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Biochemical and Biophysical Research Communications 313 (2004) 42–47
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Specific inhibition of hepatitis C virus replication by cyclosporin A

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Received 7 November 2003

Abstract

The difficulty in eradicating hepatitis C virus (HCV) infection is attributable to the limited treatment options against the virus. Recently, cyclosporin A (CsA), a widely used immunosuppressive drug, has been reported to be effective against HCV infection [J. Gastroenterol. 38 (2003) 567], although little is understood about the mechanism of its action against HCV. In this study, we investigated the anti-viral effects of CsA using an HCV replicon system. Human hepatoma Huh7 cells were transfected with an HCV replicon expressing a chimeric gene encoding a luciferase reporter and neomycin phosphotransferase (Huh7/Rep-Feo). Treatment of the Huh7/Rep-Feo cells with CsA resulted in suppression of the replication of the HCV replicon in a dose-dependent manner, with an IC50 of ~0.5 μg/ml. There were no changes in the rate of cell growth or viability, suggesting that the effect of CsA against HCV is specific and not due to cytotoxicity. In contrast, FK506, another immunosuppressive drug, did not suppress HCV replication. CsA did not activate interferon-stimulated gene responses, suggesting that its action is independent of that of interferon. In conclusion, CsA inhibits HCV replication in vitro specifically at clinical concentrations. Further defining its mode of action against HCV replication potentially may be important for identifying novel molecular targets to treat HCV infection.

Keywords: Hepatitis C virus; Replicon; Cyclosporin A; FK506; ISRE; Cyclophilins

Hepatitis C virus (HCV), which infects 170 million people worldwide, is characterized by chronic liver inflammation and liver fibrogenesis, leading to end-stage liver failure and hepatocellular malignancy [1,2]. Attempts to control HCV infection have been unsatisfactory because of the limited treatment options against the virus. Present therapies against HCV infection are based on high dose administration of interferon (IFN)-α in combination with ribavirin, a synthetic guanosine analog [3,4]. However, success rates remain at around 30–40% of patients treated. Furthermore, treatment with IFN and ribavirin carries a significant risk of serious side effects. Thus, the development of new therapeutic agents is a high priority goal.

Recently, CsA, the most widely used immunosuppressive drug, was reported to be clinically effective against HCV infection [5]. A subsequent controlled trial showed that a combination of CsA with IFN is more effective than IFN monotherapy, especially in patients with a high viral load [6]. In T cells, which are the major cellular targets of CsA, CsA binds to soluble cytosolic proteins called cyclophilins, and the cyclophilin–CsA complexes block calcineurin, which inhibits stimulation of the NFAT-induced genes which are essential for the activation of T cells [7]. However, despite the clinical effectiveness of CsA, little is understood about its anti-viral mechanisms in patients with chronic hepatitis C. In particular, elucidation of the mechanism of the anti-viral effects of CsA may give new insights into the replication of HCV and elucidate potential targets for anti-HCV therapy.

In the present study, we investigated the effects of CsA on the intracellular replication of the HCV genome in vitro, using an HCV replicon system, reported

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0006-291X/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2003.11.080

recently in a cultured human hepatoma Huh7 cell line [8]. We demonstrated that CsA inhibits HCV replication in vitro substantially and specifically, and that the mechanism of action is independent of that of IFN.

Materials and methods

Drugs. CsA was purchased from Sigma (St. Louis, MO). FK506 was from Alexis Biochemicals (Lausen, Switzerland). Recombinant human IFNα-2b was obtained from Schering-Plough (Kenilworth, NJ).

Cell culture. A human hepatoma cell line, Huh7, was maintained in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal calf serum at 37 °C under 5% CO_2. Huh7 cells expressing the HCV replicon were cultured in medium containing 200 μg/ml G418 (Wako, Osaka, Japan).

HCV replicon constructs. An HCV subgenomic replicon plasmid, pHCVIbneo-delS, was derived from an infectious HCV clone, HCV-N, genotype Ib [9]. The replicon pHCVIbneo-delS was reconstructed by substituting the neomycin phosphotransferase gene with a fusion gene comprising the firefly luciferase and neomycin phosphotransferase, as described elsewhere (pRep-Feo) [10]. RNA was synthesized from pHCVIbneo-delS and pRep-Feo using T7-polymerase (Lumat LB9501; Promega) and transfected into Huh7 cells. After culture in the presence of G418, cell lines stably expressing the replicon were established (Huh7/Rep-Neo and Huh7/Rep-Feo, respectively).

Reporter constructs. A plasmid, pISRE-TA-Luc (Invitrogen, Carlsbad, CA), expressed luciferase reporter gene under control of the interferon stimulation response element (ISRE). pRL-CMV (Promega), which expresses *Renilla* luciferase gene under the control of the cytomegalovirus early promoter/enhancer, was used as a control for transfection efficiency of pISRE-TA-Luc. A plasmid, pECMV/IRES-Rluc, was used as a control to analyze translation efficiency mediated by an encephalomyocarditis virus internal ribosome entry site (ECMV-IRES) which mediates translation of HCV non-structure gene of replicon constructs, pHCVIbneo-delS and pRep-Feo. The pECMV/IRES-Rluc expressed mRNA consisted of ECMV-IRES and *Renilla* luciferase reporter gene under control of cytomegalovirus early promoter/enhancer.

Luciferase assays. Luciferase activities were quantified using a luminometer (Promega) and the Bright-Glo Luciferase Assay System (Promega). Assays were performed in triplicate and the results were expressed as means ± SD as percentages of the controls.

Northern blotting. Total cellular RNA was extracted from cells using ISOGEN (Wako). The RNA was separated by denaturing agarose–formaldehyde–gel electrophoresis and transferred to a Hybond-N^+ nylon membrane (Amersham–Pharmacia Biotech). The upper part of the membrane, which contained the HCV replicon RNA, was hybridized with a digoxigenin-labeled probe that was specific for the full-length replicon sequence, and the lower part of the membrane was hybridized with a probe specific for β-actin. The signals were detected in a chemiluminescence reaction using a Digoxigenin Luminescent Detection Kit (Roche, Mannheim, Germany) and visualized using a Fluoro-Imager (Roche).

Western blotting. Ten micrograms of total cell lysate was separated using NuPAGE 4–12% Bis–Tris gels (Invitrogen) and blotted onto an Immobilon polyvinylidene difluoride membrane (Roche). The membrane was incubated with a monoclonal anti-NS5A antibody (BioDesign, Saco, ME) and detection was carried out in a chemiluminescence reaction (BM Chemiluminescence Blotting Substrate; Roche).

MTS assays. To evaluate cytotoxicity, MTS (dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium) assays were performed using a CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) according to manufacturer's directions.

Statistical analyses. Statistical analyses were performed using Student's *t* test; *p* values less than 0.05 were considered as statistically significant.

Results

Suppression of hepatitis C virus replication by cyclosporin A (Figs. 1 and 2)

To assess the effects of CsA on the intracellular replication of the HCV genome, Huh7/Rep-Feo cells were cultured with various concentrations of CsA in the medium. The luciferase activities of the Huh7/Rep-Feo

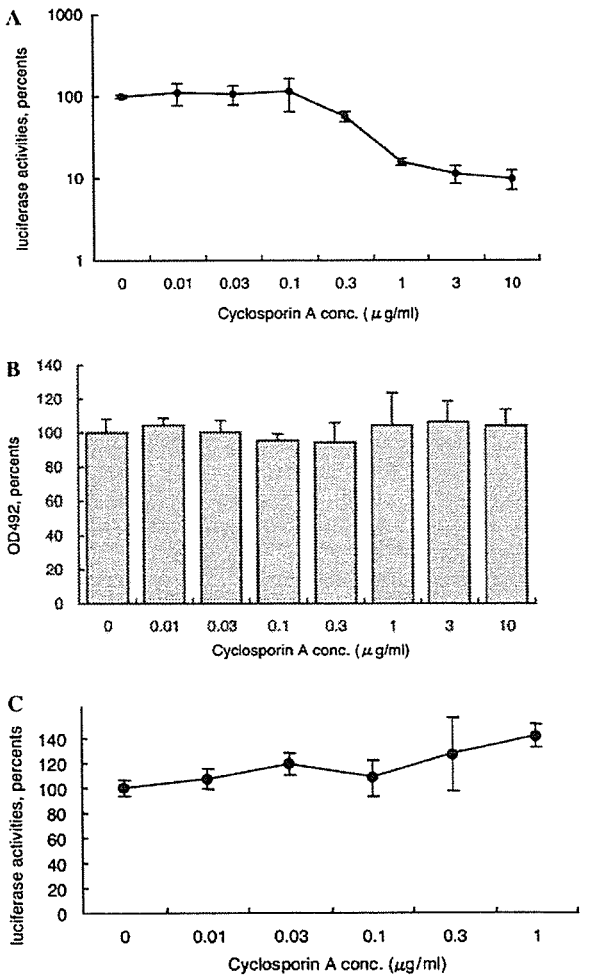

Fig. 1. Suppression of hepatitis C virus replication by cyclosporin A. (A) Huh7/Rep-Feo cells, which constitutively express a hepatitis C virus replicon, enable the quantification of replication levels through the measurement of luciferase activity. Relative log (dose)–response curves in the presence of various concentrations of CsA. Luciferase assays were performed in triplicate. Error bars indicate means ± 2SD. (B) MTS assay of Huh7/Rep-Feo cells cultured with the concentrations of CsA indicated. (C) A plasmid, pECMV/IRES-Rluc, was used to analyze the translation efficiency mediated by an encephalomyocarditis virus internal ribosome entry site (ECMV-IRES) which mediates translation of HCV non-structure gene of replicon constructs. The pECMV/IRES-Rluc was transfected into Huh7 cells. The transfected cells were cultured in the presence of indicated concentrations of CsA and luciferase activities were measured at 48 h of transfection. The assays were done in triplicate. Error bars indicate means ± 2SD.

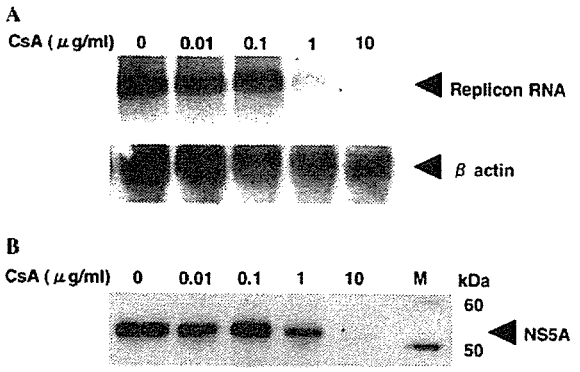

Fig. 2. Suppression of replicon RNA and NS 5A synthesis by CsA. (A) Northern hybridization. The replicon cells described by Seeger were cultured in the presence of the amounts of CsA indicated and harvested at 48 h after exposure. Ten micrograms of total cellular RNA was electrophoresed in each lane. The upper part of the membrane containing the HCV replicon RNA was hybridized with a digoxigenin-labeled probe specific for the replicon sequence, and the lower part was hybridized with a β-actin probe. Lane 1, Naïve replicon; lane 2, 0.01 μg/ml CsA; lane 3, 0.1 μg/ml CsA; lane 4, 1 μg/ml CsA; and lane 5, 10 μg/ml CsA. (B) Western blotting. Ten micrograms of total cellular protein was electrophoresed in each lane. Monoclonal anti-NS5A antibody and polyclonal anti-luciferase antibody were used as the primary antibodies. Lane 1, sample without CsA; lanes 2–5, cells cultured with CsA at concentrations of 0.01 (2), 0.1 (3), 1 (4), and 10 μg/ml (5). Lane 5, protein size marker, MagicMark (Invitrogen).

cells showed that the replication of the HCV replicon was suppressed by CsA in a dose-dependent manner. The luciferase activities were 56% and 16% of the control at CsA concentrations of 0.3 and 1 μg/ml, respectively (Fig. 1A). The MTS assay did not show any effect on cell viablilty or replication (Fig. 1B). Moreover, efficiency of the EMCV-IRES-mediated translation was not affected by CsA (Fig. 1C). These results suggest that the decrease in HCV replication is due to a specific suppressive effect of CsA on HCV replication, and not due to cytotoxicity of CsA or an artificial effect on the EMCV-IRES which direct translation of HCV non-structure protein of the replicon.

In Northern blot analysis (Fig. 2A), levels of the replicon RNA, which were detectable in CsA-negative control cells, decreased substantially following treatment with CsA at concentrations of 1 and 10 μg/ml. Densitometric analysis of the replicon RNA showed that the intracellular levels of the replicon RNA in Huh7/Rep-Feo correlated well with the luciferase activities (data not shown). Similarly, in Western blotting (Fig. 2B), the HCV non-structural protein, NS5A, translated from the HCV replicon, decreased by corresponding amounts in response to treatment with CsA.

Absence of an inhibitory effect of FK506 (Fig. 3)

FK506 (tacrolimus), another immunosuppressive agent, shares many mechanisms of action with CsA.

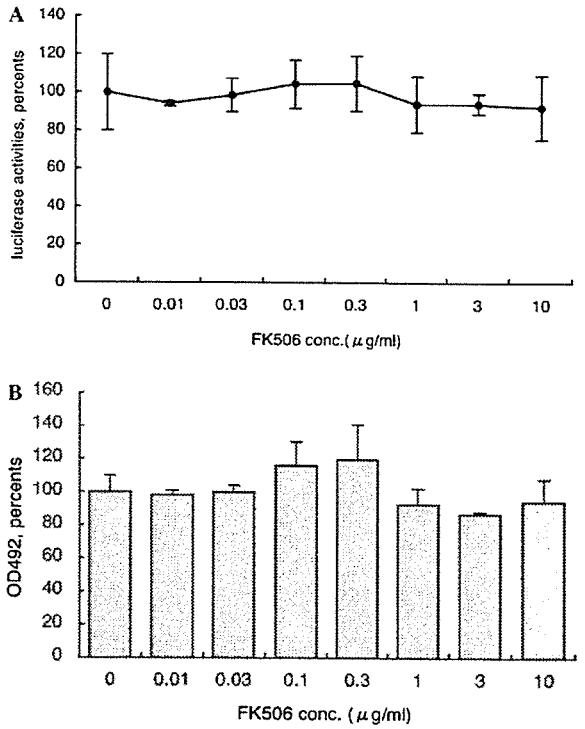

Fig. 3. Absence of an inhibitory effect of FK506. (A) To assess the effects of another immunosuppressive agent, FK506, Huh7/Rep-Feo cells were cultured with various concentrations of FK506 in the medium, and luciferase assays were performed after 48 h of culture. Luciferase assays were performed in triplicate. Error bars indicate means ± 2SD. (B) MTS assay of Huh7/Rep-Feo cells cultured with the concentrations of FK506 indicated.

FK506 is another inhibitor for the calcineurin/NFAT pathway and also blocks the activation of the JNK and p38 pathways in T cells [11]. To assess the potential effect of FK506, Huh7/Rep-Feo cells were cultured with FK506 at 0.01–10 μg/ml. The luciferase activities of the cells did not show a significant effect of FK506 on HCV replication at concentrations covering the range achievable clinically.

Cyclosporin A does not elicit an interferon-stimulated response (Fig. 4)

It has been reported that the HCV replicon is highly sensitive to IFN [12]. To determine whether the action of CsA on HCV subgenomic replication involves activation of IFN-stimulated gene responses, the ISRE-luciferase plasmids were transfected into Huh7/Rep-Feo cells and cultured in the presence of CsA and FK506 at concentrations of 0, 0.1, 1, and 10 μg/ml. As a positive control for the activation of ISRE reporter activity, the ISRE-luciferase-transfected cells were cultured with IFNα-2b at concentrations of 0, 0.1, 1, and 10 U/ml. The luciferase activities at 48 h after transfection showed that there were no significant effects of CsA and FK506 on

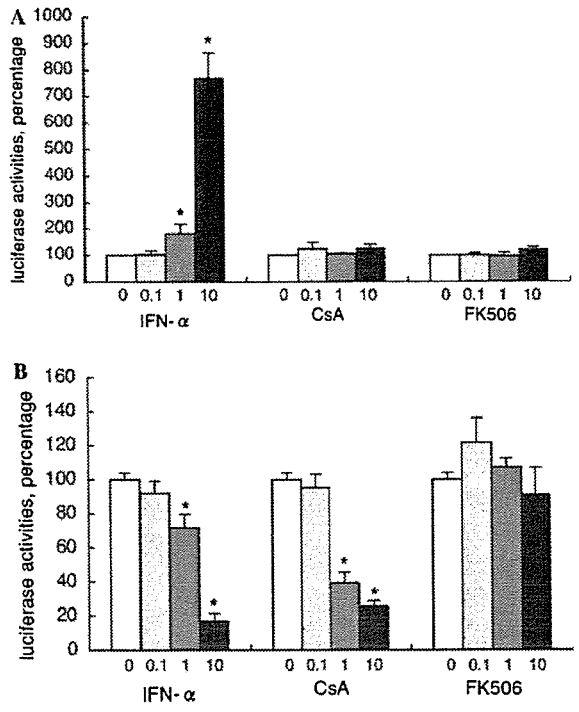

Fig. 4. Cyclosporin A does not elicit an interferon response. (A) ISRE-responsive luciferase reporter plasmids were transfected into cells containing replicon in the presence of the indicated concentrations of IFN (U/ml), CsA, and FK506 (μg/ml) in the culture medium. Luciferase assays were carried out 48 h after transfection. Values are presented as percentages of drug-negative controls. Luciferase assays were performed in triplicate. Error bars indicate means ± 2SD. *p value <0.05. (B) Huh7/Rep-Feo cells were cultured with the indicated concentrations of IFN (U/ml), CsA, and FK506 (μg/ml). Luciferase activities of the cell lysates were carried out after 24 h of exposure. Luciferase assays were performed in triplicate. Error bars indicate means ± 2SD.

ISRE-promoter activities, while IFNα-2b had significant effects (Fig. 4A). As reported above, the luciferase activities of the Huh7/Rep-Feo cells showed that the replication of the HCV replicon was suppressed by IFNα-2b, as well as CsA, in a dose-dependent manner, but not by FK506 in this condition (Fig. 4B). These results suggest that the action of CsA on the intracellular replication of HCV replicon is independent of the IFN pathway.

Discussion

Our present results demonstrate that CsA inhibits the intracellular replication of an HCV subgenomic replicon at clinically achievable drug concentrations. The Northern and Western blot analyses revealed that both RNA synthesis and its translation were reduced by CsA in a dose-dependent manner. Treatment of Huh7 cells with CsA did not activate IFN-stimulated gene responses, suggesting that the mechanisms of action of CsA are independent of those of IFN. In addition, FK506, another drug used frequently and which shares an immunosuppressive mechanism with CsA, did not show any inhibitory effect on HCV replication, suggesting that the anti-HCV effect of CsA is not associated with immunosuppressive activity.

CsA and FK506, although structurally dissimilar, have a similar mode of immunosuppressive action in preventing induction of inflammatory gene expression in activated T cells. Both CsA and FK506 bind specifically to a family of soluble cytosolic proteins called immunophilins [7]. CsA is bound by cyclophilins and FK506 is bound by the FK506 binding proteins (FKBPs). The cyclophilin–CsA and FKBPs–FK506 complexes inhibit the phosphatase activity of calcineurin, that mediates phosphorylation, and nuclear translocation of the transcription factor, NFAT, critical in the expression of cytokines and their receptors, respectively [7,13,14]. In our present study, contrary to the effect of CsA on HCV replication, FK506, which has an immunosuppressive activity 100-fold greater than that of CsA [15,16], did not show an inhibitory effect on HCV replication. The findings demonstrate that the anti-viral action of CsA against HCV is not through suppression of NFAT-regulated gene responses but through distinct mechanisms that are not shared with FK506.

Another function of cyclophilins and FKBPs, which CsA and FK506, respectively, are able to block, is a peptidyl–prolyl *cis–trans* isomerase (PPIase) activity. PPIase catalyzes the *cis–trans* conversion of imide peptide preceding prolines [17], and acts as a molecular chaperone, accelerating the slow steps of correct post-translational folding of some proteins [18]. Cyclophilins are present in every compartment of the cell, including the cytoplasm, endoplasmic reticulum (ER), and nucleus [19]. The maturation steps of HCV proteins include processing of the polyprotein by an ER-membrane-bound signal peptidase and by two viral serine proteases [20]. Recent studies have demonstrated the localization of viral non-structural proteins in the ER forming microscopic intracellular structures, called "membranous webs," which is characterized by convoluted ER membranes [21]. Moreover, folding and assembly of HCV structural and non-structural proteins require interaction with ER chaperone proteins such as calreticulin, BiP, and HSP90 [22,23]. Collectively, it is speculated that certain chaperone activities, such as those of cyclophilins, may be crucial for the processing and maturation of the viral proteins and for viral replication. Thus, one most likely mechanism of action against HCV is blocking of the PPIase activities of cyclophilins. The striking difference between the significant effects observed with CsA and the lack of effect of FK506 may be explained by the different enzymatic properties of cyclophilins and FKBPs [24]. Cyclophilins are non-specific PPIases and are able to isomerize all X-Pro bonds.

On the other hand, FKBPs catalyze the isomerization of a limited set of X-Pro bonds. Therefore, inhibition of FKBPs can be overridden by the action of cyclophilins, whereas inhibition of cyclophilins cannot be substituted by the limited substrate specificity of FKBPs [25].

In addition to the anti-viral activity of CsA against HCV, confirmed by the present results, CsA has been reported to show anti-viral effects against HIV-1 through blocking the activities of cyclophilin A [19]. Cyclophilin A binds to the viral *gag* protein with high affinity, and CsA competes with the *gag* protein for the same binding site on cyclophilin A [26]. Moreover, cyclophilin A is packaged into HIV-1 virions and catalyzes *cis–trans* isomerization of the viral capsid protein as a molecular chaperone [27]. It has been shown that the anti-HIV action of CsA does correlate not with the immunosuppressive potential of the compounds but with their capacity to bind to cyclophilins [28]. These reports demonstrate that the anti-viral action of CsA against HIV-1 is through inhibition of the PPIase activities of cellular cyclophilins, as against HCV suggested in this study.

End-stage HCV liver cirrhosis is a major indication for liver transplantation, accounting for approximately 50% of cases in the US and in Europe [29]. However, viral recurrence occurs in all recipients and HCV-graft hepatitis develops in one-third [30]. Decompensated graft cirrhosis following re-infection with HCV is the main cause of death post-transplantation [31]. Long-term immunosuppression is essential for patients who have undergone transplantation. The two most frequently used drugs are CsA and FK506 and usage of FK506 has increased from 0% before 1996 to nearly 80% after 1999 [31], because the early safety and efficacy of an FK506 regimen after liver transplantation has been shown in two multicenter trials [32,33]. More recently, it has been reported that FK506-based immunosuppression is preferable to cyclosporin A during the first year following liver transplantation [34]. As for transplantation for HCV cirrhosis, however, recent studies from two institutes in Spain and the USA report that disease progression after transplantation has accelerated in recent years [29,31], although the reasons for the worsening outcome are under question. The anti-viral activity of CsA against HCV, demonstrated in this study, should be taken into account when selecting the immunosuppressive regimen for the optimum outcome of HCV-infected recipients.

The expanding applications of CsA, however, may cause substantial problems. Especially, an undesired immunosuppression that may lead to an immunocompromised status and may interfere with the effects of anti-infectious drugs such as IFN. Some cyclosporine analogs that fail to block T cell activation are still able to inhibit the PPIase activity [16,35]. Some of these non-immunosuppressive cyclosporine analogs were, in fact, equal or even superior in anti-HIV activity to immunosuppressive types [36]. Therefore, one solution to overcome the problem due to immunosuppression would be to consider the use of such cyclosporine analogs.

In conclusion, CsA inhibits HCV replication in vitro substantially and specifically. Considering the limited therapy options against HCV infection and the unsatisfactory therapy outcome, with half of the patients unable to eradicate the virus, CsA potentially becomes available as an effective treatment against HCV infection. Further defining the mechanism of action of CsA against HCV replication potentially may be important for identifying novel molecular targets to terminate HCV infection.

Acknowledgments

We thank Dr. Christoph Seeger of Fox Chase Cancer Center, Philadelphia, PA, for providing the replicon plasmid, pHCV1bneodelS. We are indebted to Dr. Yoji Ikawa for helpful comments and discussions. This study was supported by grants from the Japan Society for the Promotion of Science.

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GASTROENTEROLOGY 2005;129:1031–1041

Suppression of Hepatitis C Virus Replication by Cyclosporin A Is Mediated by Blockade of Cyclophilins

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Background & Aims: Cyclosporin A specifically suppresses hepatitis C virus (HCV) replication in vitro at clinically achievable concentrations. In this study, we investigated the mechanisms of action of cyclosporin A against HCV replication. *Methods:* The in vitro effects of cyclosporin A on HCV replication were analyzed using an HCV replicon system that expresses chimeric luciferase reporter protein. *Results:* The significant effects of cyclosporin A on expression of an HCV replicon and the absence of such effects of FK506, which shares mechanisms of action with cyclosporin A, suggested the involvement of intracellular ligands of cyclosporin A, the cyclophilins. Transient and stable knockdown of the expression of cytoplasmic cyclophilins A, B, and C by short hairpin RNA–expressing vectors suppressed HCV replication significantly. A cyclosporin analogue, cyclosporin D, which lacks immunosuppressive activity but exhibits cyclophilin binding, induced a similar suppression of HCV replication. Furthermore, cyclosporin A treatment of Huh7 cells induced an unfolded protein response exemplified by expression of cellular BiP/GRP78. Treatment of cells with thapsigargin and mercaptoethanol, which induce the unfolded protein responses, suppressed HCV replication, suggesting that the cyclosporin-induced unfolded protein responses might contribute to the suppression of HCV protein processing and replication. *Conclusions:* The anti-HCV activity of cyclosporin A is mediated through a specific blockade of cyclophilins, and these molecules may constitute novel targets for anti-HCV therapeutics.

Hepatitis C virus (HCV) infection, which affects 170 million people worldwide, is characterized by chronic liver inflammation and fibrogenesis, leading to end-stage liver failure and hepatocellular malignancy.[1] The difficulty in eradicating HCV is attributable partly to limited treatment options. Therapies with interferon alfa, with or without ribavirin in combination, are the only approved regimens that have shown efficacy.[2–4] The success rates of treatment, however, are at best 50%, with the most effective regimen being pegylated interferon alfa and ribavirin.[5] Furthermore, these therapies carry a substantial risk of serious side effects and in quite a considerable proportion of patients require premature discontinuation because of side effects.[6] Given this situation, the development of safe and effective therapies against HCV is our high-priority goal.

Cyclosporin A (CsA), a neutral cyclic undecapeptide that was isolated from the fungus *Hypocladium inflatum gams*, has been used widely for the treatment of allograft rejection, graft-versus-host disease, and various inflammatory diseases.[7,8] We and other researchers have reported that CsA substantially and specifically inhibits intracellular HCV replication in vitro.[9,10] Using our chimeric reporter HCV replicon system,[11] the 50% inhibitory concentration of CsA was found to be ~0.5 μg/mL, which is within clinically achievable concentrations. FK506 and rapamycin, which share pharmacologic mechanisms that suppress T-cell activation, did not show any inhibitory effect on HCV replication, suggesting that the anti-HCV effect of CsA is distinct from its immunosuppressive activity.

In this study, we have investigated further the mechanisms of action of CsA against HCV replication. Here, we show that the antiviral action of CsA is mediated by blockade of actions of cellular CsA-binding proteins, the cyclophilins.

Abbreviations used in this paper: BSD, blasticidin S; CsA, cyclosporin A; CsD, cyclosporin D; CypA, cyclophilin A; CypB, cyclophilin B; CypC, cyclophilin C; ER, endoplasmic reticulum; Fluc, firefly luciferase; His, polyhistidine; IRES, internal ribosome entry site; ISRE, interferon stimulation response element; MTS, dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium; NFAT, nuclear factor of activated T cells; PPIase, peptidyl prolyl *cis-trans* isomerase; Rluc, *renilla* luciferase; shRNA, short hairpin RNA.

0016-5085/05/$30.00
doi:10.1053/j.gastro.2005.06.031

Materials and Methods

Drugs and Chemicals

CsA was purchased from Sigma Chemical Co (St Louis, MO). FK506 was from Alexis Biochemicals (Lausen, Switzerland). Recombinant human interferon alfa-2b was obtained from Schering-Plough (Kenilworth, NJ). Cyclosporin D (CsD) is a cyclosporin analogue that has valine at position 2 of CsA instead of L-α-aminobutyric acid. CsD was provided by Novartis Pharma Inc (Tokyo, Japan).

Cell Culture

Huh7 and 293 cells were maintained in Dulbecco's modified Eagle medium (Sigma Chemical Co) supplemented with 10% fetal calf serum at 37°C under 5% CO_2. Huh7 cells expressing HCV replicon were cultured in medium containing 500 μg/mL G418 (Wako, Osaka, Japan).

HCV Replicons

An HCV subgenomic replicon plasmid, pRep-Feo (Figure 1*A*), was derived from pRep-Neo (originally pHCVIb-neo-delS[12]). The pRep-Feo expresses a fusion gene comprising firefly luciferase (Fluc) and neomycin phosphotransferase, as described elsewhere.[11,13] Another replicon plasmid, pRep-BSD, expresses the blasticidin S (BSD) resistance gene. Replicon RNA was synthesized in vitro by T7-RNA polymerase (Promega, Madison, WI) and transfected into Huh7 cells by electroporation.[11] After culture in the presence of G418 (Wako), cell lines stably expressing the replicons were established (Huh7/Rep-Neo and Huh7/Rep-Feo, respectively).

Retrovirus Vectors Expressing Short Hairpin RNA

Oligodeoxyribonucleotides encoding short hairpin RNA (shRNA) sequences were synthesized and cloned just downstream of human U6 promoter in the plasmid pUC19, and the U6-shRNA cassette was subcloned into a retrovirus plasmid vector, pLNCX2 (Clontech, Palo Alto, CA). Sequences of the shRNAs were as follows: cyclophilin A (CypA) shRNA#3, 5′- GCA ATG TCG AAG AAC ACG GTG GGG TTG ACG GAG CTC GGT CAG CCT CAT CGT GTT CTT CGG CAT TGC TTT TTT -3′; CypA shRNA#441, 5′- GTG ATC TTC TTG CTG GTC TTG CCA TTC CTG GAG CTC GAG GAG TGG CAG GAT CAG CAG GAA GAT CAC TTT TTT -3′; cyclophilin B (CypB) shRNA#294, 5′- GTG AAG TCT CCG CCC TGG ATC ATG AAG TCG GAG CTC GGG CTT CAT GAT CCG GGG CGG AGG CTT CAT TTT TTT -3′; CypB shRNA#467, 5′- GCT TGC CAT CTA GCC AGG CTG TCT TGA CTG GAG CTC GAG TCG AGG CAG CCT GGT TAG ATG GCG AGC TTT TTT -3′; cyclophilin C (CypC) shRNA#0, 5′- GGT AGC AGC AGC CGA GGA CCC GGG CCC ATG GAG CTC GAT GGG CCT GGG TCT TCG GCT GCT GCT GCT TTT TTT -3′; and CypC shRNA#291, 5′- GTG CCA TCT CCA GTG GTG ATG TCA CCT CCG GAG CTC GGG AGG TGG CAT CAT CAT TGG AGG TGG CAC TTT TTT -3′.

Six negative control shRNA vectors were used that had reverse sequences of the respective targets: CypA shRNA#3 rev, CypA shRNA#441 rev, CypB shRNA#294 rev, CypBi#467 rev, CypCi#0 rev, and CypCi#291 rev. Another negative control, shRNA control, directed toward an unrelated target, the Machado–Joseph disease gene. A positive control shRNA vector, HCV shRNA, directed toward the 5′-untranslated region of HCV RNA.[13] The retrovirus plasmids were transfected into a packaging cell line, Retro Pack PT67 (Clontech), and the culture supernatant was applied to Huh7 cells with 4 μg/mL polybrene (Sigma Chemical Co). Huh7 cell lines stably expressing shRNA were established by culture in the presence of 300 μg/mL G418.

Reporter and Expression Plasmids

A plasmid, pISRE-TA-Luc (Invitrogen, Carlsbad, CA), expressed the Fluc gene under the control of the interferon stimulation response element (ISRE).[14] A nuclear factor of activated T cells (NFAT) reporter plasmid, pNFAT-Luc, was purchased from Stratagene (La Jolla, CA). A plasmid, pRL-CMV (Promega), which expresses the *renilla* luciferase (Rluc) gene under the control of the cytomegalovirus early promoter/enhancer, was used to normalize transfection efficiency. A plasmid, pCIneo-Rluc-IRES-Fluc, was constructed to analyze HCV internal ribosome entry site (IRES)-mediated translation efficiency (Figure 1*B*). The plasmid expressed a bicistronic RNA, in which Rluc was translated in a cap-dependent manner and Fluc was translated by HCV IRES-mediated initiation. A plasmid, pcDNA-NS3-5, expressed the HCV nonstructural gene spanning from NS3 to 5 of the HC-J4 clone (Figure 1*C*).[15]

Cyclophilin-Expressing Plasmid Vectors

The coding sequences of human cytoplasmic CypA, CypB, and CypC were amplified by reverse-transcription polymerase chain reaction using the following polymerase chain reaction primers: CypA-S, 5′- CAC CAT GGT CAA CCC CAC CGT GTT CTT CGA -3′; CypA-AS, 5′- TTC GAG TTG TCC ACA GTC AGC AAT GGT GAT -3′; CypB-S, 5′- CAC CAT GAA GGT GCT CCT TGC CGC CGC CCT -3′; CypB-AS, 5′- CTC CTT GGC GAT GGC AAA GGG CTT CTC CAC -3′; CypC-S, 5′- CAC CAT GGG CCC GGG TCC TCG GCT GCT GCT -3′; CypC-AS, 5′- CCA ATC AGC GAT CTC AAC CAC AAA AGG CGT -3′. The amplicons were cloned into pcDNA 3.1 using the Directional TOPO Expression Kit (Invitrogen) to make pcDNA-CypA, -CypB, and -CypC, respectively. The expressed proteins contained a C-terminal polyhistidine (His) tag, which allowed their detection by anti-His antibodies (Invitrogen).

Transfection of Plasmids

Transfection of plasmids was performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.

Luciferase Assays

Luciferase activities were measured using a luminometer (Lumat LB9501; Promega) using the Bright-Glo Luciferase Assay System (Promega) or the Dual-Luciferase Reporter Assay System (Promega). Assays were performed in triplicate.

Northern Blotting

Total cellular RNA was extracted from cells using Isogen (Wako). The RNA was separated by denaturing agarose-formaldehyde gel electrophoresis and transferred to a Hybond-N+ nylon membrane (Amersham Biosciences Corp, Piscataway, NJ). The upper part of the membrane, which contained the HCV replicon RNA, was hybridized with a digoxigenin-labeled probe that was specific for the full-length replicon sequence, and the lower part of the membrane was hybridized with a probe specific for β-actin. The signals were detected in a chemiluminescence reaction using the Digoxigenin Luminescent Detection Kit (Roche, Mannheim, Germany) and were visualized using a Fluoro-Imager (Roche).

Western Blotting

Ten micrograms of total cell lysate was separated using NuPAGE 4%–12% Bis-Tris gels (Invitrogen) and blotted onto an Immobilon polyvinylidene difluoride membrane (Roche). The membrane was incubated with antibodies against NS5A (BioDesign, Saco, ME), anti-His (C Term; Invitrogen), β-actin (Sigma Chemical Co), and BiP/GRP78 (BD Biosciences, Franklin Lakes, NJ) and then incubated with peroxidase-conjugated secondary antibodies. Immunoreactions were performed using a BM Chemiluminescence Blotting Substrate (Roche) and visualized by Lumi-Imager F1 (Roche) according to the manufacturer's protocol.

Stable Colony Formation Assays

Cells were transfected with a replicon, Rep-BSD, and cultured in the presence of 150 μg/mL BSD (Invitrogen) in the medium. BSD-resistant cell colonies were obtained after ~2 weeks in culture. The colonies were stained with neutral red solution (Invitrogen) and counted.

Dimethylthiazol Carboxymethoxyphenyl Sulfophenyl Tetrazolium Assays

To evaluate cell viability, dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium (MTS) assays were per-

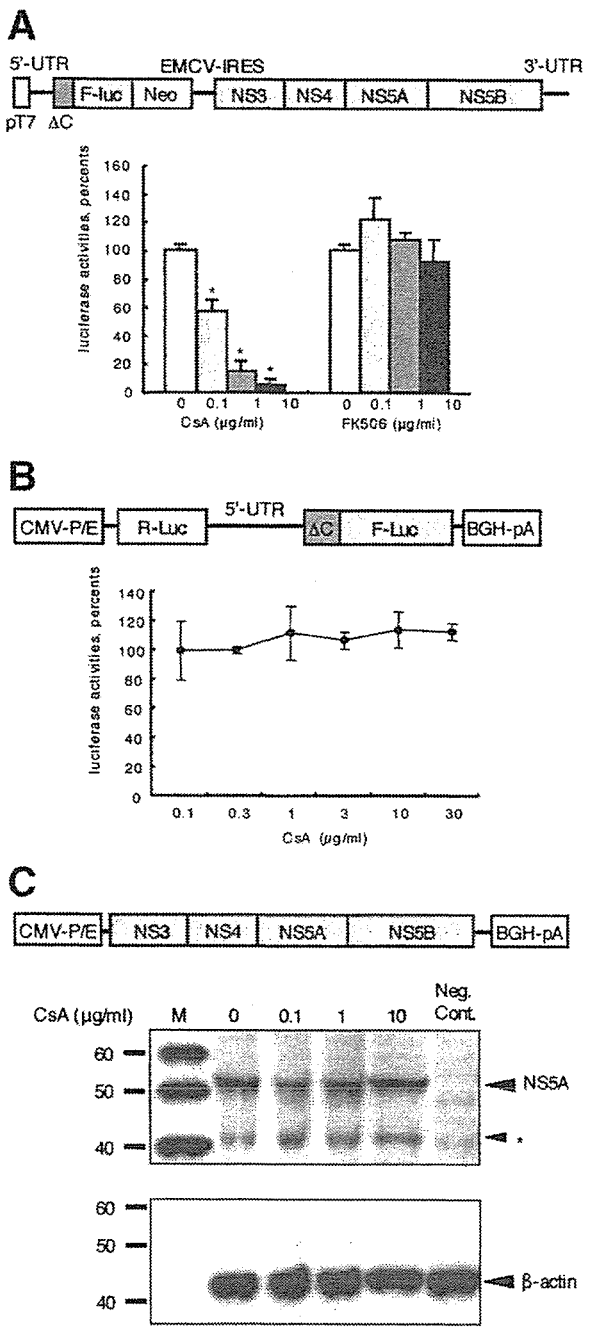


Figure 1. CsA does not influence HCV IRES-mediated translation or the processing of HCV nonstructural proteins. (*A*) Huh7/Rep-Feo cells that constitutively express the HCV Feo replicon (*top*) were cultured in the presence of CsA and FK506 at concentrations of 0, 0.1, 1, and 10 μg/mL. The internal luciferase activities were measured after 48 hours of culture. Assays were performed in triplicate. *Error bars* indicate mean ± 2 SD. **P* values of less than .05. (*B*) A bicistronic reporter gene plasmid, pCIneo-Rluc-IRES-Fluc (*top*), was stably transfected into Huh7 cells (Huh7/neo-Rluc IRES-Fluc). The cells were cultured with CsA at the concentrations indicated, and dual luciferase activities were measured after 48 hours of treatment. Values are displayed as ratios of Fluc to Rluc. *Error bars* indicate mean ± 2 SD. (*C*) Western blotting. An expression plasmid, pcDNA-NS3-5 (*top*), which expressed HCV nonstructural genes NS3–NS5, was used. The plasmid was transfected into 293 cells. At 24 hours after transfection, the cells were treated with the amounts of CsA indicated (0, 0.1, 1, and 10 μg/mL). The cells were harvested after 48 hours of treatment. The cell lysate was separated on NuPAGE 4%–12% Bis-Tris gels, transferred onto a polyvinylidene difluoride membrane, and incubated with a monoclonal anti-NS5A antibody or an anti–β-actin antibody. *Lane 1*, protein size markers. The pcDNA-NS3-5 was transfected into 293 cells and cultured in the absence (*lane 2*) or the presence of CsA at 0.1, 1 and 10 μg/mL, respectively (*lanes 3–5*). Neg. Cont., untransfected 293 cells. The *asterisk* indicates partially cleaved NS5A protein.

formed using a CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer's directions.

Statistical Analyses

Statistical analyses were performed using Student *t* test; *P* values of less than .05 was considered statistically significant.

Results

CsA Does Not Suppress Translation of HCV RNA or Posttranslational Processing of the Viral Protein

Treatment of Huh7/Rep-Feo cells with CsA at concentrations of 0, 0.1, 1, and 10 μg/mL resulted in a dose-dependent decrease in internal luciferase activities as reported (Figure 1*A*). The luciferase activities were 57% and 15% of the CsA-negative control at concentrations of 0.1 and 1 μg/mL, respectively. FK506, in contrast, did not suppress expression of the replicon at concentrations 10–100 times above the clinical range of 5–15 μg/mL.[16] We investigated which stage of the HCV replication cycle is affected by CsA. To determine whether CsA suppresses HCV IRES-dependent translation, we used an Huh7 cell line that had been stably transfected with pCIneo-Rluc IRES-Fluc (Huh7/neo-Rluc IRES-Fluc; Figure 1*B*). Treatment of these cells with CsA resulted in no significant change of the internal luciferase activities at concentrations of CsA that suppressed expression of the HCV replicon. We next determined whether CsA suppresses posttranslational self-cleavage of the viral nonstructural polyprotein. A plasmid, pcDNA-NS3-5, was transfected into 293 cells and cultured in the presence of CsA. Western blotting of the cell lysate showed that the expression levels of NS5A and other NS proteins did not change significantly (Figure 1*C*). These results suggested that the effect of CsA on HCV replication does not involve suppression of IRES-mediated viral protein synthesis or a blockade of posttranslational cleavage of the nonstructural proteins.

CsA Does Not Activate Interferon-Stimulated Genes or NFAT-Induced Genes in Huh7 Cells

To determine whether the action of CsA and FK506 involves activation of interferon-stimulated gene responses in the host Huh7 cells, the ISRE-luciferase reporter plasmid, pISRE-TA-Luc, was transfected into Huh7/Rep-Neo cells and cultured in the presence of CsA and FK506 at concentrations of 0, 0.1, 1, and 10 μg/mL. In contrast to interferon, which elevated ISRE-promoter activities significantly, CsA and FK506 showed no effects on the luciferase activities at 48 hours after transfection (Figure 2*A*). These results suggest that the action of CsA on the intracellular replication of the HCV rep-

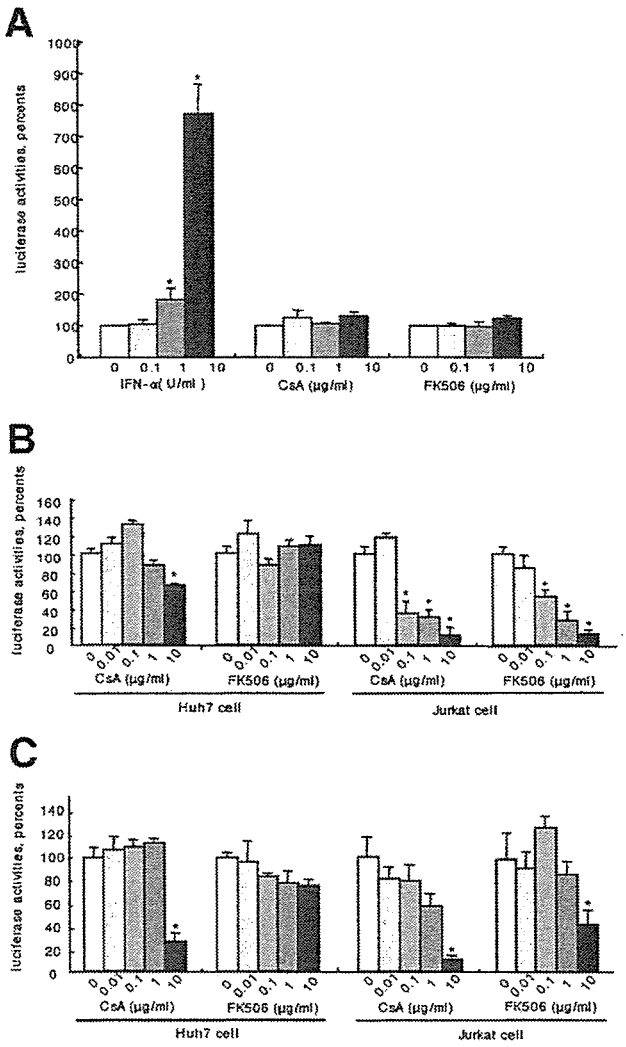


Figure 2. CsA does not affect interferon-stimulated responses and NFAT activities. (*A*) The ISRE-luciferase plasmids were transfected into Huh7/Rep-Neo cells and cultured in the presence of CsA and FK506 at concentrations of 0, 0.1, 1, and 10 μg/mL. As a positive control, cells were treated with interferon alfa at concentrations of 0, 0.1, 1, and 10 U/mL. Luciferase activities were measured at 48 hours after transfection. **P* values of less than .05. (*B*) The NFAT-luciferase plasmid was transfected into Huh7 cells and into Jurkat cells and cultured in the presence of CsA or FK506 at concentrations of 0, 0.01, 0.1, 1, or 10 μg/mL. A plasmid, pRL-CMV, which expresses the *renilla* luciferase gene under the control of the cytomegalovirus early promoter/enhancer, was used to monitor transfection efficiency. Luciferase activities were measured at 48 hours after transfection. Values are displayed as ratios of Fluc to Rluc. *Error bars* indicate mean ± 2 SD. **P* values of less than .05. (*C*) The control *renilla* luciferase activities in each sample. Assays were performed in triplicate. *Error bars* indicate mean ± 2 SD. **P* values of less than .05.

licon does not involve activation of interferon-stimulated genes.

The principal mechanism of the immune suppressive action of CsA and FK506 is to inhibit antigen-stimulated activation of cellular calcineurin, a phosphatase that catalyzes the dephosphorylation and nuclear translocation of a transcription factor, NFAT, to induce expression of inflammatory cytokines and their receptors.[8,17,18] To study the effects of CsA and FK506 on the calcineurin-NFAT pathway in Huh7 cells, the NFAT-luciferase reporter plasmid, pNFAT-luc, was transfected into cells and treated with CsA and FK506 at concentrations of 0, 0.01, 0.1, 1, and 10 μg/mL. CsA or FK506 treatment of T lymphocyte–derived Jurkat cells significantly suppressed NFAT activities. On the contrary, neither CsA nor FK506 showed effects on the NFAT-luciferase activities in Huh7/Rep-Neo cells (Figure 2*B*). Although the NFAT-luciferase activity was apparently decreased at 10 μg/mL of CsA, which was ~20 times higher than the clinically available concentration (0.1–0.5 μg/mL),[19] the control *renilla* luciferase activities expressed from a cotransfected plasmid, pRL-CMV, tended to decrease at this concentration. These results show that CsA does not influence NFAT-mediated signaling pathways in Huh7 cells and that the presence of the anti-HCV effect of CsA in the absence of the NFAT suppression indicates that the action of CsA on HCV is independent of the calcineurin/NFAT-mediated cell signaling pathway.

Knockdown of Cyclophilins Suppresses HCV Replication

Because of the differing effects of CsA and FK506 on HCV replication and the lack of effect of both drugs on the calcineurin/NFAT pathways in Huh7 cells, we speculated that the antiviral action of CsA against HCV may be mediated by cellular cyclophilins, a family of proteins that show specific binding to CsA. Cyclophilins possess a peptidyl prolyl *cis-trans* isomerase (PPIase) activity, which catalyzes *cis-trans* conversion of peptide bonds preceding proline and contributes to the correct protein folding as a molecular cochaperone.[20–22] Cyclophilins comprise at least 15 subtypes and are present in every compartment of the cell, including the cytoplasm, endoplasmic reticulum (ER), and nucleus.[23] Among them, we have investigated CypA, CypB, and CypC, which localize predominantly in the cytoplasm or microsome fraction where the HCV replicase complex is located.[24] To study effects of the cyclophilin expression on the replication of the HCV replicon, we suppressed expression of CypA, CypB, and CypC using plasmid vectors expressing shRNA.

Huh7/Rep-Feo cells were transfected with shRNA-expression vectors CypA shRNA#3, CypA shRNA#441, CypB shRNA#294, CypB shRNA#467, CypC shRNA#0, and CypC shRNA#291. Western blotting revealed that expression of the respective cyclophilins was suppressed by the shRNA vectors (Figure 3*B*). Concomitantly, the luciferase activities of Huh7/Rep-Feo cells were suppressed significantly by the transfection of each cyclophilin-directed shRNA. On the contrary, transfection of shRNAs that have reverse sequences of the corresponding shRNA vectors did not show any significant effects of HCV replication (Figure 3*A*). To investigate further the effects of the cyclophilin knockdown on HCV replication, we quantified the colony-forming ability of the HCV replicon in the cell lines in which the cyclophilins were stably knocked down. The 6 cyclophilin-directed shRNA-expression vectors were transfected individually into Huh7 cells by the retroviral transduction, and stable knocked-down cell lines were established for each cyclophilin. A replicon, Rep-BSD, was transfected into these 6 cell lines and into cells transfected with negative and positive control shRNAs and cultured in the presence of BSD. As shown in Figure 4, numbers of the BSD-resistant colonies were ~700 and ~800 in naive Huh7 and Huh7/shRNA negative control, respectively; however, as observed in the transient transfection assays (Figure 3), numbers of the BSD-resistant colonies were markedly reduced in the cells stably transfected with shRNA for CypA, CypB, and CypC (Figure 4).

Inhibition of HCV Replication by CsD

CsD is a cyclosporin analogue that lacks the immunosuppressive activity of CsA (<10%).[25] CsD shows binding to cyclophilin but has attenuated effects on the calcineurin/NFAT pathway. Treatment of Huh7/Rep-Feo with CsD showed that the luciferase activities were inhibited significantly, as much as for CsA (Figure 5*A*). Also, replicon RNA in Huh7/Rep-Feo, which was detectable in CsA-negative control cells by Northern blotting (Figure 5*B*), decreased substantially following treatment with CsD at concentrations of 1 and 3 μg/mL. Similarly, by Western blotting (Figure 5*C*), the expression of HCV NS5A protein was shown to be reduced by corresponding amounts following treatment with CsD and with CsA.

Unfolded Protein Response Agents Suppress HCV Replication

It has been reported that cyclosporins induce a cellular stress response called the unfolded protein response, which is elicited by accumulation of misfolded proteins in the ER.[26–28] Because the replicase complex of

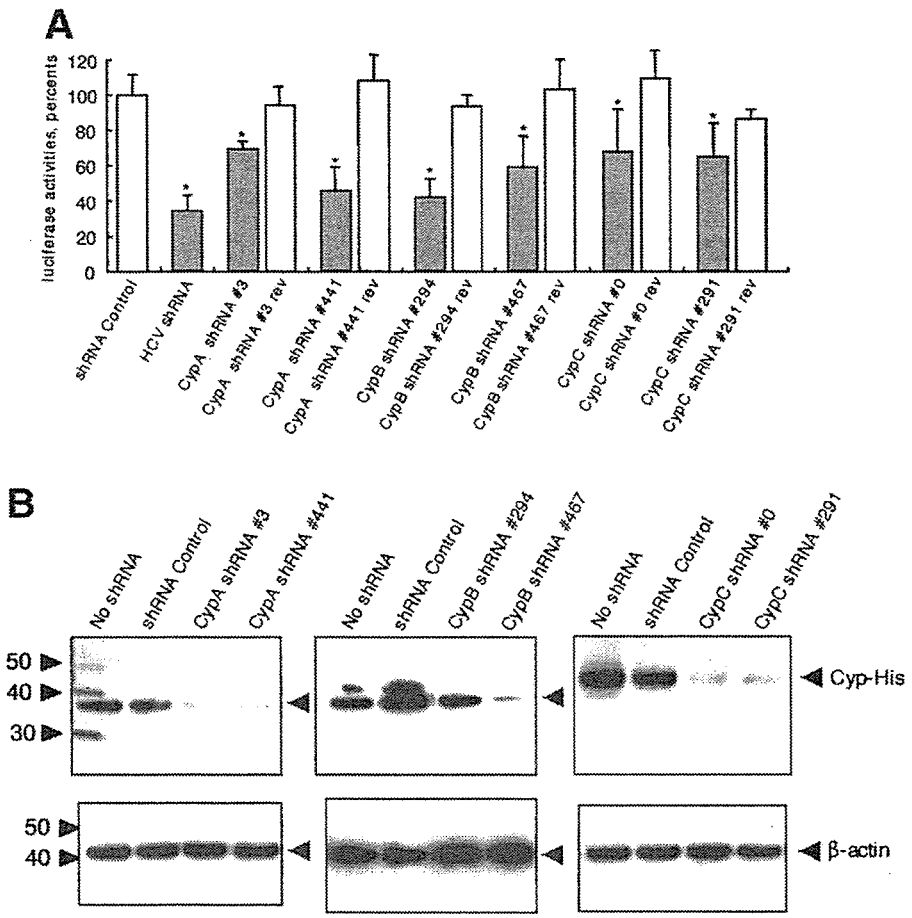


Figure 3. Knockdown of cyclophilins suppresses HCV replication. (*A*) Huh7/Rep-Feo cells were transfected with shRNA-expression plasmid vectors CypA shRNA#3, CypA shRNA#441, CypB shRNA#294, CypB shRNA#467, CypC shRNA#0, and CypC shRNA#291, which are directed against different regions of the respective cyclophilin genes. As negative and positive controls, shRNA control or an HCV shRNA was used as described.[13] We also transfected negative controls shRNA-expression plasmids that have reverse sequences of the corresponding shRNA vectors. Luciferase assays were performed at 48 hours after transfection. *Error bars* indicate mean ± 2 SD. **P* values of less than .05. (*B*) Western blotting. Cotransfection of the indicated shRNA-expression vectors and the cyclophilin-expressing vectors into Huh7 cells was performed. The expression of each cyclophilin protein contained a C-terminal His tag, which allowed its detection by anti-His antibodies. Ten micrograms of total cellular protein was separated by polyacrylamide gel electrophoresis and transferred onto the membrane. The membrane was incubated with anti-His antibodies or anti–β-actin antibodies (Sigma Chemical Co).

HCV RNA is localized in the ER,[24] we explored the possibility that drug-induced disruption of ER function may cause alterations in HCV replication. Western blotting revealed that treatment of Huh7 cells with CsA resulted in increased expression of BiP/GRP78 as reported (Figure 6*A*), while treatment with FK506 did not affect BiP/GRP78 expression. We next treated Huh7/Rep-Feo cells with reagents that induce an unfolded protein response: thapsigargin, mercaptoethanol, and tunicamycin. Treatment of the cells with thapsigargin, which causes depletion of calcium in the ER by inhibition of the Ca^{2+} adenosine triphosphatase transporter irreversibly, and mercaptoethanol, which disrupts disulfide bond formation, resulted in significant decreases in the internal luciferase activities to 79% and 38% of the control at concentrations of 0.001 and 0.003 μg/mL of thapsigargin and to 61% and 17% of the control at concentrations of 0.01 and 0.03 μg/mL of mercaptoethanol, respectively (Figure 6*B*). MTS assays did not show significant cytotoxic effects at those concentrations, suggesting that these effects are not due to cytotoxic cell death (Figure 6*C*). On the contrary, treatment with tunicamycin, which inhibits asparagines (N)-linked glycosylation, did not show significant effects on HCV replication at concentrations below the cytotoxic level.

Discussion

Several recent reports suggest that CsA is clinically effective in the treatment of HCV infection[29] and

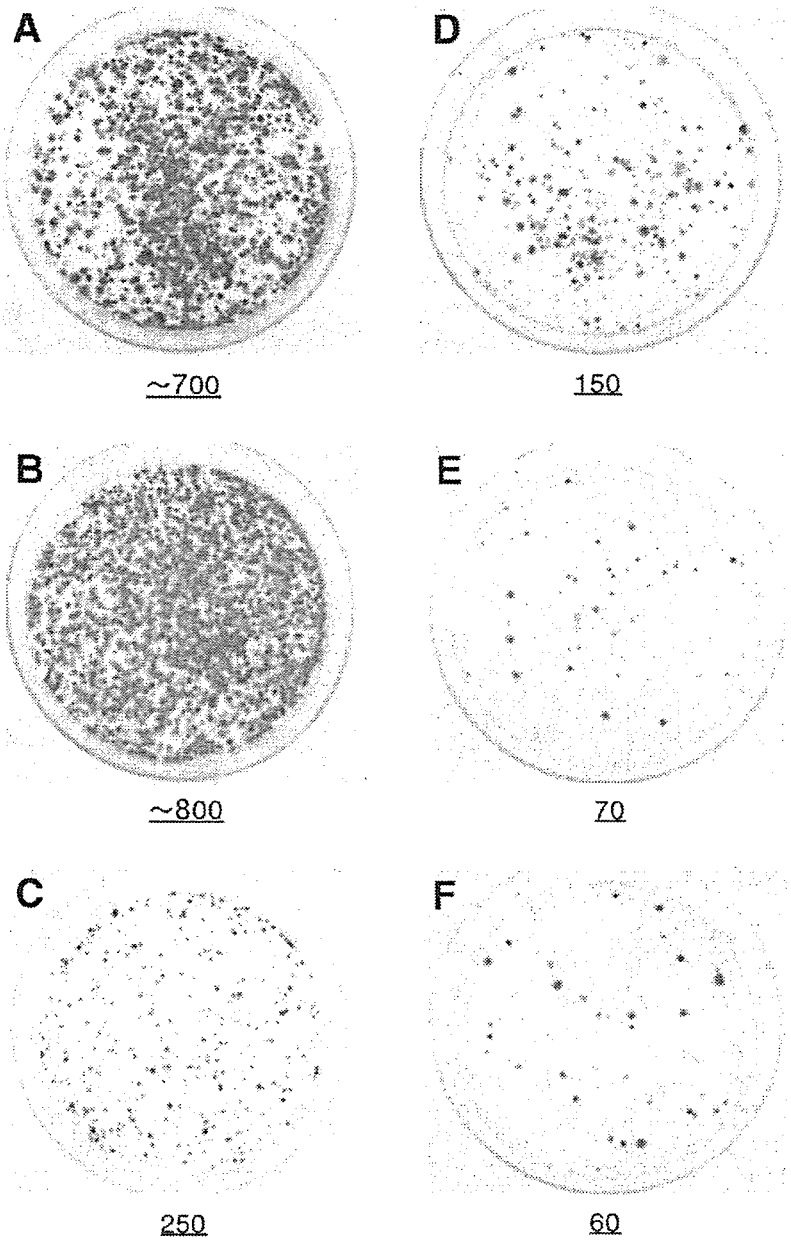
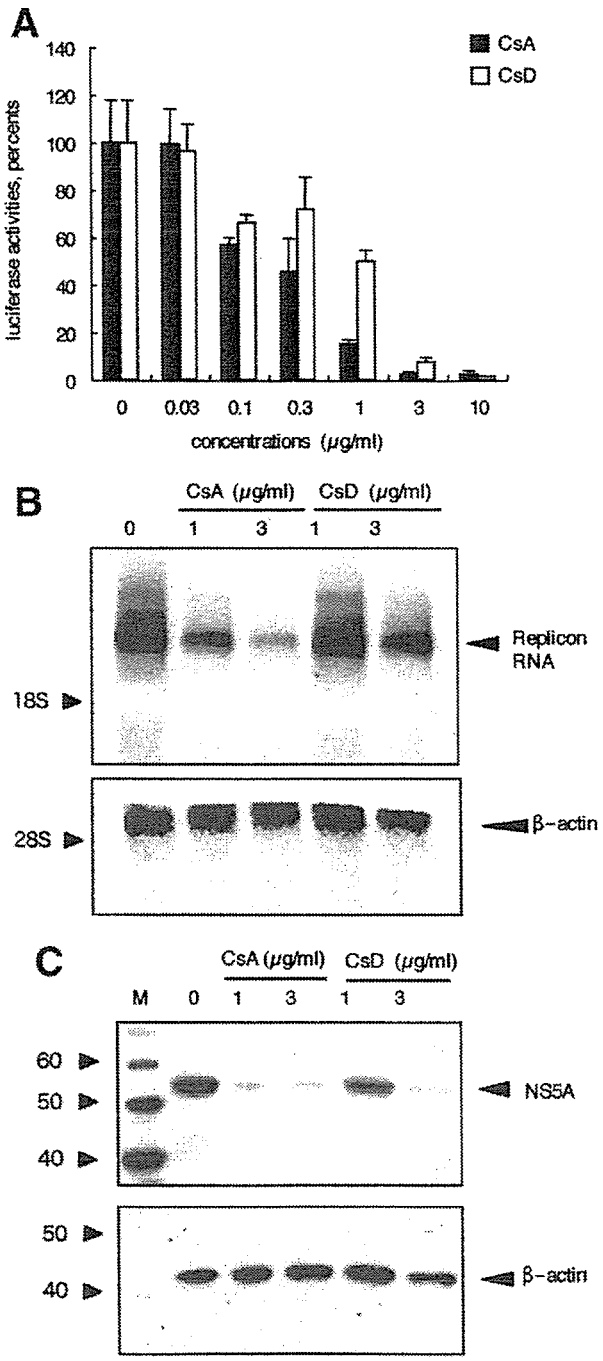


Figure 4. The replicon colony-forming activities of cell lines in which cyclophilins have been stably knocked down by shRNA. Colony-forming activities of HCV replicon were quantified in the cell lines in which cyclophilins were stably knocked down by shRNA-expression vectors. The 6 cyclophilin-directed shRNA-expression vectors were stably transfected into Huh7 cells by retroviral transduction. A replicon, Rep-BSD, was transfected into the cell lines, respectively, and along with cell lines transfected with negative and positive control shRNAs cultured in the presence of BSD. Two weeks after the transfection, the cell colonies were visualized by treating with neutral red solution. Numbers below the plates refer to the approximate numbers of the cell colonies. (*A*) Naive Huh7, (*B*) Huh7/shRNA control, (*C*) Huh7/HCV shRNA#331, (*D*) Huh7/CypA shRNA#3, (*E*) Huh7/CypB shRNA#294, and (*F*) Huh7/CypC shRNA#291.

that CsA has antiviral activity against HCV in vitro.[9,10] Watashi et al reported the in vitro effect of CsA on HCV replication using an HCV replicon system and a cultured cell line that supports HCV replication.[9] However, little is understood about the mechanisms of its action against HCV replication and the mechanism of its clinical efficacy. Our present results show that the action of CsA did not involve the calcineurin/NFAT-mediated pathway that is shared with FK506 (Figure 2*B*), showing that the anti-HCV effect of CsA is not associated with its immunosuppressive activity. On the other hand, knockdown of CypA, CypB, and CypC expression by shRNA suppressed HCV replication substantially (Figures 3 and 4). Furthermore, CsD, an analogue of cyclosporin with weak immunosuppressive activity but that retains the ability to bind cyclophilins, was similarly effective in suppressing HCV replication (Figure 5). Collectively, it was shown that the anti-HCV action of the cyclosporins is through blockade of the activities of cellular cyclophilins.

In our results, both CsA and FK506 suppressed NFAT-mediated luciferase reporter activities in a T lymphocyte–derived cell line, while neither CsA nor FK506 suppressed NFAT activities in hepatoma-derived Huh7 cells (Figure 2*B*). It has been reported that CsA shows divergent effects of NFAT-mediated luciferase activity

among cells of different origins, possibly because NFAT does not participate in autoregulatory activation of its own promoter in CsA-nonresponding cells.[9,30,31] With these findings, although our results may not completely exclude the partial involvement of the NFAT-mediated pathway, the major action of CsA against HCV replication is not through the calcineurin/NFAT pathway but through cyclophilin-mediated mechanisms.



The PPIase activity of cyclophilins has been reported by Takahashi et al and other researchers.[20–22] Through this activity, cyclophilins contribute to the maturation of several proteins, such as carbonic anhydrase[32] and the human immunodeficiency virus *gag* protein.[33,34] Furthermore, the enzymatic activity of cyclophilins may underlie several other functions of these proteins, including cell signaling, mitochondrial function, molecular chaperone activity, RNA splicing, stress response, gene expression, and regulation of kinase activity.[21,35] As for HCV, the viral structural and nonstructural proteins are processed from a single polyprotein of 3000 amino acids by ER membrane-bound signal peptidases and by 2 self-coded serine proteases.[36] It has been reported that folding and assembly of HCV proteins require interaction with ER chaperone proteins such as calreticulin, BiP, and HSP90.[37,38] In this study, the replication levels of the HCV replicon decreased substantially following the knockdown of CypA, CypB, and CypC. Moreover, our preliminary data have shown that overexpression of cyclophilins attenuated the effects of CsA on HCV replication. These results imply that the effects of CsA on HCV replication involve functional blockade of cyclophilins and, more importantly, that constitutive expression of the cytoplasmic cyclophilins such as CypA, CypB, and CypC may be necessary for HCV replication, possibly through assisting functional maturation of the viral proteins as molecular co-chaperone proteins. Although it is still unclear how cyclophilins support the processing of HCV proteins and the replication of the viral genome, these molecules could be potential targets to counteract HCV infection and replication.

One third of nascent proteins are transported to the ER, where they are subjected to posttranslational modifications such as folding, glycosylation, and oligomer-

Figure 5. Suppression of HCV replication by CsD. (*A*) Huh7/Rep-Feo cells were cultured with the concentrations of CsA (*closed boxes*) indicated or an analogue of cyclosporin, CsD (*open boxes*), in the medium. Luciferase assays were performed after 48 hours of culture. Luciferase assays were performed in triplicate. *Error bars* indicate mean ± 2 SD. (*B*) Northern blotting. Huh7/Rep-Neo was cultured with the concentrations of CsA or CsD indicated, and RNA was extracted after 48 hours of culture. Ten micrograms of total cellular RNA was applied to each lane. The upper part of the membrane containing the HCV replicon RNA was hybridized with a digoxigenin-labeled probe specific for the replicon sequence, and the lower part was hybridized with a β-actin probe. *Lane 1*, replicon alone; *lane 2*, 1 μg/mL CsA; *lane 3*, 3 μg/mL CsA; *lane 4*, 1 μg/mL CsD, and *lane 5*, 3 μg/mLCsD. (*C*) Western blotting. Ten micrograms of total cellular protein was separated by polyacrylamide gel electrophoresis and transferred onto the membrane. The membrane was incubated with a monoclonal anti-NS5A antibody or an anti–β-actin antibody. *Lane 1*, protein size markers; *lane 2*, replicon alone; *lane 3*, 1 μg/mL CsA; *lane 4*, 3 μg/mL CsA; *lane 5*, 1 μg/mL CsD; and *lane 6*, 3 μg/mL CsD.

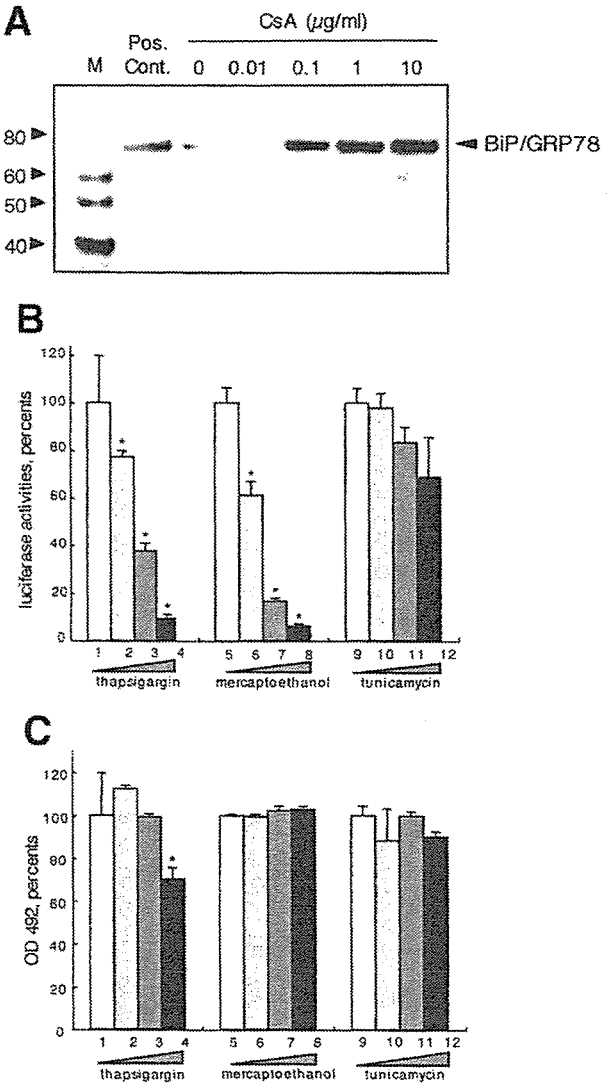


Figure 6. Unfolded protein response following CsA treatment and its effects on HCV replication. (*A*) Huh7 cells were cultured in the presence of CsA at the concentrations indicated, and the cells were harvested at 48 hours after the treatment. Western blotting was performed using anti-Bip/GRP78 antibody. *Lane 1*, protein size markers; *lane 2*, HepG2 lysate as a positive control; *lane 3*, Huh7 alone; *lane 4*, 0.01 μg/mL CsA; *lane 5*, 0.1 μg/mL CsA; *lane 6*, 1 μg/mL CsA; and *lane 7*, 10 μg/mL CsA. (*B*) Huh7/Rep-Feo cells were cultured with various concentrations of thapsigargin, mercaptoethanol, and tunicamycin in the medium. Luciferase assays were performed in triplicate at 48 hours after treatment with each drug. Error bars indicate mean ± 2 SD. Columns 1–4: cells cultured with thapsigargin at concentrations of 0 (column 1), 0.001 (column 2), 0.003 (column 3), and 0.01 μg/mL (column 4). Columns 5–8: cells cultured with mercaptoethanol at concentrations of 0 (column 5), 0.01 (column 6), 0.1 (column 7), and 1 μg/mL (column 8). Columns 9–12: cells cultured with tunicamycin at concentrations of 0 (column 9), 0.01 (column 10), 0.03 (column 11), and 0.1 μg/mL (column 12). **P* values of less than .05. (*C*) MTS assay of Huh7/Rep-Feo cells cultured with the various concentrations of ER stress agents. MTS assays at 48 hours after treatment with each drug were performed in triplicate. *Error bars* indicate mean ± 2 SD. Columns 1–12: cells cultured with the drugs that correspond to those in *B*. **P* values of less than .05.

ization and are exported to various cellular compartments. Various cellular stresses such as heat shock, ischemia, hypoxia, and viral infection and changes in calcium homeostasis prevent protein folding and maturation in the ER and result in the accumulation of misfolded proteins.[39,40] These defective proteins trigger the unfolded protein response and cause induction of molecular chaperone proteins, suppression of translation, and apoptotic cell death.[27,28,41] Paslaru et al reported that treatment of HeLa cells with CsA induced an unfolded protein response that is characterized by synthesis of a stress protein, BiP/GRP78, located inside the ER.[26] Because PPIases support the correct folding of a sufficient number of proteins, their inhibition ought to lead to the accumulation of denatured proteins in various cell compartments. In this study, treatment with thapsigargin, an inhibitor of the Ca^{2+} adenosine triphosphatase (ATPase) transporter, and mercaptoethanol, which disrupts disulfide bond formation, suppressed HCV replication. These results imply that the effects of CsA on HCV replication may involve induction of an unfolded protein response to a level below the cytotoxic range and that the selective alteration of ER function may abrogate HCV replication.

The expanding applications of CsA to infectious diseases such as HCV may cause substantial problems, particularly undesired immunosuppression and possible interference with the effects of anti-infectious agents such as interferon. One solution to overcome these problems is to use cyclosporin analogues.[42,43] Some types of cyclosporin analogues show attenuated effects in terms of blocking T-cell activation but retain activity against the PPIase activity of cyclophilins. These nonimmunosuppressive cyclosporin analogues have equal or even superior effects against in vitro human immunodeficiency virus replication compared with the immunosuppressive CsA.[44] As we have shown, HCV replication was successfully inhibited by CsD, which has weak immunosuppressive activity.[25,45] The inhibition of T-cell activation by cyclosporin analogues is a function of the binding capacities to cyclophilins, whose binding to the residues of cyclosporine is known to be critical for their immunosuppressive activity in the decreasing order of CsA to CsD.[46] However, in this study, the inhibitory effects of CsA and CsD on HCV replication were found to be similar (Figure 5), showing that the anti-HCV effects did not correlate with immunosuppressive activity. Another cyclosporin analogue, NIM811 ([methyl-Ile4]-cyclosporin), has been reported to be a similar inhibitor of CypA-mediated human immunodeficiency virus 1 replication than the parental CsA.[47] Watashi et al confirmed that NIM811 also is effective against HCV replication in

vitro.[9] The availability of nonimmunosuppressive cyclophilin inhibitors that are less toxic than CsA might hold promise of novel antiviral drugs. These drugs should be reevaluated clinically in the light of the new findings presented here.

Given the current status of limited therapy options against HCV infection and the unsatisfactory outcome of therapy, screening of nonimmunosuppressive cyclosporin analogues or agents targeting cellular cyclophilins may be important to develop novel antiviral therapies. In addition, further investigations of the action of cyclophilins on the expression, processing, or maturation of HCV proteins may elucidate new aspects of the viral infection and replication.

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Received September 10, 2004. Accepted June 9, 2005.

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Supported by grants 15590629 and 16590580 from the Japan Society for the Promotion of Science and in part by a grant from the Viral Hepatitis Research Foundation of Japan.

Journal of Gastroenterology and Hepatology (2005) **20**, 1401–1409 DOI: 10.1111/j.1440-1746.2005.04024.x

HEPATOLOGY

Mutations in the NS5B region of the hepatitis C virus genome correlate with clinical outcomes of interferon-alpha plus ribavirin combination therapy

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Abstract

Background and Aim: Combination treatments of interferon-alpha (IFN) and ribavirin (RBV) are more effective than those of IFN alone in hepatitis C virus (HCV) infection. However, mechanisms of the action of the combination regimen are not well understood. To elucidate the viral genetic basis of IFN plus RBV combination therapy, genetic variabilities of HCV-1b were analyzed.

Methods: We performed pair-wise comparisons of full-length HCV genomic sequences in three patients' sera before and after initiation of IFN plus RBV treatment. Subsequently, we analyzed amino acid sequences of the NS5B region, which codes for the viral RNA-dependent RNA polymerase, and compared these with the outcomes of the therapy in 81 patients.

Results: Analysis of the entire HCV sequence in patients who received IFN plus RBV therapy did not show consistent amino acid changes between before and after the initiation of the therapy. NS5B sequence analyses revealed that mutations at positions 300–358 of NS5B, including polymerase motif B to E, occurred more frequently in a group of patients exhibiting a sustained viral response (SVR) or an end-of-treatment response (ETR) compared with a group of patients exhibiting a non-response (NR). Closer examination revealed that mutations at aa 309, 333, 338 and 355 of NS5B occurred significantly more frequently in the SVR plus ETR group than in the NR group ($P = 0.0004$). Multivariate analysis showed that the number of mutations at these four sites was an independent predictor of SVR plus ETR *versus* NR.

Conclusions: Particular amino acid changes in the NS5B region of HCV may correlate with outcomes of IFN plus RBV combination therapy.

Key words: amino acid sequence, error catastrophe, RNA-dependent RNA polymerase, transition.

INTRODUCTION

Hepatitis C virus (HCV) is a major causative agent of chronic hepatitis, which can lead to liver cirrhosis and hepatocellular malignancy.[1,2] Interferon (IFN) is the agent of choice for treating HCV infection. However, IFN monotherapy produces sustained virological responses in only 15–20% of patients treated, most of whom relapse after completion of the therapy.[3,4] Several recent studies of combination therapy with IFN alpha 2b and ribavirin (RBV) have shown that the regimen induces higher sustained virological responses than IFN monotherapy. Unfortunately, 50–60% of patients still do not respond to the combination therapy.[5–8]

RBV is a synthetic guanosine analog with broad antiviral actions *in vitro* against various DNA and RNA

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Accepted for publication 8 September 2004.

viruses.[9,10] At present, four mechanisms of action have been postulated: (i) immune modulatory effects by a switching of T-cell phenotype from Th2 to Th1 that induces type 1 cytokine responses;[11-13] (ii) inhibition of inosine monophosphate dehydrogenase (IMPDH) leading to depletion of cellular GMP pool;[14] (iii) mutagenic activity against RNA viruses that induces misincorporation of RBV triphosphate into viral RNA leading to error prone replication of viral genome;[15-18] and (iv) inhibition of the activity of HCV NS5B RNA-dependent RNA polymerase (RdRp).[19,20] However, it has not been fully understood which mechanisms of actions of RBV are effective against HCV infection.

Certain genetic structures of viruses may affect the sensitivity to their therapeutic drugs. Nucleoside analogs are widely used against viruses such as human immunodeficiency virus type 1 (HIV) and hepatitis B virus (HBV).[21,22] The antiviral effect of those reagents arises from the inhibition of viral DNA/RNA polymerase activity. However, single or multiple mutation(s) in the viral polymerase confer drug resistance and help the drug resistant strains emerge.[22-30] Also in HCV infection, the INF sensitivity determining region (ISDR) of HCV genome, which we have previously identified, critically determines the virological response to IFN and the treatment outcomes.[31,32] As to RBV, one study of five HCV genotype 1a patients who had undergone RBV monotherapy has reported one mutation in NS5B that may correlate with RBV sensitivity.[33] These findings make us speculate that genetic variability of HCV NS5B region, which codes for RdRp, may correlate with sensitivity to RBV and may influence the outcomes of IFN plus RBV combination therapy.

In the present study, we first analyzed effects of RBV on HCV genomic structure and the viral genetic basis of RBV resistance by performing pair-wise comparisons of full-length HCV genomic sequences in patient sera before and after initiation of IFN plus RBV treatment. Subsequently, we have investigated a hypothesis that genomic variability of HCV RdRp may confer resistance or susceptibility to RBV and may correlate with the outcomes of IFN plus RBV combination therapy. Thus, we analyzed amino acid sequences of the NS5B region and the outcomes of IFN plus RBV combination therapy in 81 patients, and found that certain amino acid variations in the NS5B region may associate with the treatment outcomes.

METHODS

Patients of interferon plus ribavirin non-responders

Three patients infected with HCV, genotype 1b, were studied. All patients were non-responders to combination therapy with IFN alfa-2b (Intron A, Schering Plough, Kenilworth, NJ, USA), 6 million units three times per week plus RBV (Rebetoron, Schering Plough), 800 mg/day (> 12.1 mg/kgBW) for 24 weeks. Serum samples were obtained before treatment and at 12 weeks after initiation of the treatment, and pair-wise comparisons of the consensus sequences of full-length HCV genomes were performed. As controls for the IFN plus RBV therapy data, we analyzed our previously published HCV sequence data for three non-responders of IFN monotherapy[32] (deposited with the DDBJ/GenBank/EMBL data libraries under accession number D50483, D50480, D50485, D50481, D50484 and D50482).

RNA extraction, reverse transcription-polymerase chain reaction and direct sequencing

RNA was extracted from patient sera by the modified acid guanidinium thiocyanate-phenol-chloroform (AGPC) method,[34] using ISOGEN reagent (Wako Pure Chemical Industries, Osaka, Japan), and reverse transcription polymerase chain reaction (RT-PCR) was performed as previously described.[32] Full-length HCV genomes were amplified by nested PCR with 21 partially overlapping sets of primers, as previously reported.[32] M13-forward and M13-reverse sequencing primer sequences were attached to the 5′-termini of sense and antisense nested PCR primers. Each PCR product was purified by a spin filtration column (Suprec-02; Takara). Both strands of the PCR products were cycle sequenced with the PRISM dye termination kit (Applied Biosystems, Tokyo, Japan) according to the manufacturer's instructions, and consensus nucleotide sequences were determined by an automated DNA sequencer model 373 A (Applied Biosystems).

Sequence analyses

Nucleotide sequencing analysis was performed with a software program (MEGA version 2.1) to calculate values for d_N (non-synonymous substitution), d_S (synonymous substitution), d_N/d_S ratios, and the number of point mutations.

Clinical outcome of combination therapy

Patients were placed into one of three outcome groups.

- Sustained virologic response (SVR): HCV-RNA was not detectable by RT-PCR for 6 months following completion of the therapy.
- End-of-treatment response (ETR): HCV-RNA was not detected at the end of the treatment, but reappeared within 6 months thereafter.
- Non-response (NR): HCV-RNA did not disappear during the treatment.

Nucleoside sequencing analyses of the NS5b region

Amino acid mutations in the conserved motifs (motif A, B, C, D, E, F)[35-38] in NS5B RdRp were retrospectively analyzed in 81 HCV genotype 1b patients who were

treated with IFN alfa-2b, 6 million units three times per week plus RBV, 800 mg/day (> 12.1 mg/kgBW) for 24 weeks. All patients had biopsy-proven chronic hepatitis with positive serum HCV antibodies and serum HCV-RNA. RNA was extracted from sera of the patients before treatment. NS5B region, including motifs A to F, was amplified by RT-PCR and sequences corresponding to nucleotides 7730–8874 of HCV-J were determined.[32] The deduced amino acid sequences of all patients were aligned and compared with consensus sequences for mutations and analyzed for correlation between amino acid mutations of NS5B and the clinical outcome of the combination therapy.

Statistical analyses

Comparisons of differences in categorical data between groups were performed using the χ^2 test and Fishers exact test. Distributions of continuous variables were analyzed by the Mann–Whitney *U*-test for two groups and by the Kruskal–Wallis test or Scheffé method for three groups. Multivariate analysis was carried out by multiple logistic regression analysis. *P*-values of less than 0.05 were defined as statistically significant.

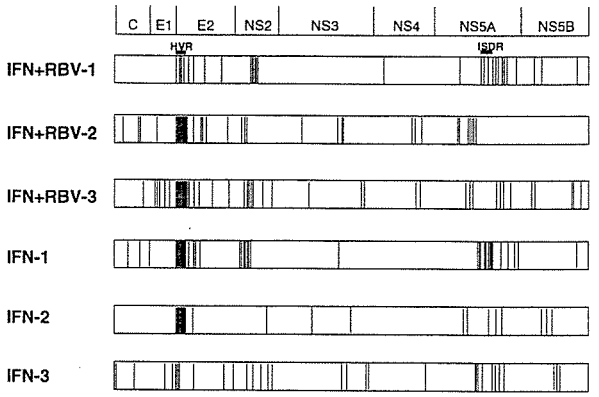

Figure 1 Schematic representation of the distribution of mutations in amino acid residues during the combination therapy and interferon (IFN) monotherapy. Distributions of amino acid changes in the entire hepatitis C virus (HCV) genome in patient serum before treatment and 12 weeks after initiation of treatment are shown. The upper three data are from patients treated with IFN/ribavirin (RBV) combination therapy (IFN + RBV 1–3), and the lower three data are those treated with IFN monotherapy (IFN 1–3). Vertical lines in each HCV polyproteins show position of amino acid differences during the therapy.

RESULTS

Pair-wise comparisons of the full-length HCV genome in three patients before and after initiation of IFN/RBV treatment

HCV genomes from the three study patients comprised 9423 nucleotides and contained an open reading frame of 3010 amino acids. In patient one, 31 amino acid changes were found in the HCV genome. These amino acid changes were clustered in the E2-hypervariable regions (8 of 31) and the NS5A regions (11 of 31). Before treatment, the INF-sensitivity determining lesion (ISDR)[31,32] were 'mutant' type with five amino acid changes compared with consensus sequence, which changed to 'intermediate' type with two amino acid changes after the initiation of treatment. In patient two, 37 amino acid changes were found in the entire HCV genome. The changes were exclusively found in the E2-hypervariable region (16 out of 37 amino acids), while there was no change in the ISDR. In patient three, 56 amino acid changes were found. The changes were exclusively found in the E2 region (24 out of 56 amino acids). Distribution of amino acid changes during the therapy in the three patients treated with combination therapy and three non-responders to IFN monotherapy are illustrated in Figure 1. The numbers of nucleotide changes for the three study patients were 88, 130 and 272, respectively. The d_N/d_S ratios were 0.195, 0.148 and 0.099, respectively. Among the three control subjects who received IFN monotherapy, the numbers of nucleotide changes were 138, 160 and 175, respectively. The d_N/d_S ratios were 0.158, 0.061 and 0.089, respectively. As shown in Figure 2, d_N/d_S ratios tended to be higher in the E2 region than in the other regions during

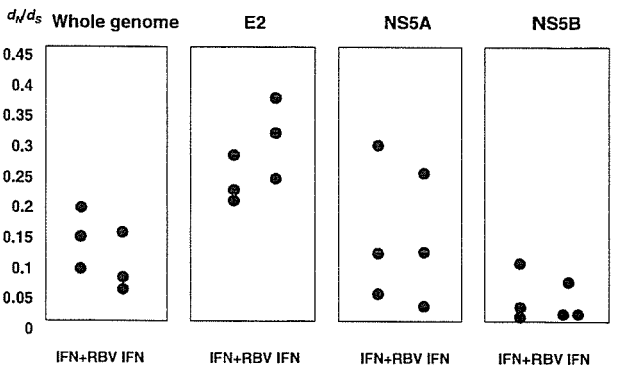

Figure 2 Ratio of non-synonymous to synonymous distances for the E2, NS5A, NS5B and whole hepatitis C virus (HCV) genome. The d_N/d_S ratio in E2 region tended to be higher than other regions during interferon (IFN) monotherapy and during combination therapy. All pairwise d_N/d_S ratios were calculated using MEGA version 2.1 for each subject.

both IFN monotherapy and combination therapy. The numbers of transitional mutations in patients who received the combination therapy had 71 (80.2% of total mutations), 104 (80.0%) and 218 (80.1%) transitional mutations, respectively, and in patients who received IFN monotherapy these were 108 (78.3%), 131 (81.9%) and 130 (75.4%), respectively. The proportion of transitions among IFN monotherapy patients did not differ from the proportion among combination therapy patients.

Two studies have observed two key transitions, C-to-U and G-to-A, in genomic sequences of RBV-treated RNA viruses.[17,18] In the present study, C-to-U and G-to-A mutations comprised 35.5%, 40.6% and 58% of