ator for the acute phase response of the liver as well as in liver regeneration.[9,10]

IL-6–type cytokines stimulate tyrosine phosphorylation of signal transducer and activator of transcription (STAT)1 and STAT3, which can form STAT3 and STAT1 homodimers as well as STAT3/STAT1 heterodimers. However, the importance of the detected STAT1 phosphorylation by IL-6–type cytokines remains elusive. For example, IL-6 and oncostatinM (OSM) only seem to induce an IFN-γ–like response in STAT3 knockout cells.[11,12] There are multiple reasons for this inefficient STAT1 response.[13] Not only is STAT1 tyrosine phosphorylation after IL-6–type cytokine stimulation very transient,[11,14] but additionally, most of the phosphorylated STAT1 seems to be trapped in STAT1/STAT3 heterodimers.[13]

We describe a function of IL-27 in hepatoma cells and hepatocytes. We show that IL-27 elicits an efficient STAT1 response and leads to the expression of IFN-γ–regulated genes in these cells.

Materials and Methods

Cell Culture. The human hepatoma cell line HepG2 (DSMZ) was maintained in Dulbecco's modified Eagle medium/Nut. MixF-12 medium with Glutamax supplemented with 10% fetal bovine serum, 100 mg/L streptomycin, and 60 mg/L penicillin. The human hepatocyte cell line PH5CH8 was described previously.[15]

Isolation and Cultivation of Rat Hepatocytes. Hepatocytes were isolated from adult male Sprague-Dawley rats as described before.[16] Details are provided as Supporting Information.

Cell Lysis, Preparation of Nuclear Extracts for Electrophoretic Mobility Shift Assay, Western Blot Analysis, and Antibodies. All of these procedures were performed as previously described.[13] The antibodies used are listed as Supporting Information.

Viral Infections and Plaque Assay. Fowl plague virus (FPV) was propagated and used as described previously.[17] For infection, 7×10^5 HepG2 cells were left untreated or were pretreated with 50 ng/mL IL-27 for 18 hours. Cells were then washed with phosphate-buffered saline followed by incubation with FPV (0.001 multiplicity of infection) diluted in phosphate-buffered saline/BA (phosphate-buffered saline containing 0.2% bovine serum albumin, 1 mM $MgCl_2$, 0.9 mM $CaCl_2$, 100 U/mL penicillin, and 0.1 mg/mL streptomycin) for 30 minutes at 37°C. The inoculum was aspirated, and cells were incubated for 24 hours with infection medium containing 0.2% bovine serum albumin and antibiotics supplemented either with or without 50 ng/mL IL-27. As a positive control for antiviral activity, infections were performed in the presence of 1000 U/mL interferon-alpha (IFN-α) for 24 hours. Plaque assays were performed as described previously.[18] Results are given as plaque-forming units per milliliter, and standard deviations are represented as error bars.

Statistical Analysis. The statistical analysis was performed using a Student t test. $P < 0.05$ was regarded as being statistically significant.

Results

IL-27 Acts on Human Hepatoma Cells and Cultured Human Hepatocytes. By screening different cell lines for their response to IL-27, we observed that human hepatoma cells are sensitive to IL-27. HepG2 cells were stimulated with increasing amounts of IL-27, and tyrosine phosphorylation of STAT3 (pY705) and STAT1 (pY701) was assessed by western blot analysis. Stimulation of HepG2 cells for 15 minutes with IL-27 leads to a phosphorylation of both STAT3 and STAT1 in a dose-dependent manner (Fig. 1A). As a control, HepG2 cells were also stimulated with IL-6. STAT1 as well as STAT3 tyrosine phosphorylation occurred on treatment of these cells with IL-6 for 15 minutes. The levels of phosphorylated STAT1 and STAT3 were higher in IL-6–stimulated HepG2 cells than in those treated with IL-27. We next investigated the kinetics of STAT phosphorylation on IL-27 stimulation in HepG2 cells and in the human hepatocyte cell line PH5CH8. Both of these cell lines express the IL-27 receptor WSX-1 on their surface (Supporting Fig. 1). As a control, the cells were also stimulated with IL-6, IFN-γ, and IFN-α. IL-27 induces a sustained phosphorylation of STAT1 and STAT3 (Fig. 1B,C; lanes 2-5) in both HepG2 and PH5CH8 cells, whereas IL-6 only leads to a sustained, albeit more pronounced, STAT3 phosphorylation (lanes 10-13). STAT1 activation after IL-6 stimulation was very transient (Supporting Fig. 2). However, a prominent STAT1 phosphorylation was observed on stimulation of the cells with interferons (lanes 6-9 and lanes 14-17), and STAT2 phosphorylation was only observed on treatment with IFN-α. Of note, up-regulation of both STAT1 and STAT2 was observed when the cells were stimulated with IL-27, IFN-γ, or IFN-α, an indication for an efficient STAT1 activation.

IL-27 Leads to a Prolonged STAT1 and STAT3 Activation in Liver Cells. As we previously reported, the STAT1 phosphorylation observed on treatment of hepatoma cells and primary human macrophages with IL-6–type cytokines such as IL-6 and OSM does not necessarily lead to the formation of active STAT1 ho-

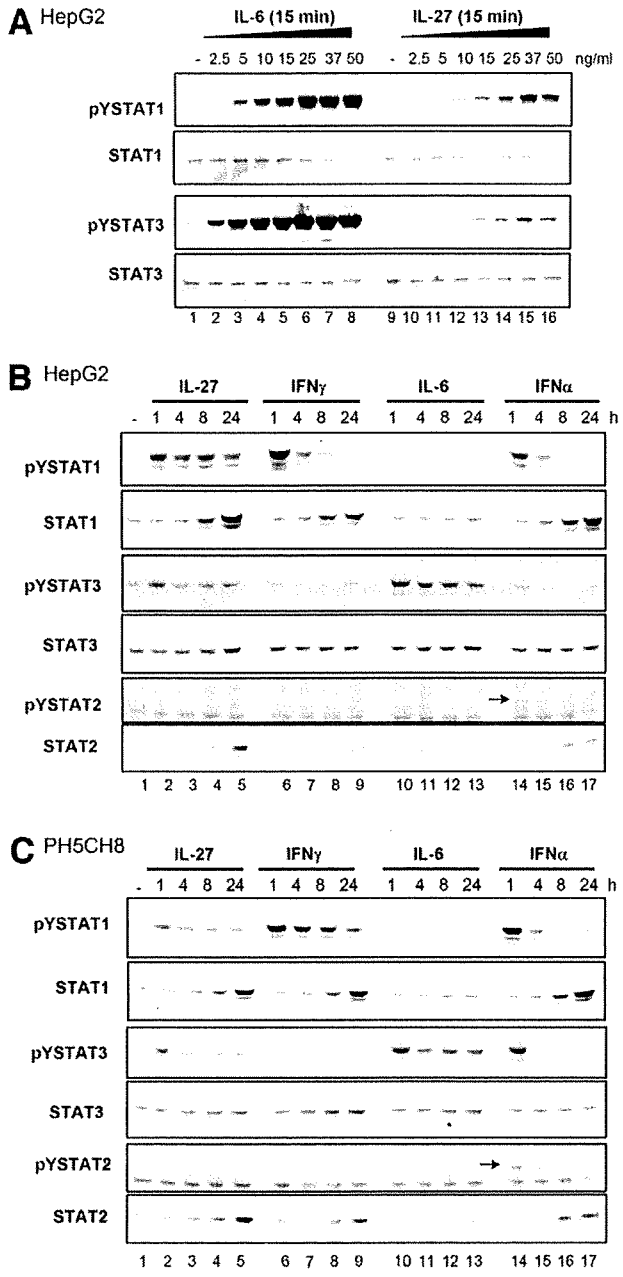

Fig. 1. IL-27 phosphorylates STAT1 and STAT3 in hepatoma cells and hepatocytes. (A) HepG2 cells were stimulated with the indicated amounts of IL-6 and IL-27. After 15 minutes, the cells were lysed, proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and tyrosine phosphorylation of STAT1, and STAT3 was detected by western blot analysis using phospho-specific antibodies for pY701-STAT1 and pY705-STAT3. Equal loading of the samples was assessed by stripping and reprobing the blot with antibodies recognizing STAT1 and STAT3. (B, C) Western blot analysis showing STAT1, STAT2, and STAT3 phosphorylation on stimulation of HepG2 hepatoma cells (B) or the cultured hepatocyte cell line PH5CH8 (C) with 20 ng/mL IL-27, IFN-γ, IL-6, or IFN-α for up to 24 hours. Western blot analysis was performed as described above.

modimers. Most of the phosphorylated STAT1 is rather trapped in STAT1/STAT3 heterodimeric complexes.[13] Thus, we performed electrophoretic mobility shift assays to examine whether phosphorylated STAT1 is forming homodimers on treatment of liver cells with IL-27 (Fig. 2). As controls, we used cells stimulated with IL-6, IFN-γ, or IFN-α. On stimulation of HepG2 or PH5CH8 cells with IL-27, the sustained formation of STAT1/STAT1 (lanes 2-5) complexes shows that IL-27 induces a persistent STAT1 activation. Although STAT3 homodimers also can be detected, STAT3 activation is weak if compared with IL-6. Of note, STAT1/STAT1 dimers are hardly observed on stimulation of the cells with IL-6 at these time points, indicating that the prominent but transient STAT1 phosphorylation observed 15 minutes after stimulation (Fig. 1A) is not translated into a STAT1 response. IL-27, however, should be capable of inducing STAT3 as well as STAT1 responses, because both factors are activated in a sustained manner and bind DNA in their homodimeric form. As expected, IFN-γ and IFN-α mainly induced the formation of STAT1 homodimers. Overall, both interferons show a more sustained STAT1 activation in PH5CH8 cells than in HepG2 cells (Figs. 1B,C, 2).

Compared with IL-6, IL-27 Does Not Induce the STAT3-Dependent Genes γ-Fibrinogen and Hepcidin. To assess whether IL-27 induces STAT3-dependent genes, we investigated the induction of the acute-phase protein genes γ-fibrinogen and hepcidin in both HepG2− and PH5CH8 cells (Supporting Fig. 3). In contrast to IL-6, neither IL-27, IFN-γ, nor IFN-α seem to induce significant levels of these genes.

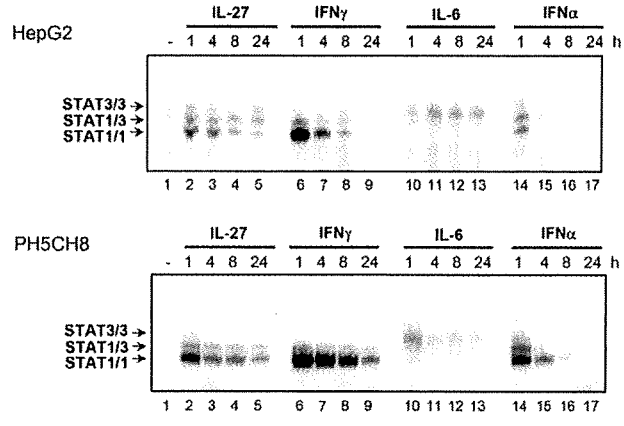

Fig. 2. IL-27 leads to a sustained STAT1 and STAT3 activation. HepG2 cells and PH5CH8 cells were stimulated with 20 ng/mL IL-27, IFN-γ, IL-6, or IFN-α for the times indicated, and nuclear extracts were prepared. These were analyzed by electrophoretic mobility shift assays, and STAT3/3, STAT3/1 and STAT1/1 dimer species were visualized by autoradiography.

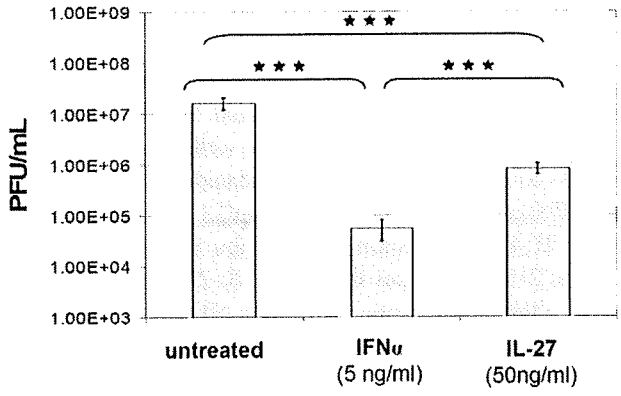

Fig. 3. IL-27 displays antiviral activity. HepG2 cells were pretreated with 50 ng/mL IL-27 for 18 hours before infection with FPV for 24 hours. The infection medium also contained 50 ng/mL IL-27. Antiviral activity was monitored by plaque assay. As a positive control, cells were infected in the presence of 5 ng/mL (1000 U/mL) IFN-α for 24 hours. Results are given in plaque-forming units per milliliter, and standard deviations are represented as error bars (n = 4; ***P < 0.001).

IL-27 Displays Antiviral Activity. Because IL-27 leads to a sustained STAT1 activation, we investigated possible antiviral activities of IL-27 by performing infection assays with HepG2 cells using the fowl plague virus FPV. HepG2 cells were pretreated with IL-27 for 18 hours before infection. Cells were then infected with FPV for 24 hours in the presence or absence of IL-27. As a positive control, infection assays were also performed in the presence of IFN-α, a cytokine that is well known to suppress virus replication. Figure 3 shows that treatment of HepG2 cells with IL-27 reduces the amount of progeny viruses after 24 hours of infection. Similar albeit less pronounced effects could be observed with a human influenza virus A7Puerto-Rico/8/34 isolate (data not shown).

IL-27 Mediates IFN-γ–like, STAT1-Dependent Responses in Liver Cells. To further investigate whether IL-27 induces an efficient STAT1 response, we investigated whether IL-27 would regulate STAT1-dependent gene transcription and thereby mediate interferon-like responses. We performed reporter gene assays in HepG2 and PH5CH8 cells and found IL-27 to prominently induce an interferon response factor (IRF)-1 promotor luciferase construct (Supporting Fig. 4). We then performed western blot analyses in these cells to monitor STAT1-dependent protein expression on treatment of these cells with IL-27, IL-6, IFN-γ, and IFN-α for different times. Figure 4A (HepG2) and 4B (PH5CH8) show that IL-27 up-regulates the STAT1-dependent genes STAT1 (lanes 4, 5), STAT2 (lanes 4, 5), and IRF-1 (lanes 2-5). Up-regulation of these genes also can be observed on treatment of the cells with IFN-γ or IFN-α, although the STAT2 up-regulation is barely detectable in HepG2 cells stimulated with IFN-γ (lane 9). In contrast, IL-6 fails to up-regulate any of the investigated STAT1-dependent genes. We further checked whether IL-27 would induce other interferon-regulated genes such as guanylate binding protein 2 (GBP2) and myxovirus resistance A (MxA), which are regulated by IFN-γ and IFN-α, respectively. Both genes are implicated in the antiviral response after interferon treatment of cells.[19] IL-27 up-regulates GBP2 in a similar manner to IFN-γ (lanes 5 and 9), whereas IFN-α does not induce GPB2. In contrast, IFN-α leads to a prominent up-regulation of MxA protein expression (lanes 15-17), whereas IL-27 only shows a weak induction (lane 5). IFN-γ only induces MxA expression in PH5CH8 cells (lane 9). Because MxA is known to be regulated by type I interferons, we investigated whether its IL-27–mediated induction could be attributable to the up-regulation of IFN-α or IFN-β by IL-27 (Supporting Fig. 5). However, stimulation of HepG2 cells with IL-27 in the presence of neutralizing antibodies directed against IFN-α or IFN-β did not affect IL-27–mediated induction of MxA, suggesting a direct induction by IL-27.

Because the IL-27–regulated transcription factor IRF-1 was recently reported to play a central role in the regulation of the antiviral protein RNA helicase retinoic acid–inducible gene-I (RIG-I),[20] we investigated whether IL-27 can induce RIG-I expression. We therefore monitored RIG-I induction in HepG2 and PH5CH8 cells by real-time polymerase chain reaction and western blot analysis. We detected a relatively weak twofold to sixfold increase in RIG-I messenger RNA expression (Supporting Fig. 6), whereas RIG-I protein up-regulation was hardly detectable after 24 hours by western blot (Fig. 4A,B; lane 5). In comparison, RIG-I protein was clearly induced on treatment of both cell lines with IFN-α (lanes 15-17).

Because we found both STAT1 and STAT2 to be up-regulated on stimulation with IL-27 (Fig. 4) and phosphorylation of these factors after treatment with IFN-α, we investigated whether prestimulation with IL-27 could enhance subsequent IFN-α–mediated signaling in PH5CH8 cells. We found that pretreatment with IL-27 enhances subsequent STAT1 and STAT2 phosphorylation on IFN-α treatment (Supporting Fig. 7A) and also induces the expression of IRF-9 (Supporting Fig. 7B), which forms the transcription factor complex interferon-stimulated gene factor 3 together with pYSTAT1 and pYSTAT2. However, we did not detect increased expression of the IFN-α–regulated genes RIG-I and MxA (Supporting Fig. 7C).

IL-27 Acts on Primary Rat Hepatocytes. To verify whether IL-27 also acts on hepatocytes in primary culture, we isolated primary rat hepatocytes and stimulated these cells with IL-27, IFN-γ, or IL-6 for different times. Treatment of

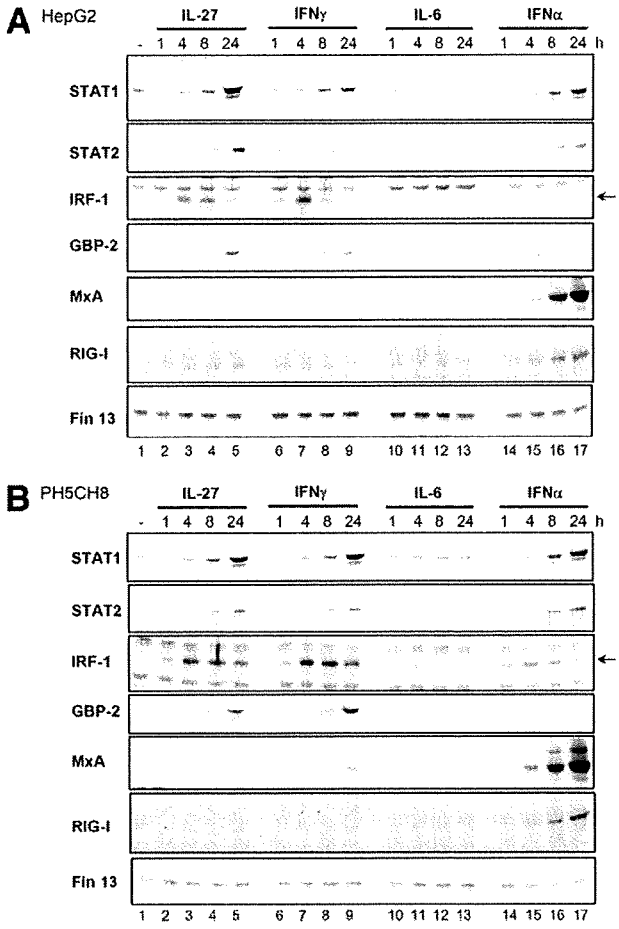

Fig. 4. IL-27 mediates STAT1 responses in hepatoma cells and cultured hepatocytes. (A, B) Western blot analysis monitoring up-regulation of STAT1, STAT2, IRF-1, GBP2, MxA, and RIG-I protein expression on stimulation of HepG2 cells (A) or PH5CH8 cells (B) with 20 ng/mL IL-27, IFN-γ, IL-6, or IFN-α for up to 24 hours. Expression levels of Fin13 are provided to compare the protein amount in the samples.

these cells with IL-27 induces a sustained phosphorylation of both STAT1 and STAT3 (Fig. 5), showing that primary hepatocytes respond to IL-27. Whereas the IL-27–mediated STAT1 phosphorylation is comparable to the one obtained after treatment with IFN-γ, the STAT3 response is much weaker than the one initiated by IL-6, confirming the results we obtained in the cell lines.

Discussion

Within the gastrointestinal system, a role for IL-27 was reported in the context of concanavalin-induced hepatitis,[21] Crohn disease,[22] as well as colon carcinoma.[23] However, all of these studies highlight IL-27 functions in infiltrating immune cells such as T cells and natural killer cells. IL-27 also acts on liver cells, namely, human hepatoma cells, cultured human hepatocytes, and primary rat hepatocytes. We find IL-27 to induce a sustained activation of STAT1 and STAT3 in these cells (Figs. 1 and 5).

IL-6–type cytokine signaling is characterized by a sustained STAT3 activation mediated via the different contributing receptor chains gp130, leukemia inhibitory factor receptor, and oncostatinM receptor. This STAT3 activation is of primordial importance for various functions in the liver such as the acute-phase response and liver regeneration. We therefore investigated the potency of the STAT3 activation mediated by IL-27, the new member of the IL-6–type cytokine family. For this, we selected γ-fibrinogen and hepcidin, two type II acute-phase proteins that are synthesized by hepatocytes in response to IL-6. In our experiments, IL-27 (as well as IFN-γ) was not able to lead to a significant induction of these genes (Supporting Fig. 3). These results suggest that IL-27 at most very weakly contributes to the acute-phase response of the liver and that it is in general a quite weak initiator of STAT3 responses in parenchymal liver cells. Because of the weak but sustained STAT3 activation that we observe after IL-27 stimulation (Figs. 1 and 2), it may be of interest to investigate a larger panel of acute-phase proteins to further dissect the potential contribution of IL-27 to the expression of type II acute-phase response genes.

However, the observed activation of STAT1 on stimulation of hepatoma cells and hepatocytes with IL-27 was of special interest because IL-6–type cytokines such as IL-6 and OSM fail to induce an efficient STAT1 response despite the fact that STAT1 phosphorylation is observed.[11-13] Thus, the detection of mere STAT1 phosphorylation (as detected after a 15-minute stimulation in Fig. 1A) does not allow drawing conclusions about STAT1 activity. Together with the very

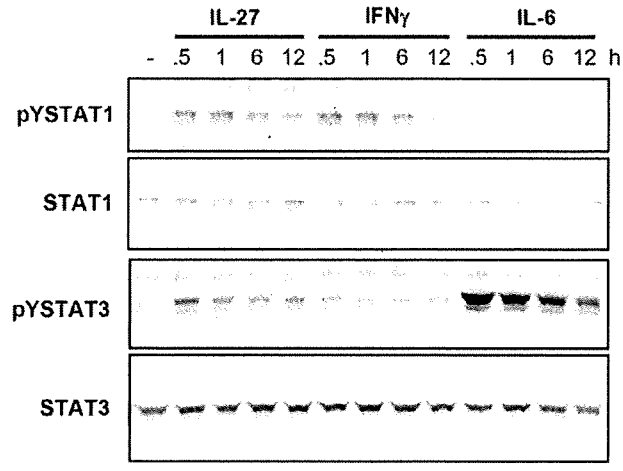

Fig. 5. IL-27 induces STAT1 and STAT3 tyrosine phosphorylation in primary hepatocytes. Primary rat hepatocytes were treated with 20 ng/mL IL-27, IL-6, or IFN-γ for the times indicated. Phosphorylation of STAT1 and STAT3 was monitored by western blot analysis.

transient phosphorylation of STAT1 after stimulation of hepatoma cells with IL-6, the fact that most of the phosphorylated STAT1 is found in STAT1/STAT3 heterodimers contributes to the lack of STAT1-dependent gene induction after treatment of cells with IL-6.[13] It also provides an explanation for the fact that IL-6 and OSM induce an interferon-like response in STAT3 knockout cells,[11,12] because the lack of STAT3 prevents the formation of heterodimers and thereby favors STAT1 homodimer formation. Here we show that IL-27 leads to a sustained STAT1 activation characterized by the formation of STAT1 homodimers (Fig. 2). Investigating whether the observed STAT1 activation translates to the induction of STAT1 target genes, we show that IL-27 up-regulates STAT1, STAT2, IRF-1, and IRF-9 protein expression (Fig. 4; Supporting Fig. 7B). This induction is comparable to the up-regulation after stimulation of these cells with IFN-γ or IFN-α, with the exception of an impaired STAT2 up-regulation by IFN-γ in HepG2 cells (Fig. 4A). This shows that IL-27 mounts an efficient STAT1 response and can mediate interferon-like responses in liver cells. This result corroborates previous data obtained in CD4+T cells and macrophages that highlight the importance of STAT1 for distinct biological activities mediated by IL-27.[4,24,25]

It is an interesting thought that the extent of STAT1 and STAT3 activation may be differently regulated as STAT3 responses are mediated via the gp130 receptor chain, whereas STAT1 responses will most likely only efficiently be mediated via the IL-27–specific WSX-1 receptor chain. This may lead to case-sensitive STAT1 or STAT3 responses. For example, it was recently reported that IL-27 activates both STAT1 and STAT3 in early activated T cells, whereas it displays a preferential activation of STAT3 in fully activated CD4+T cells.[26] The reasons for this may be manifold and could involve regulatory proteins or may even solely be attributable to different expression levels of STAT1 and STAT3 and thus to different distributions of STAT-dimer species.

The prolonged activation of STAT1 as well as the up-regulation of STAT1-dependent genes led us to investigate whether IL-27 possesses antiviral activity. We performed a plaque assay in hepatoma cells to assess the antiviral potency of IL-27 and show that, similarly to IFN-α, IL-27 reduces the amount of progeny viruses in HepG2 cells (Fig. 3). This result suggests that IL-27 can mediate antiviral effects in the liver.

To further assess the IL-27–mediated regulation of proteins involved in host resistance to pathogens, we then investigated the regulation of RIG-I, MxA, and GBP2 on stimulation of HepG2 and PH5CH8 cells with IL-27. The RNA helicase RIG-I is induced by retinoic acid as well as interferons and constitutes the first line of defense against viral infections by sensing viral double-stranded RNA.[27] Because it was recently shown that IRF-1 plays a central role in the regulation of RIG-I expression, we investigated whether IL-27 would induce this sensor for viral double-stranded RNA. Although we detected an increase in RIG-I messenger RNA levels on IL-27 stimulation in both human hepatoma cells and cultured human hepatocytes (Supporting Fig. 6), RIG-I protein was barely detectable. This shows that although IRF-1 expression may be necessary for RIG-I induction,[20] its expression alone is not sufficient. One may speculate that additional cellular signals may lead to an up-regulation of RIG-I protein expression by IL-27 and IFN-γ. Investigating the induction of the IFN-γ–induced protein GBP2 and the IFN-α–regulated MxA protein, we found IL-27 to up-regulate both proteins. IL-27 regulated these genes in a manner comparable to that of IFN-γ treatment. Because MxA is a gene regulated by type I interferons, we tested whether its induction after IL-27 treatment could be mediated through the induction of type I interferons. Experiments with neutralizing antibodies against IFN-α and IFN-β1 did not affect MxA nor GPB2 and STAT1 induction, suggesting that the observed regulation by IL-27 does not involve type I interferon production (Supporting Fig. 5).

Furthermore, we tested whether IL-27 could prime cells for a subsequent IFN-α stimulation (Supporting Fig. 7). Most interestingly, we found that prestimulation with IL-27 enhances subsequent IFN-α–mediated STAT1 and STAT2 phosphorylation and also up-regulates IRF-9. However, we did not detect increased expression of the IFN-α–regulated proteins RIG-I and MxA at the different doses of IFN-α tested. Nevertheless, it would be of interest to investigate other genes induced by the transcription factor complex interferon-stimulated gene factor 3 composed of pYSTAT1, pYSTAT2, and IRF-9.

Recent reports suggest that IL-27 can have antiviral activities in peripheral blood mononuclear cells, CD4+T cells, and macrophages and can inhibit human immunodeficiency virus 1 replication.[25,28] It was shown that IL-27 significantly induces interferon-inducible antiviral genes such as myxovirus protein 1, 2′-5′-oligoadenylate synthetase 2 and RNA-dependent protein kinase in macrophages, suggesting that IL-27 inhibits human immunodeficiency virus replication by eliciting an interferon-like response.[25] Together with our data, this suggests that IL-27 can elicit a multifaceted antiviral response.

The results presented in this study suggest that IL-27 may be a potential candidate for studies on combination therapies against hepatitis C. The standard care for a chronic hepatitis C infection is a combination therapy of IFN-α plus ribavirin. For a standard treatment, the response rate is approximately 50% for patients with hepatitis C virus genotype 1 and abut 80% for genotypes 2 and 3.[29] For the development of future

therapies, interests are focusing on combination therapies with different classes of anti–hepatitis C virus drugs such as protease or polymerase inhibitors. Furthermore, novel IFN-based products are being developed.[29] Our results that IL-27 acts on hepatocytes and hepatoma cells and displays IFN-like signaling in these cells as well as the antiviral effects of IL-27 observed in immune cells[25,28] indicate that treatment with IL-27 could be used in the therapy of hepatitis C virus infection.

Taken together, we present data showing that IL-27 acts on hepatocytes and hepatoma cells, elicits IFN-γ–like STAT1-mediated responses in these cells, and is able to regulate genes involved in host resistance to pathogens.

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Original Article

Development of a hepatitis C virus relapse model using genome-length hepatitis C virus ribonucleic acid-harboring cells possessing the interferon-α-resistance phenotype

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Aim: The cure rate of current interferon (IFN) therapy is limited to approximately 50% and most of the relapses after therapy are caused by genotype-1. To develop a relapse model in cell culture, we attempted to obtain genome-length hepatitis C virus ribonucleic acid (HCV RNA) harboring cells possessing the IFN-α-resistance phenotype from previously established OR6 cells, which enabled the luciferase reporter assay for monitoring of HCV RNA replication.

Methods: The IFN-α-resistant HCV RNA-harboring cells and control cells were obtained by the treatment of OR6 cells with and without IFN-α, respectively. Then, we examined the relapse of HCV in IFN-α-resistant HCV RNA-harboring cells.

Results: Only type I IFN (α and β) showed significantly different anti-HCV activity between IFN-α-resistant HCV RNA-harboring cells and control cells. There was no significant difference in the anti-HCV activity of IFN-γ, fluvastatin, or cyclosporine A between the two types of cells. Furthermore, we showed that fluvastatin or cyclosporine A in combination with IFN-α could prevent the relapse after therapy in the IFN-α-resistant HCV RNA-harboring cells.

Conclusion: We developed a HCV relapse model in cell culture using IFN-α-resistant HCV RNA-harboring cells. Thus anti-HCV reagents, which have a mechanism different from IFN-α, were shown to be useful for preventing a relapse of IFN-α-resistant HCV.

Key words: cyclosporine A, hepatitis C virus, interferon-α, interferon-γ, statin

INTRODUCTION

HEPATITIS C VIRUS (HCV) is a major cause of chronic liver disease, which progresses to liver cirrhosis and hepatocellular carcinoma.[1] Because approximately 170 million individuals are estimated to be infected with HCV worldwide, this infection is a major global health problem. HCV belongs to the family *Flaviviridae*, whose genome consists of a positive-stranded 9.6 kilobase ribonucleic acid (RNA) and encodes a large polyprotein precursor of about 3000 amino acid residues.[2,3] This polyprotein is processed by a combination of the host and viral proteases into at least ten proteins: the core, envelope 1 (E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B.[4–6]

The present standard therapy of pegylated interferon (PEG IFN) with ribavirin achieves a sustained virological response (SVR) in approximately 50% of patients with chronic hepatitis C (CHC). The relapse after IFN therapy is a serious problem, especially in patients with genotype-1 HCV infection. The most significant event in HCV research has been the development of a cell culture system. The subgenomic replicon system enables robust HCV RNA replication in hepatoma cells.[7–14] Recently, the complete life cycle of HCV has been achieved using a genotype-2a strain, JFH1.[15] These HCV cell culture systems have promoted the discovery and development of anti-HCV reagents. Previously, we developed a genome-length HCV RNA (strain O of genotype-1b) replication system (OR6) with renilla luciferase (RL) as

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a reporter.[8] Using this OR6 assay system, we found that mizoribine,[16] as an immunosuppressant, and fluvastatin (FLV)[17,18] and pitavastatin,[19,20] as the reagents for hypercholesterolemia, suppressed genome-length HCV RNA replication.

We previously established an IFN-resistant cell culture model from the genotype-1b 1B-1 strain,[21] while Noguchi *et al.*[22] established a similar model from the genotype-1b Con1 strain. Both cell culture models were developed using subgenomic HCV replicons. Recently, it was reported in an epidemiological study that the 70th amino acid (AA) and 91st AA in core is significant for IFN resistance.[23] Therefore, we developed IFN-α-resistant genome-length HCV RNA-harboring cells using OR6 cells.

In the present study, we developed a relapse cell culture model using IFN-α-resistant genome-length HCV RNA-harboring cells. We also showed that anti-HCV reagents, which exhibited a mechanism different from IFN-α, efficiently prevented the relapse of IFN-α-resistant HCV.

MATERIALS AND METHODS

Reagents

HUMAN IFN-α AND human IFN-γ were purchased from Sigma–Aldrich (St. Louis, MO, USA), and cyclosporine A (CsA) and FLV were purchased from Calbiochem (Los Angeles, CA, USA). IFN-β was kindly provided by Toray Industries, Inc. (Tokyo, Japan).

Cell culture

OR6 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin, streptomycin, and 0.3 mg/mL of G418 (Geneticin; Invitrogen, Carlsbad, CA, USA) and passaged twice a week at a 5:1 split ratio. OR6c cells are cured OR6 cells from which genome-length HCV RNA has been eliminated by IFN-α treatment (500 IU/mL for 2 weeks) without G418, as previously described.[8]

Luciferase reporter assay

For the RL assay, 2×10^4 OR6 cells were plated in 24-well plates at least in triplicate for each assay and were cultured for 24 h. The cells were then treated with reagents for 72 h, harvested with renilla lysis reagent (Promega, Madison, WI, USA), and subjected to the RL assay according to the manufacturer's protocol.

Immunofluorescence analysis

Immunofluorescence analysis was carried out according to a method described previously.[9] The OR6 cells were treated with the reagents for 96 h as described above. The primary antibody used to detect core was anti-core (CP11; Institute of Immunology, Tokyo, Japan) and the secondary antibody was Cy3-conjugated anti-mouse antibody (Jackson ImmunoResearch). The nucleus was stained with 4′, 6-diamidine-2′-phenylindole (Sigma). The cells were photographed under a confocal laser scanning microscope (LSM510; Carl Zeiss Inc., Tokyo).

Dual-luciferase reporter assay

For the dual-luciferase assay, firefly luciferase vector, pISRE-Luc (Stratagene, La Jolla, CA) was used. The reporter assay was carried out as previously described.[24] The experiments were carried out in at least triplicate.

RNA transfection and selection of G418-resistant cells

For electroporation, OR6c cells were washed twice with ice-cold phosphate buffered saline (PBS) and resuspended at 10^7 cells/mL in PBS. RNA was mixed with 500 μL of the cell suspension in a cuvette with a gap width of 0.2 cm (Bio-Rad, Hercules, CA, USA). The mixture was immediately subjected to two pulses of current at 1.2 kV, 25 μF and maximum resistance. After 10 min of incubation at room temperature, the cells were seeded into 10-cm dishes and selected in complete DMEM with 0.3 mg/mL of G418.

Reverse transcription polymerase chain reaction and direct sequencing

Sequence analysis of HCV RNA was carried out by the direct sequencing method. Total RNA from each cell lines were extracted by using a Qiagen RNeasy® Mini kit (Tokyo, Japan). Two primers (O-9559R: 5′-TCTGCAG AGAGGCCAGTATCAGC-3′; O-6215R: 5′-TCATTGATC CACTGGTGGAGTCTC-3′) were used for reverse transcription (RT). The three parts of HCV were amplified by polymerase chain reaction (PCR) using proofreading KOD-plus DNA polymerase (Toyobo, Osaka, Japan): the first part covered the core to the NS3 region, the second part covered the NS3 region to the NS4B region, and the third part covered the NS5A region to the NS5B region. PCR was carried out using the following three pairs of primers: EMCV: 5′-AGGTACCCCATTGTATGG GATCTG-3′ and O-3540R: 5′-ATGCAGGTCGCCAAGAA GGACTG-3′ for the first part; O-3427: 5′-TCACG

GCCTATTCCCAACAGACG-3′ and O-6215R: 5′-TCAT TGATCCACTGGTGGAGTCTC-3′ for the second part; and O-5968: 5′-CCGAGGACCTAGTCAACTTGCTC-3′ and O-9382R: 5′-CCTATTGGCCTGGAGTGTTTAGC-3′ for the third part. The lengths of the three PCR products were 3356 bp, 2812 bp, and 3437 bp, respectively. The PCR products were sequenced in both the sense and antisense directions using Big Dye terminator cycle sequencing on an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

Western blot analysis

Preparation of the cell lysate, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and immunoblotting were carried out as previously described.[10] The antibodies used in the present study were those against core (Institute of Immunology, Tokyo, Japan) and β-actin (Sigma). Immunocomplexes on the membranes were detected by an enhanced chemiluminescence assay (Renaissance; Perkin Elmer Life Science, Wellesley, MA, USA).

Statistical analysis

Statistical comparison of luciferase activities among the various treatment groups was carried out using Student's *t*-test. *P*-values of less than 0.05 were considered statistically significant.

RESULTS

The relapse of HCV RNA replication after IFN-α treatment

WE FIRST EXAMINED the dose and duration of IFN-α treatment required to eliminate HCV. OR6 cells were treated with IFN-α (1, 10, and 100 IU/mL) for 2 or 4 weeks and subjected to a luciferase assay as shown in the experimental design (Fig. 1a).

After 2-week IFN-α treatment, 1 IU/mL of IFN-α failed to reduce HCV to the background level, which was designated as the luciferase activity of cured OR6c cells (less than 1000 luciferase units; Fig. 1b, left panel). Two-week IFN-α treatment at 10 IU/mL decreased HCV to below the background level, but HCV RNA was increased after the cessation of the therapy, in a manner corresponding to a relapse in clinical therapy (Fig. 1b). Finally, 2-week IFN-α treatment at 100 IU/mL decreased HCV to below the background level, and there was no relapse after therapy (Fig. 1b).

In regard to the 4-week treatment duration, 1 IU/mL of IFN-α failed to reduce HCV to the background level (Fig. 1b, right panel). IFN treatment at 10 or 100 IU/mL for 4 weeks reduced HCV to below the background level and prevented a relapse after therapy (Fig. 1b, right panel). Together, these results indicate that longer treatment duration is more effective for preventing the relapse after treatment. As shown in Table 1, the luciferase value ratios (72 h/0 h) less than 1.1 are required to prevent relapse. Interestingly, at the dose of 1 IU/mL IFN-α, 4-week treatment resulted in a smaller luciferase value ratio than did 2 week-treatment (5.6 *vs* 19.8). These results indicate that longer treatment is more effective at preventing HCV RNA replication, even if the IFN-α cannot reduce HCV to an undetectable level.

We further characterized the effects of dose and duration in IFN-α treatment using a more sensitive colony formation assay. The colony formation assay could detect the remaining HCV RNA even within individual cells. As shown in Fig. 1c, IFN-α-treated cells were subjected to G418 selection after 1 week of recovery culture. At a dose of 100 IU/mL IFN-α, neither 2- nor 4-week treatments produced any colonies after 3 weeks of G418 selection (Fig. 1d). In contrast, under the 10 IU/mL IFN-α condition, both 2- and 4-week treatments produced G418-resistant colonies (Fig. 1d). Although the relapse assay 4 week-treatment with IFN-α at 10 IU/mL prevented relapse, latent HCV RNA produced G418-resistant colonies. Therefore, at least 100 IU/mL of IFN-α treatment for 2 weeks is needed to achieve the complete elimination of HCV RNA. The production rates of G418-resistant colonies are summarized in

Figure 1 The relapse and complete hepatitis C virus ribonucleic acid (HCV RNA) elimination after interferon (IFN)-α treatment in cell culture. (a) Schema of the experimental design for the relapse cell culture model in IFN-α treatment. OR6 cells were treated with IFN-α (1, 10, and 100 IU/mL) for 2 or 4 weeks without G418. At the end of the treatment, cells were cultured without IFN-α for 0, 24, 48, and 72 h and subjected to luciferase assay. (b) Transient luciferase assay for the cells at the end of the 2-week treatment (left panel) and 4-week treatment (right panel). (c) Schema of the experimental design for the complete HCV elimination by colony formation. The OR6 cells were treated with IFN-α (1, 10, and 100 IU/mL) for 2 or 4 weeks without G418. At the end of the treatment, cells were cultured without IFN-α for 1 week as the recovery culture. Then the cells were treated with 0.3 mg/mL of G418 for 3 weeks. (d) At the end of the G418 selection in (c), the colonies were stained by trypan blue.

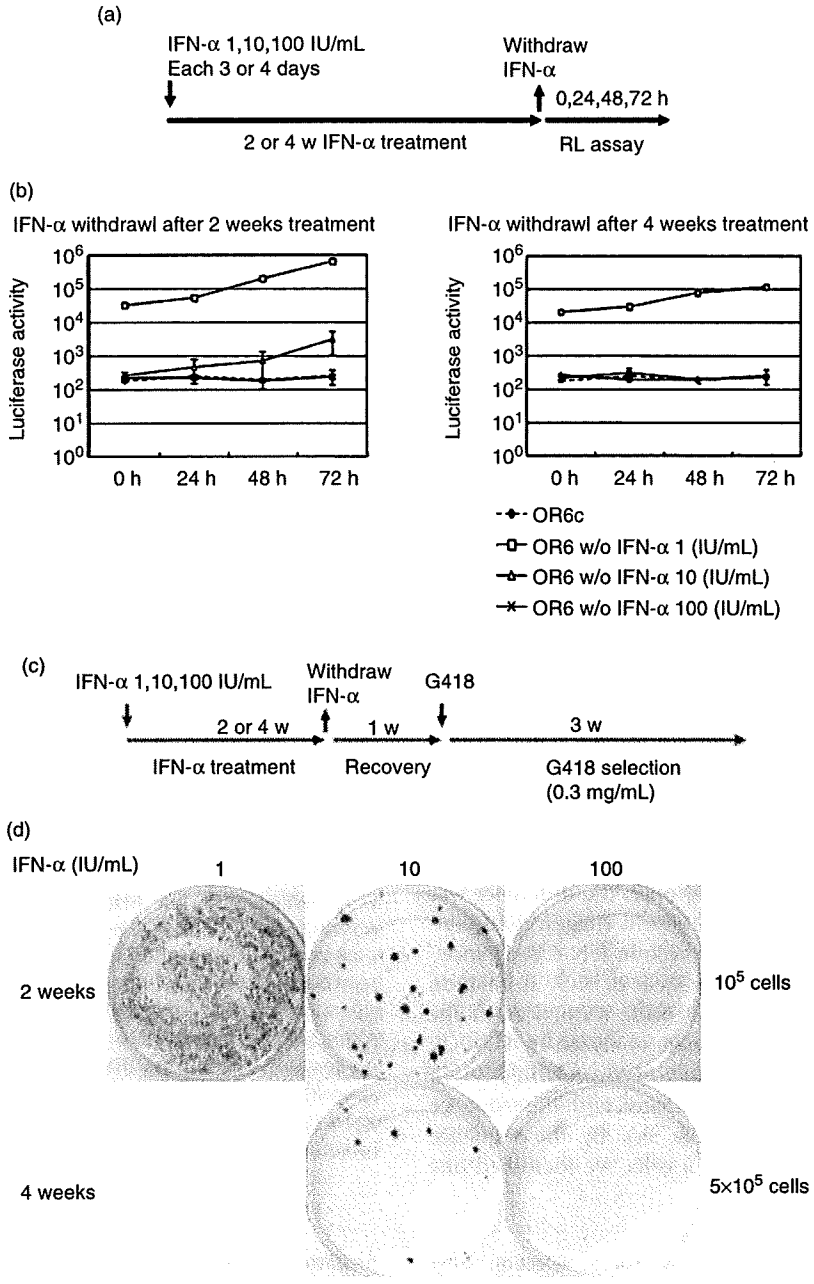


Table 2. Inhibition of the G418-resistant colony might correspond to SVR in the clinical therapy of CHC. These results suggest that the IFN-α-resistant HCV RNA-harboring cells were useful as a SVR model as well as a relapse model.

Characterization of IFN-α-resistant, HCV RNA-harboring cells

Based on the conditions regarding IFN-α resistance, we designed an experiment for the characterization of

Table 1 72 h/0 h Luciferase value ratio

		IFN-α (IU/mL) withdrawal					
		2 weeks			4 weeks		
OR6c	OR6	1	10	100	1	10	100
1.4		19.8	11.7	1.1	5.6	0.9	1.1

IFN, interferon.

IFN-α-resistant HCV RNA-harboring cells, as shown in Figure 2a and b. For the production of control cells (OR6(C)) and IFN-α-resistant HCV RNA-harboring cells (OR6(R)), OR6 cells were untreated or treated with 5 IU/mL of IFN-α for 2 weeks. The cells were then treated with G418 (0.3 mg/mL) for 3 weeks after 1 week of recovery culture. These cells were the first generation of control cells (OR6(C)) and IFN-α-resistant HCV RNA-harboring cells (OR6(R)). Then, we introduced total RNA from OR6(C) cells or OR6(R) cells into naïve cured OR6c cells for the development of the second-generation control cells (OR6(2C)) and IFN-α-resistant cells (OR6(2R)) (Fig. 2a,b).

The first generations of OR6(C) cells and OR6(R) cells were treated with IFN-α (0, 1, 2, 4, 8, and 16 IU/mL) for 72 h. OR6(R) cells were significantly less sensitive to IFN-α than OR6(C) cells (Fig. 2c). Then, the second generations of OR6(2C) cells and OR6(2R) cells were treated with IFN-α. OR6(2R) cells were less sensitive to IFN-α than OR6(2C) cells, although the difference was not significant (Fig. 2c). These results suggest that cellular factors might play a major role in IFN-α resistance.

We next examined the sequences of HCV in relation to IFN-α resistance. The nucleotide sequences of the open reading frames (ORF) were analyzed by RT-PCR direct sequencing. The AA mutations of HCV in OR6(C) cells and OR6(R) cells were compared with the sequence in the original OR6 cells (Fig. 3a). By the sequence analysis of HCV from OR6(C) cells, we identified one

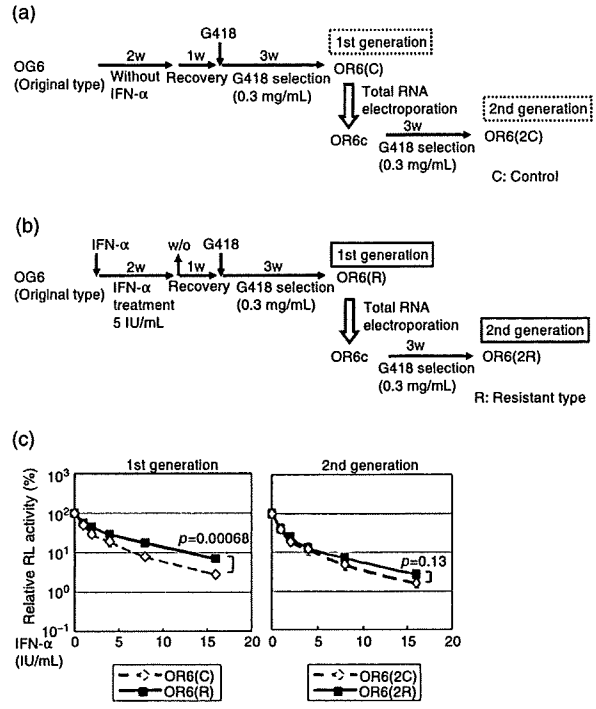


Figure 2 Establishment of the relapse cell culture model. (a and b) Schema for the establishment of the control cells (a) and interferon (IFN)-α-resistant cells (b). OR6 cells were treated without or with IFN-α (5 IU/mL) for 2 weeks. After 1 week of recovery culture without IFN-α, the cells were subjected to G418 selection for 3 weeks. The established cell lines were designated as the first generation of the control cells (OR6(C)) (a) and the IFN-α-resistant cells (OR6(R)) (b). The total ribose nucleic acid (RNA) from OR6(C) or OR6(R) were transfected into naïve OR6c for the development of second generation control cells (OR6(2C)) and IFN-α-resistant cells (OR6(2R)) by 3 week G418 treatment. (c) The sensitivity of the control cells and the IFN-α-resistant cells to IFN-α. The control cells and the IFN-α-resistant cells were treated with IFN-α (0, 1, 2, 4, 8, and 16 IU/mL) for 72 h using first generation cells (left panel) and second generation cells (right panel).

Table 2 Numbers of colonies by G418 selection after interferon-α treatment

	Numbers of colonies ($/10^5$ Cells)	
	IFN-α (IU/mL)	
	10	100
2 weeks	27.6 ± 6.7	0
4 weeks	0.9 ± 0.3	0

IFN, interferon.

mutation, I65M, in the core; one mutation, Q501R, in E2; two mutations, P1115L and T1286A, in NS3; and two mutations, A1821G and G1926, in NS4B (Fig. 3b). In the HCV sequence from OR6(R) cells, we identified two mutations, Y276H and M324V, in E1, and one mutation, E1202G, in NS3 (Fig. 3c). Although the core and NS5A were intensively investigated in relation to IFN resistance, we did not identify any mutations in these regions of HCV from IFN-resistant HCV RNA-harboring cells.

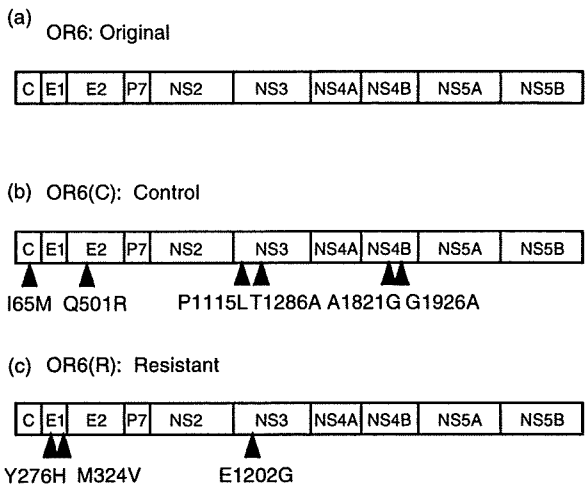


Figure 3 The sequence analysis for hepatitis C virus (HCV) in interferon (IFN)-α-resistant cells. HCV open reading frames were determined by the RT–PCR direct sequencing methods from the genome-length HCV RNA derived from the original OR6, the control, and IFN-α-resistant cells. The arrows indicate the AA substitutions in the control cells and IFN-α-resistant cells compared with original OR6.

IFN-α-resistant HCV RNA-harboring cells are resistant to IFN-β but not to IFN-γ, CsA (cyclosporine A), or FLV (fluvastatin)

We next examined the sensitivities of the established IFN-α-resistant HCV-harboring cells to anti-HCV reagents. IFN-β belongs to the type I IFN family and shares a common JAK-STAT signaling pathway with IFN-α. Therefore, we examined the sensitivity of HCV to IFN-β in OR6(C) cells and OR6(R) cells. OR6(C) cells and OR6(R) cells were treated with IFN-β (0, 0.125, 0.25, 0.5, 1, 2, and 4 IU/mL) as well as IFN-α (0, 0.25, 0.5, 1, 2, and 4 IU/mL) for 72 h. HCV in OR6(R) cells was significantly less sensitive to both IFN-α and IFN-β than that in OR6(C) cells (Fig. 4a). These results suggest that IFN-α-resistant HCV-harboring cells are also resistant to IFN-β, which shares a similar anti-HCV mechanism with IFN-α.

Next, we investigated the sensitivity of HCV in OR6(R) cells to the type II IFN, IFN-γ. IFN-γ exhibits its anti-HCV activity via a signaling pathway different from that of the type I IFN and induces unique IFN-stimulated genes. OR6(C) cells and OR6(R) cells were treated with IFN-γ (0, 0.125, 0.25, 0.5, 1, 2, and 4 IU/mL) for 72 h. There was no significant difference in the sensitivity to IFN-γ between these cells (Fig. 4b, left panel).

Then, we investigated the other well-characterized anti-HCV reagents, FLV and CsA, for their sensitivity using IFN-α-resistant HCV RNA-harboring cells.[17,25–29] OR6(C) cells and OR6(R) cells were treated with FLV (0, 0.25, 0.5, 1, 2, 4, 8, and 16 μM) or CsA (0, 0.031, 0.062, 0.125, 0.25, 0.5, and 1 μg/mL) for 72 h. There were no significant differences in the sensitivity to FLV or CsA between these cells (Fig. 4b, middle and right panel). We also evaluated and compared the EC_{50} values of the anti-HCV reagents between OR6(C) cells and OR6(R) cells (Table 3). The averages and standard deviations were calculated from the three independent experiments and subjected to Student's *t*-test. The EC_{50} of type I IFN in OR6(R) cells was approximately twice as high as that in OR6(C) cells, whereas there were no significant differences between the two types of cells in sensitivity to IFN-γ, FLV and CsA. Collectively, these results suggest that anti-HCV reagents that have inhibitory mechanisms different from that of IFN-α are useful for the treatment of IFN-α-resistant HCV-RNA harboring cells.

Using the immunofluoresence analysis, we also showed the effect of anti-HCV reagents on core expression in OR6(C) and OR6(R) cells (Fig. 4c). The expression of core in OR6(C) cells were relatively lower than that in OR6(R) after treatment with IFN-α (4 IU/mL) or IFN-β (2 IU/mL). In contrast, there were no differences between OR6(C) and OR6(R) cells after treatment with IFN-γ (4 IU/mL), FLV (10 μM) or CsA (1 μg/mL).

To clarify whether the different effects of type I IFN observed between OR6(C) and OR6 (R) cells are dependent on the cellular potentials of the type I IFN signaling pathway, we carried out a dual-luciferase reporter assay using a type I IFN-inducible ISRE promoter. The response of OR6(C) cells to IFN-α (0, 1, 10 and 100 IU/mL) was stronger than that of OR6(R) cells (Fig. 4d). This result suggests that low sensitivity of OR6(R) cells to IFN-α might be caused by the weak induction of interferon stimulated genes.

Development and characterization of a relapse model using IFN-α-resistant HCV RNA-harboring cells

We next attempted to develop a relapse model of IFN treatment using IFN-α-resistant HCV RNA-harboring cells. The experimental design is shown in Figure 5a. OR6 cells were treated with IFN-α alone or in combination with other anti-HCV reagents for 72 h. After the withdrawal of the reagents, HCV RNA levels were monitored for 72 h by luciferase activity. Western blot analysis was also carried out using the cell lysate 120 h after withdrawal of the reagents. First, we examined the concentration of IFN-α required to prevent the relapse after treatment of OR6(C) cells and OR6(R) cells for 72 h.

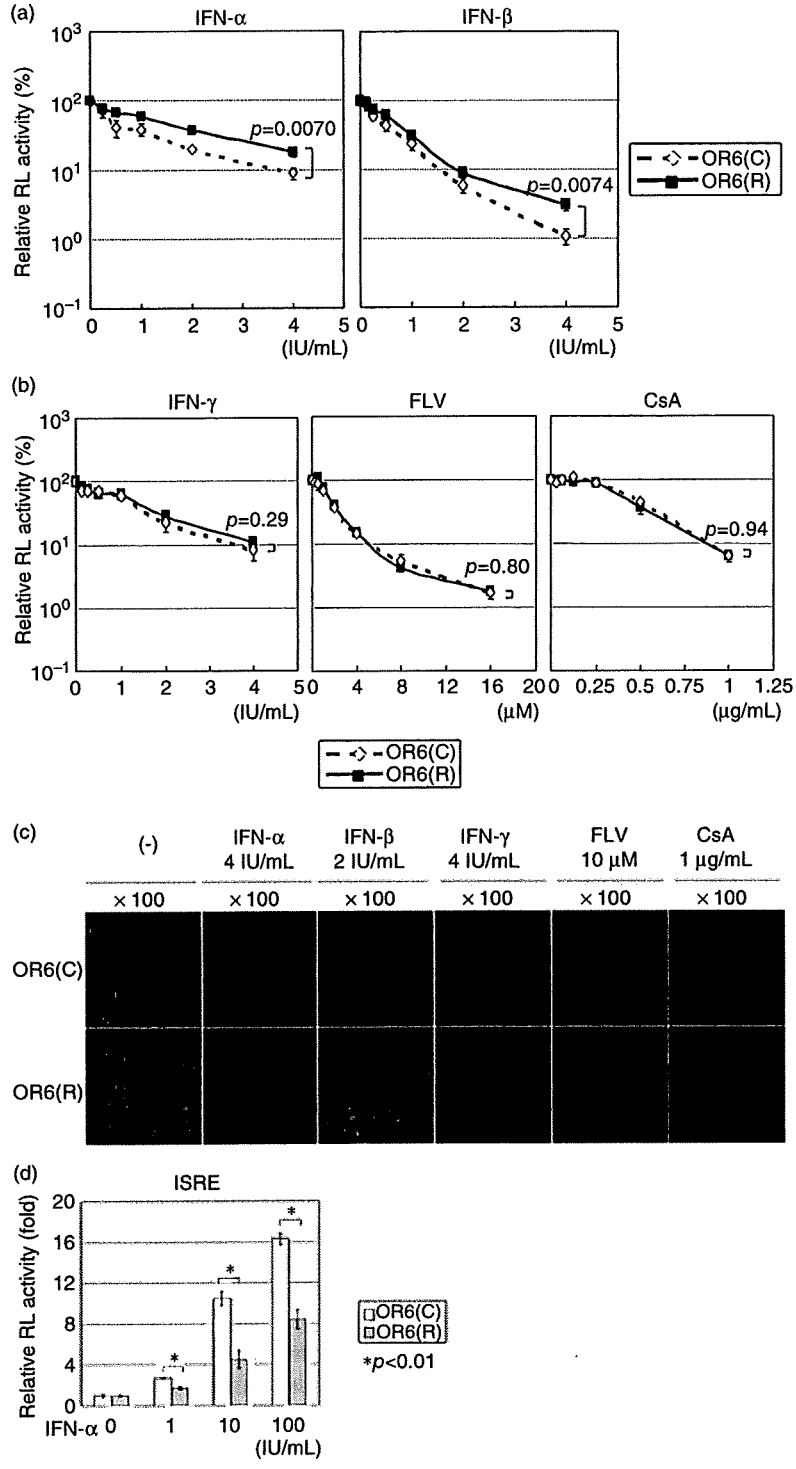


Table 3 EC_{50} values of anti-hepatitis C virus reagents in OR6(C) and OR6(R) cells

	OR6(C)	OR6(R)	*P*-value
IFN-α (IU/mL)	0.60 ± 0.20	1.32 ± 0.16	0.0077
IFN-β (IU/mL)	0.38 ± 0.12	0.66 ± 0.11	0.047
IFN-γ (IU/mL)	1.21 ± 0.16	1.30 ± 0.13	0.46
FLV (μM)	1.50 ± 0.20	1.65 ± 0.10	0.44
CsA (μg/mL)	0.46 ± 0.05	0.42 ± 0.06	0.50

CsA, cyclosporine A; FLV, fluvastatin; IFN, interferon.

For this purpose, a high concentration of IFN-α was needed: 250 IU/mL for OR6(C) cells and 1000 IU/mL for OR6(R) cells (Fig. 5b). Two hundred and fifty IU/mL of IFN-α could not prevent the relapse of HCV in OR6(R) cells (Fig. 5b). In Western blot analysis, 250 and 1000 IU/mL of IFN-α were needed to inhibit core expression in OR6(C) cells and OR6(R) cells, respectively (Fig. 5c). In contrast, 250 IU/mL of IFN-α was not sufficient to inhibit the core expression in OR6(R) cells (Fig. 5c). These results suggest that a fourfold higher dose of IFN-α is needed to prevent the relapse of HCV in OR6(R) cells compared with OR6(C) cells.

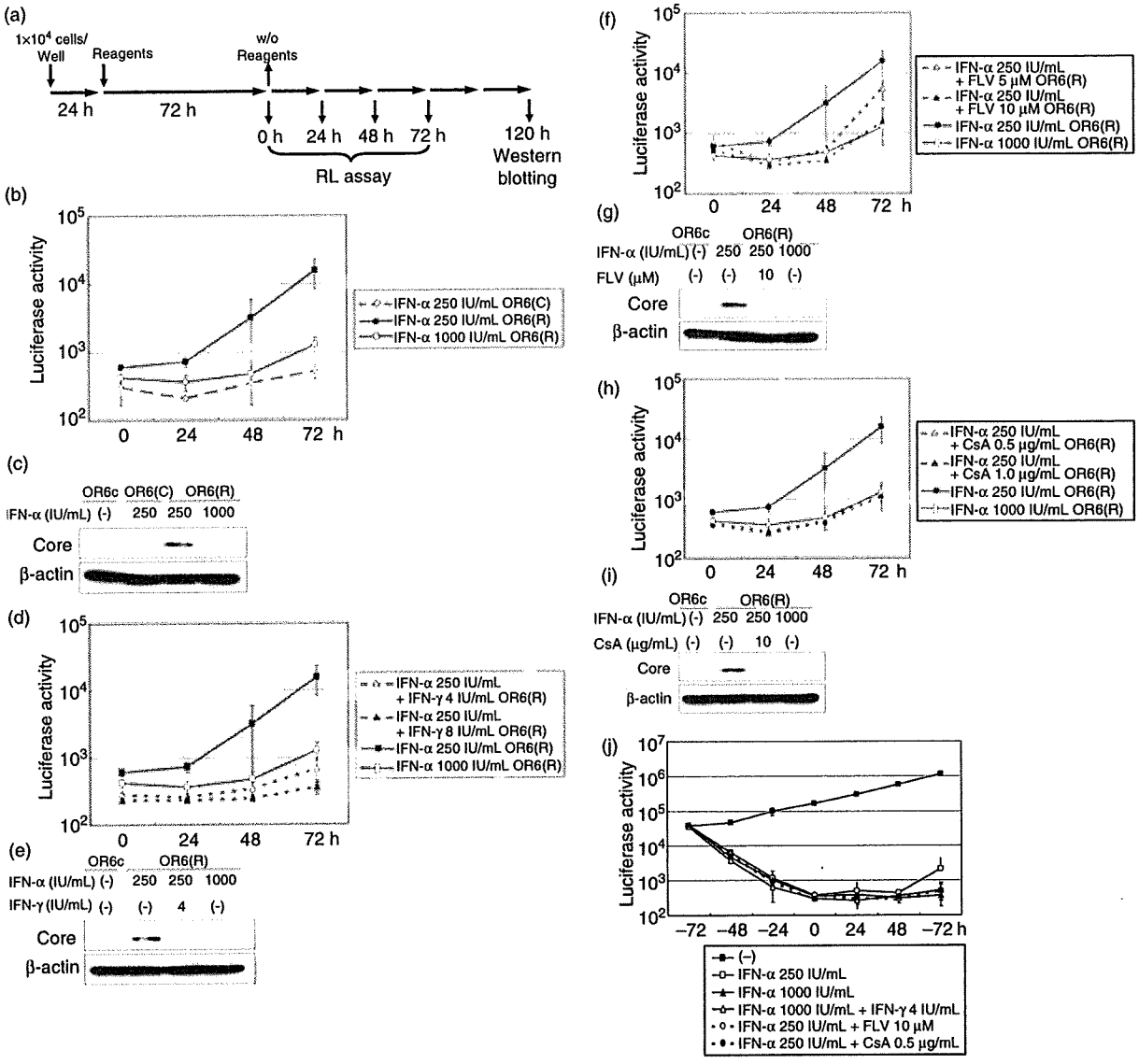
We adopted a concentration of 250 IU/mL of IFN-α for the relapse model as a cotreatment with anti-HCV reagents to prevent the relapse of HCV in OR6(R) cells. IFN-γ (4 and 8 IU/mL) in combination with IFN-α (250 IU/mL) could prevent the relapse of HCV in OR6(R) cells (Fig. 5d). This effect of the combination treatment was also confirmed by Western blot analysis (Fig. 5e). FLV (5 and 10 μM) or CsA (0.5 and 1 μg/mL) in combination with IFN-α (250 IU/mL) was also sufficient to prevent the relapse of HCV in OR6(R) cells in luciferase assay (Fig. 5f,h). These effects of the combination treatment were also confirmed by Western blot analysis (Fig. 5g,i). These results suggest that anti-HCV reagents that have an inhibitory mechanism different from that of type I IFN are useful to prevent the relapse of IFN-α-resistant HCV.

We examined the time course before and after withdrawal of anti-HCV reagents in OR6(R) cells. Luciferase activities decreased in OR6(R) cells after treatment of IFN-α (250 and 1000 IU/mL) alone and IFN-α (250 IU/mL) in combination with IFN-γ (4 IU/mL), FLV (10 μM), or CsA (0.5 μg/mL) for 72 h (Fig. 5j). In contrast, only IFN-α (250 IU/mL) treatment in its own caused relapse of HCV RNA replication after withdrawal of the anti-HCV reagents (Fig. 5j). This result in our model might correspond to the transient response in IFN treatment for patients with CHC.

DISCUSSION

PREVIOUSLY, WE REPORTED two IFN-resistant subgenomic HCV replicon-harboring cell lines from the 1B-1 strain: the severely IFN-resistant replicon cell lines (βR-series cells) and the partially IFN-resistant cell lines (αR-series cells).[30,31] The severe and mild IFN resistance of βR-series cells and αR-series cells are caused by the functional disruption of type I IFN receptors and the epigenetic silencing of interferon stimulated genes (ISG), respectively. These IFN-resistant cell lines were established with a high concentration of IFN-α (100–2000 IU/mL) and/or IFN-β (400–1000 IU/mL). In the present study, we treated the OR6 cells with a low concentration of IFN-α (5 IU/mL) to establish IFN-resistant genome-length HCV RNA-harboring cells. The total RNA from control cells or IFN-α-resistant cells were introduced into naïve OR6c cells to establish the second generation of control cells (OR6(2C)) or IFN-resistant cells (OR6(2R)). OR6(2R) cells were less sensitive to IFN-α than OR6(2C), although the difference was not significant. More recently, Noguchi *et al.*[22] reported that IFN-α-resistant HCV replicon-harboring cells were isolated by treatment with a relatively low concentration of IFN-α (10 and 30 IU/mL). In that study, the difference in IFN-α resistance between the second generation control and IFN-α-resistant cells decreased compared

Figure 4 The sensitivities of hepatitis C virus (HCV) in the interferon (IFN)-α-resistant cells to anti-HCV reagents. (a) The sensitivity of HCV in the IFN-α-resistant cells to type I IFNs. The control cells and the IFN-α-resistant cells were treated with IFN-α (0, 0.25, 0.5, 1, 2, and 4 IU/mL) (left panel) and IFN-β (0, 0.125, 0.25, 0.5, 1, 2, and 4 IU/mL) (right panel), respectively, for 72 h. Then these cells were subjected to luciferase assay. (b) The sensitivity of HCV in the IFN-α-resistant cells to IFN-γ, FLV, and CsA. The control cells and the IFN-α-resistant cells were treated with IFN-γ (0, 0.125, 0.25, 0.5, 1, 2, and 4 IU/mL; left panel) and FLV (0, 0.25, 0.5, 1, 2, 4, 8, and 16 μM; middle panel), and CsA (0, 0.031, 0.062, 0.125, 0.25, 0.5, and 1.0 μg/mL; right panel), respectively, for 72 h. Then these cells were subjected to luciferase assay. (c) Expression of core in the control cells and the IFN-α-resistant cells. The control cells and the IFN-α-resistant cells were treated with IFN-α (0 and 4 IU/mL), IFN-β (2 IU/mL), IFN-γ (4 IU/mL), FLV (10 μM), and CsA (1 μg/mL) for 72 h and subjected to immunofluorescence analysis. (d) Dual-luciferase reporter assay of IFN-α. The control cells and the IFN-α-resistant cells were treated with IFN-α (0, 1, 10, and 100 IU/mL) for 6 h and subjected to ISRE reporter assay.



with the difference between first generation control and IFN-α-resistant cells. Together with these studies, the present results suggest that cellular factors play a predominant role in the IFN-resistance phenotype in cell culture.

The present study is the first report on the establishment of IFN-resistant genome-length HCV RNA-harboring cells, because previous IFN-resistant cells were established using subgenomic replicons.[21,22,32,33] Recently, it was reported that the 70th AA (Q or H) and 91st AA (M) in the core region are responsible for the resistance to PEG IFN and ribavirin therapy.[23] Core AA (70th/91st) of the original HCV-O sequence in OR6 is (Q/L). Therefore, the original HCV-O sequence is a partially resistant type based on this criterion of the core sequence (70th/91st). We could not detect any change in these AA positions in the core region after IFN treatment in IFN-resistant cells. To clarify the role of the core sequence (70th/91st) in IFN resistance, we are now planning to make the HCV-O with wild and mutant type sequences at 70th/91st in the core region. These HCV will provide us with important information regard-

Figure 5 The prevention of the relapse of hepatitis C virus (HCV) in interferon (IFN)-α-resistant cells. (a) Schema of the relapse cell culture assay. The control cells (OR6(C)) or the IFN-α-resistant cells (OR6(R)) were treated with IFN-α alone or in combination with IFN-γ, FLV, or CsA for 72 h. Then the cells were cultured for 0, 24, 48, and 72 h for luciferase assay and for 120 h for western blot analysis. (b and c) Luciferase assay and western blot analysis for the cells treated with IFN-α. The control cells were treated with IFN-α (250 IU/mL) for 72 h and subjected to luciferase assay. The IFN-α-resistant cells were treated with IFN-α at 250 IU/mL and 1000 IU/mL for 72 h and subjected to luciferase assay. The cells treated with the conditions above were further cultured for 48 h and subjected to western blot analysis using the core antibody. OR6c was used as a negative control (lane 1). OR6(C) and OR6(R) treated with IFN-α under the conditions shown above were also subjected to western blot analysis (lanes 2–4). (d and e) Luciferase assay and western blot analysis for the cells treated with IFN-α alone or in combination with IFN-γ. OR6(R) were treated with IFN-α (250 IU/mL and 1000 IU/mL) alone, or IFN-α (250 IU/mL) and IFN-γ (4 IU/mL) and subjected to luciferase assay and western blotting analysis. (f and g) Luciferase assay and western blot analysis for the cells treated with IFN-α alone or in combination with FLV. OR6(R) were treated with IFN-α (250 IU/mL and 1000 IU/mL) alone, or IFN-α (250 IU/mL) and FLV (10 μM) and subjected to luciferase assay and western blot analysis. (h and i) Luciferase assay and western blot analysis for the cells treated with IFN-α alone or in combination with CsA. OR6(R) were treated with IFN-α (250 IU/mL and 1000 IU/mL) alone, or IFN-α (250 IU/mL) and CsA (0.5 μg/mL) and subjected to luciferase assay and western blot assay. (j) Time course of HCV RNA levels before and after withdrawal of anti-HCV reagents in IFN-α resistant cells. OR6(R) were treated with IFN-α (250 IU/mL and 1000 IU/mL) alone, or IFN-α (250 IU/mL) in combination with IFN-γ (4 IU/mL), FLV (10 μM), or CsA (0.5 μg/mL) for 72 h. Then, anti-HCV reagents were removed and the cells were further cultured for 72 h.

ing IFN-resistant mechanism related to core sequences. The IFN-sensitivity-determining region (ISDR) in NS5A has also been reported as the region responsible for IFN resistance.[34] There is no AA substitution in ISDR in the original HCV-O sequence. Therefore, the original HCV-O sequence is a resistant-type based on the criterion of the ISDR. More recently, El-Shamy *et al.*[35] reported that the sequence variation in IFN/ribavirin resistant-determining region (IRRDR) (2334–2379 AA in NS5A) could correlate with SVR on IFN/ribavirin therapy. In the original HCV-O sequence, there were four AA substitutions in IRRDR. Therefore, original HCV-O sequence is a resistant-type based on the criterion of the IRRDR. We could not detect any mutations in NS5A in IFN-resistant cells. In a previous study using subgenomic 1B-1 and Con1 replicon, AA substitutions were frequently seen in NS5A.[21,22] The NS3 is another region in which frequent mutations occurred in 1B-1 and Con1 replicons with the IFN-resistance phenotype. The mutations P1115L and E1202G in NS3 have been identified in HCV sequences of OR6(C) cells and OR6(R) cells, respectively. We previously reported that these mutations are adaptive mutations, and that E1202G has a stronger enhancing effect on HCV RNA replication than P1115L.[36] This difference in the effects of adaptive mutations might be at least partly responsible for the diverse sensitivity of HCV to IFN-α. In the present study, we couldn't identify mutations responsible for IFN-resistance, because the effect of the viral mutations on IFN-resistance seems to be not so strong compared with the cellular factor and the possible combinations of mutations are so large.

The advantages of using the reporter HCV RNA system are its precision and high-sensitivity for monitoring HCV RNA replication. The system is especially useful for the detection of HCV RNA replication at near undetectable levels. The monitoring of HCV relapse after combination treatment with IFN-α and anti-HCV reagents is a good example of the use of this assay system. Therefore, we tried to develop a relapse cell culture model using the established IFN-α-resistant cells. The IFN-α-resistant HCV RNA-harboring cells are less sensitive to IFN-α and IFN-β than control cells. However, both cell lines possessed similar sensitivity to IFN-γ, CsA, and FLV. The luciferase assay revealed that 250 and 1000 IU/mL of IFN-α was needed to prevent the relapse of HCV in control cells and IFN-α-resistant cells, respectively. Four IU/mL of IFN-γ, 0.5 μg/mL of CsA, or 5 μM of FLV could help to prevent the relapse of HCV in combination with 250 IU/mL of IFN-α. These results suggest that IFN-γ, CsA, and FLV are good partners with IFN-α in combination therapy to prevent the relapse of IFN-α-resistant HCV.

The relapse model in the present study corresponds with a relapse just after the end of IFN treatment in clinical therapy. The evaluation for the relapse required only 72 h by luciferase assay. As we described in the first section of the Results, we need an additional 3 weeks to confirm the complete elimination of HCV or SVR in the cell culture model by colony formation assay. To further evaluate the complete elimination, of HCV, a more sensitive colony formation assay is needed. For example, although 4 weeks of IFN-α treatment at 10 IU achieved the inhibition of the relapse, G418-resistant colonies

emerged after 3 weeks. We are currently planning a study on the SVR cell culture model using established IFN-α-resistant HCV RNA-harboring cells.

To use our cell culture system as the relapse model, there are some limitations derived from the differences between cell culture and humans: (i) SVR in patients with CHC is assessed by the serum HCV RNA. However, our system monitors the intracellular HCV RNA levels; and (ii) HuH-7 cells lack several innate immunities, such as TRL3 in cell surface. To date, we don't have a genotype 1b HCV infection system and cell lines other than HuH-7 cells, which support robust HCV RNA replication. The development of a genotype 1b HCV infection system and cell line will overcome the limitation and improve our present relapse model.

In conclusion, we established IFN-α-resistant, HCV-harboring cells from genome-length HCV RNA-harboring OR6 cells. In addition, we developed a relapse model using the IFN-α-resistant HCV RNA-harboring cells. The anti-HCV reagents, which exhibited a mechanism different from IFN-α efficiently, could prevent the relapse of HCV in IFN-α-resistant HCV RNA-harboring cells. This cell culture model should prove useful for the study of relapse after IFN therapy.

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Hypoxia-Inducible Factor 1α Is Up-Regulated by Oncostatin M and Participates in Oncostatin M Signaling

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The interleukin-6–type cytokine oncostatin M (OSM) acts via the Janus kinase/signal transducer and activator of transcription pathway as well as via activation of mitogen-activated protein kinases and is known to critically regulate processes such as liver development and regeneration, hematopoiesis, and angiogenesis, which are also determined by hypoxia with the hypoxia-inducible factor 1α (HIF1α) as a key component. Here we show that treatment of hepatocytes and hepatoma cells with OSM leads to an increased protein level of HIF1α under normoxic and hypoxic conditions. Furthermore, the OSM-dependent HIF1α increase is mediated via Janus kinase/signal transducer and activator of transcription 3 and mitogen-activated protein kinase kinase/extracellular signal-regulated kinase 1/2 pathways. OSM-mediated HIF1α up-regulation did not result from an increase in HIF1α protein stability but from increased transcription from the *HIF1α* gene. In addition, we show that the OSM-induced *HIF1α* gene transcription and the resulting enhanced HIF1α protein levels are important for the OSM-dependent vascular endothelial growth factor and plasminogen activator inhibitor 1 gene induction associated with several diseases. *Conclusion:* HIF1α levels increase significantly after treatment of hepatocytes and hepatoma cells with OSM, and HIF1α contributes to OSM downstream signaling events, pointing to a cross-talk between cytokine and hypoxia signaling in processes such as liver development and regeneration. (HEPATOLOGY 2009;50:253-260.)

Oncostatin M (OSM) is an interleukin (IL)-6–type cytokine produced by monocytes and macrophages, T cells, and several other cell types. OSM receptors are widely expressed and are composed of the common signal transducer gp130 in complex with the LIFR or the OSMR. OSM has pleiotropic effects that in part overlap with those of other IL-6–type cytokines; examples include inflammation, neurogenesis, regulation of cell proliferation, and fibrosis.[1-3] In addition, OSM plays a crucial role in the orchestration of hematopoiesis and liver development.[3]

Upon OSM-induced receptor clustering, Janus kinases—mainly Janus kinase 1—are activated, phosphorylate tyrosines within the receptor that recruit other signaling proteins with matching SH2 domains such as signal transducers and activators of transcription (STATs) or adapter proteins for the mitogen-activated protein kinases to the receptor. The major signaling cascades activated by OSM include STAT3 as well as the extracellular signal-regulated kinase (Erk) 1/2 and p38 pathways.[2-4]

Several processes such as hematopoiesis, angiogenesis, liver development, metabolism, inflammation, and tu-

Abbreviations: Erk, extracellular signal-regulated kinase; HIF, hypoxia-inducible factor; HIF1α, hypoxia-inducible factor 1α; HRE, hypoxia response element; IL, interleukin; mRNA, messenger RNA; OSM, oncostatin M; PAI1, plasminogen activator inhibitor 1; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; siRNA, small interfering RNA; STAT, signal transducer and activator of transcription; TNF-α, tumor necrosis factor α; VEGF, vascular endothelial growth factor.

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morigenesis are also crucially influenced by the ambient oxygen tension of the tissue. Hypoxia-inducible factors (HIFs) act as master regulators for the expression of genes essential in a hypoxic microenvironment. The best characterized factor is HIF1, which regulates more than 100 genes. HIF1 is formed by dimerization of the oxygen-sensitive hypoxia-inducible factor 1α (HIF1α) subunit with the constitutively expressed beta-subunit, which is also known as ARNT. Under normoxic conditions, the HIF1α subunit is hydroxylated and quickly degraded via the proteasome. Under hypoxic conditions, the activity of the hydroxylases is reduced, and HIF1α protein is stabilized and can bind to hypoxia-response elements (HREs) within the regulatory areas of HIF target genes and efficiently recruit cofactors.[5]

In addition to hypoxia, HIF1α has also been shown to be up-regulated under normoxia in response to growth factors, thrombin, lipopolysaccharide, angiotensin II, insulin, or the cytokines IL-1 and tumor necrosis factor α (TNF-α).[6] Although several details have been unraveled regarding the ability of hypoxia to stabilize HIF1α, the mechanisms by which those factors (especially cytokines) induce HIF1α have not been fully elucidated.

Interestingly, OSM has been described to orchestrate the hypoxia-influenced processes of hematopoiesis, angiogenesis, liver development, and regeneration.[3,7] This suggests a possible cross-talk of the OSM and hypoxia signaling pathways. The expression of the HIF1 target genes vascular endothelial growth factor (VEGF) and plasminogen activator inhibitor 1 (PAI1)—which are crucial for angiogenesis and tissue remodeling, respectively—can also be up-regulated by the cytokine OSM.[8-12] OSM supports *in vitro* differentiation of fetal hepatic cells into liver-like structures, which is paralleled by enhanced VEGF expression.[11] Moreover, OSM mediates differentiation of oval cells into hepatocytes.[13] OSM plays a crucial, nonredundant role in liver regeneration, as shown for OSM receptor knock-out mice after partial hepatectomy or CCl_4 treatment,[14] and OSM gene therapy attenuates liver damage induced by dimethylnitrosamine.[15] Interestingly, HIF1α is also expressed during liver regeneration,[16] and HIF1α supports the growth of hepatoma cells *in vivo* and *in vitro*.[17,18]

Because both OSM and HIF1α play a pivotal role in liver-related processes (development, regeneration, carcinogenesis), the aim of the present study was to investigate whether the OSM signaling pathway has an impact on the HIF1 system in hepatoma cells and hepatocytes.

Materials and Methods

Cell Culture and Reagents. HepG2 hepatoma cells were maintained in DMEM/NUT-MIX-F12 medium (Lonza) supplemented with 10% fetal bovine serum (PAA), 100 mg/L streptomycin, and 60 mg/L penicillin (Cytogen). The human hepatocyte cell line PH5CH8 has been described.[19] Cells were grown at 37°C in a water-saturated atmosphere at 5% CO_2. Hypoxia treatment was performed at 37°C in a water-saturated atmosphere at 5% CO_2 and 6% oxygen. HepG2 cells were transfected using the Fugene reagent (Roche) according to the manufacturer's recommendations. Cotransfections of small interfering RNA (siRNA) and reporter gene constructs are described in the Supporting Information. Human recombinant OSM was obtained from Peprotech. Actinomycin D and cycloheximide were obtained from Calbiochem. Stattic[20] was from Sigma.

Western Blot Analysis and Antibodies. All steps of cell lysis and immunoprecipitation were performed at 4°C using ice cold buffers. Cells were lysed on a dish with lysis buffer containing 30 mM Tris/HCl (pH 6.7), 5% glycerol, 2.5% mercaptoethanol, and 1% sodium dodecyl sulfate. The lysates were further analyzed via sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting. Antibodies against HIF1α, STAT3, STAT1, and Fin13 were obtained from BD Transduction Laboratories. Antibodies against phospho-STAT3, phospho-STAT1, Erk1/2, phospho-Erk1/2, p38, and phospho-p38 were obtained from Cell Signaling. ECL signals were detected as described.[21] Before reprobing, blots were stripped as described.[4]

Reporter Gene Assays. HepG2 cells were transfected with 1 μg of the β-galactosidase control plasmid (pCH110, Amersham Biosciences) and 1.5 μg of the respective reporter gene construct. Twenty-four hours after transfection, the cells were treated with the different stimuli as described in the figure legends. Cell lysis and luciferase assays were performed using the Promega luciferase assay system (Promega, Madison, WI) (see Supporting Information for further details). All experiments were performed at least in triplicate, and biological triplicates were also performed within one experiment. Luciferase activity values were normalized to transfection efficiency monitored by the cotransfected β-galactosidase expression vector. For some experiments the luciferase activity values after OSM stimulation were additionally normalized to values from control unstimulated cells.

Quantitative Real-Time Polymerase Chain Reaction. The exact protocol is described in the Supporting Information. Total RNA was extracted using the RNeasy Mini Kit (Macherey Nagel) according to the manufacturer's instructions. The concentration of isolated RNA was measured using a NanoDrop spectrophotometer. One microgram of total RNA was reverse-transcribed with a ThermoScript RT-PCR System (Invitrogen). Quantita-