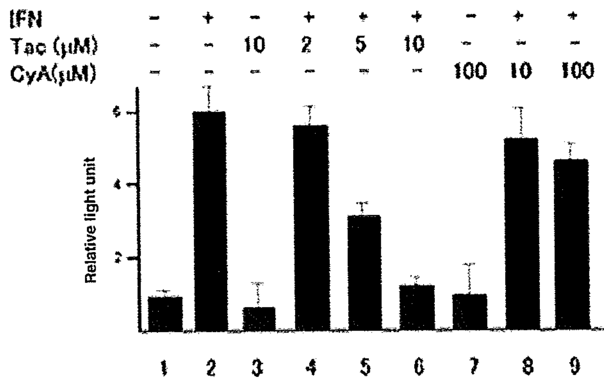


**Figure 2. Suppression effect of calcineurin inhibitors on IFN-α-induced reporter gene assay. HuH-7 cells transfected with reporter gene (pISRE-Luc and pRL-SV40) were either untreated (lanes 1, 2) or pretreated with Tac (lanes 3-6) or CyA (lanes 7-9) for 16 hours, followed by IFN-α 100 IU/mL (lanes 2, 4-6, 8, and 9)or absence (lanes 3 and 7). Six hours later, the relative ISRE-luciferase activity (n = 4) was determined as described in Materials and Methods. The data are expressed as the mean ± SD and are representative examples of four similar experiments.**

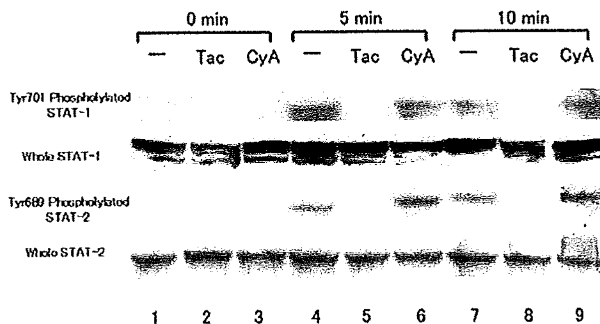


**Figure 3. Effect of calcineurin inhibitors on STAT-1and STAT-2. After pretreatment with 10 μmol/L Tac (lanes 2, 5, and 8) and 100 μmol/L CyA (lanes 3, 6, and 9) for 16 hours, Hc cells were untreated (lane 1) or treated with 100 IU/mL IFN-α (lanes 4-9) for the indicated periods and phosphorylated STAT-1 at tyrosine-701 residue (first panel), expression of STAT-1 (second panel), phosphorylated STAT-2 at tyrosine-689 residue (third panel), and expression of STAT-2 (fourth panel) were analyzed by western blotting. The density of each band was quantified and the nuclear translocation rate was calculated by the following: nuclear intensity (N)/[nuclear intensity (N) + cytoplasmic intensity (C)].**

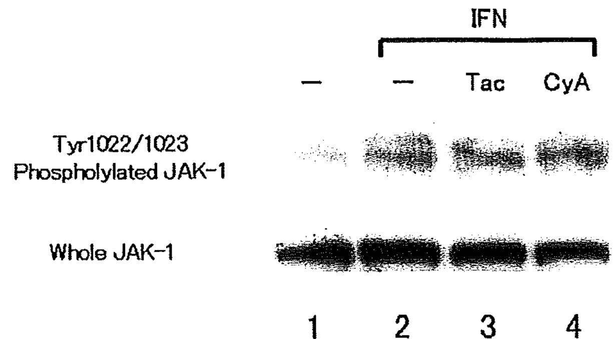


**Figure 4. Evaluation of IFN-α-induced phosphorylated JAK-1 by calcineurin inhibitors. After pretreatment of 10 μmol/L Tac (lane 3) and 100 μmol/L CyA (lane 4) for 16 hours, Hc cells were untreated (lane 1) or treated with 100 IU/mL IFN-α (lanes 2-4) for 3 minutes, then phosphorylated JAK-1 at tyrosine-1022/1023 residue (first panel) and expression of JAK-1 (second panel) were analyzed by western blotting.**

| | Control | | IFN | | Tac+IFN | | CyA+IFN | |
|---|---|---|---|---|---|---|---|---|
| | N | C | N | C | N | C | N | C |
| Tyr701 Phospholylated STAT-1 | | | | | | | | |
| relative intensity | 0 | 0 | 21 | 48 | 3 | 16 | 8 | 42 |
| N/N+C (%) | 0 | | 31% | | 15% | | 16% | |
| Tyr689 Phospholylated STAT-2 | | | | | | | | |
| relative intensity | 0 | 0 | 9 | 0.3 | 2 | 0 | 3 | 1 |
| N/N+C (%) | 0 | | 96% | | 100% | | 75% | |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |

**Figure 5. Alternation of distribution of IFN-α-induced phosphorylated STAT-1 and STAT-2 by calcineurin inhibitors. Hc cells were pretreated with absence (lanes 1-4) or presence of 10 μmol/L Tac (lanes 5 and 6) or 100 μmol/L CyA (lanes 7 and 8). And then, Hc cells were stimulated by 500 IU/L IFN-α (lanes 3-8) for 10 minutes. Hc cells harvested by extraction kit of nucleus (lanes 1, 3, 5, and 7) and cytoplasm (lanes 2, 4, 6, and 8). Phosphorylated STAT-1 at tyrosine-701 residue (upper panel) and phosphorylated STAT-2 at tyrosine-689 residue (lower panel) were analyzed by western blotting.**

STAT-1 and -2 (Fig. 3). IFN-α clearly induced the tyrosine phosphorylation of STAT-1 and -2, but Tac and CyA could not. However, when the Hc cells were pretreated with Tac, but not CyA, before IFN-α stimulation, the levels of tyrosine phosphorylated STAT-1 and -2 were clearly lower than those induced by IFN-α alone. In the case of pretreatment with CyA, the IFN-α-induced tyrosine phosphorylation levels were similar to IFN-α alone. Then, the cells were changed from Hc cells to HuH-7 cells and a similar experiment was done. The inhibitory effect of Tac to IFN-α-induced STAT-1 and -2 tyrosine phosphorylation was the same (data not shown).

When we performed western blotting of phosphorylated JAK-1 under the same conditions, Tac and CyA did not decrease the IFN-induced JAK-1 phosphorylation (Fig. 4).

## Influence of Calcineurin Inhibitors on IFN-α-Induced Nuclear Translocation of Tyrosine Phosphorylated STATs

For transcription of the IFN-α-induced antiviral gene, the ISGF-3 complex, including activated STAT-1, STAT-2, and p48, could be translocated to the nucleus. Initially, we detected tyrosine phosphorylated STAT-1 and -2 extracted it from the nucleus and cytoplasm by western blotting. In this experiment, detectable band intensities were quantified by National Institutes of Health image software and we evaluated the nuclear translocation rate of activated STAT-1 and -2 (Fig. 5).

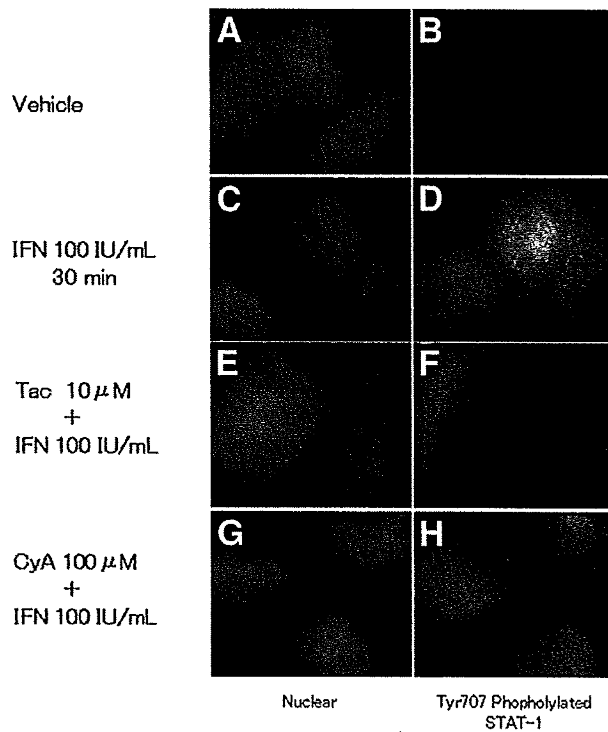


**Figure 6. Inhibition of IFN-α-induced nuclear translocation of phosphorylated STAT-1 by calcineurin inhibitor. Hc cells were pretreated in the absence (A-D) or presence of 10 μmol/L Tac (E,F) or 100 μmol/L CyA (G,H). After pretreatment, Hc cells were stimulated by 100 IU/L IFN-α (C-H) for 30 minutes. Thereafter, the cells were fixed, permeabilized, processed for immunofluorescence (B,D,F,H) and Hoechst staining (A,C,E,G), and visualized with fluorescence microscopy. The results shown are from one representative experiment from a total of three performed.**

The total IFN-α-stimulated tyrosine phosphorylated STAT-1 was decreased by pretreatment with Tac; furthermore, the nuclear translocation rate of activated STAT-1 was inhibited both by pretreatment with Tac and CyA. However, in the case of pretreatment with Tac and CyA, there was no effect on the nuclear translocation of tyrosine phosphorylated STAT-2. Secondarily, we evaluated the location of tyrosine phosphorylated STAT-1 by fluorescence immunohistochemistry of cultured Hc cells (Fig. 6).The IFN-α-induced nuclear translocation of tyrosine phosphorylated STAT-1 was observed, but its translocation was inhibited by pretreatment with Tac. Along with the nuclear translocation rate of activated STAT-1 by western blotting (Fig. 5), pretreatment with Tac also attenuated the nuclear staining of activated STAT-1 compared to IFN-α alone, but did not attenuate the expression of activated STAT-1 by immunohistochemistry.

## Inhibitory Effect of Tac on IFN-α-Induced Anti-HCV Efficiency

To examine the effect of calcineurin inhibitors on IFN-α, we used the full-length HCV replication system, OR6 cells. The cells were treated with IFN-α after 16 hours in

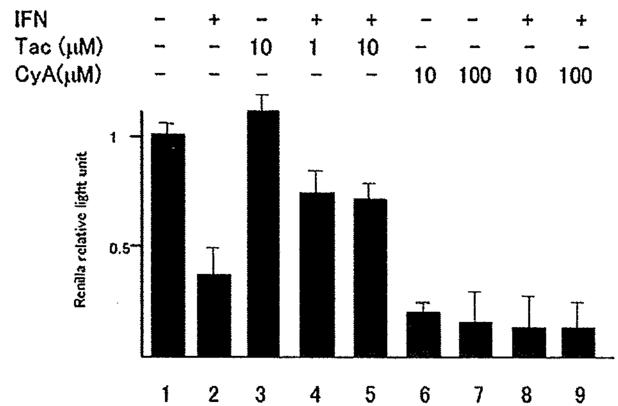


**Figure 7. Alternation of IFN-α-suppressed HCV replication by Tac. OR6 cells, full-length replicon system, were treated with 100 IU/mL of IFN-α in the absence (lane 2) or presence of pretreatment (lanes 4, 5, 8, and 9). Indicated concentration of calcineurin inhibitor alone was lanes 3, 6, and 7, lane 1 was not treated with IFN-α and calcineurin inhibitors. One day later, *Rennila* luciferase activity was determined by luminometer.**

the presence or absence of pretreated Tac or CyA (Fig. 7). IFN-α or CyA alone repressed the *Renilla* luciferase activity, which is well correlated with HCV-RNA concentration in OR6 cells.[19] In contrast, Tac alone had little effect on *Renilla* luciferase activity. However, pretreatment with Tac attenuated the IFN-α-induced repression of *Renilla* luciferase activity (Fig. 7; lane 2 versus lanes 4 and 5), but pretreatment with CyA did not (Fig. 7; lanes 8 and 9).

## DISCUSSION

We herein show that calcineurin inhibitors, especially Tac, are negative regulators of IFN signaling in the hepatocyte, and the greatest cause of this phenomenon is phosphorylation of STAT-1, next to inhibition of nuclear translocation of STAT-1. Disturbance of STAT-1 phosphorylation caused diminished ISRE-containing promoter activity, for example PKR and STAT-1, and antiviral protein expression declined. Pretreatment with Tac diminished the replication inhibitory effect of IFN-α. This phenomenon has a detrimental effect on IFN therapy after HCV-related liver transplantation. In our experiments, we speculated that Tac is not better suited for posttransplantation IFN therapy than CyA, but it did not report that IFN-a response is different between Tac and CyA in human study in previous time. When the alternative of potent immunosuppressant for prevention of rejection, or antiviral-activity for HCV reactivation is weighed, we might need to consider other factors in choosing between Tac and CyA. We had compared high concentration CyA with low concentration Tac, since rejection was controlled by serum trough values of tacrolimus of 5 ng/mL and of cyclosporin of 100 ng/mL in our hospital in the period of stability after liver transplantation.

Recently, the difference between Tac and CyA has been regarded in another function than immunosuppression, and we presume that this discrepancy de-

pended on differences of "immunophilins." Immunophilins are a ubiquitous family of proteins. All cells contain several members of this family, which bind specific calcineurin inhibitors and participate in many cellular functions.[22] Tac has been reported to have neuroprotection,[23] but CyA did not, whereas CyA had anti-HCV action,[24-26] but Tac did not. Tac binds specific FK506 binding protein members of the immunophilin family, whereas cyclosporin binds a different subset of immunophilins (cyclophilins). FK506 binding protein and CyP have the same function as peptidyl prolyl *cis-trans* isomerase and they inhibited the nuclear translocation of nuclear factor of activated T cells (NF-AT). Despite this common pathway, the cell protection activity has been reported to require the induction of heat shock protein 70 by Tac but not CyA,[27] and the anti-HCV activity contributed to a specific blockade of CyP B by CyA.[25] The differences in the medical effects for immunosuppression between Tac and CyA require attention, when these immunosuppressants are used in posttransplantation-related HCV infection.

In our study, the IFN-induced tyrosine phosphorylated STAT-1 and -2 both decreased after the administration of Tac, but Tac is known essentially for the inhibition of serine/threonine protein phosphatase. Calcineurin, regardless of independent Jak-1 tyrosine phosphorylation, and CyA did not have such a tyrosine phosphatase action against STAT-1 and -2. We could not resolve this phosphatase mechanism, but we speculated that Tac induced the tyrosine phosphatase kinase and inhibited tyrosine phosphorylation of STAT-1 and -2. Tac did not induce suppression of cytokines signaling-1 and 3, Jak inhibitors, by western blotting in our study (data not shown); however, we could not rule out the induction of other types of tyrosine phosphatase. Previous studies described that suppressor of cytokines signaling-1, 3 and SH2-containing protein tyrosine phosphatase inhibited NF-AT activation,[28-30] and therefore the relationship between Tac and tyrosine phosphatase might be reconsidered. Barat and Tremblay[31] and Zhu and McKeon[32] previously described the protein-tyrosine phosphatase inhibitor bisperoxovanadium as a potent activator of T cell receptor signaling, and SH2-containing protein tyrosine phosphatase-1, T cell protein-tyrosine phosphatase, Tac, and CyA are inhibitors of such activation. We were interested in the inhibition of protein-tyrosine phosphatase inhibitor by Tac and CyA, because Tac and CyA possessed the same action as SH2-containing protein tyrosine phosphatase-1 and protein-tyrosine phosphatase.[32] Furthermore, this action of Tac was stronger than CyA.[31] From these studies, we assume that Tac has tyrosine phosphatase action in the hepatocyte and inhibits tyrosine phosphorylation of STAT-1 and -2.

The inhibition of IFN-induced antiviral proteins by Tac, and the inhibition of nuclear trafficking of tyrosine phosphorylated STAT-1, is the common phenomenon between Tac and CyA in this study. This phenomenon was observed in the western blotting findings (Fig. 3) and immunohistochemistry of the cultured cells (Fig. 6).

NF-AT activation requires the suppression of Crm1-dependent export from nucleus to cytoplasm by calcineurin,[33] and the presence of importin, bounded to calcineurin, in the nucleus.[34] In IFN-induced Jak-STAT signaling, nuclear trafficking of ISGF-3 requires suppression of Crm1 and binding importin[18] in the same fashion as NF-AT. Calcineurin inhibitors bind to immunophilin and inhibit dephosphorylation of NF-AT, then they inhibit the transcription activity of NF-AT. In addition to such action, it might be considered that the nuclear trafficking of NF-AT is regulated by the calcineurin inhibitor and immunophilin complex. We speculated that the decrease of the nuclear import of tyrosine phosphorylated STAT-1 is the function, the calcineurin inhibitor and immunophilin complex modified Crm1 and importin in the same fashion as NF-AT. Then, we recognized that the mechanisms of diminished tyrosine phosphorylation STATs and nuclear translocation STAT-1 were different.

Presently, there is no definite opinion regarding the selection of calcineurin inhibitors for liver transplantation.[6] However, reports of inhibition of HCV replication by CyA in vitro were noted recently[24-26] and the result were same in our full-length replicon system (Fig. 7). In our data, we consider that CyA has the effect of, not only the previously reported anti-HCV replication action itself, but it creates much less interference with IFN treatment for HCV reactivated after liver transplantation than does Tac. It has been reported that CyA increased the chance of a sustained viral response after liver transplantation.[35] However, we used care with our data, because both Tac and CyA inhibit the nuclear translocation of tyrosine phosphorylated STAT-1. Our data revealed that when an excess of CyA was used after liver transplantation, it resulted in a decrease in the amount of IFN-induced antiviral protein, because of inhibition of nuclear transportation of tyrosine phosphorylation STAT-1 (Figs. 5 and 6). The immunosuppression levels of Tac and CyA have already been reported to decrease significantly in patients responding favorably to anti-HCV therapy post–liver transplantation.[36] In this study, we therefore considered it necessary to pay attention to an excess dose of CyA, when IFN treatment for reactivation of HCV is required.

In conclusion, Tac has been shown to influence the tyrosine phosphorylation of STAT-1, and the result was a decline in antiviral protein PKR. In addition, Tac and CyA have been shown to interfere with the translocation of STAT-1. We speculated that posttransplantation immunosuppression is part of the reason for IFN resistance to HCV reinfection of the graft liver. As the course, calcineurin inhibitors, especially Tac, were pointed out in this study, and we clarified a part of the IFN resistance. Although the mechanism of inhibition of IFN signaling has not yet been fully investigated, it is necessary to compare the antirejection action of Tac to the anti-HCV action of CyA when selecting calcineurin inhibitors.

## REFERENCES

1. Perz JF, Armstrong GL, Farrington LA, Hutin YJ, Bell BP. The contributions of hepatitis B virus and hepatitis C virus

infection to cirrhosis and primary liver cancer worldwide. J Hepatol 2006;45:529-538.
2. Forman LM, Lewis JD, Berlin JA, Feldman HI, Lucey MR. The association between hepatitis C infection and survival after orthotopic liver transplantation. Gastroenterology 2002;122:889-896.
3. Berenguer M, Prieto M, San Juan F, Rayon JM, Martinez F, Carrasco D, et al. Contribution of donor age to the recent decrease in patient survival among HCV-infected liver transplant recipients. Hepatology 2002;36:202-210.
4. Berenguer M, Ferrell L, Watson J, Prieto M, Kim M, Rayon M, et al. HCV-related fibrosis progression following liver transplantation: increase in recent years. J Hepatol 2000; 32:673-684.
5. Berenguer M, Crippin J, Gish R, Bass N, Bostrom A, Netto G, et al. A model to predict severe HCV-related disease following liver transplantation. Hepatology 2003;38:34-41.
6. Everson GT. Impact of immunosuppressive therapy on recurrence of hepatitis C. Liver Transpl 2002;8:S19–S27.
7. Gane E. Treatment of recurrent hepatitis C. Liver Transpl 2002;8:S28–S37.
8. Davis GL, Wong JB, McHutchison JG, Manns MP, Harvey J, Albrecht J. Early virologic response to treatment with peginterferon alfa-2b plus ribavirin in patients with chronic hepatitis C. Hepatology 2003;38:645-652.
9. Castells L, Vargas V, Allende H, Bilbao I, Luis Lazaro J, Margarit C, et al. Combined treatment with pegylated interferon (alpha-2b) and ribavirin in the acute phase of hepatitis C virus recurrence after liver transplantation. J Hepatol 2005;43:53-59.
10. Heydtmann M, Freshwater D, Dudley T, Lai V, Palmer S, Hubscher S, et al. Pegylated interferon alpha-2b for patients with HCV recurrence and graft fibrosis. following liver transplantation. Am J Transplant 2006;6:825-833.
11. Bizollon T, Pradat P, Mabrut JY, Chevallier M, Adham M, Radenne S, et al. Benefit of sustained virological response to combination therapy on graft survival of liver transplanted patients with recurrent chronic hepatitis C. Am J Transplant 2005;5:1909-1913.
12. Abdelmalek MF, Firpi RJ, Soldevila-Pico C, Reed AI, Hemming AW, Liu C, et al. Sustained viral response to interferon and ribavirin in liver transplant recipients with recurrent hepatitis C. Liver Transpl 2004;10:199-207.
13. Hu X, Li WP, Meng C, Ivashkiv LB. Inhibition of IFN-gamma signaling by glucocorticoids. J Immunol 2003; 170:4833-4839.
14. Kimura T, Kadokawa Y, Harada H, Matsumoto M, Sato M, Kashiwazaki Y, et al. Essential and non-redundant roles of p48 (ISGF3 gamma) and IRF-1 in both type I and type II interferon responses, as revealed by gene targeting studies. Genes Cells 1996;1:115-124.
15. Kuhen KL, Vessey JW, Samuel CE. Mechanism of interferon action: identification of essential positions within the novel 15-base-pair KCS element required for transcriptional activation of the RNA-dependent protein kinase pkr gene. J Virol 1998;72:9934-9939.
16. Brierley MM, Fish EN. Stats: multifaceted regulators of transcription. J Interferon Cytokine Res 2005;25:733-744.
17. Ogata H, Kobayashi T, Chinen T, Takaki H, Sanada T, Minoda Y, et al. Deletion of the SOCS3 gene in liver parenchymal cells promotes hepatitis-induced hepatocarcinogenesis. Gastroenterology 2006;131:179-193.
18. Reich NC, Liu L. Tracking STAT nuclear traffic. Nat Rev Immunol 2006;6:602-612.
19. 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.
19. Ichikawa T, Nakao K, Nakata K, Yamashita M, Hamasaki K, Shigeno M, et al. Involvement of IL-1beta and IL-10 in IFN-alpha-mediated antiviral gene induction in human hepatoma cells. Biochem Biophys Res Commun 2002; 294:414-422.
20. Nishimura D, Ishikawa H, Matsumoto K, Shibata H, Motoyoshi Y, Fukuta M, et al. DHMEQ, a novel NF-kappaB inhibitor, induces apoptosis and cell-cycle arrest in human hepatoma cells. Int J Oncol 2006;29:713-719.
21. Takahashi N. Macrolide compounds as inhibitors of the intracellular signal transmission pathway: the mechanism of actions of rapamycin and FK506. Jpn J Antibiot 2000;53(Suppl A):62-67.
22. Keswani SC, Chander B, Hasan C, Griffin JW, McArthur JC, Hoke A. FK506 is neuroprotective in a model of antiretroviral toxic neuropathy. Ann Neurol 2003;53:57-64.
23. Watashi K, Hijikata M, Hosaka M, Yamaji M, Shimotohno K. Cyclosporin A suppresses replication of hepatitis C virus genome in cultured hepatocytes. Hepatology 2003; 38:1282-1288.
24. Watashi K, Ishii N, Hijikata M, Inoue D, Murata T, Miyanari Y, Shimotohno K. Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase. Mol Cell 2005; 19:111-122.
25. Henry SD, Metselaar HJ, Lonsdale RC, Kok A, Haagmans BL, Tilanus HW, et al. Mycophenolic acid inhibits hepatitis C virus replication and acts in synergy with cyclosporin A and interferon-alpha. Gastroenterology 2006;131:1452-1462.
26. Kaibori M, Inoue T, Tu W, Oda M, Kwon AH, Kamiyama Y, et al. FK506, but not cyclosporin A, prevents mitochondrial dysfunction during hypoxia in rat hepatocytes. Life Sci 2001;69:17-26.
27. Matsuda T, Yamamoto T, Kishi H, Yoshimura A, Muraguchi A. SOCS-1 can suppress CD3zeta- and Syk-mediated NF-AT activation in a non-lymphoid cell line. FEBS Lett 2000;472:235-240.
28. Banerjee A, Banks AS, Nawijn MC, Chen XP, Rothman PB. Cutting edge: suppressor of cytokine signaling 3 inhibits activation of NFATp. J Immunol 2002;168:4277-4281.
29. Su MW, Yu CL, Burakoff SJ, Jin YJ. Targeting Src homology 2 domain-containing tyrosine phosphatase (SHP-1) into lipid rafts inhibits CD3-induced T cell activation. J Immunol 2001;166:3975-3982.
30. Fortin JF, Barbeau B, Robichaud GA, Pare ME, Lemieux AM, Tremblay MJ. Regulation of nuclear factor of activated T cells by phosphotyrosyl-specific phosphatase activity: a positive effect on HIV-1 long terminal repeat-driven transcription and a possible implication of SHP-1. Blood 2001;97:2390-2400.
31. Barat C, Tremblay MJ. Treatment of human T cells with bisperoxovanadium phosphotyrosyl phosphatase inhibitors leads to activation of cyclooxygenase-2 gene. J Biol Chem 2003;278:6992-7000.
32. Zhu J, McKeon F. NF-AT activation requires suppression of Crm1-dependent export by calcineurin. Nature 1999; 398:256-260.
33. Hallhuber M, Burkard N, Wu R, Buch MH, Engelhardt S, Hein L, et al. Inhibition of nuclear import of calcineurin prevents myocardial hypertrophy. Circ Res 2006;99:626-635.
35. Firpi RJ, Zhu H, Morelli G, Abdelmalek MF, Soldevila-Pico C, Machicao VI, et al. Cyclosporine suppresses hepatitis C virus in vitro and increases the chance of a sustained virological response after liver transplantation. Liver Transpl 2006;12:51-57.
36. Kugelmas M, Osgood MJ, Trotter JF, Bak T, Wachs M, Forman L, et al. Hepatitis C virus therapy, hepatocyte drug metabolism, and risk for acute cellular rejection. Liver Transpl 2003;9:1159-1165.

Journal of Medical Virology 80:632–639 (2008)

# Possible Molecular Mechanism of the Relationship Between NS5B Polymorphisms and Early Clearance of Hepatitis C Virus During Interferon Plus Ribavirin Treatment

**Mitsuyasu Nakamura,[1] Hidetsugu Saito,[1]* Masanori Ikeda,[2] Shinichiro Tada,[1] Naoki Kumagai,[3] Nobuyuki Kato,[2] Kunitada Shimotohno,[4] and Toshifumi Hibi[1]**

[1]*Division of Gastroenterology and Hepatology, Department of Internal Medicine, School of Medicine, Keio University, Shinanomachi, Shinjuku-ku, Tokyo, Japan*
[2]*Department of Molecular Biology, Okayama University Graduate School of Medicine and Dentistry, Shikada-cho, Okayama, Japan*
[3]*Research Center for Liver Diseases, The Kitasato Institute, Shirogane, Minato-ku, Tokyo, Japan*
[4]*Center for Integrated Medical Research, School of Medicine, Keio University, Shianomachi, Shinjuku-ku, Tokyo, Japan*

We previously reported the relationship between viral polymerase polymorphisms and the initial decline in viral load induced by interferon-α and ribavirin therapy in genotype 1b-related chronic hepatitis C patients. The presence of E124K and I85V of NS5B was closely associated with viral clearance at 8 weeks of treatment. The aim of this study was to investigate the mechanisms by which this polymorphism of NS5B protein affects early viral clearance. We used a replicon system derived from strain O, genotype 1b virus. Three mutants (V85I), (K124E), and (V85I/K124E) were introduced to the replicon. OR6c, a derivative of HuH7 cells, was transfected with the replicon including a luciferase reporter gene. Luciferase activities were measured 72 hr post-transfection. All three mutants showed higher luciferase activity than that of the wild type, and the V85I mutant showed the highest activity. This result was also confirmed by neomycin gene-containing replicons with same mutations. All replicons were down-regulated by ribavirin, but the level of reduction in the V85I mutant was the lowest. Our results suggested that this mutation at least partly contributes to resistance to early viral clearance during interferon and ribavirin combination therapy. ***J. Med. Virol. 80:632–639, 2008.*** © 2008 Wiley-Liss, Inc.

**KEY WORDS:** hepatitis C virus; NS5B polymorphism; replicon; interferon and ribavirin combination therapy; viral proliferation

## INTRODUCTION

With an estimated 170 million infected individuals, hepatitis C virus (HCV) has a major impact on public health. An estimated 65–80% of the individuals infected with HCV develop persistent infection while 20–50% develop cirrhosis and 5% develop hepatocellular carcinoma (HCC) [Liang et al., 2000; Gao et al., 2004]. Until recently, interferon (IFN)-α and IFN-β were the only available treatments for HCV infection, although only 10–15% of treated subjects achieved sustained viral eradication with IFN monotherapy, and early viral clearance after initiation of IFN monotherapy was correlated with sustained viral clearance [Saito et al., 2000].

The current approved treatment for HCV infection is pegylated IFN-α (peg-IFN) in combination with ribavirin (RBV). This combination therapy leads to viral clearance in 50–80% of cases, depending on the infecting HCV genotype, and 50% of patients with HCV genotype 1b and high baseline levels of viral RNA do not achieve a sustained virological response with the combination therapy after 48 weeks [Manns et al., 2001; Fried et al., 2002; Feld and Hoofnagle, 2005]. Several prior studies have attempted to predict the efficacy of IFN plus RBV combination therapy. A quantitative measurement of HCV viremia or the initial decline in viral load is a reliable marker for early prediction of the therapeutic response to IFN and RBV combination therapy [Zeuzem et al., 1998; Bouvier-Alias et al., 2002; Takahashi et al., 2005; Lukasiewicz et al., 2007].

RBV is a broad-spectrum nucleoside analogue antiviral drug which is especially noted for its actions

Grant sponsor: Viral Hepatitis Research Foundation of Japan.

*Correspondence to: Hidetsugu Saito, Department of Internal Medicine, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan.
E-mail: hsaito@sc.itc.keio.ac.jp

Accepted 28 December 2007
DOI 10.1002/jmv.21125
Published online in Wiley InterScience
(www.interscience.wiley.com)

© 2008 WILEY-LISS, INC.

WILEY InterScience DISCOVER SOMETHING GREAT

against RNA viruses and exhibits in vitro activity against some DNA and RNA viruses, including certain members of *Flaviviridae* [Sidwell et al., 1972]. It has recently been demonstrated that the antiviral activity of RBV can result from the ability of a viral RNA-dependent RNA polymerase (RdRP) to utilize RBV triphosphate and to incorporate this nucleotide into the viral genome with reduced specificity, thereby mutagenizing the genome and decreasing the yield of infectious virus [Crotty et al., 2000; Lanford et al., 2003]. Moreover, RBV exhibits an antiviral effect through a mechanism of error-prone replication in the HCV subgenomic replication system [Contreras et al., 2002]. Although RBV by itself cannot decrease serum HCV RNA levels in patients, it has been demonstrated that combination therapy with RBV and either IFN-α or peg-IFN yields a higher sustained response rate than is achieved with IFN-α monotherapy [Pol et al., 2000; Poynard et al., 2000; Saracco et al., 2001].

We previously reported the relationship between viral RdRP polymorphisms and the initial decline in viral load induced by IFN-α and RBV therapy in genotype 1b-related chronic hepatitis C patients [Kumagai et al., 2004]. Substitution of glutamic acid to lysine at the 124th position (E124K) and of isoleucine to valine at the 85th position (I85V) of NS5B was closely associated with viral clearance at 8 weeks of treatment.

In this study, we used the genotype 1b HCV replicon system [Ikeda et al., 2005] to generate NS5B mutants (E124K, I85V, and both) and we compared the replication activity with that of the wild-type replicon and to analyze how this polymorphism of NS5B protein affects early viral clearance during combination therapy with IFN and RBV. We also examined the significance of NS5B polymorphisms in the RBV-induced decrease in viral replication. We concluded that the identified polymorphism of NS5B partly affects viral replication.

## MATERIALS AND METHODS

### Cell Culture System

OR6 cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Invitrogen Life Technology, Carlsbad, CA) supplemented with 10% fetal calf serum, penicillin, and streptomycin (complete DMEM), in addition to G418 (300 μg/ml; Geneticin, Invitrogen), and were then passaged twice a week at a 5:1 split ratio. OR6c cells are cured OR6 cells from which genome-length HCV RNA was eliminated by IFN-α treatment (500 IU/ml for 2 weeks) without G418, as previously described [Ikeda et al., 2005].

### Plasmids

The plasmids pON/C-5B/KE (Fig. 1a) and pHCV-O were described previously [Ikeda et al., 2005]. This plasmid includes the adaptive mutation of K1609E of NS3 to enhance the efficiency of replication, this adaptive mutation was reported by Lohman et al. (22) The plasmid pON/C-5B/KE contains neomycin phosphotransferase (Neo) downstream of HCV IRES and the full length HCV-O polyprotein coding sequence downstream of encephalomyocarditis virus (EMCV) IRES. To introduce a pON/C-5B/KE/(V85I), pON/C-5B/KE/(K124E), pON/C-5B/KE/(V85I&K124E), we first made PCR fragments including the partial NS5B region with the primers 5′-ggatcccgatctcagcgacgg-3′ and 5′-tctagaggtcccattcgccattac-3′. This 2.4-kb fragment was subcloned into pSTBlue1 Blunt vector (Novagen, Madison, WI) to generate pSTBlue-1MN002. Each vector expressing the V85I mutant, K124E mutant, and V85I&K124E double mutant of HCV-O was generated by Quick Change mutagenesis (Stratagene, La Jolla, CA) to generate pSTBlueMN002(V85I), pSTBlueMN002(K124E) pSTBlueMN002(V85I&K124E). Next, pON/C-5B was

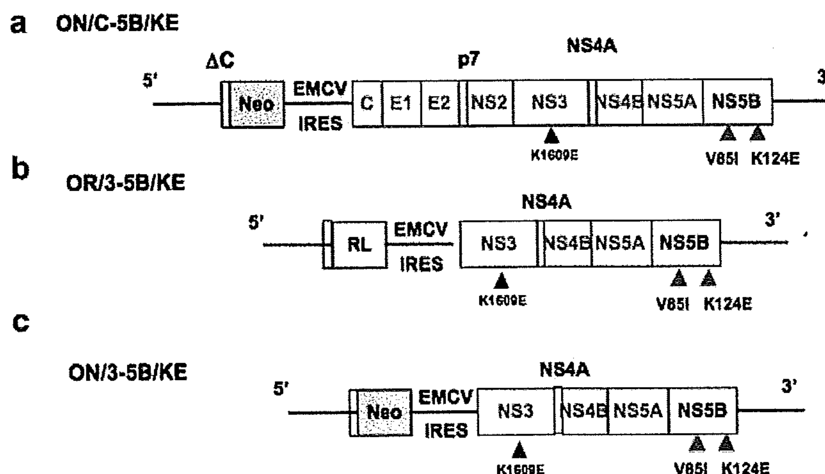


Fig. 1. **a**: Organization of genome-length HCV RNA derived from HCV-O. Open reading frames, untranslated regions, EMCV IRES, and Neo genes are depicted as shaded boxes, thin lines, thick lines, and open boxes, respectively. ΔC indicates the 12 N-terminal amino acid residues of the core as a part of IRES. This construct also contains adaptive mutation K1609E which is indicated by a black triangle. We use this construct as a wild type. Grey triangle is the position of 85 and 124 in NS5B which we generated mutation to the replicon for this experiments. **b**: The construct of the reporter subgenomic HCV replicon carries the renilla luciferase gene (RL). **c**: The construct of the reporter subgenomic HCV replicon carries the Neo gene.

digested with *Sna*b1 and *Xba*1 and subcloned into pSTBlue-1 to create pSTBlueMN001. All of the pSTBlue-MN002mutants were digested with *Bam*H1 and *Xba*1, which were subcloned into pSTBlueMN001 to create pSTBlueMN001mutant. The pSTBlueMN001 mutants were digested with *Sna*b1 and *Xba*1 and re-ligated in pON/C-5B/KE to introduce pON/C-5B/KE/(V85I), pON/C-5B/KE/(K124E), pON/C-5B/(V85I&K124E). The plasmids pOR/3-5B/KE/(V85I), pOR/3-5B/KE/(K124E) and pOR/3-5B/KE/(V85I&K124E), were constructed from pOR/3-5B/KE (Fig. 1b) by swapping for fragments of pSTBlueMN001 mutants digested with *Sna*b1 and *Xba*1. DNA sequencing of the manipulated regions of the plasmids verified all mutations.

### RNA Transfection and Selection of G418-Resistant Cells

For electroporation, OR6c cells were washed twice with ice-cold phosphate buffered saline (PBS) and resuspended at $10^7$ cells/ml in PBS. Twenty microgram of ON/C-5B/KE or its mutant derived RNA was mixed with 500 µl of the cell suspension in a cuvette with a gap width of 0.2 cm (Bio-Rad, Hercules, CA). The mixture was immediately subjected to two pulses of current at 1.2 kV, 25 µF, and maximum resistance. Following 10 min of incubation at room temperature, cells were seeded into 10-cm dishes. Cells were selected in complete DMEM with 300 µg/ml G418. About 3 weeks after transfection and G418 selection, cells were fixed and stained with Coomassie brilliant blue (0.6 g/l in 50% methanol–10% acetic acid) and the number of colonies was counted.

### Transient-Replication Assays With Luciferase Replicons

OR6c cells were transfected by electroporation as the same protocol described above using 20 µg of OR/3-5B/KE or its mutants derived RNAs carrying the renilla luciferase (RL) gene. After addition of 2 ml of complete DMEM, $2 \times 10^4$ of aliquot OR6c cells were plated in 24-well plates at least in triplicate for each assay and harvested at various time points with renilla lysis reagent (Promega KK, Tokyo, Japan) and subjected to the RL assay according to the manufacturer's protocol (Promega). Values obtained with cells harvested 6 hr after electroporation were used to correct for the transfection efficiency.

### IFN and Ribavirin Treatment

To monitor the anti-HCV effect of IFN and RBV on replication, OR6c cells were transfected by electroporation using 10 µg of OR/3-5B/KE derived RNAs as described elsewhere [Crotty et al., 2000]. OR6c cells ($2 \times 10^4$/well) were plated onto 24-well plates at least in triplicate for each assay and cultured for 4 hr. Then the cells were treated with IFN at a final concentration of 1, 2, 4, 10, and 20 units/ml or RBV at a final concentration of 50,100, and 200 µM for 72 hr, harvested with renilla lysis reagent (Promega), and assayed for luciferase activity according to the manufacturer's protocol. We also studied about the additional effect of RBV (100 µM) on IFN (1 u/ml).

### Cell Viability

We checked toxic effect of IFN and RBV. Effect of IFN (1 and 4 units/ml), and RBV (50 and 100 µM) on cell viability was investigated. To examine the cytotoxic effect of IFN and RBV on OR6c cells with OR/3-5B/KE replicon RNA, the cells were seeded at a density of $2 \times 10^5$ cells per dish onto 6-well plates. After 24-hr culture, the cells were treated with IFN or RBV at final concentrations of 2 and 4 units/ml or 50 and 100 µM, respectively, in the absence of G418. After incubation for 72 hr, the number of viable cells was counted in an improved Neubauer-type hemocytometer after trypan blue dye (Invitrogen) treatment.

### Indirect Immunofluorescence

Cells were grown on four-well chamber slides until 70–80% confluent, washed three times with PBS, and fixed in methanol–acetone (1:1, v/v) for 10 min at room temperature. Dilutions of primary murine monoclonal antibody to residues 21–40 of the core protein (2Zcp11; Tokushu Men-eki Institute, Tokyo) (1:1,000), were prepared in PBS containing 3% bovine serum albumin and incubated with fixed cells for 2 hr at room temperature. After additional washes with PBS, specific antibody binding was detected with a goat anti-mouse immunoglobulin G-fluorescein isothiocyanate-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) diluted 1:500. Cells were washed with PBS, and mounted in DAKO Fluorescent mounting medium (DAKO Japan, Tokyo, Japan) prior to examination using a Zeiss AxioPlan2 fluorescence microscope.

### Statistical Analysis

Difference in relative luciferase activity among mutant replicons and differences in anti-HCV activity of RBV among mutant replicons were tested using Student's *t*-test and Mann–Whitney *U*-test as appropriate. *P*-values <0.05 were considered statistically significant.

## RESULTS

### Mutation in NS5B Enhances Levels of Replication on Transient Assay

To investigate whether the mutations in NS5B of the HCV genome affect replication, we used subgenomic HCV replicons with the renilla luciferase gene for transient assay [Ikeda et al., 2005]. In a previous study [Kumagai et al., 2004], substitution of glutamic acid at the 124th position with lysine and substitution of isoleucine at the 85th position with valine in NS5B yielded a complete match with the population of good

responders (5 out of 5 patients). We introduced mutations to two different types of replicon to obtain ON/C-5B/KE(V85I), ON/C-5B/KE/(K124E), ON/C-5B/KE (V85I&K124E), OR/3-5B/KE/(V85I), OR/3-5B/KE(K124E), and OR/3-5B/KE(V85I&K124E) as described in Materials and Methods Section. The subgenomic replicons with V85I showed higher replication activity than the wild-type replicon in OR6c cells (Fig. 2). Also the replicon with K124E and the replicon with V85I&K124E showed slightly higher replication activity than the wild type, but the replicon with K124E single amino acid mutation did not show statistically higher replication activity than the wild type (Fig. 2). We initially expected that double mutations (V85I&K124E) would lead to better replication than either of the single mutations (V85I or K124E), but interestingly the V85I mutation on NS5B replicated best. This result indicated that the level of replication was affected by amino acid substitution at the 85th position.

## Mutation in NS5B Enhances the Efficiency of Colony Formation in Cured Cells

In colony formation assay, we used cured subgenomic replicon cells (OR6c), since cured cells enhanced colony formation of the replicon more efficiently than did parental HuH-7 cells. We examined the effect of these mutations in full-length replicon, ON/C-5B/KE by a colony formation assay. In the initial experiment, we introduced each 20 μg of RNA derived from the ON/C-5B/KE, ON/C-5B/KE/(V85I), ON/C-5B/KE/(K124E), and ON/C-5B/KE/(V85I&K124E) into OR6c cells. After 3 weeks of G418 selection at a concentration of 300 μg/ml, only one colony was obtained and the same result was obtained with ON/C-5B/KE(K124E) and ON/C-5B/KE/(V85I&K124E) transcripts. In repeated experiments, the number of G418-resistant colonies was reproducibly one or zero, but when ON/C-5B/KE/(V85I) transcripts was electroporated, G418 resistant 4–6 colonies was obtained in repeated experiments. These results also confirm that the replication level of ON/C-5B//KE/(V85I) is higher than that of ON/C-5B/KE, ON/C-5B/KE/(K124E) and ON/C-5B/KE/(V85I&K124E).

As the efficiency of colony formation with full-length replicon (ON/C-5B/KE; Fig. 1a) was quite low, we investigated colony formation with subgenomic replicon, ON/3-5B/KE (Fig. 1c). Figure 3 shows the representative result of three independent colony formation assays. The efficiency of colony formation of ON/3-5B/KE was better than that of full-length replicon and the colony formation of in vitro transcript of ON/3-5B/KE, ON/3-5B//KE/(V85I), ON/3-5B/KE/(K124E) and ON/3-5B/KE/(V85I&K124E) was 39 (61), 157 (132), 44 (54), and 134 (107), respectively (the numbers in parentheses show another set of result). The efficiency of colony formation of ON/3-5B//KE/(V85I) was greater than that of ON/C-5B/KE and it showed a similar result with that obtained from genome-length replicon.

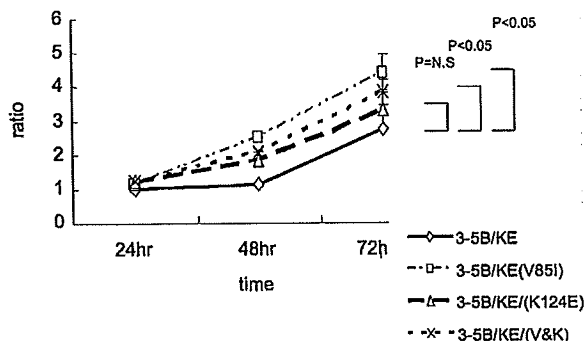


Fig. 2. Effect of amino acid substitutions in NS5B on transient replication activity of replicon. The replication activity of reporter subgenomic HCV replicon with mutation of V85I, K124E, or V85I and K124E (V&K) was compared with that of wild-type in OR6c cells (transient transfection). After 72 hr of transfection, the Renilla luciferase (RL) assay was performed as described in the Materials and Methods section. The relative RL activity (ratio) of mutants was calculated in comparison to that of subgenomic replicon of wild-type (assigned as 1). The data indicate means ± SD of triplicates from three independent experiments. 3-5B/KE: OR/3-5B/KE, 3-5B/KE(V85I): OR/3-5B(K124E), 3-5B/KE/(K124E): OR3-5B/KE/(K124E), 3-5B/KE/(V&K): OR/3-5B/KE/(V85I&K124E).

## Inhibition of HCV RNA Replication by IFN and RBV

We examined the inhibitory effect of IFN and RBV on the replication of OR/3-5B/KE. In this experiment, the subgenomic replicon system was used. OR6c cells were treated with IFN at concentrations of 1–20 μM (Fig. 4) and RBV at concentrations of 50, 100, and 200 μM (Fig. 5) after transfection of OR/3-5B/KE derived RNA. Since it is important to know how IFN and RBV treatment is toxic to the cells, we examined cell viability after treatment with 50 and 100 μM of RBV or 2 and 4 units/ml of IFN. The cell viability of OR6c was not

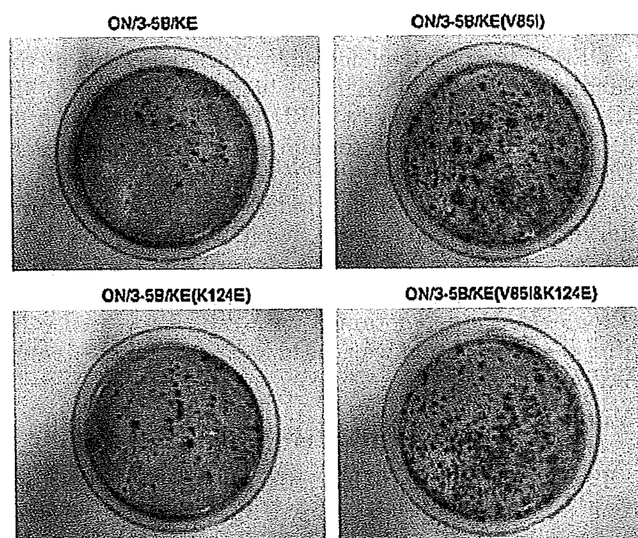


Fig. 3. Colony formation assay of OR6c cells transfected with wild-type and three different mutant replicons. A representative result of colony formation assay using subgenomic replicon RNA (ON/3-5B/KE) system. The efficacy of colony formation was much higher than that of full-length replicon RNA (ON/C-5B/KE). In this series of photographs, colony forming unit of ON/3-5B/KE, ON/3-5B//KE/(V85I), ON/3-5B/KE/(K124E) and ON/3-5B/KE/(V85I&K124E) was 2.7/μg, 6.7/μg, 3.0/μg and 5.4/μg, respectively.

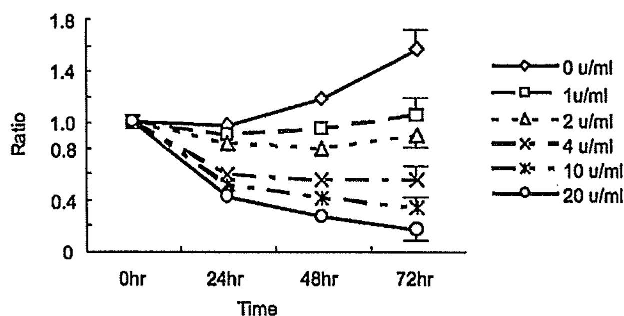


Fig. 4. Dose-dependent inhibition of replication by interferon-α (IFN). OR6 cells were transfected with wild-type replicon (OR/3-5B/KE). Inhibition of HCV RNA replication in the OR6c cell treated with IFN-α was shown at the indicated time (24, 48, and 72 hr) from the start of treatment. The cells were treated with IFN-α (0, 1, 2, 4, 10, and 20 u/ml), and the Renilla luciferase assay was performed as described in Materials and Methods Section. The relative luciferase activity (%) calculated at each point, where the luciferase activity of non-treated cells at 0 hr was assigned to be 100%, is presented. The data indicate means ± SD of triplicates from three independent experiments.

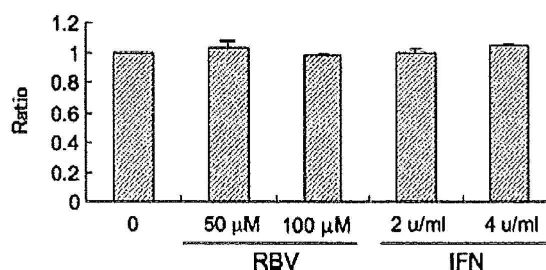


Fig. 6. Cytotoxicity of ribavirin (RBV) or interferon-α (IFN) on replicon RNA in OR6c cells. OR6c cells with OR/3-5B/KE RNA were cultured in the absence or presence of RBV or IFN (50 and 100 μM or 2 and 4 u/ml) for 72 hr, and then the cell viability was determined as described in Materials and Methods Section. The relative cell viability (%) calculated at each point, when viability of non-treated cells was assigned to be 100%, is presented. The data indicate means ± SD of triplicates from three independent experiments.

changed by these treatments (Fig. 6), indicating that both IFN and RBV were not toxic to the cells at the indicated concentrations. As shown in Figures 4 and 5, the inhibition of HCV RNA replication occurred in a dose-dependent manner with IFN or RBV treatments. RBV at a concentration of 100 μM inhibited replication of RNA (Fig. 5), but was not toxic to OR6c cells (Fig. 6).

The inhibitory effect of 100 μM RBV on RNA replication in each mutant was also examined. Various biological effect of IFN has been investigated and its effect on cell cycle or cell-differentiation is strong, and we focused on the effect of mutants on RBV treatment. To see this effect, we compared between IFN alone and IFN + RBV. As shown in Figure 7, no difference between three mutants was seen in the treatment with 1 unit/ml of IFN. The proliferation of each mutant RNA was similarly reduced to around a ratio of 0.6. On the other hand, addition of 100 μM of RBV was differently affected by each mutation pattern (Fig. 8). The single mutant with V85I and double mutants with V85I and K124E were significantly increased in RNA proliferation. The degree of inhibition by RBV in OR/3-5B/KE(V85I) and OR/3-5B/KE(V85I&K124E) was significantly lower than that in OR/3-5B/KE, although the difference of OR/3-5B/KE(K124E) was not significant.

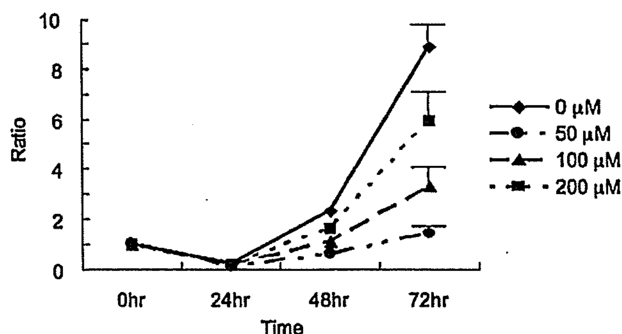


Fig. 5. Dose-dependent inhibition of HCV RNA replication by ribavirin. OR6 cells were transfected with wild-type replicon (OR/3-5B/KE) and treated with ribavirin at concentrations of 50, 100, and 200 μM for 72 hr. Inhibition of HCV RNA replication in the OR6c cell treated with ribavirin (RBV) was shown at the indicated time (24, 48, and 72 hr) from the start of treatment. The relative luciferase activity (%) calculated at each point, where the luciferase activity of non-treated cells at 0 hr was assigned to be 100%, is presented. The data indicate means ± SD of triplicates from three independent experiments.

### Indirect Immunofluorescence

To confirm the presence of replicating full-length RNAs in cells selected for G418 resistance following transfection with ON/C-5B/KE(V85I), one G418-resistant cell colony was selected at random and clonally cultured. We confirmed HCV protein expression by indirect immunofluorescence imaging and observed core protein in the replicon cells (OR6) (Fig. 9c), HCV core protein was demonstrated in the clonally isolated cell line selected after transfection with ON/C-5B/KE(V85I)

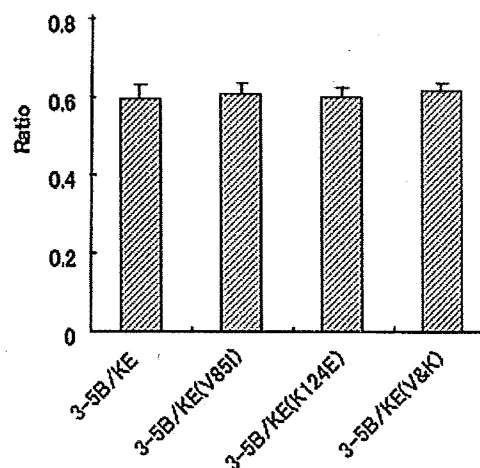


Fig. 7. Effect of interferon-α (IFN) on the subgenomic HCV replicon possessing the Renilla luciferase reporter. The replication levels of the subgenomic HCV replicons were monitored by luciferase reporter assay with IFN 0 u/ml or IFN 1 u/ml for 72 hr. Renilla luciferase assay was performed as described in Materials and Methods Section. The relative luciferase unit with IFN (1 u/ml) treatment were calculated, where the luciferase unit without IFN treatment was assigned to be 1, and compared between wild type (OR/3-5B/KE) and other three mutants (OR/3-5B/KE(V85I), OR/3-5B/KE(K124E), OR/3-5B/KE(V&K)). The data indicate means ± SD of triplicates from two independent experiments.

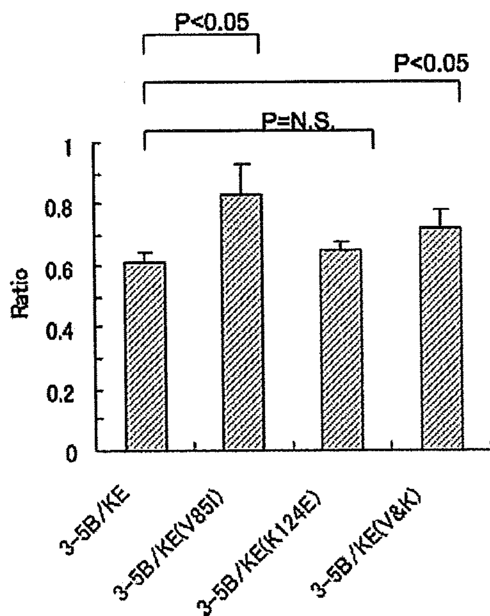


Fig. 8. Effect of interferon-α (IFN) and ribavirin (RBV) combination treatment on the replication levels of the subgenomic HCV replicon possessing the Renilla luciferase reporter. The replication levels of the subgenomic HCV replicons were monitored by luciferase reporter assay with IFN 1 u/ml or IFN 1 u/ml and ribavirin 100 μM for 72 hr. The relative luciferase unit of IFN 1 u/ml and ribavirin 100 mM treatment were calculated, where the luciferase unit of IFN 1 u/ml treatment was assigned to be 1, and compared in wild type (OR/3-5B/KE) and other three mutants (OR/3-5B/KE(V85I), OR/3-5B/KE(K124E), OR/3-5B/KE(V&K)). The data indicate means ± SD of triplicates from three independent experiments.

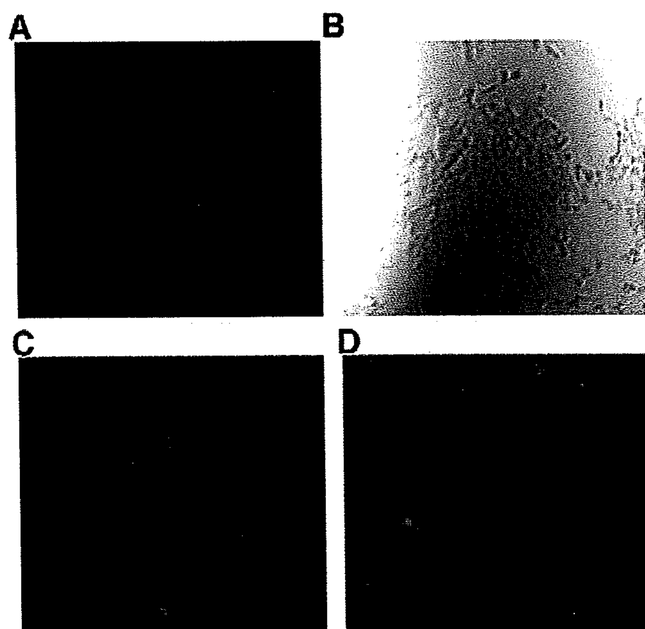


Fig. 9. Indirect immunofluorescence detection of HCV core antigen in normal OR6c cells (cured cell) (**a**), OR6 cells (wild-type HCV replicon) (**c**), and a clonally isolated cell line selected following transfection of OR6c cells with ON/3-5B/V85I (cell line 1) (**d**) and the correspondent phase-contrast microscopic photograph of OR6c cells (**b**).

(Fig. 9d), while it was not observed in the cured cell line (OR6C) (Fig. 9a,b).

## DISCUSSION

Predictive factors for a sustained viral response (SVR) in IFN monotherapy or combination therapy have been vigorously investigated in prior studies. In addition to several host and viral factors, such as HCV genotypes, baseline viral load, stage of fibrosis, gender, age, and obesity [Saito et al., 2000, 2006], disappearance of serum HCV RNA during the early phase of therapy or a rapid decrease in HCV RNA levels are significant factors for achieving a SVR [Ferenci et al., 2001]. In our previous study, two distinct amino acid substitutions in the NS5B region of the HCV genome correlated with early viral responses in combination therapy [Kumagai et al., 2004]. NS5B of the HCV genome codes for RdRP, which regulates viral replication. Thus, the detected mutations might increase replication efficacy of HCV or induce resistance to the anti-viral effect of RBV, which could lead to resistance to therapy in the early phase. It was thought that the HCV replicon system would be a good tool for examining the correlation between viral mutation and replication capability. One of the mutation-introduced replicons (V85I) showed a higher replication activity than that of the wild type, and, consistent with our previous clinical study, this mutant was resistant to in vitro RBV treatment. The present study is the first to examine the precise relationship between such mutations and clinical data on the early clearance of HCV during IFN and RBV combination therapy. The mutations of V85I and K124E in NS5B have never been reported in the replicon system.

We investigated the effect of both IFN and RBV on the wild type and three mutants in NS5B at non-toxic concentration to the host cell (Fig. 6). One unit of IFN did not affect the replication of mutants (Fig. 7) but RBV significantly affected the replication of three mutants in the presence of IFN (Fig. 8). These results indicated that the polymorphism of NS5B affect sensitivity to RBV treatment. Although it has been known in the clinical setting that HCV RNA levels are not changed in patients with chronic hepatitis C during RBV monotherapy, our in vitro results showed the reduction of HCV RNA replication with RBV treatment. It was reported that serum levels of RBV in patients with chronic hepatitis C under IFN + RBV combination therapy was very low such as $10^{-14}$ mM [Naka et al., 2005], however, we can examine the anti-viral effect of much higher levels of RBV on the replicon system without a direct toxic effect of RBV in HuH7 cells. The possibility of a difference between circulating HCV particles and the replicon system in terms of RBV sensitivity may still exist, but this question will be further investigated using a recently developed cell culture system.

We used a dicistronic genome length and subgenome HCV RNA replication systems, which were established previously using HCV RNA from HCV-O infected in non-neoplastic human hepatocyte PH5CH8 cells. For the

cells into which genome-length and subgenomic HCV RNA were introduced, we chose the cloned cell line OR6c, prepared by IFN treatment from subgenomic HCV replicon-supporting cells, since OR6c had a higher efficiency of colony formation (ECF) than its parental HuH-7 cell line in a study of subgenomic HCV replicons [Blight et al., 2002]. It is known that the efficiency of colony formation is unstable, so that the luciferase activity and the colony-forming unit are always discrepant. The impact of ON/C-5B/KE(V85I) on colony formation was about 4 times that of the wild-type replicon in genome length and subgenomic RNAs, and the V85I mutation in NS5B showed 1.5 times higher replication activity in luciferase assay than the wild type in the subgenomic replicon system. Young et al. reported an RBV-resistant NS5B mutation during RBV monotherapy [Young et al., 2003], but this phenylalanine to tyrosine amino acid substitution located at the 415th position in NS5B differed from our amino acid substitution. Replicon cells were selected after G418 exposure, and the replication may be amplified by this selection culture. We sequenced the NS5B region, which includes the 85th and 124th nucleotide portions, from some clones 2 months after G418 selection culture, and we did not found significant mutations. From the present in vitro study and previous clinical study, it may be concluded that at least V85I mutation in NS5B increases viral replication that may cause resistance to RBV treatment.

Two of the patients in the clinical study [Kumagai et al., 2004] had previously been treated with IFN-α monotherapy in our previous study: one patient (Pt 3) has V85 and K124 in the HCV RdRP and the other (Pt 7) had I85 and E124. The former was a good responder to IFN-α and RBV combination therapy, but the latter was not. This result indicated that I85V and E124K substitutions did not affect the response to IFN-α monotherapy, because both types had failed to respond previously to IFN monotherapy. Therefore, we surmised that this amino acid substitution influenced the response to RBV anti-viral activity, which prompted us to examine the effect of RBV on viral replication. Several mechanisms of anti-viral activity of RBV have been proposed [Tam et al., 1999; Maag et al., 2001; Lau et al., 2002], but it is unclear why only the V85I single amino acid substitution induced replication better than the wild type. As shown previously [Kumagai et al., 2004], the 85th amino acid of HCV RdRP is distant from the active site of polymerase but is located near the RNA primer binding site, and this substitution may influence nucleotide misincorporation during polymerization. This 85th position is more important than the 124th position for replication of HCV-O.

This study is the first to examine whether NS5B polymorphism affects the replication efficiency and anti-HCV effect of RBV in an HCV RNA replicon system. It will be interesting to know whether these mutations in other genotypes (genotypes 2 and 3) replicate more efficiently and are more resistant than genotype 1b to RBV alone. Our data suggested that during clinical use of RBV, several mutations in the HCV genome might occur, such as in the isoleucine residue at the 85th position of HCV NS5B, which then affect viral replication and RBV resistance. This viral mutation may be one of the reasons for the failure in early viral clearance by IFN and RBV. There are, however, many factors that influence the success of IFN and RBV combination therapy. The resistance or sensitivity to IFN or peg-IFN, not to RBV, might also affect the early viral response, and many factors in both viral and host sides are known to affect IFN responsiveness, such as NS5A mutations [Enomoto et al., 1996], immunological status [Saito et al., 2000], or irf-1 gene promoter polymorphisms [Saito et al., 2002, 2005]. Together, these factors might determine the efficacy of anti-viral therapy in vivo, and the present in vitro data provides evidence partially supporting our clinical observations that NS5B polymorphisms are associated with early viral clearance during IFN and RBV therapy. However, it is unclear whether this single mutation occurs with peg-IFN plus RBV combination therapy and further studies are necessary. Nevertheless, our report is useful for modeling targets for antiviral compounds for the treatment of HCV.

## REFERENCES


Blight KJ, McKeating JA, Rice CM. 2002. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. J Virol 76:13001–13014.

Bouvier-Alias M, Patel K, Dahari H, Beaucourt S, Larderie P, Blatt L, Hezode C, Picchio G, Dhumeaux D, Neumann AU, McHutchison JG, Pawlotsky JM. 2002. Clinical utility of total HCV core antigen quantification: A new indirect marker of HCV replication. Hepatology 36:211–218.

Contreras AM, Hiasa Y, He W, Terella A, Schmidt EV, Chung RT. 2002. Viral RNA mutations are region specific and increased by ribavirin in a full-length hepatitis C virus replication system. J Virol 76: 8505–8517.

Crotty S, Maag D, Arnold JJ, Zhong W, Lau JY, Hong Z, Andino R, Cameron CE. 2000. The broad-spectrum antiviral ribonucleoside ribavirin is an RNA virus mutagen. Nat Med 6:1375–1379.

Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, Ogura Y, Izumi N, Marumo F, Sato C. 1996. Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. N Engl J Med 334:77–81.

Feld JJ, Hoofnagle JH. 2005. Mechanism of action of interferon and ribavirin in treatment of hepatitis C. Nature 436:967–972.

Ferenci P, Brunner H, Nachbaur K, Datz C, Gschwantler M, Hofer H, Stauber R, Hackl F, Jessner W, Rosenbeiger M, Munda-Steindl P, Hegenbarth K, Gangl A, Vogel W. 2001. Combination of interferon induction therapy and ribavirin in chronic hepatitis C. Hepatology 34:1006–1011.

Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales FL, Jr., Haussinger D, Diago M, Carosi G, Dhumeaux D, Craxi A, Lin A, Hoffman J, Yu J. 2002. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 347:975–982.

Gao B, Hong F, Radaeva S. 2004. Host factors and failure of interferon-alpha treatment in hepatitis C virus. Hepatology 39:880–890.

Ikeda M, Abe K, Dansako H, Nakamura T, Naka K, Kato N. 2005. 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 329:1350–1359.

Kumagai N, Takahashi N, Kinoshita M, Tsunematsu S, Tsuchimoto K, Saito H, Ishii H. 2004. Polymorphisms of NS5B protein relates to early clearance of hepatitis C virus by interferon plus ribavirin: A pilot study. J Viral Hepat 11:225–235.

Lanford RE, Guerra B, Lee H, Averett DR, Pfeiffer B, Chavez D, Notvall L, Bigger C. 2003. Antiviral effect and virus-host

interactions in response to alpha interferon, gamma interferon, poly(i)-poly(c), tumor necrosis factor alpha, and ribavirin in hepatitis C virus subgenomic replicons. J Virol 77:1092–1104.

Lau JY, Tam RC, Liang TJ, Hong Z. 2002. Mechanism of action of ribavirin in the combination treatment of chronic HCV infection. Hepatology 35:1002–1009.

Liang TJ, Rehermann B, Seeff LB, Hoofnagle JH. 2000. Pathogenesis, natural history, treatment, and prevention of hepatitis C. Ann Intern Med 132:296–305.

Lohmann V, Korner F, Dobierzewska A, Bartenschlager R. 2001. Mutations in hepatitis C virus RNAs conferring cell culture adaptation. J Virol 75:1437–1449.

Lukasiewicz E, Hellstrand K, Westin J, Ferrari C, Neumann AU, Pawlotsky JM, Schalm SW, Zeuzem S, Veldt BJ, Hansen BE, Verhey-Hart E, Lagging M. 2007. Predicting treatment outcome following 24 weeks peginterferon alpha-2a/ribavirin therapy in patients infected with HCV genotype 1: Utility of HCV-RNA at day 0, day 22, day 29, and week 6. Hepatology 45:258–259.

Maag D, Castro C, Hong Z, Cameron CE. 2001. Hepatitis C virus RNA-dependent RNA polymerase (NS5B) as a mediator of the antiviral activity of ribavirin. J Biol Chem 276:46094–46098.

Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M, Albrecht JK. 2001. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: A randomised trial. Lancet 358:958–965.

Naka K, Ikeda M, Abe K, Dansako H, Kato N. 2005. Mizoribine inhibits hepatitis C virus RNA replication: Effect of combination with interferon-α. Biochem Biophys Res Commun 330:871–879.

Pol S, Nalpas B, Bourliere M, Couzigou P, Tran A, Abergel A, Zarski JP, Berthelot P, Brechot C. 2000. Combination of ribavirin and interferon-alfa surpasses high doses of interferon-alfa alone in patients with genotype-1b-related chronic hepatitis C. Hepatology 31:1338–1344.

Poynard T, McHutchison J, Goodman Z, Ling MH, Albrecht J. 2000. Is an "a la carte" combination interferon alfa-2b plus ribavirin regimen possible for the first line treatment in patients with chronic hepatitis C? The ALGOVIRC Project Group. Hepatology 31:211–218.

Saito H, Ebinuma H, Satoh I, Miyaguchi S, Tada S, Iwabuchi N, Kumagai N, Tsuchimoto K, Morizane T, Ishii H. 2000. Immunological and virological predictors of outcome during interferon-alpha therapy of chronic hepatitis C. J Viral Hepat 7:64–74.

Saito H, Tada S, Wakabayashi K, Nakamoto N, Takahashi M, Nakamura M, Ebinuma H, Ishii H. 2002. The detection of IRF-1 promoter polymorphisms and their possible contribution to T helper 1 response in chronic hepatitis C. J Interferon Cytokine Res 22: 693–700.

Saito H, Tada S, Nakamoto N, Kitamura K, Horikawa H, Kurita S, Ebinuma H, Ishii H, Takahashi M, Tanaka S, Hibi T. 2005. Contribution of Irf-1 promoter polymorphisms to the Th1-type cell response and interferon-beta monotherapy for chronic hepatitis C. Hepatol Res 32:25–32.

Saito H, Tada S, Ebinuma H, Ishii H, Kashiwazaki K, Takahashi M, Tsukada N, Nishida J, Tanaka S, Shiozaki H, Hibi T. 2006. Role of erythrocytes as a reservoir for ribavirin and relationship with adverse reactions in the early phase of interferon combination therapy for chronic hepatitis C virus infections. J Clin Microbiol 44: 3562–3568.

Saracco G, Ciancio A, Olivero A, Smedile A, Roffi L, Croce G, Colletta C, Cariti G, Andreoni M, Biglino A, Calleri G, Maggi G, Tappero GF, Orsi PG, Terreni N, Macor A, Di Napoli A, Rinaldi E, Ciccone G, Rizzetto M. 2001. A randomized 4-arm multicenter study of interferon alfa-2b plus ribavirin in the treatment of patients with chronic hepatitis C not responding to interferon alone. Hepatology 34:133–138.

Sidwell RW, Huffman JH, Khare GP, Allen LB, Witkowski JT, Robins RK. 1972. Broad-spectrum antiviral activity of Virazole: 1-beta-D-ribofuranosyl-1,2,4-triazole-3-carboxamide. Science 177:705–706.

Takahashi M, Saito H, Higashimoto M, Atsukawa K, Ishii H. 2005. Benefit of hepatitis C virus core antigen assay in prediction of therapeutic response to interferon and ribavirin combination therapy. J Clin Microbiol 43:186–191.

Tam RC, Pai B, Bard J, Lim C, Averett DR, Phan UT, Milovanovic T. 1999. Ribavirin polarizes human T cell responses towards a Type 1 cytokine profile. J Hepatol 30:376–382.

Young KC, Lindsay KL, Lee KJ, Liu WC, He JW, Milstein SL, Lai MM. 2003. Identification of a ribavirin-resistant NS5B mutation of hepatitis C virus during ribavirin monotherapy. Hepatology 38: 869–878.

Zeuzem S, Lee JH, Franke A, Ruster B, Prummer O, Herrmann G, Roth WK. 1998. Quantification of the initial decline of serum hepatitis C virus RNA and response to interferon alfa. Hepatology 27:1149–1156.

Liver International ISSN 1478-3223

BASIC STUDIES

# Mitochondrial electron transport inhibition in full genomic hepatitis C virus replicon cells is restored by reducing viral replication

Mie Ando[1], Masaaki Korenaga[2], Keisuke Hino[1], Masanori Ikeda[3], Nobuyuki Kato[3], Sohji Nishina[2], Isao Hidaka[2] and Isao Sakaida[2]

1 Department of Basic Laboratory Sciences, Yamaguchi University Graduate School of Medicine, Yamaguchi, Japan
2 Department of Gastroenterology and Hepatology, Yamaguchi University Graduate School of Medicine, Yamaguchi, Japan
3 Department of Molecular Biology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan

**Keywords**
fluvastatin – interferon – oxidative stress – reactive oxygen species

**Correspondence**
Keisuke Hino, MD, PhD, Department of Hepatology and Pancreatology, Kawasaki Medical University, 577 Matsushima Kurashiki, Okayama 701-0192, Japan
Tel: +81 86 4621111
Fax: +81 86 4641196
e-mail: khino@med.kawasaki-m.ac.jp

Received 25 September 2007
Accepted 30 January 2008

DOI:10.1111/j.1478-3231.2008.01720.x

## Abstract

*Background/Aim:* Hepatitis C virus (HCV) core protein has been shown to inhibit mitochondrial electron transport and to increase reactive oxygen species (ROS) *in vitro* and *in vivo*. The aim of this study was to investigate whether inhibiting HCV replication could restore the mitochondrial redox state and electron transport activity. *Methods:* We measured ROS, mitochondrial reduced glutathione content, and mitochondrial complex I, II, III and IV activities and protein expression in full genomic HCV replicon cells and cured cells that had been prepared by eliminating HCV RNA from replicon cells by interferon (IFN)-α treatment. *Results:* Cured cells had significantly lower ROS production and greater mitochondrial glutathione content than replicon cells. Complete inhibition of HCV replication by IFN-α restored complex I and IV activities by 20–30% ($P < 0.01$) and complex I expression ($P < 0.05$). Treatment with fluvastatin, one of the 3-hydroxy-3-methylglutaryl co-enzyme A reductase inhibitors, which is known to have anti-HCV activity, partially inhibited core protein expression and restored complex I activity in full genomic HCV replicon cells to a lesser degree ($P < 0.05$). *Conclusions:* Our results show that the mitochondrial redox state and electron transport activity can be restored by reducing HCV replication.

Hepatitis C virus (HCV) causes acute and chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC) (1). Because current antiviral treatment can only eliminate the virus in about 50% of patients (2, 3), therapies to reduce disease progression in chronically infected individuals would be of great benefit. In this respect, it is still a matter of debate whether reduction of HCV replication, even if not eliminating HCV, is beneficial to the outcome of disease. Although the mechanisms of its pathogenesis are incompletely understood, there have been several lines of evidence suggesting that oxidative stress is present in chronic hepatitis C to a greater degree than in other inflammatory liver diseases and is closely related to disease progression (4, 5). We and others have shown that HCV core protein induces the production of reactive oxygen species (ROS) (6–8) and that mitochondrial electron transport inhibition by HCV is associated with ROS production (9). Therefore, whether reduction of HCV replication restores mitochondrial electron transport activity is of interest in exploring treatments to reduce disease progression in HCV-associated chronic liver disease.

Establishment of the HCV subgenomic replicon has made it possible to assess the antiviral activities of interferon (IFN) and other reagents *in vitro* (10). We also developed a genome-length HCV RNA replication reporter system (11) and found that different statins, which are 3-hydroxy-3-methylglutaryl co-enzyme A reductase inhibitors, have different anti-HCV profiles while using this reporter system (12). In the present study, we chose to use fluvastatin, which exhibited the strongest inhibition of HCV replication among the statins (12), to reduce HCV replication in full genomic HCV replicon cells without complete inhibition. The aim of this study was to examine whether mitochondrial electron transport activity was restored by reduction of HCV replication.



© 2008 The Authors. Journal compilation © 2008 Blackwell Munksgaard

## Materials and methods

### Cell cultures

Full genomic HCV replicon cells were described in detail by Ikeda *et al.* (11). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin, streptomycin and G418 (300 μg/ml; Calbiochem, Darmstadt, Germany) and passaged twice a week at a 5:1 split ratio. Cured cells were established by eliminating genome-length HCV RNA from replicon cells by IFN-α treatment (500 IU/ml for 2 weeks; Sigma-Aldrich, St Louis, MO, USA) without G418, as described (11). In some experiments, full genomic HCV replicon cells were incubated in the presence of 10 μmol/L fluvastatin (Novartis Pharmaceutical, Tokyo, Japan) for 96 h.

### Measurement of reactive oxygen species

The cellular ROS level was measured by oxidation of the cell-permeable, oxidation-sensitive fluorogenic precursor dihydrodichlorocarboxyfluorescein diacetate (DCFDA; Molecular Probes Inc., Eugene, OR, USA). Cells in six-well plates were treated with tertiary butyl hydroperoxide (t-BOOH) for 5 h or not, followed by a 30-min incubation with DCFDA (500 nmol/L final concentration) at 37 °C. Fluorescence was measured with a CytoFluorII fluorescence plate reader (PerSeptive Biosystems, Framingham, MA, USA) at an excitation wavelength of 486 nm and an emission wavelength of 530 nm as described (7).

Localization of ROS production on the subcellular level was observed with a Zeiss (Oberkochen, Germany) LSM5 Pascal inverted laser scanning confocal microscope. Cells were pre-incubated with 5 μmol of hydroxyphenyl florescein (HPF, Alexis Corporation, Lausen, Switzerland) (13) for 5 min at 37 °C. They were then imaged at 30-s intervals after treatment with 10 nmol/L t-BOOH. The green fluorescence of HPF (excitation, 488 nm; emission, 505–530 nm) was observed after excitation with an argon–krypton laser.

### Isolation of mitochondria

Mitochondrial pellets were obtained as described previously with some modification (7, 9). Briefly, harvested cells were centrifuged at 500*g* for 5 min. The pellets were homogenized with 25 strokes using a Dounce homogenizer (Wheaton Science Products, Millville, NJ, USA) and a tight-fitting pestle with isolation buffer [70 mM sucrose, 1 mM $KH_2PO_4$, 5 mM HEPES, 220 mM mannitol, 5 mM sodium succinate and 0.1% bovine serum albumin (BSA), pH 7.4]. The homogenate was centrifuged at 1330*g* for 5 min at 4 °C. The supernatant fraction was retained, whereas the pellet was washed with isolation buffer and centrifuged again. The combined supernatant fractions were centrifuged at 1000*g* for 15 min at 4 °C to obtain a crude mitochondrial pellet. Purified mitochondria were prepared by sucrose gradient (1.5 M sucrose and 1 M sucrose) centrifugation as described (14) with some modification. An aliquot was removed for determination of the protein concentration with the Bio-Rad protein DC assay kit (Bio-Rad, Hercules, CA, USA), using BSA as the standard.

### Measurement of reduced glutathione content

Crude mitochondrial samples (3–4 mg of mitochondrial protein) were sonicated using a Sonifier cell disruptor 200 (VWR Scientific, Danbury, CT, USA) for 15 s at power setting 3 in ice-cold 5% metaphosphoric acid and centrifuged at 3000*g* at 4 °C for 10 min. The concentration of reduced glutathione was measured by the thioester method using a GSH-400 kit (Oxis International Inc., Portland, OR, USA).

### Immunoblotting

Crude mitochondrial pellets were suspended in lysis buffer (T-PER Tissue Extraction Reagent; Pierce, Rockford, IL, USA) and centrifuged at 10 000*g* for 15 min at 4 °C. The supernatant (20 μg of protein) was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis on 16% gel. The proteins were electrophoretically blotted onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA), blocked overnight at 4 °C with 5% skim milk and 0.1% Tween 20 in Tris-buffered saline, and subsequently incubated for 1 h at room temperature with an anti-hepatitis C core protein antibody (1:1000, Affinity Bio Reagents, Golden, CO, USA), anti-OxPhos complex I antibody (1:1000), anti-OxPhos complex II antibody (1:2000), anti-OxPhos complex III antibody (1:2500) or anti-OxPhos complex IV antibody (1:1000, Molecular Probes Inc). The membranes were washed, incubated with appropriate secondary antibodies and detected with ECL™ Western blot detection reagents (Amersham Biosciences, Piscataway, NJ, USA). The degree of protein expression was expressed as the normalized quotient, which was derived by dividing the intensity of the blot density of each protein by that of β-actin protein.

### Measurement of complex I, II, III and IV activities

Submitochondrial particles were prepared from mitochondria by incubation for 3 min at 37 °C followed by sonication in a microcentrifuge tube immersed in ice water. Forty micrograms of submitochondrial


© 2008 The Authors. Journal compilation © 2008 Blackwell Munksgaard

particles was pelleted at 15 000*g* for 10 min. Enzyme activity assays were performed at 25 °C by a previously established method (15). Complex I [nicotinamide adenine dinucleotide (NADH)-decylubiquinone oxidoreductase] activity was measured as the initial (5 min) rate of decrease of $A_{340}$ using the acceptor 2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzoquinone (DB 80 μM) and 200 μM NADH as the donor in 10 mM Tris (pH 8.0) buffer containing 1 mg/ml BSA, 0.24 mM KCN and 0.4 μM antimycin A. Complex II (succinate decylubiquinone 2,6-dichlorophenolindophenol reductase) activity was measured at 600 nm using 80 μM DCPIP as the acceptor and 10 mM succinate as the donor in 10 mM $KH_2PO_4$ (pH 7.8), 1 mg/ml BSA, 2 mM EDTA, in the presence of 0.24 mM KCN, 4 μM rotenone, 0.2 mM ATP and 0.4 μM antimycin A. Complex III (ubiquinol cytochrome *c* reductase) activity was measured at 550 nm using 40 μM oxidized cytochrome *c* as the acceptor and 80 μM decylubiquinol as the donor in 10 mM $KH_2PO_4$ (pH 7.8), 1 mg/ml BSA, 2 mM EDTA, in the presence of 0.24 mM KCN, 4 μM rotenone and 0.2 mM ATP for 2 min. Complex IV (cytochrome *c* oxidase) activity was measured using a cytochrome *c* oxidase assay kit (Sigma-Aldrich), following the manufacturer's instructions.

### Statistical analysis

Quantitative values are expressed as mean ± standard deviation. Two groups were compared by the Student *t*-test. A $P$ value of $< 0.05$ was considered to be significant. Two groups among multiple groups were compared by the rank-based, Kruskal–Wallis analysis of variance test followed by Scheffe's test.

## Results

### Increased reactive oxygen species production and mitochondrial oxidant status in full genomic hepatitis C virus replicon cells

To assess the effect of HCV replication on ROS production, we used the ROS-sensitive fluorescent probe DCFDA. As compared with cured cells, HCV replication increased ROS 1.4-fold (Fig. 1A). Because HCV infection results in an inflammatory response and an increase in the basal oxidative stress, we next determined the effect of an exogenous oxidant, 500 nmol/L t-BOOH, on ROS production. This treatment had no effect on cured cells, but increased ROS production in full genomic HCV replicon cells to a level 2.5-fold greater than that of cured cells (Fig. 1A; $P < 0.01$). Cells were then imaged by confocal microscopy at 30-s intervals after exposure to HPF, which is more sensitive to ROS production than DCFDA. As shown in Figure 1B, treatment with t-BOOH significantly increased the oxidized fluorescent product as time passed in full genomic HCV replicon cells, but not in cured cells ($P < 0.0005$). Thus, a small volume of exogenous oxidant (10 nmol/L) that did not induce ROS production in cured cells significantly increased ROS production in full genomic HCV replicon cells.

We previously demonstrated, by confocal microscopy, that the mitochondria are the primary site of initial ROS production in cells expressing HCV core protein and cytochrome P450 2E1 (7). Because confocal microscopic images of the oxidized fluorescent product in replicon cells were almost the same as those in our previous study, we measured mitochondrial reduced glutathione content to assess mitochondrial antioxidant capacity. The level of mitochondrial reduced glutathione was significantly lower in full genomic HCV replicon cells than in cured cells (Fig. 1C; $P < 0.05$), suggesting that HCV replication was responsible for the mitochondrial oxidant status and sensitized to exogenous oxidative stress.

### Restoration of mitochondrial electron transport activity by complete inhibition of hepatitis C virus replication

Our previous study has demonstrated that core protein causes oxidation of the glutathione pool, increases ROS production and inhibits complex I activity (9). Because increased ROS production and mitochondrial oxidant status were found in full genomic HCV replicon cells as well, we next measured complex I, II, III and IV activities in submitochondrial particles to determine whether inhibiting HCV replication restored mitochondrial electron transport activity. Complete inhibition of HCV replication by IFN-α restored complex I and IV activities by 20–30% ($P < 0.01$) (Fig. 2). However, complex II and III activities were not changed after treatment with IFN-α in these cells (Fig. 2).

We further assessed the expression levels of complexes I, II, III and IV in full genomic HCV replicon cells and cured cells. As shown in Figure 3, immunoblotting revealed that complete inhibition of HCV replication by IFN-α restored the complex I expression as well ($P < 0.05$). Although the complex IV activity was restored by IFN-α, the expression of complex IV did not change after complete inhibition of HCV replication. Thus, it should be noted that both activity and expression of complex I were restored by completely inhibiting HCV replication in full genomic HCV


© 2008 The Authors. Journal compilation © 2008 Blackwell Munksgaard

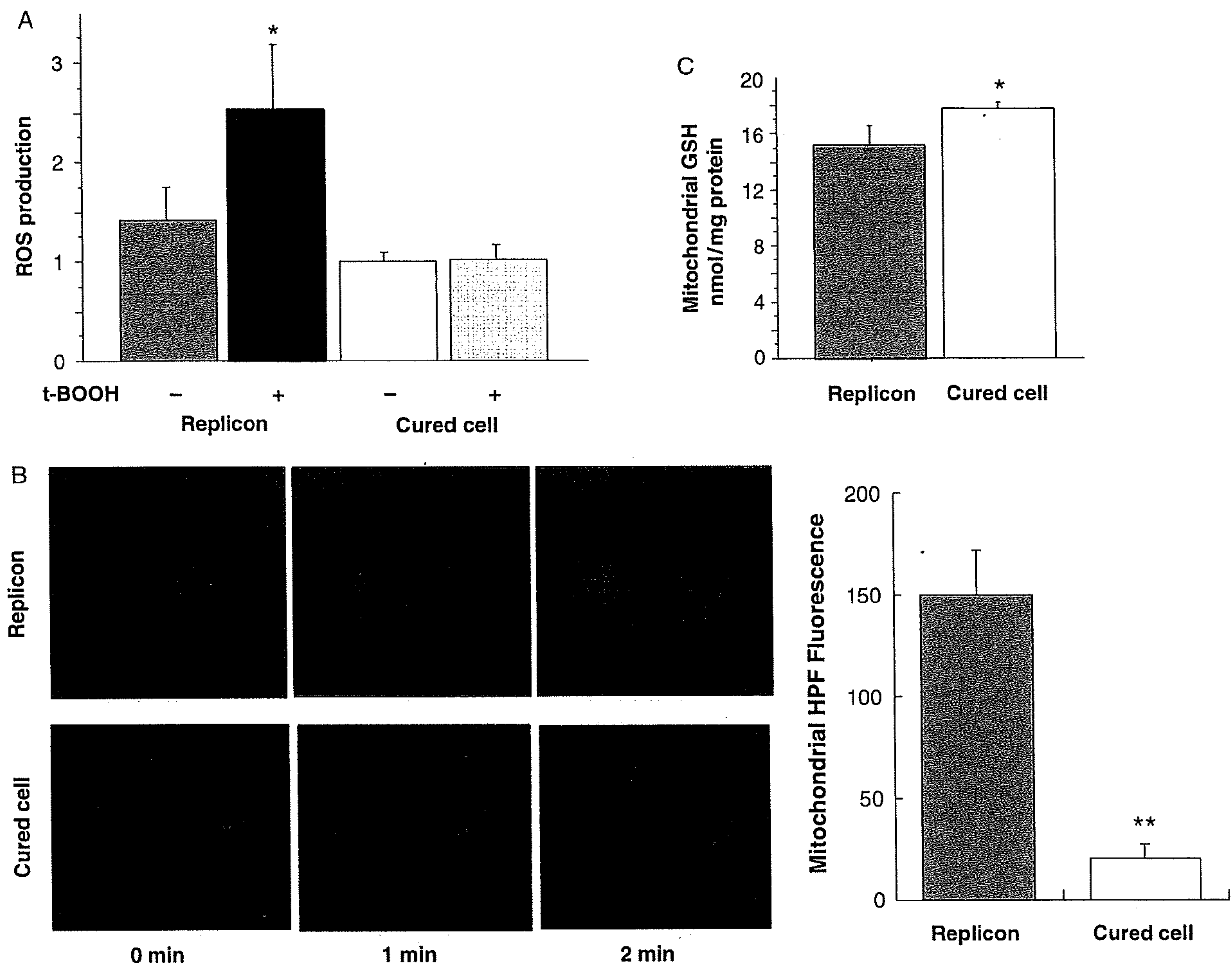


**Fig. 1.** Effects of HCV replication on ROS production and mitochondrial reduced glutathione level. (A) ROS production was measured by oxidation of DCFDA in HCV replicon cells and cured cells under control conditions or after 5-h incubation with t-BOOH (500 nmol/L). $^{*}P < 0.01$ compared with untreated cured cells. (B) Confocal images of ROS generation. HCV replicon cells and cured cells were pre-incubated with HPF, subsequently treated with t-BOOH (10 nmol/L) and imaged at 30-s intervals. The increase in HPF fluorescence intensity 2 min after treatment with t-BOOH was compared between HCV replicon cells and cured cells. $^{**}P < 0.0005$ compared with HCV replicon cells. (C) Reduced glutathione content was measured in crude mitochondrial fractions prepared from HCV replicon cells and cured cells. $^{*}P < 0.05$ compared with HCV replicon cells. DCFDA, dihydrodichlorocarboxyfluorescein diacetate; HCV, hepatitis C virus; HPF, hydroxyphenyl florescein; ROS, reactive oxygen species.

replicon cells, even though the significance of reduced complex IV activity remains elusive.

### Incompletely inhibited hepatitis C virus replication partially restores mitochondrial electron transport activity

Fluvastatin, a 3-hydroxy-3-methylglutaryl co-enzyme A reductase inhibitor, has been shown to have an inhibitory effect on HCV replication in the present full genomic HCV replicon cells (12). We used fluvastatin for partially inhibiting HCV replication in full genomic HCV replicon cells, because it has a lesser inhibitory effect on HCV replication than IFN-α (12). In fact, expression of core protein was present in mitochondria, but was significantly lowered by treatment with fluvastatin in full genomic HCV replicon cells ($P < 0.05$; Fig. 4A). Partial inhibition of HCV replication restored complex I activity by ~13% ($P < 0.05$; Fig. 4B). Although statins including fluvastatin are known to have an anti-oxidative effect (16, 17), treatment with fluvastatin did not improve complex I activity in cured cells (Fig. 4B), suggesting that this activity was restored by its inhibitory effect on HCV replication rather than its anti-oxidative property. However, partial inhibition of HCV replication did


© 2008 The Authors. Journal compilation © 2008 Blackwell Munksgaard