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Evaluation of the anti-hepatitis C virus effects of cyclophilin inhibitors, cyclosporin A, and NIM811

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Abstract

Hepatitis C virus (HCV) is a major causative agent of hepatocellular carcinoma. We recently discovered that the immunosuppressant cyclosporin A (CsA) and its analogue lacking immunosuppressive function, NIM811, strongly suppress the replication of HCV in cell culture. Inhibition of a cellular replication cofactor, cyclophilin (CyP) B, is critical for its anti-HCV effects. Here, we explored the potential use of CyP inhibitors for HCV treatment by analyzing the HCV replicon system. Treatment with CsA and NIM811 for 7 days reduced HCV RNA levels by 2–3 logs, and treatment for 3 weeks reduced HCV RNA to undetectable levels. NIM811 exerted higher anti-HCV activity than CsA at lower concentrations. Both CyP inhibitors rapidly reduced HCV RNA levels even further in combination with IFNα without modifying the IFNα signal transduction pathway. In conclusion, CyP inhibitors may provide a novel strategy for anti-HCV treatment.
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Keywords: HCV; Cyclosporin; NIM811; Interferon; Cyclophilin; Cyclosporine; Replication; Replicon; Inhibitor; Therapy

Hepatitis C virus (HCV), which is associated with non-A and non-B hepatitis [1], is a major causative agent of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC). Worldwide, HCV infection constitutes a serious health threat, and is estimated to affect more than 200 million individuals and cause approximately 280,000 deaths per year [2,3]. The current standard therapy for chronic HCV infection is interferon (IFN) or pegylated IFN, either alone or in combination with ribavirin [4,5]. Because treatment with these agents fails to produce sustained virus elimination in about half the total patients [6,7], however, alternative and more effective strategies to treat hepatitis C are needed.

We recently discovered that an immunosuppressant, cyclosporin A (CsA), and its nonimmunosuppressive analogue, NIM811, suppress HCV genome replication in a cell culture system. The maximum effect of each cyclosporin was comparable to that of IFNα. The anti-HCV effects of the cyclosporins correlated with cyclophilin (CyP) inhibition [8]. We also revealed that CyPB, one of the cellular targets of CsA, regulated HCV replication through its interaction with viral RNA-dependent RNA polymerase NS5B [9]. Cyclosporins suppressed HCV replication by dissociating CyPB from NS5B. These properties recommended the CyP inhibitors as agents for clinical use, especially considering the fact that hepatitis C treatment should preferably suppress the emergence of drug-resistant viruses. Because the CyP inhibitors specifically target a cellular factor, they are expected to exert robust anti-HCV activities with a low risk of developing drug resistance (see Discussion). Therefore, it will assist in the development of new anti-HCV strategies to investigate the effects of cyclosporins on HCV replication in a cell culture system.

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In this report, we examined in detail the anti-HCV effects of CsA and NIM811 and the effects of the cyclosporins in combination with IFNα, using an HCV replicon system [10]. Treatment with CsA and NIM811 reduced HCV RNA in the replicon system. NIM811 was a more potent anti-HCV agent than CsA. We observed further reduction of HCV RNA using a combination of either CsA or NIM811 with IFNα, and detected little to no increase in cytotoxicity. In addition, HCV RNA was reduced to background level after 21 days of treatment with each cyclosporin. Based on these results, CyP inhibitors could potentially serve as a new class of anti-HCV agents.

Materials and methods

Compounds. CsA, IFNα, and ribavirin were purchased from Sigma, Otsuka Pharmaceutical Co., Ltd., and Calbiochem, respectively. NIM811 was generously provided by Novartis (Basel, Switzerland).

Cell Culture. NNC and LMH14 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) with 10% fetal bovine serum, nonessential amino acids (Invitrogen), and L-glutamine (Invitrogen) in the presence of 500 μg/ml G418 (Invitrogen). LucNeo#2 cells were generated by selecting Huh7 cells transfected with LNMH14 RNA in the presence of 800 μg/ml G418.

Plasmid construction. The pLNMH14 plasmid was constructed as follows. The luciferase gene was amplified from pLMH14 [11] by polymerase chain reaction (PCR) with the primers sspfor 5′-AATATTATTG AAGCATTTATCAGGG-3′ and lucneorev 5′-GAACCTGCGTGCAAT CCATCTTGCAATTTGGACTTTCCGCCCTTC-3′. The gene for neomycin phosphotransferase (Neo^r) was amplified by PCR from pMH14 using the primers lucneofor 5′-GAAGGGCGGAAAGTCCAAATTGC AAGATGGATTGCACGCAGGTTC-3′and neonotrev 5′-CAATTGTT ACCGCGGCCGCTGGAGGATC-3′. Both cDNA fragments were annealed, followed by PCR amplification using the primers sspfor and neonotrev. The amplified DNA fragment was digested with *Ssp*I and *Afl*II and cloned into pMH14.

In vitro RNA synthesis. LNMH14 RNAs were prepared by in vitro transcription using a MEGAscript T7 kit (Ambion), as described previously [12].

Synergy and antagonism analysis. The effects of drug combinations were evaluated using the Loewe additivity model, in which data were analyzed with CalcuSyn software (Biosoft, Ferguson, Mo.), a computer program based on the method of Chou and Talalay [13]. After converting the dose-effect curves for each drug or drug combination to median-effect plots, the program calculated a combination index (CI) value based on the following equation: $[(D)_1/(D_x)_1] + [(D)_2/(D_x)_2] + [(D)_1(D)_2/(D_x)_1(D_x)_2]$, where $(D_x)_1$ and $(D_x)_2$ are the doses of drugs 1 and 2, respectively, that have the same x effect when used in combination. CI values of <1, 1, and >1 indicate synergy, an additive effect, and antagonism, respectively.

Colony formation assay. NNC cells were treated with drugs (CsA and NIM811 alone or in combination with IFNα) in the presence of 500 μg/ml G418 for 2 weeks, followed by fixation and staining with crystal violet.

Real-time RT-PCR analysis. The 5′-nontranslated region of HCV RNA was quantified using an ABI PRISM 7500 sequence detector (Applied Biosystems), as previously described [8].

RT-PCR analysis. RT-PCR was performed as described previously [8] using the following primer sets: 5′-TGACGCTGACCTGGTTGTCTT-3′ and 5′-CAGGCTTCCAGCTGTCTCCTAA-3′ to detect mRNA for 2′,5′-oligoadenylate synthetase (2′,5′-OAS), 5′-CCGCAGCCAAATTAGC TGTT-3′, and 5′-GGCCTATGTAATTCCCCATGG-3′ to detect double-strand RNA-dependent protein kinase (PKR), and 5′-TGGAGGGATCT CGCTCCTGG-3′ and 5′-ATGGGGAAGGTGAAGGTCGG-3′ to detect glyceraldehydes-3-phosphate dehydrogenase (GAPDH).

Results

Response of HCV genome replication to treatment with CsA and NIM811

We previously reported that CsA and its nonimmunosuppressive derivative, NIM811, strongly suppress HCV genome replication in the replicon system [8,10]. To characterize the anti-HCV effect profile of cyclosporins, we first examined in detail the cyclosporin responses of HCV replicons. Consistent with previous results, HCV RNA levels in NNC cells, which harbor full-genomic HCV replicons, were decreased by over 2 logs following treatment with 1–3 μg/ml of either CsA or NIM811 for 7 days (Fig. 1A). In this assay, NIM811 tended to decrease HCV RNA more strongly than CsA at lower concentrations; the decreasing effect of NIM811 on HCV RNA at 0.5 μg/ml was about 1 log higher than that of CsA at the same concentration.

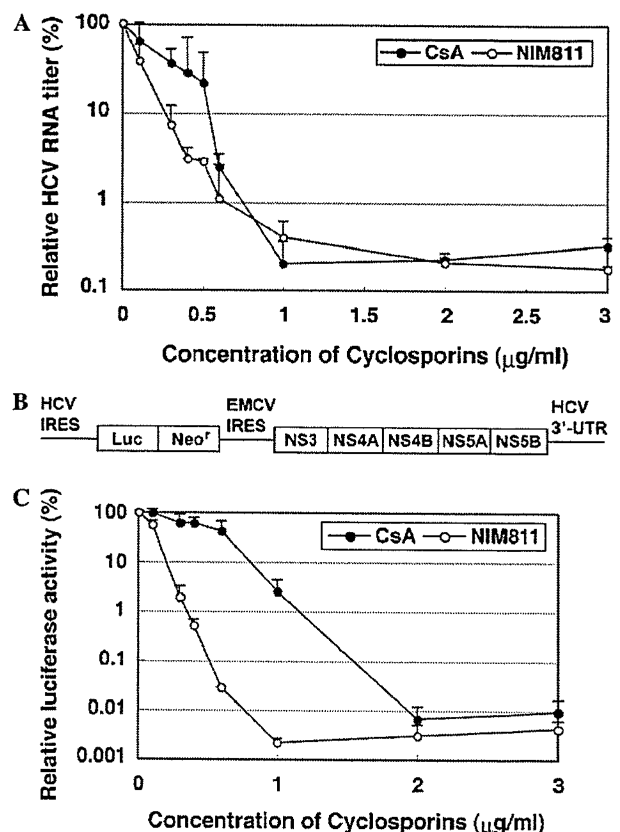

Fig. 1. Response curve of HCV RNA titers vs. the concentration of CsA and its nonimmunosuppressive analogue, NIM811. (A) NNC cells, harboring the HCV full genome replicon, were treated with either CsA or NIM811 for 7 days, and the HCV RNA extracted from these cells was quantified by real-time RT-PCR. The data represent percentages of HCV RNA levels in cells either untreated or treated with CsA or NIM811. (B) Schematic representation of the RNA construct carried in LucNeo#2 cells. LucNeo#2 cells were established as described in the Materials and methods. HCV replication can be monitored by measuring the activity of the resulting luciferase activity. (C) Luciferase activities were measured in the lysates of cells treated with either CsA or NIM811 for 7 days. The data show the means of the results from three independent experiments, with the standard deviation values indicated by error bars.

To confirm this result in another experimental system with higher sensitivity to antiviral agents, we performed a luciferase assay, which quantifies the activity of luciferase driven from a subgenomic HCV replicon construct (Fig. 1B). The maximum effect of treatment with each cyclosporin was a drop in luciferase activity of more than 4 logs (Fig. 1C). The difference in magnitude of suppression between Figs. 1A and C is likely due to differences in the experimental systems, because the response of the full genome replicon to CsA is similar to that of the subgenomic replicon [10]. Also, in this assay, the decreasing effect of NIM811 on HCV RNA at 0.5 μg/ml was approximately 2 logs higher than that of CsA at the same concentration. These results suggest that the anti-HCV effect of NIM811 is more potent than that of CsA, especially at lower concentrations.

Analysis of cotreatment with IFNα and either CsA or NIM811

We examined the effect on HCV replication of cotreatment with both IFNα and a cyclosporin by treating NNC cells for 7 days with varying concentrations of a cyclosporin and IFNα. The combination of each cyclosporin with IFNα showed a greater decrease in HCV RNA levels compared to each compound alone (Figs. 2A and C), with little to no increase in cytotoxicity (Figs. 2B and D). The amplification of the IFNα-induced anti-HCV effects of NIM811 was stronger than that of CsA (Figs. 2A and C). This effect was further demonstrated using a colony formation assay (Fig. 3). Cells treated with IFNα (3 and 10 IU/ml) or each cyclosporin (0.5 and 0.7 μg/ml) survived under G418 selection similarly to untreated control cells, but a drastic reduction of the colony formation resulting from replicating HCV was apparent following cotreatment with IFNα and either CsA or NIM811. These data suggest that combination treatment of cyclosporins with IFNα exhibits a stronger antiviral effect than single treatments.

The next question is whether the antiviral effect of the combination treatment is synergistic or additive. We therefore analyzed the data in Fig. 2A, obtained by cotreatment with IFNα and CsA, using Loewe additivity models [14] and a computer program, CalcuSyn [13]. Fig. 4 shows the analysis results of the combination effects of IFNα (in units per milliliter) and CsA (in micrograms per milliliter) at a fixed ratio of 100:1. Fig. 4A presents a conservative isobologram, illustrating lines that represent the effective doses (ED_x) of the two compounds that would be required to attain X% inhibition if the combination were simply additive. The actual experimental doses inducing 50 (filled triangle), 90 (filled square), and 99 (filled circle) % inhibition obtained in the data of Fig. 2A were more than, nearly equal to, and less than, respectively, the expected doses from ED curves which showed the additive interaction between the two compounds. This result indicates antagonistic, nearly additive, and synergistic effects for ED_{50}, ED_{90}, and ED_{99}, respectively, between CsA and IFNα. Combination effect was further examined in Fig. 4B by the calculation of a CI value (In this figure, more than, equal to, and less than 1 of CI value indicate antagonistic, additive, and synergistic effect, respectively). CI values of the combination effects of IFNα and CsA at the fixed ratio of 100:1 in the experiment shown in Fig. 2A were >1 in lower fractional effect and <1 in higher fractional effect, indicating a synergistic effect at high fractional effect levels. A stronger synergistic effect was observed at the dose

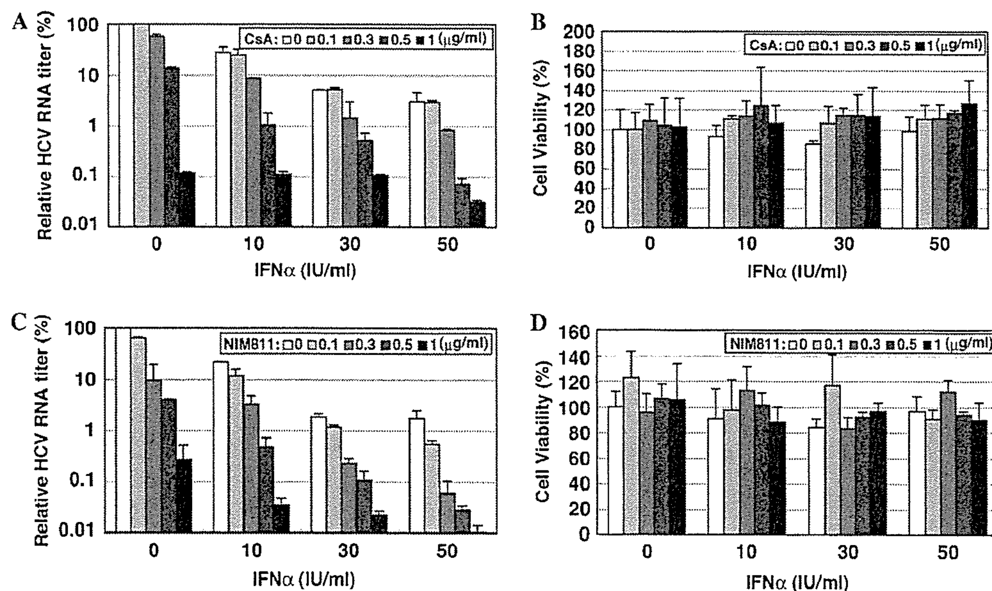

Fig. 2. The effects on HCV replication of cotreatment with both IFNα and cyclosporins. (A,C) NNC cells were treated with varying concentrations of either CsA (A) or NIM811 (C) in combination with various concentrations of IFNα for 7 days. HCV RNA levels were determined by real-time RT-PCR and are shown as percentages of the level in cells untreated (control). (B,D) The numbers of NNC cells treated with either CsA (B) or NIIM811 (D) in combination with IFNα for 2 days were determined to show the cytotoxicity of the drugs. The data represent means of the results from three independent experiments, with the standard deviation values indicated by error bars.

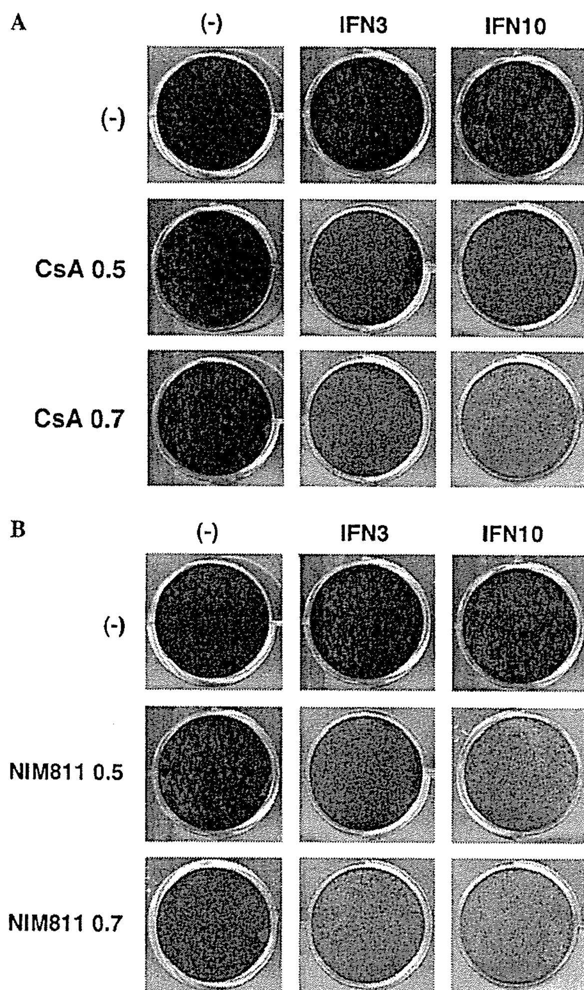

Fig. 3. Colony formation assay showing the effects of cotreatment with IFNα and cyclosporins, CsA (A) or NIM811 (B). NNC cells were treated with cyclosporins in combination with IFNα at the indicated doses in the presence of 500 μg/ml G418. After 2 weeks in culture, cells were fixed and stained with crystal violet.

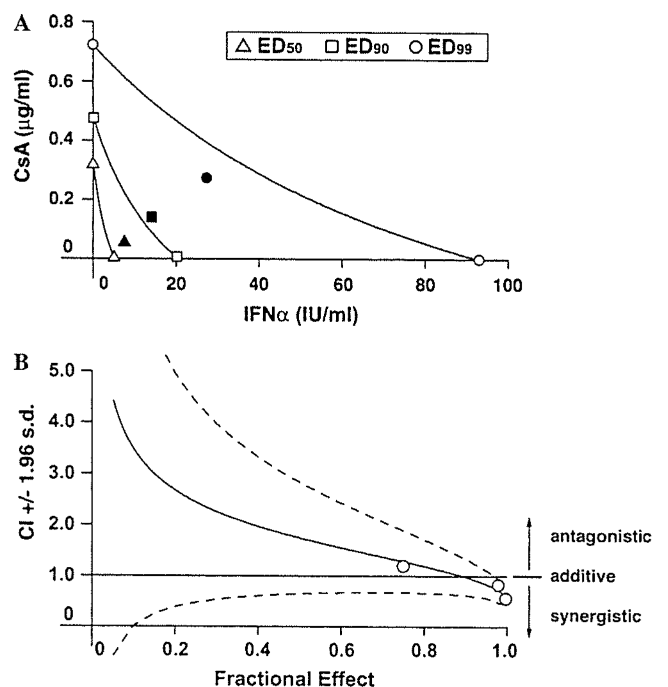

Fig. 4. Analysis of the combination treatment of CsA and IFNα using a Loewe additivity model. (A) Conservative isobologram determined by analyzing the data shown in Fig. 2A using the CalcuSyn program. The lines represent the effective doses (ED_x) of two drugs that would be required to attain X% inhibition if the effects of combination were simply additive, and the filled dots (filled triangles, squares, and circles for ED_{50}, $_{90}$, and $_{99}$, respectively) are the actual doses used to accomplish these inhibition effects obtained from the data of Fig. 2A. (B) The combination index (CI) was calculated and plotted as the solid curve versus the percent inhibition (i.e., the fractional effect). Two dotted curves represent the 95% confidence intervals (1.96 standard deviations) of the CI.

region providing higher antiviral effects than ED_{99} (data not shown). The data clearly show that the stronger the antiviral effect, the more synergistic the effect of cotreatment becomes, though the cotreatment shows additive to antagonistic interactions at relatively low concentrations. A similar result was obtained by analyzing cotreatment of IFNα and NIM811 (data not shown). Based on our computational analysis, to induce a synergistic antiviral effect by cotreating with IFNα and cyclosporins, it is important to use doses representing more than 90% inhibition. Because the ED_{90} of NIM811 is less than that of CsA, NIM811 more strongly potentiates the antiviral effects of IFNα than does CsA at the same cotreatment dose.

The antiviral effects of cyclosporins alone or in combination with IFNα were sustained for over 10 days

To analyze the anti-HCV kinetics of the cyclosporins and cotreatment with a cyclosporin and IFNα, we treated cells with either cyclosporin, IFNα, or ribavirin alone, or IFNα in combination with either a cyclosporin or ribavirin for 3, 5, 7, and 10 days and measured the quantity of HCV replicon RNA (Fig. 5). CsA and NIM811, at 0.5 μg/ml, both decreased HCV replicon RNA in a time-dependent manner, resulting in about 2 and 2 logs reduction, respectively, of HCV RNA titers after 10 days of treatment, similar to 10 IU/ml IFNα. On the other hand, the combination of 10 IU/ml IFNα with 0.5 μg/ml CsA or NIM811 led to greater than 3 and around 4 logs reduction, respectively, of HCV RNA after 10 days of treatment. These effects were greater than that of cotreatment with IFNα and ribavirin (200 μM, which was the highest dose without significant cytotoxicity). Three weeks of treatment with CsA or NIM811 reduced HCV RNA to below detectable levels as assayed by real-time RT-PCR (data not shown). These results indicate that the strong antiviral effects of cyclosporin and NIM811 alones or in combination with IFNα were sustained over time and that viruses were eventually eliminated.

Cotreatment with CsA augmented the anti-HCV effects of IFNα without enhancing the IFNα signal transduction pathway

To investigate the mechanisms of action for the enhancing effects of cyclosporins on the anti-HCV activity of

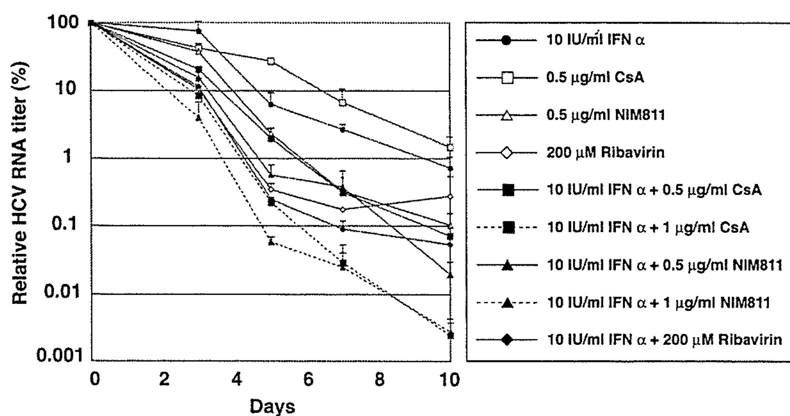

Fig. 5. Time course for the reduction of HCV RNA levels in NNC cells treated with CsA, NIM811, IFNα, or ribavirin. The levels of HCV RNA in the cells treated with the compounds for 3, 5, 7, and 10 days were determined by real-time RT-PCR and plotted vs. the days of treatment. The data represent the means of the results of three independent experiments.

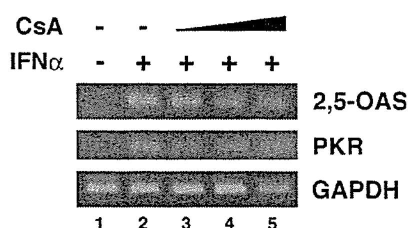

Fig. 6. Effects of the addition of CsA on the IFNα signal transduction pathway. NNC cells were treated either without (lane 1) or with 100 IU/ml IFNα (lanes 2–5) in combination with 0.5 μg/ml (lane 3), 1 μg/ml (lane 4), and 3 μg/ml (lane 5) CsA for 2 days. The mRNAs of 2′,5′-oligoadenylate synthetase (2′,5′-OAS) (upper panel), double-strand RNA-dependent protein kinase (PKR) (middle panel), and glyceraldehydes-3-phophate dehydrogenase (GAPDH) as an internal control (lower panels) were detected by RT-PCR.

IFNα, we examined the IFNα signal transduction pathway following the addition of a cyclosporin in NNC cells. The IFNα-induced upregulation of mRNA coding for 2′,5′-oligoadenylate synthetase (2′,5′-OAS) and double-strand RNA-dependent protein kinase (PKR), which are antiviral proteins downstream of IFNα, were not augmented by the cyclosporin cotreatment (Fig. 6). This result suggests that the IFNα-induced transcriptional activation was not altered by the cyclosporin treatment.

Discussion

We previously reported [8] that CsA and NIM811 suppress HCV replication. In the present study, we evaluated the anti-HCV effects of CsA and NIM811 in detail and revealed that these compounds achieve multiple-log reduction of HCV RNA levels in a cell culture system. NIM811 exhibited a more potent anti-HCV activity than did CsA, especially at relatively low concentrations. We previously demonstrated [9] that CyPB is a cellular replication cofactor that regulates the function of NS5B. CsA suppressed HCV replication via the dissociation of CyPB from NS5B [9]. In addition, NIM811 is reported to bind CyP with higher affinity (about 2-fold) than does CsA [15]. Taken together, the stronger anti-HCV activity of NIM811 over CsA at low concentrations may be due to NIM811's higher binding affinity to CyPB. In actuality, the strength of suppression of cyclosporins against vaccinia virus correlates with their inhibition/binding activities to CyP [16], in agreement with the above explanation. The higher anti-HCV activity of NIM811 at relatively low concentrations may be important for anti-HCV therapies in vivo because the trough level of CsA in the peripheral blood during the employment of CsA as an immunosuppressive agent during liver transplantation is 0.2–0.3 μg/ml (Peak cyclosporin levels are 0.8–2.3 μg/ml) [17–19]. Thus, NIM811 may eliminate HCV at the concentrations that are permissive in vivo, although other factors, such as pharmacodynamics and side effects, must be validated. Moreover, CsA might exert some pro-viral effects due to its immunosuppressive activity against T lymphocytes [20–22] in addition to its antiviral effects in hepatocytes. Thus, NIM811, which has little immunosuppressive function [15,23], is expected to be preferable to CsA for eliminating HCV in vivo.

Combining antiviral compounds that have different targets is effective in suppressing the emergence of drug-resistant viruses, as illustrated by the example of human immunodeficiency virus. Highly active antiretroviral combination therapy, which consists of a nucleoside backbone plus either a nonnucleoside reverse transcriptase inhibitor or a protease inhibitor, has dramatically decreased the mortality rate of AIDS patients [24]. Combining anti-HCV drugs might be one therapeutic approach to eradicate HCV, in addition to conventional therapy using IFNα, PegIFNα, or either compound in combination with ribavirin. In this study, we showed that both CsA and NIM811 exhibited enhanced anti-HCV effects in combination with IFNα. Importantly, a recent clinical study reported that the combination use of IFNα with CsA achieved a more sustained virological response than did CsA monotherapy [25]. This elevated antiviral effect with CsA cotreatment did not modify the IFNα signal transduction pathway (Fig. 6). Past candidates with anti-HCV potential, such

as protease inhibitors or polymerase inhibitors, which are now undergoing clinical trials, directly target viral proteins and inhibit their enzymatic activity. Because cyclosporins such as CsA and NIM811 target a cellular factor, CyPB, as described above, these compounds could serve as an additional type of anti-HCV agent. Moreover, viruses resistant to cyclosporins are less likely to occur, since antiviral compounds that target cellular factors generally induce less drug resistance than those inhibiting viral proteins; this difference is due to the high mutation rates of RNA viruses [26–30]. Thus, this novel anti-HCV candidate could provide an alternative strategy to combat HCV.

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Serum-derived hepatitis C virus infectivity in interferon regulatory factor-7-suppressed human primary hepatocytes

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See Editorial, pages 1–5

***Background/Aims*: The development of an efficient in vitro infection system for HCV is important in order to develop new anti-HCV strategy. Only Huh7 hepatocyte cell lines were shown to be infected with JFH-1 fulminant HCV-2a strain and its chimeras. Here we aimed to establish a primary hepatocyte cell line that could be infected by HCV particles from patients' sera.**

***Methods*: We transduced primary human hepatocytes with human telomerase reverse transcriptase together with human papilloma virus 18/E6E7 (HPV18/E6E7) genes or simian virus large T gene (SV40 T) to immortalize cells. We also established the HPV18/E6E7-immortalized hepatocytes in which interferon regulatory factor-7 was inactivated. Finally we analyzed HCV infectivity in these cells.**

***Results*: Even after prolonged culture HPV18/E6E7-immortalized hepatocytes exhibited hepatocyte functions and marker expression and were more prone to HCV infection than SV40 T-immortalized hepatocytes. The susceptibility of HPV18/E6E7-immortalized hepatocytes to HCV infection was further improved, in particular, by impairing signaling through interferon regulatory factor-7.**

***Conclusions*: HPV18/E6E7-immortalized hepatocytes are useful for the analysis of HCV infection, anti-HCV innate immune response, and screening of antiviral agents with a variety of HCV strains.**

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***Keywords*: Immortalization; Primary hepatocytes; HCV infection; IRF-7; IRF-3; HPV18/E6E7; Innate immune response**

1. Introduction

Infection with Hepatitis C virus (HCV) is a serious problem worldwide since 3% of the world's population is chronically infected [1]. Chronic HCV may lead to liver cirrhosis and hepatocellular carcinoma. Current standard therapy utilizes the combination of pegylated interferon-α and ribavirin, which results in a sustained response in only 30–60% of patients [2–5]. Many patients, however, do not qualify for or tolerate standard therapy [6]. Thus, it is important to develop an efficient in vitro infection system for HCV to facilitate the discovery of new anti-HCV strategies. Only Huh7 cell line is permissive for replication, infection and release of the fulminant hepatitis-derived HCV-2a (JFH-1) *strain* and its chimeric derivatives [7–9]. No other hepatocyte cell lines are able to support HCV replication efficiently.

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Normal human hepatocytes are the ideal system in which to study HCV infectivity. When cultured in vitro, however, they proliferate poorly and divide only a few times [10]. Continuous proliferation could be achieved however by introducing oncogenes, such as Simian virus large tumor antigen (SV40 T) [11]. This often resulted in tumor development [12] together with numerical (aneuploidy) and structural (aberrations) chromosome abnormalities [13]. The human papilloma virus E6E7 genes (HPV/E6E7) immortalized multiple cell types that were phenotypically and functionally similar to the parental cells [14–20]. As yet, no human hepatocytes have been immortalized with HPV18/E6E7.

We established a human primary non-neoplastic hepatocyte cell line transduced with the HPV18/E6E7 that retained primary hepatocyte characteristics even after prolonged culture, and were more prone to HCV infection than those cells immortalized with SV40 T antigen. We further improved the susceptibility of HPV18/E6E7-immortalized hepatocytes to HCV infectivity by impairing interferon regulatory factor-7 (IRF-7) expression. These cells are useful to assay infectivity of HCV strains other than JFH-1, HCV replication, innate immune system engagement of HCV, and screening of anti-HCV agents. This infection system using non-neoplastic cells also suggested that IRF-7 plays an important role in eliminating HCV infection.

2. Materials and methods

2.1. Cell cultures

We obtained the approval of the Ethical Committee of Kyoto University for the use of human hepatocytes and sera obtained from HCV-positive patients. Informed consent was obtained from both the hepatocyte donor and HCV-positive patients. Primary hepatocytes (P.H.) were cultured as described [21]. HeLa, 293, Huh-7.5, and PH5CH8 cells were cultured as previously described [22]. For three-dimensional (3D) cultures, Mebiol Gel (Mebiol Inc.) was prepared according to the manufacturer's instructions.

2.2. Plasmids construction

The SV40 T, hTERT and HPV/E6E7 fragments from pAct-SVT, PCX4neo/hTERT, and pLXSN-E6E7 plasmids were inserted into pCSII-EF-RFA plasmid creating the pCSII-EF-SVT, pCSII-EF-hTERT, and pCSII-EF-E6E7 plasmids, respectively. The full-length IRF-3 and IRF-7 genes were cloned by RT-PCR using total RNA isolated from 293 cells as a template and were inserted into pcDNA3 vector. Dominant-negative forms of IRF-3 (DNIRF-3) and IRF-7 (DNIRF-7) were constructed by PCR amplification of the coding region for amino acid residues 108–427 of IRF-3 and 237-514 of IRF-7, respectively. The amplified IRF-3 fragment was cloned into pcDNA3 in frame with a FLAG epitope tag generating pcFLAG-DNIRF-3. The amplified IRF-7 fragment was cloned into pLXSH in frame with HA epitope tag generating pLXSH-HA-DNIRF-7. The pIFNβ promoter-luc and pIFNα promoter-luc plasmids were gifts from Dr. Taniguchi of the Tokyo University. The psiRNA-hIRF-3 and psiRNA-hIRF-7 plasmids were purchased from Invivogen (USA).

2.3. Immunoblot analysis

Immunoblot analysis was performed as described previously [22]. We used anti-SV40 T (Santa Cruz), anti-HPV18/E7 (Santa Cruz), anti-tubulin (Sigma), anti-FLAG (Sigma), and anti-HA (Sigma) antibodies.

2.4. Transfection, small interfering RNA silencing and luciferase assays

Transfection of plasmid DNA was performed using Effectene transfection reagent (Qiagen) as recommended by the manufacturer. The pLXSH-HA-DNIRF-7 plasmid was transfected into the HuS-E/2 clone; transfectants were selected in 100 μg/ml hygromycin B (Gibco). The psiRNA-hIRF-3 and psiRNA-hIRF-7 plasmids were separately transfected into HuS-E/2 cells followed by Zeocin (250 μg/ml) selection. After two weeks of continuous selection, cells were infected with HCV. Luciferase assays were conducted as previously described [22]. The results are presented as relative light units (RLU) normalized to the total content of protein in the cell lysates.

2.5. Reverse transcriptase polymerase chain reaction (RT-PCR) and real-time RT-PCR

Using 250 ng of total RNA as a template, we performed RT-PCR with a one-step RNA PCR kit (Takara) according to the manufacturer's instructions. The primer sets and reaction conditions used are detailed in Table 1. To measure HCV-RNA titers by real-time RT-PCR, we collected RNA from infected wells. Five hundred nanograms of total cellular RNA was analyzed for the quantity of HCV-RNA as previously described [23].

2.6. HCV infection experiment

HCV infection experiment from serum was done as mentioned before [22]. HCV-infected-serums were titrated and 1×10^5 HCV-RNA copies/ml were used for each infection experiment. Concentrated culture medium for HCV/JFH1-producing cells was prepared as previously described [7]. HCV titer in the concentrated medium was measured, adjusted and added to the cells as mentioned above.

2.7. Blocking of HCV infectivity by anti-CD81

Inhibition of HCV infectivity was performed by blocking CD81 as previously described [7].

3. Results

3.1. Establishment of immortalized primary human hepatocytes

Primary hepatocytes were isolated from liver tissue obtained from a 9-year-old male patient with Primary Hyperoxaluria who had undergone liver transplantation. Hepatocytes were left unmanipulated or transduced with CSII-EF-hTERT alone or in combination with CSII-EF-SVT or CSII-EF-E6E7 to enhance the efficiency of immortalization. After six weeks only cells transduced by the combination of hTERT and either LT or HPV18/E6E7 continued to proliferate. Initially appearing colonies with a growth advantage were picked up and expanded. SV40 T-immortalized cell clones were named HuS-T cells and given numbers from 1 to 7,

Table 1
Primer sequences and RT-PCR parameters

Genes	Primer sequence 5′–3′	PCR parameters[a]
HGF	F: AGGAGCCAGCCTGAATGATGA R: CCCTCTGATGTCCCAAGATTAGC	95, 56, 72 1 min, 45 s, 1 min
TGFα	F: ATGGTCCCCTCGGCTGGA R: GGCCTGCTTCTTCTGGCTGGCA	95, 59, 72 45 s, 30 s, 1 min
TGFβ1	F: GCCCTGGACACCAACTATTGCT R: AGGCTCCAAATGTAGGGGCAGG	95, 58, 72 45 s, 30 s, 1 min
TGFβ2	F: GATTTCCATCTACAAGACCACGAGGGACTTGC R: CAGCATCAGTTACATCGAAGGAGAGCCATTCG	95, 58, 72 45 s, 30 s, 1 min
HGFR	F: TGGTCCTTGGCGTCGTCCTC R: CTCATCATCAGCGTTATCTTC	95, 54, 72 30 s, 45 s, 1 min
EGFR	F: CTACCACCACTCTTTGAACTGGACCAAGG R: TCTATGCTCTCACCCCGTTCCAAGTATCG	95, 58, 72 45 s, 30 s, 1 min
TGFβ1R	F: CGTGCTGACATCTATGCAAT R: AGCTGCTCCATTGGCATAC	95 s, 54, 72 30 s, 45 s, 1 min
TGFβ2R	F: TGCACATCGTCCTGTGGAC R: GTCTCAAACTGCTCTGAAGTGTTC	95, 58, 72 45 s, 30 s, 1 min
FGFR	F: ATGTGGAGCTGGAAGTGCCTC R: GGTGTTATCTGTTTCTTTCTCC	95, 54, 72 30 s, 45 s, 1 min
IGF-1R	F: ACCCGGAGTACTTCAGCGCT R: CACAGAAGCTTCGTTGAGAA	95, 54, 72 30 s, 45 s, 1 min
HNF1α	F: GTGTCTACAACTGGTTTGCC R: TGTAGACACTGTCACTAAGG	95, 52, 72 45 s, 30 s, 1 min
HNF1β	F: GAAACAATGAGATCACTTCCTCC R: CTTTGTGCAATTGCCATGACTCC	95, 52, 72 1 m, 45 s, 1 min
HNF3β	F: CACCCTACGCCTTAACCAC R: GGTAGTAGGAGGTATCTGCGG	95, 56, 72 1 m, 45 s, 1 min
HNF4	F: CTGCTCGGAGCCACAAAGAGATCCATG R: ATCATCTGCCACGTGATGCTCTGCA	95, 58, 72 45 s, 30 s, 1 min
Albumin	F: AGTTTGCAGAAGTTTCCAAGTTAGTG R: AGGTCCGCCCTGTCATCAG	95, 55, 72 45 s, 30 s, 1 min
Apolipoprotein-a	F:AGGCTCGGCATTTCTGGCAG R: TATCCCAGAACTCCTGGGTC	95, 55, 72 45 s, 30 s, 1 min
HTF	F: TCGCTACAGCCTTTGCAATG R: TTGAGGGTACGGAGGAGTTCC	95, 55, 72 45 s, 30 s, 1 min
E-cadherin	F: TCCATTTCTTGGTCTACGCC R: TTTGTCCTACCGACTTCCAC	95, 55, 72 45 s, 30 s, 1 min
CYP 1B1	F: CACCAAGGCTGAGACAGTGA R: GCCAGGTAAACTCCAAGCAC	94, 57, 72 30 s, 30 s, 1 min
CYP 2C9	F: GGACAGAGACGACAAGCACA R: TGGTGGGGAGAAGGTCAAT	94, 57, 72 30 s, 30 s, 1 min
CYP 2B	F: GGCACACAGCCAAGTTTACA R: CCAGCAAAGAAGAGCGAGAG	94, 57, 72 30 s, 30 s, 1 min
CYP 3A4	F: TGTGCCTGAGAACACCAGAG R: GCAGAGGAGCCAAATCTACC	94, 57, 72 30 s, 30 s, 1 min
CYP 2E1	F: CCGCAAGCATTTTGACTACA R: GCTCCTTCACCCTTTCAGAC	94, 57, 72 30 s, 30 s, 1 min
CYP 1A1	F: AGGCTTTTACATCCCCAAGG R: GCAATGGTCTCACCGATACA	94, 57, 72 30 s, 30 s, 1 min
GAPDH	F: CCATGGAGAAGGCTGGGG R: CAAAGTTGTCATGGATGACC	95, 8, 72 45 s, 30 s, 1 min

Table 1 (*continued*)

Genes	Primer sequence 5′–3′	PCR parameters[a]
CD81	F: CTCAACTGTTGTGGCTCCAAC R: CCAATGAGGTACAGCTTCCC	95, 55, 72 45 s, 30 s, 1 min
TLR3	F: GATCTGTCTCATAATGGCTTG R: GACAGATTCCGAATGCTTGTG	95, 55, 72 45 s, 30 s, 1 min
TLR7	F: CCAGACATCTCCCCAGCGTC R: GGCAAAACAGTAGGGACGGC	95, 55, 72 45 s, 30 s, 1 min
TLR8	F: CTGTGAGTTATGCGCCGAAG R: CGGGATTTCCGTTCTGGTGC	95, 55, 72 45 s, 30 s, 1 min
Myd88	F: GGTCTCCTCCACATCCTCCC R: CCAGCTTGGTAAGCAGCTCG	95, 55, 72 45 s, 30 s, 1 min
IRF3	F: GAACCCCAAAGCCACGGATC R: CCTCCCGGGAACATATGCAC	95, 55, 72 45 s, 30 s, 1 min
IRF7	F: GTGCTGTTCGGAGAGTGGCTC R: CAGCCCAGGCCTTGAAGATG	95, 55, 72 45 s, 30 s, 1 min

CYP, cytochrome P450; EGFR, epidermal growth factor receptor; F, forward primer; FGFR, fibroblast growth factor receptor; GAPDH, glyceraldehyde phosphate dehydrogenase; HGF, hepatocyte growth factor; HGFR, hepatocyte growth factor receptor; HNF, hepatocyte nuclear factor; HTF, human transferrin; IGF-1R, insulin-like growth factor-type I receptor; IRF, interferon regulatory factor; R, reverse primer; TGF, transforming growth factor; TGFR, transforming growth factor receptor; TLR, toll like receptor.

[a] Temperatures are tabulated in the first lane in degrees celsius and the corresponding times in the second lane. Performing one-step RT-PCR, reverse transcription was carried out at 42 °C for 20 min with a pre-PCR denaturation at 95 °C for 10 min.

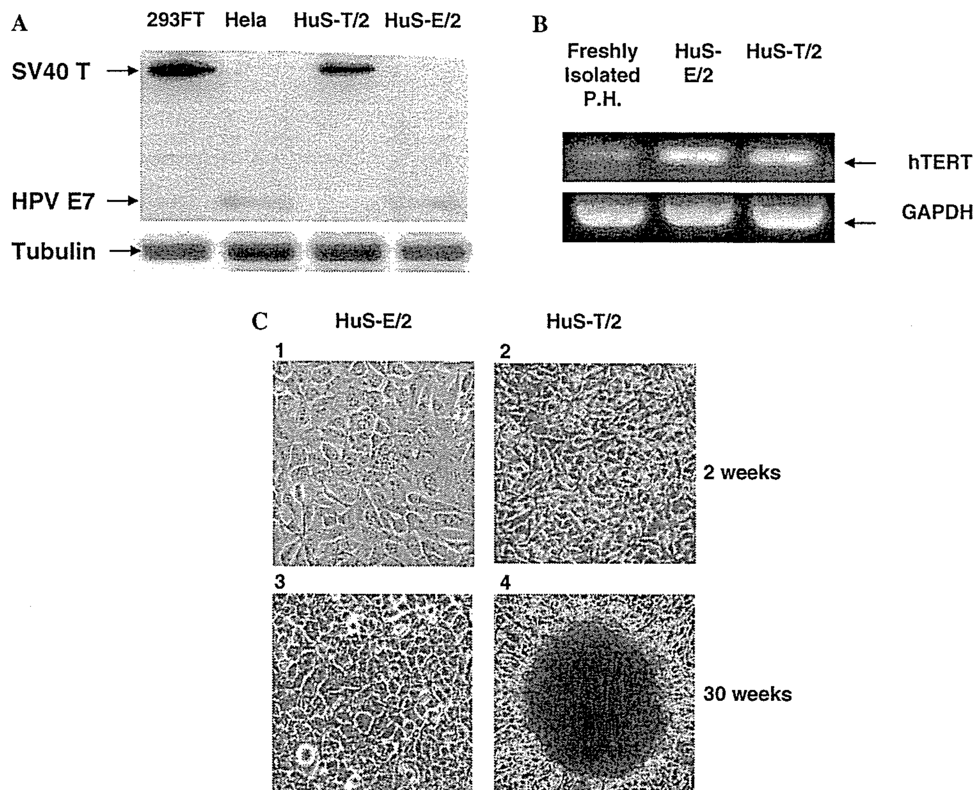

Fig. 1. (A) Immunoblot detection of SV40 T and HPV E7 expression in HuS-T/2 and HuS-E/2 cells, respectively. 293-FT and HeLa cells were used as positive controls for SV40 T and HPV E7 expression, respectively. The specific bands representing the targets are indicated. Detection of tubulin expression in all cells served as an internal control. (B) Human Telomerase Reverse Transcriptase (hTERT) expression was examined by RT-PCR in freshly isolated hepatocytes and the HuS-E/2 and HuS-T/2 cell lines. GAPDH expression was used as an internal control. The hTERT-specific bands are shown. (C) Morphological characteristics of HuS-E/2 and HuS-T/2 cells after two (panels 1 and 2) and 30 (panels 3 and 4) weeks in culture. [This figure appears in colour on the web.]

while the HPV18/E6E7-immortalized clones were named HuS-E cells and given numbers from 1 to 4. Expression of SV40 T and HPV E7 proteins was detected in the appropriate cells by immunoblot analysis (Fig. 1A). In both immortalized cell lines, expression of hTERT-mRNA was enhanced in comparison to non-transduced, freshly isolated hepatocytes as determined by RT-PCR (Fig. 1B). HuS-E cells were larger in size and exhibited slower growth than HuS-T cells (Fig. 1C).

3.2. Characterization of HuS-E and HuS-T immortalized hepatocytes

The HuS-E/2 and HuS-T/2 clones demonstrated the highest expression of hepatocyte-specific markers and transcription factors by RT-PCR (data not shown); these cells were used as representative for each group in this study. To address if HuS-E/2 and HuS-T/2 maintained similar characteristics as primary hepatocytes, they were both cultured continuously for 30 weeks and the expression profiles of a variety of growth factors (Fig. 2A), growth factor receptors (Fig. 2B), hepatocyte-specific nuclear factors (Fig. 2C), albumin, apolipoprotein-A1, transferrin (Fig. 2D), cytochrome p450 (CYP) genes (Fig. 2E), and GAPDH were compared with freshly isolated primary hepatocytes after isolation or two weeks of culture, Huh-7.5 cells, and 293 cells. After two weeks in culture, the expression of nearly all examined genes was similar between freshly isolated hepatocytes and the HuS-E/2 cell line. HuS-E/2 cells, however, exhibited higher expression of TGFβ2 (Fig. 2A), TGFβ2R, and HGFR (Fig. 2B) and lower expression of CYP 3A4 and 2C9 (Fig. 2E) in comparison to freshly isolated hepatocytes. Primary hepatocytes displayed reduced expression of TGFβ1 and TGFβ2 (Fig. 2A) and a loss of CYP1A1 expression (Fig. 2E) after two weeks of culture. HuS-E/2 cells exhibited higher expression of HGF (Fig. 2A), HGF receptor (Fig. 2B), HNF-4, (Fig. 2C), albumin, apolipoprotein-A1, HTF, and E-cadherin (Fig. 2D) in comparison to HuS-T/2 cells. Expression of CYP 3A4 (Fig. 2E) was lost from both HuS-T/2 and HuS-E/2 cells, while HuS-T/2 cells also lost the expression of HNF-1α (Fig. 2D), and CYPs 2B, 2E1 (Fig. 2E).

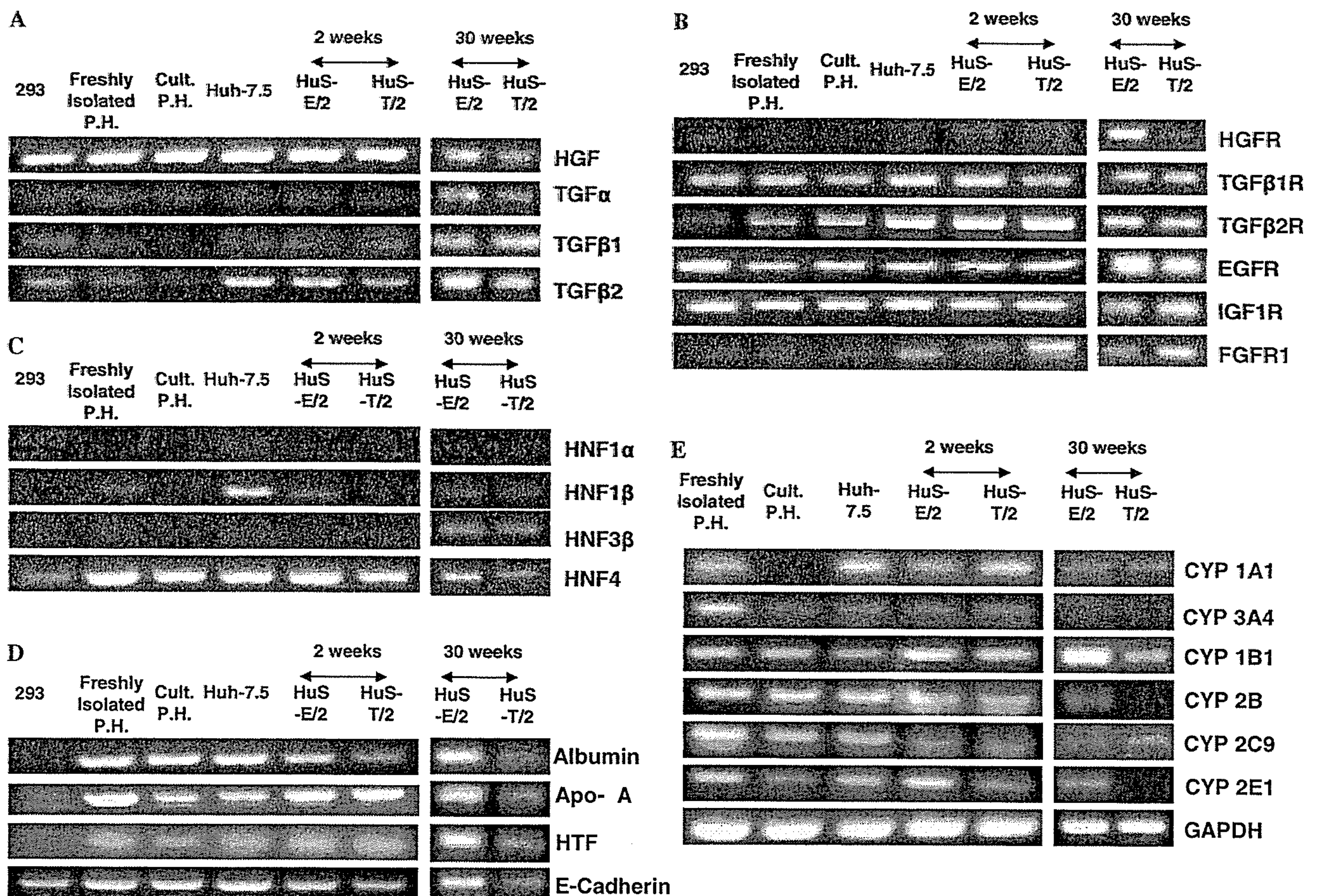

Fig. 2. Expression of the genes encoding growth factors (A), growth factor receptors (B), hepatocyte-specific nuclear factors (C), hepatocyte differentiation and functional markers (D), and CYP enzymes (E) in 293 cells, freshly isolated primary hepatocytes (P.H.), primary hepatocytes cultured for two weeks (Cult. P.H.), Huh-7.5 cells, and HuS-E/2 and HuS-T/2 cells cultured for two and 30 weeks were investigated by RT-PCR. The bands representing specific targets are indicated in the representative reactions.

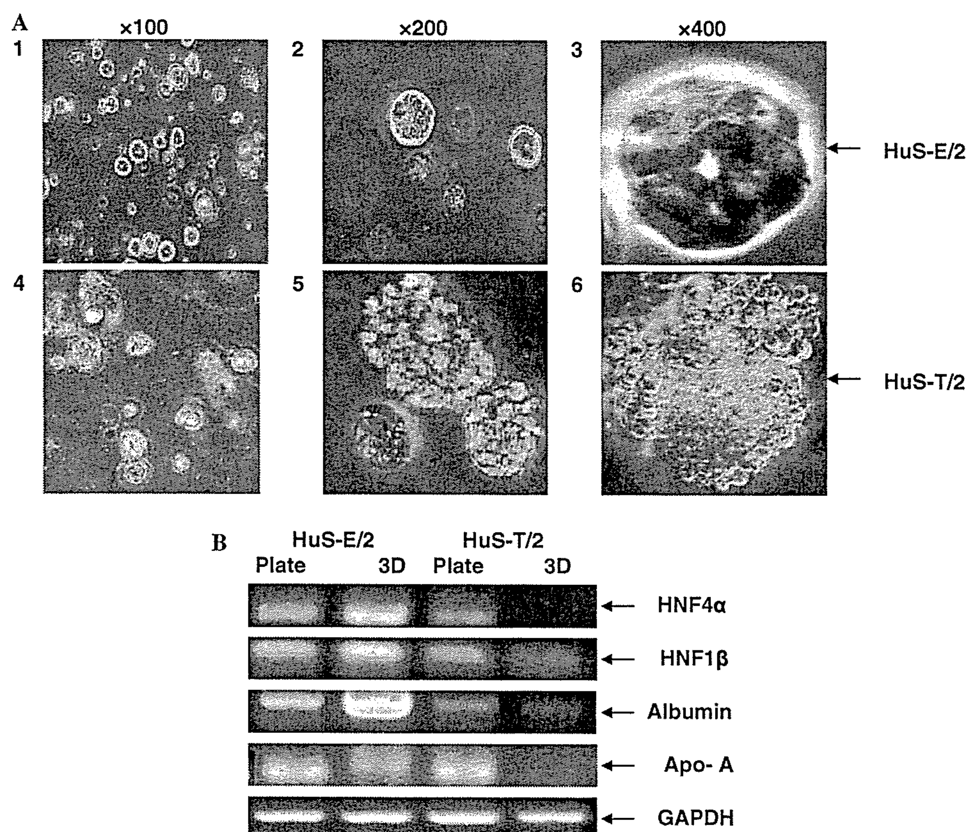

Fig. 3. (A) The morphology of HuS-E/2 and HuS-T/2 cells in 3D culture. HuSE/2 and HuS-T/2 cells were cultured in Mebiol Gel in 12-well plates at a concentration of 5×10^5 cells/well. The microscopic characteristics of these cells after one week of 3D culture are shown. (B) The expressions of HNF4α, HNF1β, albumin, and apo-A by HuS-E/2 and HuS-T/2 cells in both flat and 3D cultures are detailed. After one week of culture of HuS-E/2 and HuS-T/2 cells in flat and 3D cultures, the expressions of HNF4α, HNF1β, albumin, and apo-A were measured by RT-PCR in 250 ng total RNA.

HuS-T/2 but not in HuS-E/2 cells showed a transformed-like character starting from the 13th week of culture. This was demonstrated by continuing proliferation after confluence, pile-up formations (Fig. 1C), and proliferating in serum-deprevied condition. However, HuS-E/2 cells did not show any transformed-like characters even after 30 weeks of culture.

3.3. The characteristics of HuS-E and HuS-T immortalized hepatocytes in 3D culture

After one week in 3D culture, HuS-E/2 (Fig. 3A, panels 1, 2, and 3) cells adopted a donut-shaped structure with a central pore, while HuS-T/2 cells (Fig. 3A, panels 4, 5, and 6) displayed irregular mass formations (similar to the growth pattern of Huh-7.5 cells in 3D culture (data not shown)). In 3D culture, while the expression of HNF4, HNF1β, and albumin was enhanced in HuS-E/2, it was decreased in HuS-T/2 cells (Fig. 3B).

3.4. HCV infection to HuS-E/2

We further assessed the HCV infectivity of HuS-E- and HuS-T-derived clones by infection with HCV-1b-infected serum. Of the three HuS-E clones examined, HuS-E/2 clone demonstrated the highest infectability with HCV genotype 1b in comparison to Huh-7.5, PH5CH8 (Fig. 4A), and HuS-T cells (data not shown), which were excluded from further experiments.

3.5. Anti-CD81 blocked HCV infectivity

CD81 is involved in the entry of HCV pseudoparticles [24] and in vitro-synthesized JFH-1 [7]. To determine if authentic viral particles follow the same route of entry when infecting HuS-E/2 cells, we first examined the CD81 expression by RT-PCR. Both HuS-E/2 and HuS-T/2 cells expressed similar amounts of CD81 as freshly isolated hepatocytes and Huh-7.5 cells (Fig. 4B). Antibodies against CD81 reduced HCV infectivity of HuS-E/2 cells from the levels seen using a nonspecific control antibody, confirming the importance of CD81 in HCV infectivity (Fig. 4C).

3.6. IFNα blocked HCV infectivity

We treated HuS-E/2 cells with HCV-containing serum. Cells were then cultured in fresh medium supplemented

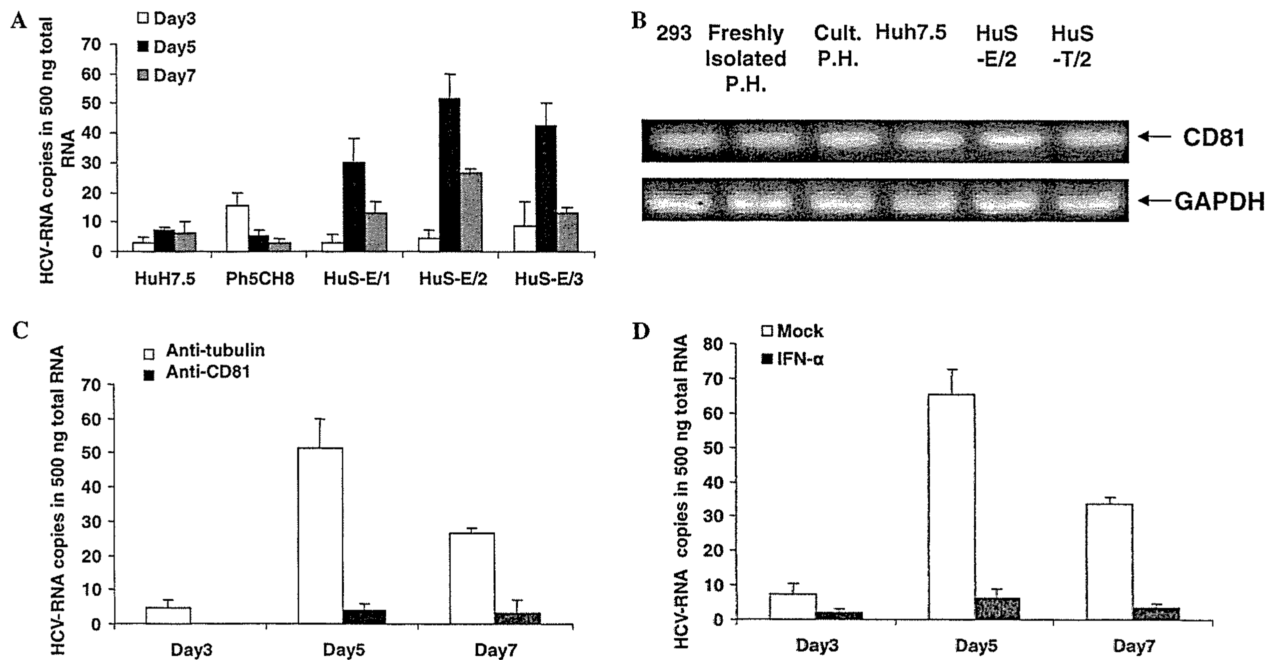

Fig. 4. (A) Serum from an HCV patient was used to infect Huh-7.5 cells, PH5CH8 cells, and three HPV E6E7-immortalized clones (HuS-E/1-3) for 24 h. After washing three times in phosphate-buffered saline (PBS), cells were cultured in fresh medium. Cells were then harvested and lysed at the indicated time points. The quantity of HCV genome RNA per 500 ng total RNA was determined by real-time RT-PCR analysis. (B) HuS-E/2 and HuS-T/2 cells both expressed CD81. Expression of CD81 (upper panel) and GAPDH as an internal control (lower panel) in 293 cells, freshly isolated P.H., cultured P.H., and Huh-7.5, HuS-E/2, and HuS-T/2 cells was investigated by RT-PCR. (C) Anti-CD81 antibodies blocked HCV infectivity. HCV infection was performed as described in (A) with the addition of CD81-specific (black bar) or anti-tubulin antibodies (control, white bar). (D) IFNα inhibits HCV multiplication in HuS-E/2 cells infected with HCV-containing serum. HuS-E/2 cells were infected with HCV as described in (A). After washing three times with PBS, cells were cultured in fresh medium supplemented with (black bar) or without (white bar) 100 U/ml IFNα.

without or with 100 U/ml IFNα. The enhancement of the HCV-RNA genome titers on the fifth day (about 10-fold) was not observed in cells treated continuously with IFNα (Fig. 4D). This result suggests that IFNα inhibited HCV replication in infected HuS-E/2 cells.

3.7. The effect of blocking IRF-3 and IRF-7 signaling on HCV infectivity

Production of interferon-alpha (IFNα) and interferon-beta (IFNβ) limits viral replication and spread, providing one of the most effective innate antiviral responses [25]. Signaling through IRF-3 and IRF-7 plays important roles in the stimulation of IFN-α/β production [25]. To determined which molecules (IRF-3 or IRF-7) play an important role in modulation of the innate immune response against HCV infection in these cells, we first detected intrinsic expression of double-stranded RNA-stimulated Toll-like receptor (TLR) 3, the downstream effector IRF-3, single-stranded RNA-stimulated TLR7, and 8, and the downstream effectors MyD88 and IRF-7 by RT-PCR. TLR3 exhibited very low expression in freshly isolated hepatocytes, Huh-7.5, HuS-E/2, and HuS-T/2 cells, while TLR7, TLR8, MyD88, and IRF-7 were easily detectable in both freshly isolated and immortalized cell lines (Fig. 5A).

The abilities of DNIRF-3 and DNIRF-7 to inhibit IFNβ and IFNα production by HuS-E/2 cells infected with Sendai virus were confirmed using assays of IFNβ or IFNα promoter-driven luciferase reporters. DNIRF-3 exhibited strong inhibition of IFNβ production (Fig. 5B) and weaker inhibition of IFNα transcription (Fig. 5C), while DNIRF-7 strongly inhibited IFNα production (Fig. 5C) and only weakly inhibited IFNβ production (Fig. 5B).

We then assessed the inhibition of HCV infectivity by DNIRF-3 and DNIRF-7. Transient transfection with DNIRF-3, DNIRF-7, or an empty vector was performed prior to HCV infection. Using Effectene reagent, the efficiency of plasmid transfection into HuS-E/2 cells was approximately 70% (data not shown). While there was no significant effect of DNIRF-3 on HCV infectivity, DNIRF-7 demonstrated a marked increase in HCV titers on days 3 and 5 after infection in comparison to control cells (Fig. 5D). To confirm that the enhancement of HCV replication by DNIRF-7 is not mediated by the impairment of IRF-3 signaling by heterodimeric interactions between IRF-3 and DNIRF-7, we performed siRNA inhibition of IRF-3 and IRF-7. The reduction of IRF-3 and IRF-7 expression by siRNA was obvious by RT-PCR (Fig. 5E). siRNA-mediated suppression of either IRF-3 or IRF-7 inhibited IFNβ and IFNα production

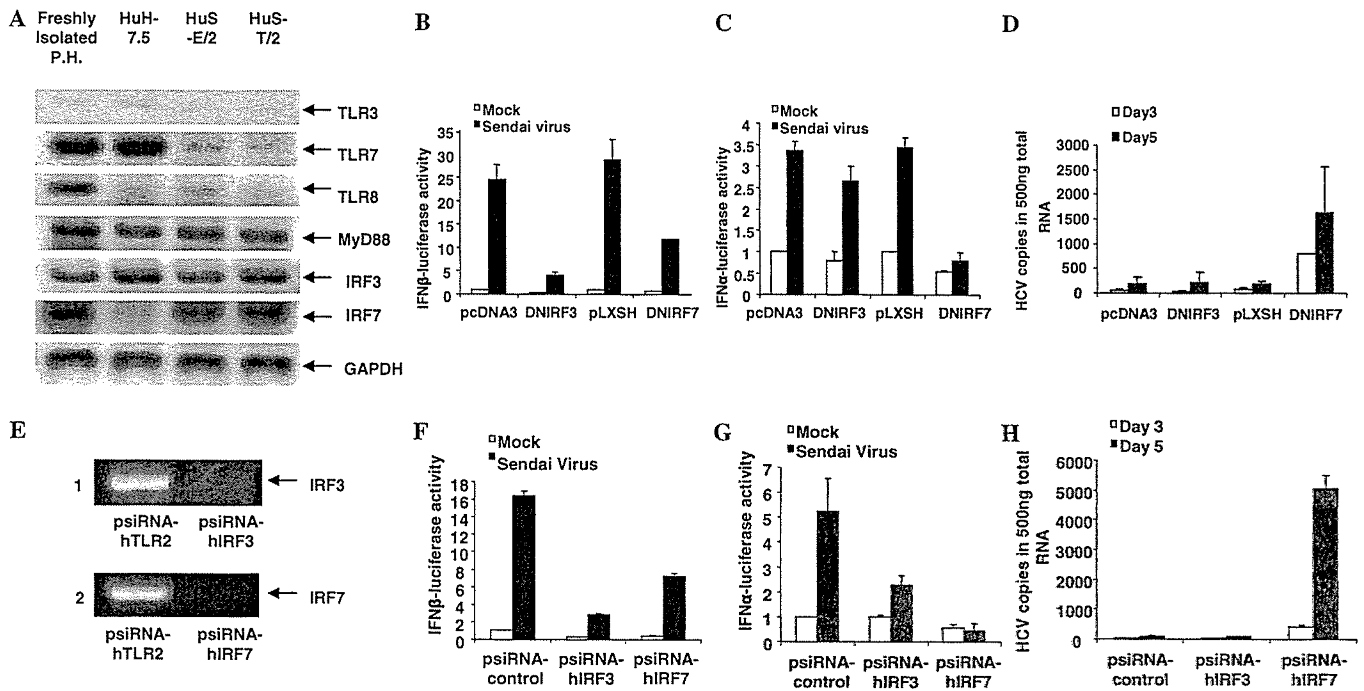

Fig. 5. (A) We examined the expression of TLR3, TLR7, TLR8, MyD88, IRF-3, and IRF-7, as well as GAPDH as an internal control in freshly isolated primary hepatocytes and Huh-7.5, HuS-E/2, and HuS-T/2 cells was investigated by RT-PCR. (B and C) HuS-E/2 cells were cotransfected with pIFNβ-luc (B) or pIFNα-luc (C) with an expression plasmid encoding DNIRF-3, DNIRF-7, or the appropriate empty vector (pcDNA3 and PLXSH, respectively). Twenty-four hours later, cells were infected (black bar) with Sendai virus or mock-infected (white bar), then analyzed for luciferase activity after 12 h. (D) IRF-7, but not IRF-3, suppression enhanced HCV infectivity of HuS-E/2 cells. HuS-E/2 cells were transiently transfected with empty pcDNA3, DNIRF-3, empty pLXSH, or DNIRF-7 plasmids. Twenty-four hours later, serum from a patient with HCV was used to infect transfected cells for 24 h. After washing, cells were cultured in fresh medium. The cells were then harvested and lysed at the indicated time points. The quantity of HCV genome RNA per 500 ng total RNA was determined by real-time RT-PCR analysis. (E) IRF-3 and IRF-7 levels were suppressed by specific siRNAs. HuS-E/2 cells were transfected with control psiRNA-hTLR2, psiRNA-hIRF-3, or psiRNA-hIRF-7, then selected with Zeocin at 250 μg/ml. Two weeks later, cells were harvested and assessed for the expression of IRF-3 and IRF-7 by RT-PCR. (F and G) HuS-E/2 cells were transfected with control psiRNA-hTLR2, psiRNA-hIRF-3, or psiRNA-hIRF-7, followed by selection in Zeocin at 250 μg/ml. Two weeks later, cells were cotransfected with pIFNβ-luc (F) or pIFNα-luc (G). Twenty-four hours later, cells were infected (black bar) with Sendai virus or mock-infected (white bar), then analyzed for luciferase activity after 12 h. (H) Transfected cells were infected with serum from HCV patient; HCV infectivity was assessed as described above.

in HuS-E/2 cells infected with Sendai virus in patterns similar to the effects seen following DNIRF-3 and DNIRF-7 expression, respectively (Figs. 5F and G). Blockade of IRF-7 expression resulted in a significantly higher titer of HCV after infection, while IRF-3 down-regulation did not have any significant effect on HCV titers (Fig. 5H). The enhancement of IRF-7 silencing by siRNA improved the infectivity of HCV (data not shown). These results suggest that IRF-7 plays the major role in the innate immune response to HCV in HuS-E/2 cells.

3.8. Establishment of stable DNIRF-7 expressing clones derived from HuS-E/2 cells

Since DNIRF-7 enhanced HCV infectivity, we transduced the plasmid encoding DNIRF-7 and a hygromycin-B resistance gene, into HuS-E/2 cells. Following selection with hygromycin-B, we obtained the HuS-E7/DN22 and HuS-E7/DN24 clones. As detected by RT-PCR, both clones demonstrated similar expression levels of albumin, apolipoprotein-A1, and HNF4 as the parental HuS-E/2 cells (Fig. 6A). The HuS-E7/DN24 clone exhibited stronger expression of DNIRF-7 than the HuS-E7/DN22 clone by immunoblotting (Fig. 6B). The induction of IFNα in HuS-E7/DN24 in response to infection with an RNA virus (Sendai virus) was low in comparison to the parental HuS-E/2 and HuS-E7/DN22 clones, as detected by IFNα-luciferase reporter assay (Fig. 6C). HuS-E7/DN24 also exhibited a higher HCV infectability in comparison to parental HuS-E/2 cells and the HuS-E7/DN22 clone (Fig. 6D).

3.9. Infection of HuS-E7/DN24 cells with different HCV genotypes

Huh7.5 and HuS-E7/DN24 cells were separately infected with serums derived from 3 different HCV-patients or by JFH-1 concentrated medium (HCV-2a). Two serums were infected by HCV-1b, while the third by HCV-2b. Inoculated virus titer was adjusted to be the same in all cases. Except for JFH-1, which efficiently

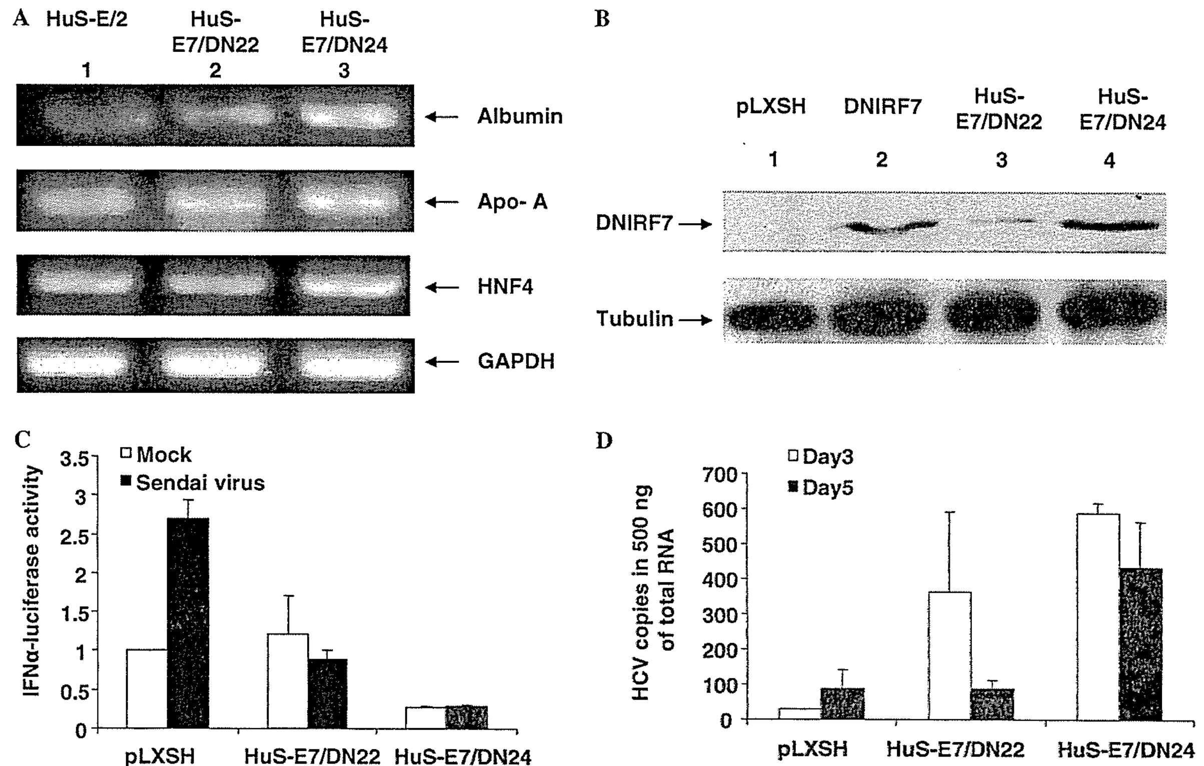

Fig. 6. (A) The pLXSH-HA-DNIRF-7 plasmid was transfected into HuS-E/2 cells, followed by selection in 100 μg/ml Hygromycin B. Two clones, HuS-E7/DN22 (lane 2) and HuS-E7/DN24 (lane 3), were obtained. We investigated the expression of albumin, apo-A, HNF4, and GAPDH as an internal control in parental HuS-E/2, HuS-E7/DN22, and HuS-E7/DN24 hepatocytes cultured for two weeks by RT-PCR. (B) Expression of HA-tagged DNIRF-7 (upper panel) and tubulin (control, lower panel) was detected by immunoblotting analysis. HuS-E/2 cells transiently transfected with either empty pLXSH vector (lane 1) or pLXSH-HA-DNIRF-7 (lane 2) were used as negative and positive controls, respectively, after 48 h. (C) HuS-E/2, HuS-E7/DN24, and HuS-E7/DN22 cells were transfected with IFNα-luc. HuS-E/2 cells were also cotransfected with pLXSH. All of these cells were then infected (black bar) or with Sendai virus or mock-infected, then analyzed for luciferase activity after 12 h. (D) HuS-E7/DN24 cells exhibited high infectivity to HCV samples derived from patient serum. HuS-E/2 cells were transiently transfected with empty pLXSH. Twenty-four hours later, serum from a recurrently transplanted HCV patient was used to infect transfected cells and HuS-E7/DN22 and HuS-E7/DN24 cells for 24 h. After washing three times, cells were cultured in fresh medium. Cells were then harvested and lysed at the indicated time points.

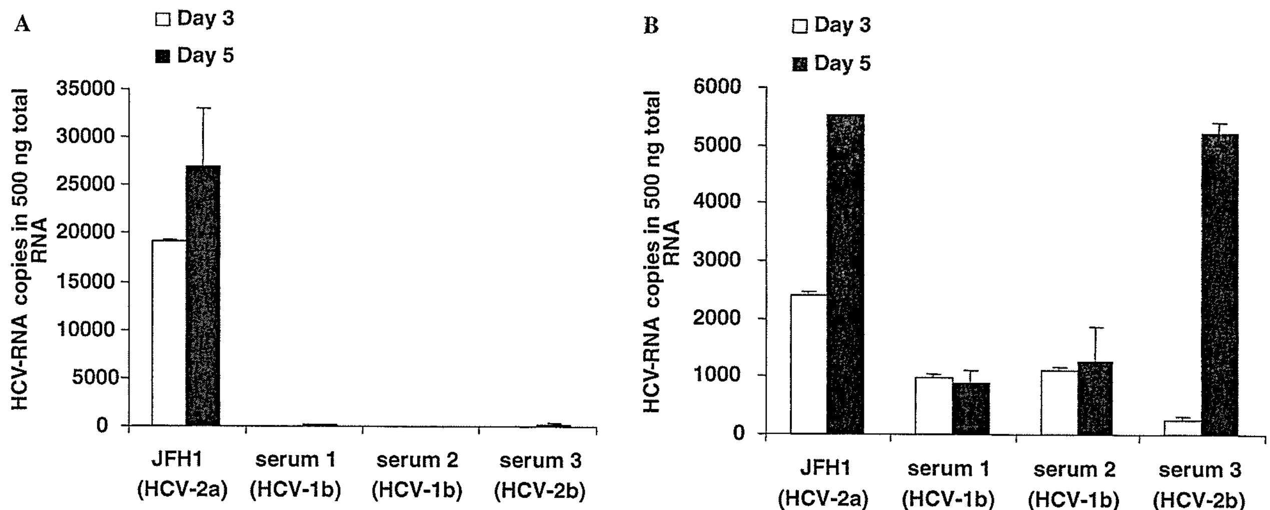

Fig. 7. The infectability of Huh-7.5 and HuS-E7/DN24 cells to different HCV genotypes. Huh-7.5 (A) and HuS-E7/DN24 (B) cells were infected with same titer of JFH1 (HCV-2a), two different HCV-1b serums and one HCV-2b serum. After removing the infected medium, the cells were washed in PBS and recultured in fresh medium. Cells were harvested and lysed at the indicated time points. The quantity of HCV genome RNA per 500 ng RNA was detected by real-time RT-PCR analysis.

replicated in Huh7.5 cells (Fig. 7A), HuS-E7/DN24 cells showed a higher and reproducible infectability for the different HCV strains than Huh7.5 cells (Fig. 7B). Similar higher infectability of HuS-E7/DN24 cells was observed with HCV-4a genotype (unpublished data). These results suggest that the high infectability of Huh-7.5 with JFH-1 is specific among the combinations of HCV strains and cell lines; while HuS-E7/DN24 cells were generally permissive to HCV-infected serum independent of HCV strains.

4. Discussion

This study demonstrates that ectopic expression of the HPV18/E6E7 genes in combination with hTERT could efficiently immortalize mature human hepatocytes, generating a cell line with stable expression of hepatocyte markers and functions for more than 30 weeks in culture. HuS-E/2 cells continuously exhibited higher expression of both HGF and HGFR than HuS-T/2 cells. This result suggests that HPV18/E6E7-immortalized hepatocytes maintain responsiveness to paracrine signals capable of inducing cell differentiation to a greater extent than SV40 T-immortalized hepatocytes. This conclusion is further supported by the increased expression of HNF4 in HuS-E/2 cells in comparison to HuS-T/2 cells. HNF4 is a major hepatocyte transcription factor, required for hepatocyte differentiation and liver-specific gene expression [26]. HNF4 drives hepatocytes differentiation by acting upstream in a transcription factor cascade that included HNF1α [27]. HuS-E/2 cells continued to express HNF1α throughout prolonged culture, while HuS-T/2 cells lost expression completely. Maintenance of hepatocellular functions was demonstrated by continuous and high expression of albumin, apolipoprotein-A, human transferrin, and E-cadherin by HuS-E/2 in comparison to HuS-T/2 cells. These differences became more pronounced in the late passages. In a similar manner, HuS-E/2 cells continued to express all of the examined CYP genes, with the exception of CYP 3A4, while HuS-T/2 cells lost expression of CYP 3A4, 1B, and 2E1 completely and displayed markedly lower expression of CYP 1B1 than HuS-E/2 cells. Thus, human hepatocytes immortalized by HPV E6/E7 transfection are phenotypically similar to primary hepatocytes, even during extended cultures.

Recently, it was reported that the JFH-1 strain and derived chimeras could only infect and propagate efficiently in Huh7.5.1 and Huh7.5 cells, both of which are subclones of Huh7 cells [7–9]. This limitation, however, may be specific to the JFH-1 **strain**, which may not accurately reflect the course of other HCV strains' infection. Thus, usage of HCV particles isolated from patient serum could be more useful to study authentic HCV infection. Using sera from HCV patients as a source of infective virus, HPV18/E6E7-immortalized cell lines exhibited higher reproducible susceptibility to HCV infection than HuS-T, PH5CH8, and Huh-7.5 cell lines.

IRF3 and IRF7 play an important role in the activation of interferon signaling [28]. We suppressed the functions of IRF-3 or IRF-7 to assess their role in HCV infectivity. In fact, we observed significant increase of HCV replication in HuS-E/2 cells bearing dominant-negative IRF7 that impaired IFN signaling. The suppression of IRF-3, however, did not have any significant effect on HCV infectivity or replication in this cell line. This may result from the blockade of IRF-3 activation by an HCV NS3/4A serine protease [29] through at least two independent pathways that inhibit the TLR3-dependent and RIG-I-dependent signaling pathways [29–33]. Although HCV was shown to inhibit basal expression levels of IRF-7 at both mRNA and protein levels and it was shown that NS5A suppresses IRF-7-induced IFNα promoter activation [34], Stimulation of TLR7 was shown to activate IRF-7 and induce suppression of HCV replicon levels in Huh-7 cells [35]. This suggests that the inhibition of IRF7 by HCV is not complete. Using IRF-7-deficient (IRF-7−/−) mice, Honda [36] demonstrated that the transcription factor IRF-7 is essential for the induction of IFNα/β genes. We established a clone stably expressing DNIRF-7 (HuS-7E/DN24), which demonstrated higher infectivity with different HCV strains than the parental HuS-E/2 clone.

In summary, we have established a human hepatocyte-derived cell line that maintains the characteristic features of primary hepatocytes by transduction with HPV18/E6E7. This cell line is highly infectable by HCV, which suggests that these cells may be useful to characterize the molecular mechanisms involved with HCV infection and to develop novel HCV treatment modalities.

Acknowledgements

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DNA and its cationic lipid complexes induce CpG motif-dependent activation of murine dendritic cells

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Summary

Unmethylated CpG motifs in bacterial DNA, but not in vertebrate DNA, are known to trigger an inflammatory response of antigen-presenting cells (APC). In this study, we investigated the cytokine release from murine dendritic cells (DC) by the addition of various types of DNA in the free or complexed form with cationic lipids. Naked plasmid DNA and *Escherichia coli* DNA with immunostimulatory unmethylated CpG motifs induced pro-inflammatory cytokine secretion from granulocyte–macrophage colony-stimulating factor (GM-CSF)-cultured bone marrow-derived DC and the DC cell-line, DC2.4 cells, though vertebrate calf thymus DNA (CT DNA) with less CpG motifs did not. These characteristics differed from mouse peritoneal resident macrophages that do not respond to any naked DNA. The amount of cytokines released from the DC was significantly increased by complex formation with cationic lipids when CpG-motif positive DNAs were used. Unlike murine macrophages or Flt-3 L cultured DC, GM-CSF DC did not release inflammatory cytokines in response to the addition of CT DNA/cationic lipid complex, suggesting that the activation is completely dependent on CpG motifs. Taken together, the results of the present study demonstrate that murine DC produce pro-inflammatory cytokines upon stimulation with CpG-containing DNAs and the responses are enhanced by cationic lipids. These results also suggest that DC are the major cells that respond to naked CpG DNA *in vivo*, although both DC and macrophages will release inflammatory cytokines after the administration of a DNA/cationic lipid complex.

Keywords: CpG motifs; dendritic cells; TLR9; DNA and DNA uptake

Introduction

It is well known that unmethylated CpG sequences (CpG motifs) in bacterial DNA, but not in vertebrate DNA, are recognized by the immune system as a danger signal.[1] Cytokines such as tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), IL-12 and interferon-α (IFN-α) are secreted from antigen presenting cells, especially macrophages or dendritic cells (DC), upon stimulation with CpG DNA and synthetic oligodeoxynucleotides (ODN) containing CpG motifs. These cytokines significantly modify the therapeutic effects of DNA-based therapies in different ways.[2] For example, in gene therapy, cytokine production generally seems inappropriate because these inflammatory cytokines significantly reduce transgene expression in target cells through their direct

Abbreviations: APC, antigen-presenting cells; DC, dendritic cells; BMDC, bone-marrow derived dendritic cell; CT DNA, calf thymus DNA; TNF-α, tumour necrosis factor-α; IL-6, interleukin-6; IL-12, interleukin-12; IFN-α, interferon-α; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; IRF, interferon regulatory factor; LPS, lipopolysaccharide; ODN, oligodeoxynucleotide; MHC, major histocompatibility complex; TLR, Toll-like receptor; JNK, c-Jun NH_2-terminal kinase; Flt-3 L DC, Flt-3ligand cultured bone-marrow dendritic cells; EC DNA, *Escherichia coli* DNA, pDNA, plasmid DNA; FL-pDNA, fluorescein labelled plasmid DNA; GM-CSF, DC; granulocyte–macrophage, colony-stimulating factor cultured dendritic cells; DNase II, deoxyribonuclease II.

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cytotoxicity and promoter attenuation.[3–5] On the other hand, it is essential for DNA vaccination because these cytokines can enhance the immune responses and profoundly affect the balance of these cytokines and the nature of the immune responses.[6–9]

DC are one of the most important cell populations as far as both innate and acquired immunity are concerned. They influence a variety of immunological responses associated with the therapeutic use of CpG DNA.[10,11] In addition to cytokine secretion, the expression of surface major histocompatibility complex (MHC) class I and II molecules as well as costimulatory molecules increases, and the maturation of DC is induced upon stimulation with CpG motifs.[12] The initial important step for all these processes associated with CpG DNA is cellular uptake because the receptor of CpG DNA, Toll-like receptor-9 (TLR9), is expressed within cells.[13,14] Our previous *in vitro* study using a DC cell line, DC2.4 cells, in mice demonstrated that DC take up pDNA via a mechanism specific to some defined polyanions[15] similar to cultured mouse peritoneal macrophages.[16,17]

There is a rapidly growing body of information about the mechanism of antigen-presenting cell (APC) activation by CpG DNA. This activation requires endosomal acidification and recognition by TLR9.[18–20] CpG DNA appears to use a TLR9 signaling pathway for NF-κB and c-Jun NH_2-terminal kinase (JNK) and IRF-7 through MyD88.[19,21] However, these proposed mechanisms are mainly based on studies using synthetic phosphorothioate CpG ODN, and there is little information about the activation induced by native DNA. Our previous study has demonstrated that, in contrast to macrophage cell lines, primary cultured mouse peritoneal macrophages secrete almost no inflammatory cytokines upon stimulation with pDNA, in spite of extensive uptake of the CpG DNA[22]. However, DNA/cationic lipid complex can activate the murine macrophages to induce inflammatory cytokines, whether they have replete CpG motifs or not.[23] Flt-3-ligand cultured bone-marrow DC (Flt-3 L DC) exhibit a different type of activation.[24,25] Upon stimulation with naked DNA, bacterial pDNA and CpG ODN stimulate Flt-3 L DC to induce cytokines IFN-α or IL-6 although vertebrate CT DNA does not. However, TLR9 in Flt-3 L DC can react when CT DNA is combined with cationic lipid N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP).[24] Methylated CpG motifs or non-canonical CpG motifs complexed with DOTAP induce the activation of TLR9 in Flt-3 L DC. Further experiments have proved that the other sequences also induce the activation of TLR9 when ODNs are translocated to endosomes by DOTAP.[25] While receptor-mediated endocytosis restricts the uptake of DNA, adsorptive endocytosis by cationic lipids does not. Thus, enhancement of DNA uptake seems to control the activation of TLR9 by vertebrate DNA. In the present study, we used a different type of DC and showed that the cells could respond to only DNA with CpG motifs even if the DNA was translocated to endosomes by cationic lipids.

Materials and methods

Chemicals

RPMI-1640 medium was obtained from Nissui Pharmaceutical (Tokyo, Japan). *Escherichia coli* DNA (EC DNA) and calf thymus DNA (CT DNA) were purchased from Sigma (St Louis, MO). Lipofectin reagent and Opti-MEM were purchased from Invitrogen (Rockville, MD). Mouse recombinant GM-CSF (rGM-CSF) and Triton-X-114 were purchased from Nacalai Tesque (Kyoto, Japan). [α-^{32}P]dCTP (3000 Ci/mmol) was obtained from Amersham (Amersham, UK). Fetal Bovine Serum (FBS) was purchased from Thermo Trace (Melbourne, Australia).

Cell culture

Male ICR mice (5 weeks) were purchased from Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). After bone marrow was flushed out of the bones of the hind legs of the mice, the cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1000 U/ml rGM-CSF. After a 4–5 day incubation at 37° in 5% CO_2-95% air, cells were collected and centrifuged at 200 *g* for 10 min. After removal of the supernatant, the cells were resuspended in 400 μl phosphate-buffered saline (PBS) containing 0·5% bovine serum albumin (BSA) per 10^8 total cells. The cell suspension was mixed thoroughly with 100 μl magnetic-activated cell sorting (MACS) CD11c MicroBeads (Miltenyi Biotec, Germany), and incubated for 15 min at 4°. After incubation, the cells were washed, centrifuged at 200 *g* for 10 min, and resuspended in 500 μl PBS containing 0·5% BSA. Then, magnetic separation with MACS was carried out to isolate the DC by selecting CD11c-positive cells from the cultured cells. These isolated cells were washed and then plated on 24-well culture plates (Falcon, Becton Dickinson, Lincoln Park, NJ) at a density of 5×10^5 cells/well and cultured for 24 hr. The murine DC2.4 cells were a gift from Dr Kenneth Rock (Department of Pathology, University of Massachusetts Medical School, MA). DC2.4 cells display dendritic morphology, express dendritic cell-specific markers, MHC molecules, and costimulatory molecules, and exhibit phagocytic activity and an antigen-presenting capacity.[26] DC2.4 cells were cultured with RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 μM non-essential amino acids, 50 μM 2-mercaptoethanol, and antibiotics. They were then plated on a 24-well culture plate at a density of 5×10^5 cells/well and cultured for 24 hr.

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