facilitates the mass screening for anti-HCV reagents. HCV RNA replicating in OR6 cells contained an adaptive mutation, K1609E, in the NS3 region. Adaptive mutations have been reported to enhance the replication level of HCV RNA in cell culture [59–61]. In the case of HCV-O, two adaptive mutations were required for robust replication of the genome-length HCV RNA replication [60]. For example, authentic HCV-O RNA with the adaptive mutations of E1202G and K1609E can robustly replicate in HuH-7 cells for 9 months or more (Ikeda et al., unpublished data).

In 2005, three groups reported infectious HCV production systems using the JFH1 strain in cell culture [6–8]. These reports showed that the life cycle of HCV could be re-constructed in HuH-7 cells, and thus became landmarks in the search for an ideal HCV cell culture system. The unique features of these systems were the origin of this strain and the cell lines. JFH1 was a genotype 2a strain derived from a patient with fulminant hepatitis and did not require any adaptive mutations for robust replication, unlike other HCV strains. The unique feature of this system was that it employed HuH-7 cells such as Huh-7.5 or Huh-lunet cells, since the parental HuH-7 cells could not support robust production of infectious HCV [6–8,62]. Recently, the genotype 1a H77-S strain was reported to produce infectious HCV in cell culture, although the production level of infectious H77-S was lower compared with that by JFH1 [63]. Interestingly, five adaptive mutations were introduced into the H77-S genome in order to enhance the efficiency of infectious virus production. The presence of these adaptive mutations is the most striking and controversial characteristic regarding the production of infectious HCV described above. Further study will be needed to understand the role of adaptive mutations on infectious virus production.

3.2. HCV life cycle

The establishment of an infectious HCV production system gradually led to clarification of the life cycle of HCV. Information regarding the HCV RNA replication has been accumulated since the development of the HCV replicon system, and the infectious HCV production system [6–8] has further provided information about the step of virus entry and release. The life cycle of HCV includes the (1) receptor binding and cell entry, (2) cytoplasmic release and uncoating, (3) IRES-mediated translation, (4) processing, (5) RNA replication, (6) packaging and assembly, (7) virion maturation, and (8) virion release. Although some of the mechanisms are still unclear, each of these steps is a target for antivirals. Among the proteins involved in these steps, the protease in step (4) and polymerase in step (5) have been especially well characterized. Specific inhibitors for these proteins have been developed and some of them are now in clinical trials for patients with CH–C [21,64].

3.3. Cellular proteins required for HCV RNA replication

Cellular proteins are required for HCV RNA replication and may determine the cell tropism of HCV. As HCV is a parasite, it utilizes the cellular proteins for its replication machinery. Therefore, cellular proteins essential for HCV RNA replication are the targets for antivirals. Using cell culture systems, several cellular proteins have been identified as effective molecules for HCV RNA replication (Table 1). La and PTB were representative molecules reported as essential host factors for HCV RNA replication [40]. Recently, an immunosuppressant, CsA, has been reported to inhibit HCV RNA replication by blocking the binding of CyPB to NS5B [28]. HSP90 and the FK-506-binding protein 8 (FKBP8) form a complex with NS5A and geldanamycin, an inhibitor of HSP90, suppressed HCV RNA replication by blocking the formation of these complex [38]. The advantage of the inhibitors targeting cellular factor is that these reagents do not affect the viral escape achieved through mutations. The high mutation rate caused by RdRp frequently produced escape mutants toward the antiviral reagents for HCV proteins. A disadvantage of the inhibitors targeting cellular factors may be that they induce side effects by inhibiting the primary roles of the cellular factors.

4. Host metabolism as anti-HCV targets

The cellular factors are the targets of the antivirals independent of the viral escape via the genetic mutations caused by RdRp. The cellular factors were synthesized in their metabolic pathways and modified by the enzymes. These enzymes are also targets in the antiviral strategy (Table 1). Furthermore, some of the reagents have already been used in the clinical treatment of the respective diseases. One of the advantages of using existing reagents is that their characterizations—including safety and side effects—have already been performed. Therefore, screening of the existing reagents for anti-HCV will be a new field of antivirals. The development of a cell culture system for HCV led to the revelation that HCV incorporates many cellular factors into the replication machinery of the virus. Now we have both the information of the HCV life cycle and the cell culture assay system—the input and output—that we need to develop a pool of antiviral reagents. Below, we will discuss the particular host cell metabolic pathways that are currently being targeted by anti-HCV reagents including more recently found pitavastatin (PTV) (Fig. 2B).

4.1. Cholesterol-biosynthesis pathway and geranylgeranylation

In the cholesterol-biosynthesis pathway, the region downstream of mevalonate branches into separate pathways for cholesterol and isoprenoid synthesis (Fig. 3). The attachment of the isoprenoid is called prenylation of the protein. Prenylation regulates a variety of cellular functions, such as growth, differentiation, and oncogenesis. Farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) are mevalonate-derived isoprenoids and are attached to the target proteins by farnesyltransferase (FTase) and geranylgeranyl transferase type I (GGTase-I), respectively. FTase and GGTase-I recognize protein substrates with a C-terminal tetrapeptide recognition motif called the CaaX box: in the case of GGTase-I, C is cysteine, a is an aliphatic amino acid, and X is leucine, isoleucine, valine, or phenylalanine. Production of mevalonate by 3-hydroxy-3-methylglutaryl coenzyme A (HMG–CoA) reductase is the rate-

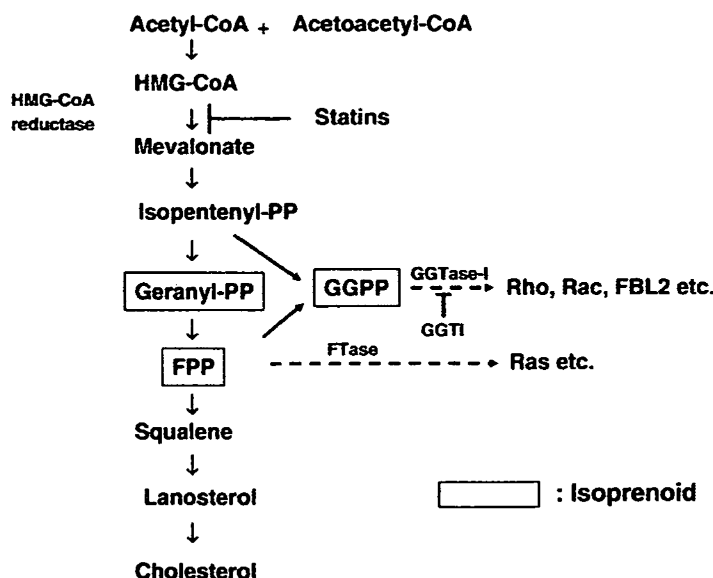

Fig. 3. Cholesterol-biosynthesis pathway. The inhibition of HMG–CoA reductase by statins leads to the suppression of mevalonate and of the production of its downstream metabolites. Decreased prenylation on the GTP-binding proteins had a significant effect on the signal transduction.

limiting step in the cholesterol biosynthesis. Statins are potent HMG–CoA reductase inhibitors and are beneficial in the prevention of coronary heart disease. Statins also inhibit the prenylation of the proteins.

Lipid metabolism is essential for the life cycle of many viruses. The cholesterol-rich lipid raft plays an important role in virus entry, replication, and assembly. HCV also forms a replication complex on the lipid raft membrane structure [65]. HCV RNA replication occurs in the lipid raft and the cholesterol supply is crucial to maintain the structure of the lipid raft [65]. Aizaki et al. [66] reported that lovastatin (LOV), one of the HMG–CoA reductase inhibitors, inhibited HCV RNA replication in HCV replicon-harboring cells.

Statins also possess the cholesterol-independent action (pleiotropic effect) [67]. Many of these pleiotropic effects are mediated by the isoprenoid. For example, inhibition of small GTP-binding proteins, Ras and Rho, whose proper membrane localization and function are dependent on prenylation, may play a significant role in the pleiotropic effect of statins. Ras and Rho are major substrates for prenylation with FPP and GGPP, respectively. GDP-bound Ras and Rho are localized in the cytoplasm. When FPP or GGPP is bound to the inactive Ras or Rho, they are translocated to the cell membrane and converted to GTP-bound active forms. Recently, Wang et al. [68] identified FBL2 as one of the geranylgeranylated cellular proteins required for HCV RNA replication. FBL2 belongs to the FBL family of proteins, all of which contains an F box and a multiple leucine-rich repeat, with the F box binding to a multicomponent ubiquitin ligase complex. Geranylgeranylated FBL2 binds to NS5A, and the resulting complex seems to be required for HCV RNA replication. In HCV replicon-harboring cells, knockdown of FBL2 by siRNA has been shown to reduce HCV RNA by 65% [68]. Depletion of the GGPP by statins may inhibit the geranylgeranylation of cellular proteins such as FBL2 and cause the anti-HCV effect in the cells.

Statins are among the most widely used reagents to lower cholesterol. One of the statins used clinically, LOV, has been well characterized and shown anti-HCV activity in cell culture. [66,69,70]. However, the anti-HCV activities of other statins remain to be clarified. Recently the anti-HCV activities of several statins were characterized using an OR6 assay system [71]. The anti-HCV activities were tested for five statins: atorvastatin (ATV), fluvastatin (FLV), pravastatin (PRV), simvastatin (SMV), and LOV. FLV exhibited the strongest anti-HCV activity (50% effective concentration to inhibit HCV RNA replication (EC_{50}): 0. 9 μM), while ATV and SMV showed moderate inhibitory effects (EC_{50}: 1.39 and 1.57 μM, respectively). However, LOV, which has been reported to inhibit HCV replication, was shown to possess the weakest anti-HCV activity (EC_{50}: 2.16 μM). More recently, we found that PTV possessed stronger anti-HCV activity than FLV (Fig. 2B). The EC_{50} of PTV was calculated as 0.45 μM. The anti-HCV activities of statins were reversed by supplying mevalonate or geranylgeraniol. However, surprisingly, PRV exhibited no anti-HCV activity, although it worked as an inhibitor for HMG–CoA reductase. Although PRV is a water-soluble reagent (the others are lipophilic), PRV induced the expression of HMG–CoA reductase by a positive feedback mechanism. There may be another mechanism underlying the depletion of GGPP by the statins. Interestingly, it has been reported that only PRV has a different effect on the induction of P450 compared with the other statins [72].

Ribavirin is the only reagent currently used with IFN-α to treat patients with CH-C [73]. In the previous study on anti-HCV activity using the OR6 assay system, the EC_{50} of ribavirin was 76 μM [74]. This concentration is much higher than the clinically achievable ribavirin concentration (10–14 μM) reported previously [75,76]. Since FLV exhibited strong anti-HCV activity, FLV was examined for its anti-HCV activity in combination with IFN-

α in OR6 cells [71]. Co-treatment of IFN-α and FLV exhibited synergistic inhibitory effects on HCV RNA replication. For example, when administered in combination with IFN-α (2 IU/ml) and FLV (5 μM), the level of HCV RNA replication was remarkably reduced to approximately 3%, compared with the effects of treatment with IFN-α alone. The combination therapy of FLV may be effective for the treatment of patients with CH–C.

It is not appropriate to further reduce the cholesterol level of CH–C patients who already have a normal cholesterol level. For these patients, statin-related anti-HCV reagents possessing no cholesterol-lowering activity would be good candidates for future clinical use. The specific inhibition of GGPP synthesis and prenylation will be worth testing, and GGTase-I inhibitor (GGTI) is one of the candidates for this purpose. Furthermore, specific inhibition of the proteins modified by GGTase-I may be more effective. FBL2 may be one of the target proteins, because its formation of a complex with NS5A is required for HCV RNA replication. Therefore, the reagents blocking the association of FBL2 with NS5A will be able to inhibit the HCV RNA replication with fewer side effects. Prenyltransferase recognizes a broad range of protein substrates with a CaaX motif. Reid et al. [77] reported a list of hypothetical prenyltransferase substrates within the human genome. Other than FBL2, the host molecules involved in HCV RNA replication may be exist in this list.

Antiviral activity of statins has also been reported in other viruses. In the respiratory syncytial virus (RSV), LOV exhibited antiviral activity via the inhibition of RhoA [78]. RhoA is activated by geranylgeranylation, and activated RhoA interacts with the F glycoprotein of RSV. FLV inhibited cytomegalovirus (CMV) replication by abolishing CMV-induced NF-κB activity, which is involved in a pathway that is crucial for CMV replication [79]. In human immunodeficiency virus (HIV), LOV and SIV reduced HIV replication via suppression of the binding between the integrin intercellular adhesion molecule 1(ICAM1) and lymphocyte function associated antigen-1 (LFA-1) [80]. Statins were recently shown to bind to LFA-1, and ICAM1-bearing viruses were reduced by statins in a dose-dependent manner. It is noteworthy that the inhibition of LFA-1 binding to ICAM-1 by statins is independent of the inhibition of HMG CoA reductase. Statins inhibited the cholesterol-biosynthesis pathway and branched prenylation pathways by depletion of mevalonate. The latter caused pleiotropic effects in growth, differentiation, and antivirals. However, an unknown function of statins may exist—for example, the binding of LFA-1 is likely independent of the cholesterol-lowering and the inhibition of prenylation. Furthermore, the finding that PRV has a different effect on the induction of P450 than the other statins has not been clearly explained by the characterization of these mechanisms of statins. A better understanding of this finding may lead to the discovery of statin-related anti-HCV reagents that do not have exhibit any cholesterol-lowering activity or inhibition of prenylation.

4.2. Sphingolipid synthesis pathway

Lipid rafts are detergent resistant membranes (DRM) and are enriched in cholesterol and sphingolipids. The active replication complex of HCV is present in lipid rafts [65]. Therefore, sphingolipid metabolism is also an antiviral target for HCV. Serine palmitoyltransferase (SPT) is the enzyme responsible for the condensation of L-serine with palmitoyl–CoA to produce 3-ketodihydrosphingosine in the first step of sphingolipid biosynthesis (Fig. 4). Myriocin, a selective inhibitor of SPT, inhibited the replication of HCV replicon [81,82].

Sakamoto et al. [81] reported that the compound NA255, which is structurally similar to myriocin, also inhibited the replication of the HCV replicon. NA255 has been identified as the secondary fungal metabolite derived from *Fusarium* sp. NA255 suppressed HCV replicon in a dose-dependent manner, and its EC_{50} was 2 nM. They further examined the involvement of the sphingolipid synthetic pathway in HCV RNA replication. Fumonisin B1, an inhibitor of dihydroceramide synthase, also suppressed the replication of HCV replicon. In mammalian cells, ceramide is synthesized in the endoplasmic reticulum (ER) and translocates to the Golgi compartment for conversion to sphingomyelin. HPA-12, an inhibitor of ceramide trafficking from the ER to the Golgi apparatus, also inhibited the replication of HCV replicon. Glycosphingolipids (GSLs) are also a component of lipid rafts, and PPMP, an inhibitor of GSL biosynthesis, also suppressed the replication of HCV replicon. Furthermore, they demonstrated that after treatment with NA255, the NS5B ratio in the DRM was markedly decreased. Interestingly, however, the DRM fraction of NS3 and NS5A were not affected. Inhibition of sphingolipid biosynthesis by NA255 disrupted the association of lipid rafts with NS5B, but not with NS3 or NS5A. They identified a helix-turn-helix motif (Glu230-Gly263) in NS5B as a sphingolipid-binding domain (SBD), which was similar in structure to the SBD of the V3 loop of HIV-1.

Umehara et al. [82] reported that myriocin suppressed HCV RNA replication *in vivo*, using HCV-infected chimeric mice with humanized livers. Myriocin reduced the HCV RNA levels in both serum and liver to 1/10–1/100 of the levels prior to the 8 day treatment. They also demonstrated that the combined treatment of myriocin with PEG-IFN reduced the HCV RNA level to less than 1/1000 of the control levels. These results

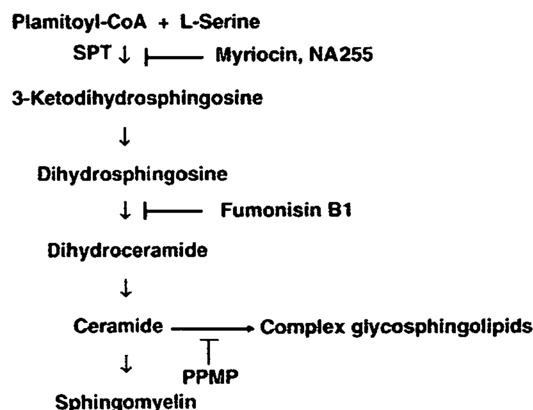

Fig. 4. Sphingolipid-biosynthesis pathway. The spingolipid-biosynthesis pathway. Myriocin and NA255 inhibited the SPT and caused the depletion of spingomyelin and glycosphingolipids.

suggest that the sphingolipid biosynthetic pathway is also a suitable target for the development of HCV therapies.

4.3. GTP-biosynthesis pathway

At the beginning of GTP-biosynthesis pathway, inosine monophosphate dehydrogenase (IMPDH) is the enzyme responsible for the conversion of inosine 5′ monophosphate (IMP) into xanthosine 5′ monophosphate (XMP) (Fig. 5). Ribavirin, mizoribine, mycophenolic acid (MPA), and VX-497 are IMPDH inhibitors and inhibit HCV RNA replication.

Ribavirin enhanced the SVR of PEG-IFN therapy from 29% to 56% compared to the PEG-IFN monotherapy [83]. However, the antiviral mechanisms of ribavirin remain to be clarified. Four possible mechanisms have been proposed [73,84]: (1) direct inhibition of RNA replication; (2) inhibition of IMPDH; (3) immunomodulation; (4) mutagenesis. Ribavirin is phosphorylated to mono-, di-, and triphosphate (RMP, RDP, and RTP, respectively). (1) RTP, an analog of GTP, is incorporated into replicating RNA by RdRp and caused termination of the RNA synthesis. (2) RMP competitively inhibits the host enzyme IMPDH, which is essential for the synthesis of GTP, and causes a depletion of the GTP pool. (3) Rivavirin has been suggested to cause immunomodulatory effects, such as the shift of Th2 to Th1 in immune response, and to induce an HCV-specific T cell response. (4) Ribavirin acts as an RNA mutagen and causes error catastrophe. In poliovirus replication, 100 μM of ribavirin increased the mutation rate from about 1.5 mutations/genome (wild type) to about 1.9 mutations/genome and resulted in a decrease of infectivity of 70% [85]. The mutation rate increased in a ribavirin dose-dependent manner: 6.9 mutations/genome and 15.5 mutations/genome at 400 μM and 1000 μM, respectively [85].

In the clinical study of CH–C, the enhancement of SVR has been observed only in combination therapy of ribavirin with IFN, but not in ribavirin monotherapy. It may be difficult to test the effect of ribavirin monotherapy, since the clinically achievable concentration of ribavirin without severe side effects such as anemia is too low (10–14 μM). However, in the cell culture model [74,86], a higher concentration of ribavirin suppressed HCV RNA replication (EC_{50}: 76 μM) [74].

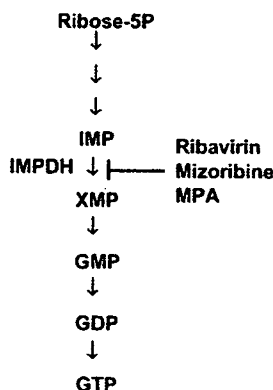

Fig. 5. GTP-biosynthesis pathway. The de novo GTP-biosynthesis pathway. Ribavirin, mizoribine, and MPA suppressed the XMP synthesis by the inhibition of IMPDH.

Mizoribine is an imidazole nucleoside that is isolated from culture medium of the mold *Eupenicillium brefeldianum* M-2166 and is structurally similar to ribavirin. Mizoribine was authorized by the Japanese Government as an immunosuppressive drug for renal transplantation; thereafter, lupus nephritis, rheumatoid arthritis, and nephritic syndrome were also added to the list of diseases for which this agent is indicated [87,88]. Based on the similarity of mizoribine to ribavirin, the anti-HCV activity of mizoribine has been tested using an OR6 assay system. The anti-HCV activity of mizoribine (EC_{50}: 99 μM) was similar to that of ribavirin [74]. Furthermore, a low dose (at least 5 μM) of mizoribine was able to enhance the antiviral activity of IFN [74]. Mizoribine was reported to exhibit antiviral activity on influenza virus types A and B [87] and recently on bovine viral diarrhea virus [89] and severe acute respiratory syndrome (SARS)-associated coronavirus [90]. The precise antiviral mechanism of mizoribine remains unclear. However, any of the four hypothesized mechanisms of ribavirin mentioned above may be possible. Since mizoribine has not been associated with severe side effects, it will be an alternative reagent for combination therapy with IFN.

Like mizoribine, MPA is used as an immunosuppressant and is known to inhibit IMPDH. It has been reported to show *in vitro* antiviral activity against dengue virus [91,92], hepatitis B virus (HBV) [93], avian reovirus [94], yellow fever virus [95], and West Nile virus [96]. The anti-HCV activity of MPA was reported by Henry et al. [97]. At clinically relevant concentrations (1.0–6.0 μg/ml), MPA inhibited HCV RNA replication to approximately 75% in a study using HCV replicon-harboring cells. Furthermore, combination treatment of MPA with CsA or IFN showed synergistic inhibition of HCV RNA replication. We also recently confirmed that the combination of CsA and mizoribine had a synergistic effect on the inhibition of HCV RNA replication (Yano et al., unpublished data). These data suggest that immunosuppressive drugs possessing anti-HCV activity, such as CsA, MPA, and mizoribine, may prevent not only the rejection of the graft but also the recurrence of HCV infection after liver transplantation, and that a combination of these drugs may be of additional benefit for such patients.

VX-497 is a reversible uncompetitive IMPDH inhibitor that is structurally unrelated to other known IMPDH inhibitors. Markland et al. [98] reported the broad-spectrum antiviral activity of VX-497.VX-497 exhibited 10- to 100-fold more potency than ribavirin against HBV, human CMV, RSV, herpes simplex virus type 1, parainfluenza 4 virus, EMCV, and Venezuelan equine encephalomyelitis virus in cell culture [98]. Zhou et al. [99] reported that VX-497 alone had only marginal effect on HCV replicon, although combination treatment with ribavirin and VX-497 enhanced anti-HCV activity. They also reported that in their HCV replicon assay system, MPA showed only a marginal anti-HCV effect [99]. This result is different from the anti-HCV effect of MPA reported by Henry et al. [97]. Further study will be needed to clarify these controversial results.

4.4. N-glycosylation pathway

HCV morphogenesis is a target of antivirals in the life cycle of the virus. The HCV envelope glycoproteins E1 and E2 are highly N-glycosylated [100]. The consensus sequence for N-glycosylation is Asn-X-Ser/Thr, where X is any amino acid except for Pro, and E1 and E2 contain 5-6 and 11 glycosylation sites, respectively. From the previous study using bovine viral diarrhea virus, inhibition of α-glucosidase is expected to prevent the proper folding and assembly of HCV. Therefore, the N-glycosylation pathway may be a novel molecular target for antivirals. Chapel et al. [101] reported an anti-HCV effect of the α-glucosidase inhibitor in the binding step using HCV virus-like particles (VLPs) derived from baculovirus. The glucose analogue deoxynojirimycin derivatives, which are α-glucosidase inhibitors, caused the retention of unprocessed, hyperglycosylated N-linked glycans on HCV glycoproteins and led to the reduction in binding of VLP to the cells [101]. These results will be examined using a recently developed infectious HCV production cell culture system. α-glucosidase inhibitor may be one of the candidates for an effective combination therapy.

4.5. STAT1 methylation

It is crucial that the SVR for patients with CH–C receiving the current standard therapy of PEG-IFN plus ribavirin is improved from the current value of about 50%. The anti-HCV effect of IFN-α is caused through the Jak-STAT signaling pathway. Duong et al. [102] proposed that hypomethylation of STAT1 by HCV protein caused the resistance to IFN therapy. Unmethylated STAT1 is less active because it can be bound and inactivated by its inhibitor, the protein inhibitor of activated STAT1 (PIAS1). Protein arginine methyltransferase 1 (PRMT1) is the enzyme responsible for the methylation of STAT1. HCV proteins induced the expression of the catalytic subunit of protein phosphatase 2A (PP2Ac), and overexpression of PP2Ac induced STAT1 hypomethylation via the inhibition of PRMT1. Finally, PIAS1 interacted with and inhibited hypomethylated STAT1 and resulted in the suppression of IFN signaling [102].

S-adenosyl-L-methionine (AdoMet) is a methyl group donor for STAT1 methylation by PRMT1. AdoMet is used for the treatment of alcoholic liver disease and is available in many countries as a nonprescription drug. Betaine has been known to raise the intracellular concentration of AdoMet and plays the central role in the recycling of AdoMet. When PP2Ac was overexpressed in HuH-7 and UHVH 57.3 cells, IFN-α signaling was suppressed [102]. However, the co-treatment of AdoMet and betaine restored the IFN-α signaling. These results suggest that the addition of AdoMet and betaine to the current standard therapy with PEG-IFN and ribavirin may enhance the SVR for patients with CH–C.

4.6. Fatty acid-biosynthesis pathway

Lipid metabolism is one of the most important pathways for HCV RNA replication. Other than cholesterol and sphingolipid synthesis, fatty acids are reported to be metabolites involved in HCV RNA replication [70,103]. However, the precise mechanisms of fatty acids on HCV RNA replication have remained unclear.

Leu et al. [103] reported that polyunsaturated fatty acids (PUFAs) inhibited HCV replicon replication. Arachidonic acid (AA), docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA) belong to PUFAs (Fig. 6) and possessed anti-HCV activity. The EC_{50} of AA was 4 μM. However, at 100 μM, α-linolenic acid, γ-linolenic acid (GLA), and linoleic acid reduced HCV RNA levels slightly, and saturated fatty acids, including oleic acid, myristic acid, palmitic acid, and steric acid, slightly enhanced HCV RNA levels. Similar results were also reported by Kapadia et al. [70] using a genome-length HCV RNA-replicating cell line.

AA produces lipid mediators such as prostaglandins (PGs), thromboxanes (Txs), leukotriens (LTs), and lipoxins (LXs) (Fig. 6). However, the antiviral activity of these eicosanoids remains unclear. In their clinical study, Hyman et al. [104]

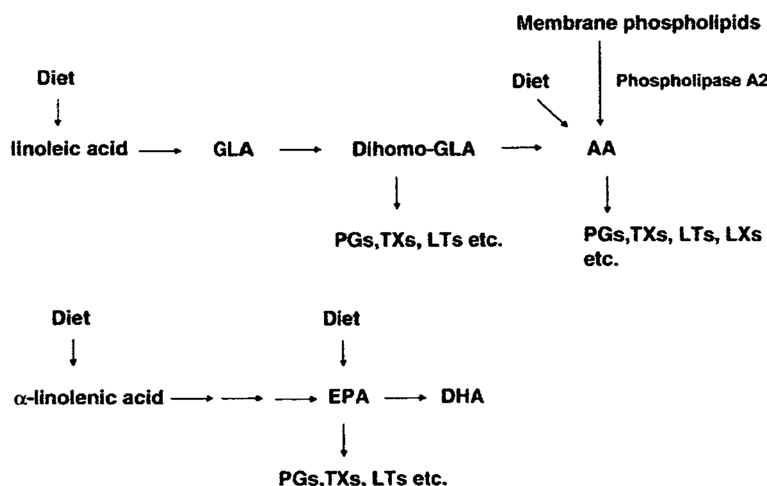

Fig. 6. Fatty acid-biosynthesis pathway. The PUFA metabolism from diet or membrane phospholipids.

reported that oral prostaglandin E2 therapy resulted in no beneficial effect on patients with CH–C. Investigation of the anti-HCV effects of the metabolites of PUFAs will lead to a new field of antivirals based on the host metabolism.

5. Conclusions

Ever since HCV was discovered to be the causative agent of non-A, non-B hepatitis virus, IFN has played the central role in treating the disease. Currently IFN has been modified by PEG and accompanied by the powerful partner, ribavirin, which boosts the anti-HCV activity of IFN. During the development of IFN therapy for patients with CH–C, the lack of a robust method of HCV RNA replication in cell culture has hampered research into the HCV life cycle and the discovery of potent new anti-HCV reagents. It is difficult to attack the Achilles' heel of HCV without information on the replication machinery of the virus. However, the development of a subgenomic replicon system by Lohmann et al. [5] partially revealed the HCV life cycle. The information about HCV RNA replication in the virus life cycle provided clues to the development of antivirals both from the standpoint of the virus and the host. A representative example is the discovery that NS3-4A inhibits innate immunity [105]. HCV runs through the cellular first defenses of the IFN-production system. NS3-4A, a serine protease, cleaved the unexpected cellular target Cardif and disrupted RIG-I signaling [106]. HCV replicon contributed to the discovery of the viral serine protease inhibitor. Surprisingly, a serine protease inhibitor, SCH6, inhibited HCV RNA replication not only by the inhibition of NS3-4A activity but also by the inhibition of the RIG-I signaling [105]. This serine protease inhibitor possesses dual functions, inhibiting both viral (NS3-4A) and cellular (Cardif) proteins involved in IFN production.

Viral and cellular molecules are the targets of antivirals. HCV RdRp caused a high mutation rate and the mutations accumulated in virus genome [107]. The high mutation rate enhances the viral evolution. As for the reagents targeting viral proteins, such as NS3-4A or NS5B, resistance to the therapy happens by the frequent mutations caused by RdRp. In fact, in the clinical trial of the NS3-4A protease inhibitor, VX-950, HCV RNA rapidly decreased within 3 days after treatment [20]. However, HCV RNA increased again at around 14 days after treatment [20]. HCV mutants may not be the problem in the anti-HCV reagent against cellular proteins, although the inhibition of the primary functions of the cellular proteins may cause side effects. In this review, host metabolic pathways are overviewed. One of the advantages of targeting host metabolism as antivirals is that multiple enzymes involved in the metabolism could become candidates for antivirals. In the strategy targeting host metabolism, we should be careful in regard to the side effects caused by inhibition of the primary function of the metabolite. To minimize these undesirable effects, pinpoint inhibition of the enzyme should be done.

Lipid metabolism is one of the important targets for antivirals among cellular factors. Very recently, we examined the effect of ordinary nutrients on HCV RNA replication [108]. Using an OR6 assay system, we found that linoleic acid possessed an anti-HCV effect and its combination with CsA exerted synergistic inhibitory effect on HCV RNA replication [108]. However, the anti-HCV mechanism of PUFAs remains unclear. An improved understanding of the anti-HCV effect of PUFAs will extend the field of host metabolism as a target of antivirals in the future.

One recent striking advance is the development of a method for infectious HCV production in cell culture. This system provides information regarding the complete life cycle of HCV and will extend our understanding of the antivirals to virus entry, assembly and release. The discovery of anti-HCV reagents targeting host metabolism in the HCV life cycle will improve the SVR in combination with IFN. Or, the development of new anti-HCV reagents could lead to the retirement of IFN in the near future.

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Serum-free cell culture system supplemented with lipid-rich albumin for hepatitis C virus (strain O of genotype 1b) replication

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Abstract

HuH-7 is a highly differentiated hepatoma cell line and the only cell line that supports robust RNA replication of the hepatitis C virus (HCV). HuH-7 cells cause cell death in serum-free culture condition. However, the effect is reversed by supplementation with selenium. Serum-free cell cultures are advantageous for vaccine development and experimental reproducibility. However, HCV RNA replication in HuH-7 cells in serum-free medium had not yet been achieved. Therefore, we tried to develop a system for robust HCV RNA replication in a serum-free cell culture. Although HuH-7 cells grew in serum-free medium in the presence of selenium, HuH-7 cells under these conditions did not support HCV RNA replication in long-term culture. Among the supplements tested, serum-free medium with lipid-rich albumin (LRA) was found to yield robust HCV RNA replication. HCV proteins were detected for more than 9 months in serum-free medium supplemented with LRA. This is the first report to demonstrate a long-term, serum-free cell culture that successfully maintained robust HCV RNA replication. This cell culture system is expected to be a useful tool for vaccine development, as well as for further investigation of cellular factors that are essential for HCV RNA replication.
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Keywords: HCV; Serum-free cell culture; Selenium; Lipid-rich albumin; Vaccine

1. Introduction

Persistent hepatitis C virus (HCV) infection causes liver fibrosis and hepatocellular carcinoma. Approximately 170 million people worldwide are infected with HCV. The combination of pegylated interferon (IFN) with ribavirin is the current standard therapy for chronic hepatitis C and yields a sustained virological response rate of about 55% (Feld and Hoofnagle, 2005). HCV, a member of the *Flaviviridae* family, is an enveloped virus with a positive single-stranded 9.6 kilobase (kb) RNA genome, which encodes a large polyprotein precursor of about 3000 amino acids (aa) (Kato et al., 1990; Tanaka et al., 1996). This polyprotein is processed by a combination of host and viral proteases into at least 10 proteins: Core, envelope 1 (E1), E2, p7, non-structural 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (Hijikata et al., 1991, 1993).

The discovery of the HCV subgenomic replicon in 1999 was a turning point for HCV RNA replication in cultured cells (Lohmann et al., 1999). Furthermore, genome-length HCV RNA replication systems were developed using N, Con1, and H strains (Blight et al., 2002; Ikeda et al., 2002; Pietschmann et al., 2002). We recently developed a genome-length HCV RNA (strain O of genotype 1b) replication reporter system (OR6) as an effective screening system (Ikeda et al., 2005). The development of infectious virus-producing cells has been a remarkable breakthrough in the fields of virology (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005).

HuH-7 is a highly differentiated hepatoma cell line that is commonly used for replication and infection studies. However, these cells have been reported by a number of groups to be heterogeneous, and the replication efficiency of this cell line differed among subclonal HuH-7 cells. Parental HuH-7 cells showed low capacity for HCV RNA replication and low susceptibility for HCV infection, but Huh7.5, Huh-Lunet, and our recently developed the RSc cells efficiently support HCV RNA replication and infection (Blight et al., 2002; Pietschmann et al., 2006; Ikeda et al., in preparation). In addition to these sub-

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clonal HuH-7 cell features, fetal bovine serum (FBS) may be another factor that affects HCV RNA replication and infection, as FBS is a pooled material containing unknown factors from different origins. Therefore, different FBS lots may affect the reproducibility of experiments conducted by different research groups. Furthermore, pathogens contained in FBS may introduce additional problems during the development of an HCV vaccine.

To resolve these issues, in this study, we investigated whether or not HCV RNA could replicate in serum-free cell culture. As HuH-7 cells produce an autocrine growth factor, hepatoma-derived growth factor, additional supplementation with growth factor seemed to be unnecessary (Nakamura et al., 1989, 1994). HuH-7 cells cause cell death in serum-free culture condition. However, when HuH-7 cells were cultured in serum-free medium supplemented with selenium, they produce a number of plasma proteins and liver-specific enzymes essential for their survival (Nakabayashi et al., 1982, 1984). Therefore, the serum-free culture of HuH-7 cells can be maintained by the addition of selenium alone. However, HCV RNA replication was not yet maintainable under these conditions.

In the present study, we found that HCV RNA replicates robustly for more than 9 months in serum-free medium supplemented with selenium and lipid-rich albumin (LRA). These results indicate the requirement of the lipid for HCV RNA replication. This cell culture system is expected to be a useful tool for the development of an HCV vaccine, and will also enhance the reproducibility of experiments, including those that evaluate anti-HCV reagents.

2. Materials and methods

2.1. Reagents

Sodium selenite (Na_2SeO_3), insulin, linoleic acid, oleic acid, IFN-α, and cyclosporine A (CsA) were purchased from Sigma–Aldrich (St. Louis, MO). Fluvastatin (FLV) and low-density lipoprotein (LDL) were purchased from Calbiochem (San Diego, CA). Lipid-rich albumin (ALBUMAX I™) was purchased from Invitrogen and is referred to as LRA in this study.

2.2. Cell cultures

The OR6 cells were cultured in Dulbecco's modified eagle's medium (DMEM; Gibco-BRL, Invitrogen Life Technology, Carlsbad, CA) supplemented with 10% FBS, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1% penicillin–streptomycin (referred to as 10% FBS medium in this study), and G418 (300 μg per ml; Geneticin, Invitrogen) in a 5% CO_2 atmosphere at 37 °C. The cells were supplied with fresh medium twice a week at a 5:1 split ratio. The serum-free medium was DMEM containing 100 nM sodium selenite (Na_2SeO_3, Sigma–Aldrich) with LRA. The cells were cultured on six-well plates in 10% FBS medium or the serum-free medium. The cells cultured in the serum-free medium were harvested at 1, 3, 6, 9, and 12 months, and were subjected to Western blot analysis.

2.3. Cell count

To examine cell growth in selenium-containing medium with 10% FBS, 2 mg per ml of LRA, or no supplementation, OR6 cells were seeded at a density of 1×10^5 cells per well onto six-well plates in the absence of G418. Then, the number of the cells was counted in an improved Neubauer-type hemotocytometer after trypan blue dye (Invitrogen) treatment.

2.4. Western blot analysis

Preparation of the cell lysate, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblot analysis with a polyvinylidene difluoride membrane were performed as described previously (Kato et al., 2003). The antibodies used in this study were those against Core (Institute of Immunology, Tokyo, Japan), NS3 (Novocastra Laboratories, UK) and β-actin (AC-15; Sigma–Aldrich). Immunocomplexes were detected with the Renaissance enhanced chemiluminescence assay (Perkin-Elmer Life Sciences, Boston, MA).

2.5. Luciferase reporter assay

A luciferase reporter assay was performed as described previously (Ikeda et al., 2006). Briefly, 2×10^4 cells were plated onto 24-well plates and cultured in 10% FBS or the serum-free medium, at least in triplicate for each assay, and the cells were cultured for 24 h. Then, the cells were treated with human IFN-α, CsA, or FLV at several concentrations for 72 h. The cells were then harvested and subjected to luciferase assay using the *Renilla* luciferase assay system (Promega). The cells were washed twice with phosphate-buffered saline and were then extracted with 100 μl of *Renilla* lysis reagent. The relative luciferase unit value in 10 μl of lysates was measured by adding 50 μl of *Renilla* luciferase assay reagent according to the manufacturer's protocol. A manual Lumat LB 9501/16 luminometer (EG&G Berthold, Bad Wildbad, Germany) was used for the detection of luciferase activity.

3. Results

3.1. Efficiency of HCV RNA replication in HuH-7 cells with supplements in serum-free medium

At the early stage of the establishment of the HuH-7 cells, the serum-free cell culture was examined; the HuH-7 cells were found to replicate continuously for more than 9 months in a chemically defined medium containing selenium (Nakabayashi et al., 1982). Furthermore, the HuH-7 cells were maintained for a period of more than 3 years in improved serum-free medium containing additional supplements, i.e., oleic acid, linoleic acid, and insulin (Nakabayashi et al., 1984). We first investigated whether these serum-free conditions would support HCV RNA replication using the OR6 reporter system. The OR6 cells supported the replication of genome-length HCV-O RNA, into which the luciferase gene had been introduced (Ikeda et al., 2005). Using this OR6 system, we were able to monitor the level of HCV RNA

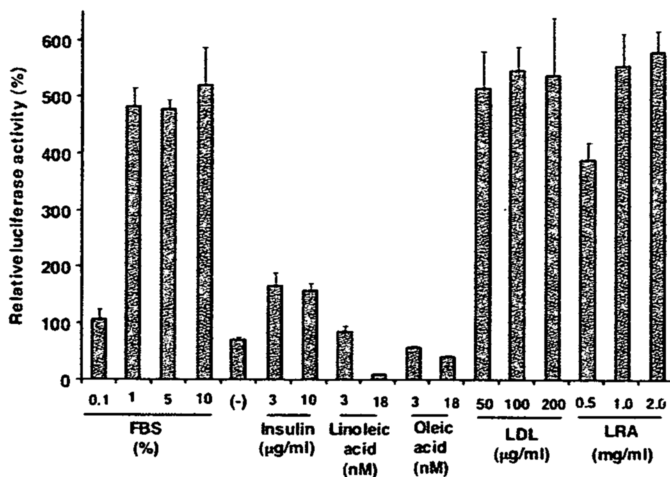

Fig. 1. HCV RNA replication in OR6 cells under different cell culture conditions. The OR6 cells were cultured in DMEM containing 100 nM sodium selenium with FBS (0.1, 1, 5, and 10%), insulin (3 and 10 μg per ml), linoleic acid (3 and 18 nM), oleic acid (3 and 18 nM), LDL (50, 100, and 200 μg per ml), or LRA (0.5, 1.0, and 2.0 mg per ml). The cells were harvested at 24 and 96 h and were subjected to luciferase assay as described in Section 2. Relative luciferase activities (%) were obtained from the value at 96 h, when the value at 24 h was assigned as 100%. The data indicate means ± standard deviations (S.D.s) from three independent experiments. (−) indicates culture in DMEM containing 100 nM sodium selenium.

replication by measuring the activity of luciferase. Luciferase activity at 96 h was five times higher than that at 24 h in 10% FBS medium (Fig. 1). However, HCV RNA replication was reduced when the OR6 cells were cultured in serum-free medium containing only selenium (Fig. 1). Serum-free medium supplemented with insulin reduced HCV RNA replication to about one-third of that observed in cultures maintained in 10% FBS medium supplemented with selenium. When used in combination with linoleic acid or oleic acid, the serum-free medium with selenium remarkably reduced HCV RNA replication (Fig. 1). However, in the serum-free medium with selenium in combination with LDL (50, 100, or 200 μg per ml) or LRA (1 or 2 mg per ml), HCV RNA replication was supported at the same level as that in 10% FBS medium, although the replication of HCV RNA was slightly low level in serum-free medium with selenium and LRA supplement at 0.5 mg per ml. These results suggest that chemically conditioned serum-free medium supplemented with selenium is not sufficient to support HCV RNA replication, but the addition of either LDL or LRA restored HCV RNA replication to almost the same level as that observed in 10% FBS medium. Thus, some of the elements essential for HCV RNA replication may be contained in LDL and LRA.

3.2. Cell growth of HuH-7 cells in selenium-containing medium supplemented with FBS or LRA

As HCV RNA replication depends on cell growth (Guo et al., 2001; Pietschmann et al., 2001), we next determined the number of cells at 24, 48, 72, and 96 h of culture. The doubling time of the OR6 cells was estimated to be approximately 29, 43, and 64 h in selenium-containing medium with 10% FBS, or 0.5, 1.0, and 2 mg per ml of LRA, or no supplementation, respectively

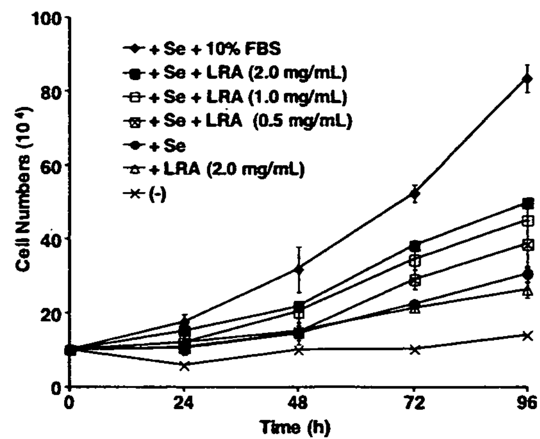

Fig. 2. Cell growth of HuH-7 cells in serum-free medium with LRA or in 10% FBS medium. The OR6 cells were plated at 1×10^5 cells per well onto six-well plates in triplicate. The cells were cultured in DMEM containing 100 nM sodium selenium with 10% FBS or 0.5, 1.0 and 2.0 mg per ml LRA or no supplement and were harvested at 24, 48, 72, and 96 h. The cells were cultured in DMEM or DMEM with LRA in the absence of selenium and were harvested at indicated time points.

(Fig. 2). Selenium or LRA containing medium enhanced the growth of OR6 cells and the combination of the selenium with LRA further enhanced the cell growth in a LRA dose-dependent manner (Fig. 2). Interestingly, the cell culture in serum-free medium supplemented with 2 mg per ml of LRA and selenium supported HCV RNA replication as efficiently as did that supplemented with 10% FBS and selenium (Fig. 1), although the cell growth of the culture in the medium with LRA and selenium was slower than that in the medium supplemented with 10% FBS and selenium. These results indicate that LRA may contain factors that enhance HCV RNA replication, and these LRA-derived factors appear to function in a manner that is not dependent on the cell growth factor.

3.3. Expression of HCV proteins in HuH-7 cells at 1 month of cell culture under various medium conditions

We continued to maintain the culture of OR6 cells for 1 month in different types of conditioned media. NS3 and Core HCV proteins were detected in the OR6 cell culture for 1 month in medium containing selenium with 10% or 5% FBS, but not with 1% or 0.5% FBS (Fig. 3A). The protein expression levels were higher in the cell culture with 10% FBS medium than in that with the 5% FBS medium. HCV proteins were not detected in the OR6 cells cultured in serum-free medium containing selenium alone (Fig. 3A). In contrast, HCV proteins were detected in LRA- and selenium-containing cell cultures. The levels of expression of HCV proteins were almost equal to those in the cell culture with selenium and 5% FBS (Fig. 3A). To further confirm the results, we performed luciferase assay for the OR6 cells cultured for 1 month after RNA transfection (Fig. 3B). These results indicated that HCV RNA replication was not maintained for more than 1 month in low concentrations (less than 1%) of FBS with selenium. However, the cell culture in serum-free medium with selenium and LRA at concentrations of 0.5, 1, or 2 mg per ml did efficiently support HCV RNA replication for more than

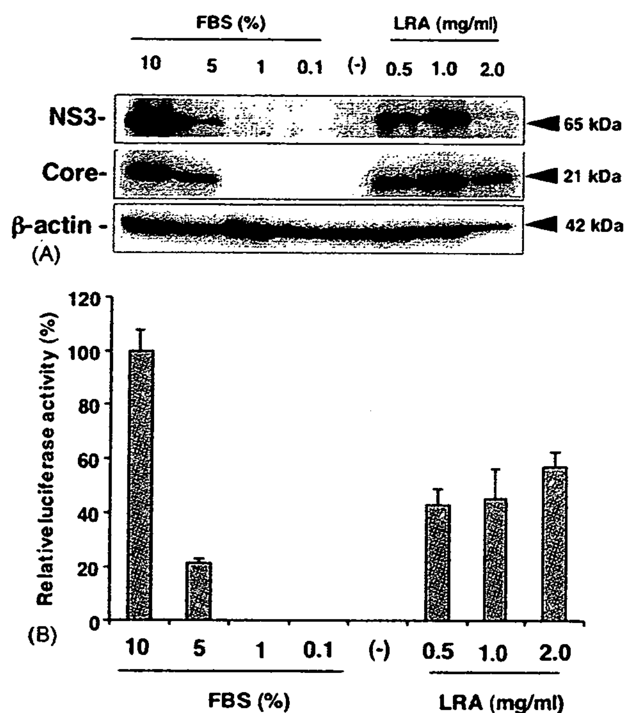

Fig. 3. Expression of HCV proteins in HuH-7 cells cultured in serum-free medium with LRA or in 10% FBS medium. OR6 cells were cultured for 30 days in DMEM containing 100 nM sodium selenium with LRA (0.5, 1.0, and 2.0 mg per ml) or FBS (0.1, 1, 5, and 10%). (−) indicates the culture in DMEM with sodium selenium. (A) The production of Core and NS3 in the OR6 cells was analyzed by immunoblotting using anti-Core and anti-NS3 antibodies. β-actin was used as a control for the amount of protein loaded per lane. (B) Relative luciferase activity was determined for the cells from 24-well plates in triplicate.

1 month. Therefore, we concluded that LRA could serve as an alternative supplement to FBS, when used in combination with selenium for HCV RNA replication.

3.4. Core expression in OR6 cells cultured in serum-free medium supplemented with LRA and selenium

Since OR6 cells cultured in serum-free medium with selenium and LRA could support HCV RNA replication at least for 1 month, we continued to culture the OR6 cells under the same conditions for 1 year. Core was detected for 9 months and for 6 months in serum-free medium containing selenium with LRA at 1.0 and 2.0 mg per ml, respectively (Fig. 4). These results suggest that serum-free cell culture supplemented with LRA supports HCV RNA replication at almost equal level to that in the culture supplemented with 10% FBS without G418 selection at least more than 9 months.

3.5. Influence of anti-HCV compounds on HCV RNA replication in OR6 cells cultured in serum-free medium supplemented with LRA and selenium

IFN is currently used as a therapeutic treatment of patients with chronic hepatitis C. Subgenomic HCV replicon-harboring cells and genome-length HCV RNA-replicating cells have been used to evaluate IFN and other candidate anti-HCV reagents, as

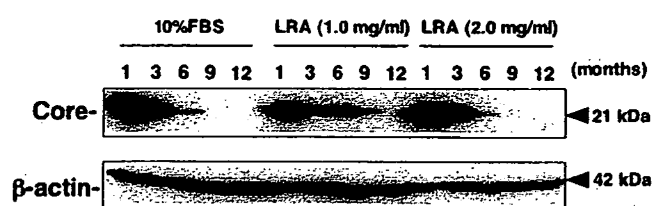

Fig. 4. Core expression in a long-term culture of OR6 cells in serum-free medium supplemented with LRA. The OR6 cells were cultured for 1 year in DMEM containing 100 nM sodium selenium with LRA at 1.0 or 2.0 mg per ml. The OR6 cells were also cultured in DMEM with 10% FBS and used as a positive control. Cells were harvested at 1, 3, 6, 9, and 12 months, and were subjected to Western blot analysis for Core using anti-Core antibody. β-actin was used as a control for the amount of protein loaded per lane.

has recently been reported in the case of CsA and the statins (Ikeda et al., 2006; Kapadia and Chisari, 2005; Watashi et al., 2003; Ye et al., 2003). However, one remaining problem with the evaluation of the anti-HCV activity of these reagents has been that FBS is derived from pooled blood materials and contains unknown cellular factors in various concentrations. These unknown factors can affect experimental results and reproducibility. Therefore, it is preferable to use a culture medium containing as few cellular factors as possible in order to ensure the reliability of the results. In this context, completely chemical conditioned medium is most ideal. Although our selenium and LRA culture system still contained animal proteins and lipids, it contained fewer unknown factors than medium containing FBS. We compared the anti-HCV activity of IFN-α, CsA, and FLV in serum-free medium with selenium and LRA, as well as in 10% FBS medium with selenium. OR6 cells were treated with these reagents for 72 h, and harvested cells were subjected to luciferase assay. When the OR6 cells were treated with IFN-α under either the 10% FBS medium condition or the selenium and LRA condition, HCV RNA replication was inhibited by IFN-α in a dose-dependent manner (range: 0.625–5 IU per ml; Fig. 5). However, the sensitivity of the cultures to IFN-α was greater in selenium and LRA medium than in 10% FBS medium (Fig. 5). When the OR6 cells were treated with CsA at low concentrations of 0.0625 and 0.125 μg per ml, sensitivity was greater in selenium and LRA medium than in 10% FBS medium, but almost identical sensitivity was observed under both medium conditions at concentrations of CsA of 0.25 and 0.5 μg per ml (Fig. 5). The most striking result was observed when the OR6 cells were treated with FLV in concentrations ranging from 1.25 to 10 μM. In contrast to the results obtained with CsA and IFN-α, the sensitivity associated with FLV was markedly reduced in the cultures treated with selenium and LRA medium, as compared to that of cultures in 10% FBS medium (Fig. 5).

4. Discussion

The development of serum-free cell culture systems will reduce the risk of contamination by infectious pathogens of animal origin in studies of vaccine development. Moreover, such systems will enhance the reproducibility of such experiments, because FBS contains unknown cellular factors that can affect the physiological state of cells. In this study, we developed a

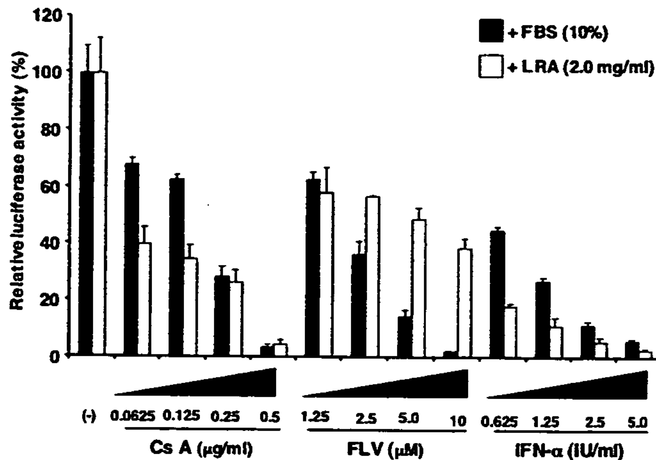

Fig. 5. Influence of anti-HCV compounds on HCV RNA replication in OR6 cells in serum-free medium supplemented with LRA or in 10% FBS medium. The OR6 cells were cultured in triplicate in 10% FBS medium with selenium (100 nM) or in serum-free medium with LRA (2 mg per ml) and selenium (100 nM). Under these cell culture conditions, the cells were treated with CsA (0.0625, 0.125, 0.25, and 0.5 μg per ml), FLV (1.25, 2.5, 5.0, and 10 μM), or IFN-α (0.625, 1.25, 2.5, and 5 IU per ml) for 72 h. Then, the cells were harvested and subjected to luciferase assay as described in Section 2. The luciferase activity of OR6 cells cultured under both medium conditions in the absence of anti-HCV compounds was assigned as 100% and is indicated by (−).

novel serum-free cell culture system supplemented with LRA that was able to support HCV RNA replication for more than 9 months. Although this cell culture system still contained animal proteins, the quantity of unknown cellular factors contained in the FBS was to a great extent reduced. The development of such a long-term cell culture is noteworthy, as it could be used for the stable mass-production of an HCV vaccine.

In a related previous study, it was reported that insulin, linoleic acid, and oleic acid enhance the growth of HuH-7 cells in serum-free cell culture (Nakabayashi et al., 1984). Therefore, we tested these supplements in HCV RNA replication experiments. Insulin was found to slightly enhance HCV RNA replication, but linoleic acid and oleic acid inhibited HCV RNA replication. In another recent study, Kapadia and co-workers reported that oleic acid enhanced HCV RNA replication in a serum-containing cell culture (Kapadia and Chisari, 2005). This discrepancy may have been due to the presence or absence of serum; moreover, some of the serum proteins may function in concert with oleic acid to support HCV RNA replication.

LDL is an exogenous source of cholesterol, and it was found to support HCV RNA replication in serum-free cell culture. When the cholesterol demand is satisfied, intrinsic mevalonate, which is a precursor of both cholesterol and non-sterol isoprenoid, is directed to non-sterol isoprenoid. Non-sterol isoprenoid is essential for the prenylation of cellular proteins that support HCV RNA replication (Ikeda et al., 2006; Kapadia and Chisari, 2005; Ye et al., 2003). For this reason, LDL may support HCV RNA replication, even in serum-free cell culture. LRA was initially developed to reduce or replace the requirement of serum supplementation by chromatographic separation from bovine plasma (Invitrogen). Here, we found that LRA supported HCV RNA replication as well as cell growth in serum-free cell culture. LRA contains free fatty acids and cholesterol associated with albumin. Therefore, cholesterol may, at least to some extent, play a role in HCV RNA replication by the mechanism described above. However, care should be taken before coming to a conclusion regarding the effects of free fatty acids on HCV RNA replication, because LRA contains a mixture of fatty acids in different states of saturation. Recent reports have demonstrated that saturated fatty acids enhance HCV RNA replication, but polyunsaturated fatty acids inhibit HCV RNA replication (Kapadia and Chisari, 2005). In addition, these diverse effects of fatty acids on HCV RNA replication in serum-containing medium cannot be simply applied to serum-free culture systems, because oleic acid has been shown to exert different effects on HCV RNA replication under serum-containing and serum-free culture conditions. To clarify the roles played by fatty acids in this context, further studies will be needed.

Here, HCV RNA replication depended on the growth of HuH-7 cells, and it has previously been shown that expression levels of HCV proteins and RNA are low in confluent cells (Guo et al., 2001; Pietschmann et al., 2001). Therefore, we examined the time course of cell growth and found that cell growth in serum-free medium with LRA was slower than that in 10% FBS medium, although the replication levels of HCV RNA were similar under both culture conditions studied. As regards HCV RNA replication and cell growth, Windisch et al. reported that HCV RNA replication in HuH-6 cells was not dependent on cell growth (Windisch et al., 2005). They demonstrated that the expression of HCV proteins was not reduced, even when the HuH-6 cells became confluent. In serum-free culture supplemented with LRA, HCV RNA replication in HuH-7 cells proceeds in a manner independent of cell growth, as was previously observed in the case of replication in HuH-6 cells.

One disadvantage associated with the use of FBS-containing cultures in virology studies is the influence exerted by unknown serum proteins, because FBS is derived from the serum pool of a bovine population. To prevent discrepancies between experiments due to differences between FBS lots, it is desirable to include only the most simple components as possible in the culture media. To this end, serum-free cultures are preferable in terms of reproducibility. Along these lines, it is expected that the use of our serum-free culture system with LRA may lead to improvements in experimental conditions for experiments in cell biology, as our culture medium contained only very simple supplements: fatty acids, cholesterol, albumin, and selenium. We tested the anti-HCV reagents CsA, FLV, and IFN-α in our serum-free culture supplemented with LRA. CsA and IFN-α were found to inhibit HCV RNA replication more efficiently in serum-free medium with LRA than in 10% FBS medium. Surprisingly, FLV inhibited HCV RNA replication less effectively in serum-free medium supplemented with LRA than it did in 10% FBS medium. One explanation for these differences may be that only FLV is a lipid metabolism-related reagent, and therefore the anti-HCV effect appeared to be antagonized by LRA. To clarify this issue, further study will be needed.

The goal of a serum-free cell culture is to develop a cell culture system containing only compounds that are of non-animal origin. Recently, a serum-free cell culture for canine

pathogenic virus production was reported using Madin Darby canine kidney cells lacking animal protein (Mochizuki, 2006). In this system, soybean peptone was used for the serum-free culture without animal protein. Canine viruses were able to grow almost as efficiently in this serum-free medium as in serum-containing medium. This plant protein-containing culture system is of the second-highest quality in terms of controlling for animal-derived pathogens in vaccine development experiments. Assessments of this animal protein-free cell culture system in terms of its usefulness for HCV RNA replication are therefore warranted.

We found HuH-7 cells supported HCV RNA replication for more than 9 months in serum-free medium supplemented with LRA at 1.0 mg per ml and selenium at 100 nM. This is the first report to describe HCV RNA replication in a long-term, serum-free culture. Recently, an infectious virus-producing cell culture system was reported using genotype 2a strain JFH1 (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). We are currently examining infectious virus production in a serum-free cell culture using the JFH1 virus. Our serum-free cell culture system may provide the useful information to the vaccine development.

In conclusion, we have established a serum-free cell culture system supplemented with LRA for the purpose of achieving HCV RNA replication. HCV proteins were detected during this series of experiments for more than 9 months. The present system has enabled an ongoing study of the production of an infectious HCV virion. Our serum-free cell culture system will yield relevant information for vaccine development, sustains only a relatively low risk of pathogenic contamination as compared to that of previous systems, and is expected to improve the reproducibility of similar experiments in the future.

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Limited suppression of the interferon-β production by hepatitis C virus serine protease in cultured human hepatocytes

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Keywords
antiviral response; hepatitis C virus; innate immune response; interferon-β; serine protease

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Toll-like receptors and RNA helicase family members [retinoic acid-inducible gene I (RIG-I) and melanoma differentiation associated gene-5 (MDA5)] play important roles in the induction of interferon-β as a major event in innate immune responses after virus infection. TRIF (adaptor protein of Toll-like receptor 3)-mediated and Cardif (adaptor protein of RIG-I or MDA5)-mediated signaling pathways contribute rapid induction of interferon-β through the activation of interferon regulatory factor-3 (IRF-3). Previously, it has been reported that the hepatitis C virus NS3-4A serine protease blocks virus-induced activation of IRF-3 in the human hepatoma cell line HuH-7, and that NS3-4A cleaves TRIF and Cardif molecules, resulting in the interruption of antiviral signaling pathways. On the other hand, it has recently been reported that non-neoplastic human hepatocyte PH5CH8 cells retain robust TRIF- and Cardif-mediated pathways, unlike HuH-7 cells, which lack a TRIF-mediated pathway. In the present study, we further investigated the effect of NS3-4A on antiviral signaling pathways. Although we confirmed that PH5CH8 cells were much more effective than HuH-7 cells for the induction of interferon-β, we obtained the unexpected result that NS3-4A could not suppress the interferon-β production induced by the TRIF-mediated pathway, although it suppressed the Cardif-mediated pathway by cleaving Cardif at the Cys508 residue. Using PH5CH8, HeLa, and HuH-7-derived cells, we further showed that NS3-4A could not cleave TRIF, in disagreement with a previous report describing the cleavage of TRIF by NS3-4A. Taken together, our findings suggest that the blocking of the interferon production by NS3-4A is not sufficient in HCV-infected hepatocyte cells.

Persistent infection by hepatitis C virus (HCV) frequently causes chronic hepatitis [1,2], which progresses to liver cirrhosis and hepatocellular carcinoma [3,4]. This is a serious health problem because approximately 170 million people are currently infected with HCV worldwide [5]. To resolve the mechanism of persistent HCV infection, it will be necessary to better understand the virus life cycle and then to develop more effective anti-HCV reagents. HCV is an enveloped positive ssRNA (9.6 kb) virus belonging to the *Flaviviridae* family [6,7]. The HCV genome encodes a large polyprotein precursor of approximately 3000 amino acid residues, which is cleaved co- and post-translationally into at least ten proteins in the order: core, envelope 1

Abbreviations
CARD, caspase recruitment domain; E1, envelope 1; EGFP, enhanced green fluorescent protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCV, hepatitis C virus; HEK293, human embryonic kidney 293; IFN, interferon; IRF-3, interferon regulatory factor 3; IKK-ε, inhibitor of κB kinase ε; MDA5, melanoma differentiation associated gene-5; MyD88, myeloid differentiation factor 88; NS2, nonstructural protein 2; RIG-I, retinoic acid-inducible gene I; siRNA, small interfering RNA; TBK, Tank-binding kinase 1; TLR, Toll-like receptor; TRIF, Toll-IL1 receptor domain-containing adaptor inducing IFN-β.

(E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B. These cleavages are mediated by the host and virally encoded serine protease located in the amino-terminal domain of NS3. Serine protease activity of NS3 requires NS4A, a protein consisting of 54 amino acid residues, to form a stable complex with the NS3 [8–10].

Virus-infected cells trigger the innate immune response by recognizing viral components, including DNA, ssRNA, dsRNA and glycoproteins. This response initiates signaling pathways leading to the induction of protective cellular genes, including type-I interferons [initially interferon (IFN)-β, and then IFN-α] and proinflammatory cytokines that directly limit viral replication. Within these signaling pathways, Toll-like receptors (TLRs) and RNA helicase family members play very important roles in the recognition of the viral components [11,12].

IFN-β is induced by dsRNA, a common intermediate in many RNA virus infections, including HCV. The viral dsRNA as well as the synthetic dsRNA analogue poly(I-C) are recognized by TLR3, which is expressed on the cell surface or in endosome vesicles [13,14]. On the other hand, it has been shown that retinoic acid-induced gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) also recognize dsRNA molecules [15–17]. A recent study showed that MDA5 and RIG-I recognize different types of dsRNA: MDA5 recognizes poly(I-C), and RIG-I recognizes *in vitro* transcribed dsRNA [18]. Very recently, it was discovered that viral 5′-triphosphate RNA is the ligand for RIG-I [19,20]. Both MDA5 and RIG-I contain DexD/H-box helicase domains that serve as intracellular cytoplasmic dsRNA and 5′-triphosphate RNA receptors, respectively [15–20]. After dsRNA is recognized, the cytoplasmic domain of TLR3 recruits TIR-domain-containing adaptor inducing IFN-β (TRIF) through a myeloid differentiation factor 88 (MyD88)-independent pathway (TRIF-mediated pathway) [21–23]. In contrast, the caspase recruitment domains (CARDs) of MDA5 or RIG-I recruit the CARD adaptor inducing IFN-β, Cardif (also known as IPS-1, MAVS, or VISA), which was recently identified as an adaptor protein located in the outer membrane of mitochondria (this recruitment is known as the Cardif-mediated pathway) [24–27].

The TRIF- and Cardif-mediated signaling pathways rapidly induce IFN-β through the phosphorylation of multiple cellular factors, including IFN regulatory factor-3 (IRF-3) and kinases, including the Tank-binding kinase 1 (TBK-1) and inhibitor of κB kinase ε (IKK-ε) [28–31]. Although IRF-3 is located in the cytoplasm in an inactive state [28,29], phosphorylation (Ser385, 386, 396, 398, 402, 405, and Thr404) of IRF-3 by TBK-1 and IKK-ε induces dimerization and nuclear translocation of IRF-3, leading to transcriptional activation of IFN-β [28–31].

Recent studies have found that several RNA virus proteins could inhibit the early signaling activation (TRIF- and Cardif-mediated pathways) leading to IFN-β production [32,33]. Regarding HCV, Foy *et al.* [33] found that NS3-4A serine protease blocked HCV-induced activation of IRF-3 in the human hepatoma cell line HuH-7. Additional studies regarding this finding have shown that NS3-4A blocks the Cardif-mediated signaling pathway by cleaving the Cardif molecule and blocking downstream IFN-β activation [24,34,35], and that TBK-1, IKK-ε, and TRIF may also be targeted for cleaving by NS3-4A [36–38]. With respect to TRIF, NS3-4A was reported to cleave this molecule in both an *in vitro* experiment using a reticulocyte lysate system and an *in vivo* experiment using human embryonic kidney 293 (HEK293) and UNS3-4A-24 osteosarcoma cells [36]. These studies suggest that NS3-4A has the ability to inhibit both TRIF- and Cardif-mediated signaling pathways.

On the other hand, we recently demonstrated that HCV proteins exhibited conflicting effects on the IFN-β production in non-neoplastic human hepatocyte PH5CH8 cells [39,40]: Core and NS5B synergistically enhanced IFN-β expression and this enhancement was dependent on the RNA-dependent RNA polymerase activity of NS5B, but NS3-4A significantly inhibited the production of IFN-β induced by the combination of Core and NS5B. Furthermore, Li *et al.* [41] recently reported that PH5CH8 cells retained robust and functionally active TRIF- and Cardif-mediated signaling pathways, unlike HuH-7 cells, which lacked the TRIF-mediated pathway [41,42]. Therefore, using poly(I-C) as an inducer of IFN-β, we investigated the effects of NS3-4A on antiviral signaling pathways in PH5CH8 cells. Our results showed that the extracellular TLR3/TRIF signaling pathway was not blocked by NS3-4A because NS3-4A did not cleave TRIF, unlike in the previous study [36].

Results

Human hepatocyte PH5CH8 cells more readily activate IFN-β transcription in response to dsRNA compared to HuH-7 cells and their sublines

Recently, Li *et al.* [41] reported that PH5CH8 cells showed a better response to dsRNA, including IFN-β induction, than other human hepatoma cell lines (HuH-7, HepG2, and Hep3B). Therefore, using a dual

luciferase reporter assay, we first confirmed that PH5CH8 cells were much more effective at inducing IFN-β than HuH-7 cells and HuH-7-derived cell sublines (O [43], Oc [43], and OR6c [44]) that can support HCV RNA replication.

When the dsRNA analog, poly(I-C), was transfected into cells using a liposome-mediated procedure (intracellular dsRNA, T-pIC), PH5CH8 cells showed a more potent (> 25-fold) activation of the IFN-β gene promoter than HuH-7 and HuH-7-derived cell lines (Fig. 1A). Furthermore, when poly(I-C) was added to the culture medium (extracellular dsRNA; M-pIC), a significant elevation (12-fold) of the IFN-β gene promoter was observed in PH5CH8 cells only (Fig. 1B). These results were confirmed by quantitative RT-PCR analysis of endogenous IFN-β mRNA induction in cells treated with poly(I-C) (T-pIC, Fig. 1C; M-pIC, Fig. 1D). In both T-pIC and M-pIC treatments, the induction level of IFN-β mRNA was markedly higher in PH5CH8 cells than in O, Oc, OR6c, and HuH-7 cells (Fig. 1C,D). Next, we carried out quantitative RT-PCR analysis of TLR3, TRIF, RIG-I, MDA5, Cardif, and IRF-3 mRNAs to clarify their expression levels in the steady state and the effects of poly(I-C)

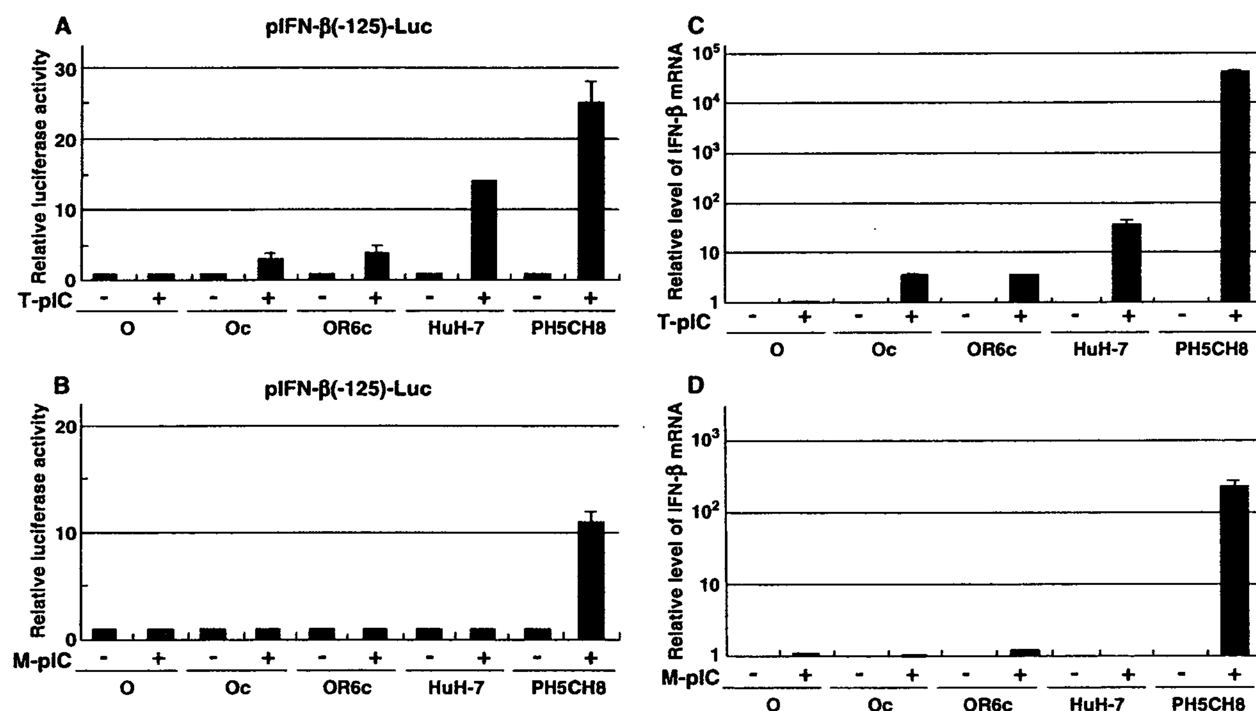

Fig. 1. PH5CH8 cells show high-level IFN-β production in response to dsRNA. (A) Dual luciferase reporter assay of the IFN-β gene promoter using the various cells treated with T-pIC. The following HuH-7-derived cell sublines were used: O, cloned cells [43] replicating genome-length HCV RNA; Oc, cured cells which were created by eliminating genome-length HCV RNA from the O cells by IFN treatment; and OR6c, cured cells which were created by eliminating genome-length HCV RNA from the cloned OR6 cells [44] by IFN treatment. Cells grown in 24-well plates were cotransfected with pIFN-β-(−125)-Luc and pRL-CMV (internal control reporter) and cultured for 42 h, and then poly(I-C) (1 μg) was transfected into the cells for 6 h before the reporter assay as described in the Experimental procedures. The relative luciferase activity was normalized to the activity of *Renilla* luciferase (internal control). The lysate of cells without poly(I-C) treatment was used as a control. Data are the means ± SD from three independent experiments, each performed in triplicate. (B) Dual luciferase reporter assay of the IFN-β gene promoter using the various cells treated with M-pIC. The dual luciferase reporter assay was performed as described in (A) except that poly(I-C) was added to the medium (50 $\mu g \cdot mL^{-1}$) for 6 h before the reporter assay. (C) Quantitative RT-PCR analysis of IFN-β mRNA in various cells treated with T-pIC. Poly(I-C) (1 μg) was transfected into the cells for 6 h before the sampling for RNA preparation. Total RNA extracted from the cells was subjected to real-time LightCycler PCR analysis using the primer set of IFN-β (202 bp). Data are the means ± SD from three independent experiments. To correct the differences in RNA quality and quantity between the samples, data were normalized using the ratio of IFN-β mRNA concentration to that of GAPDH. The IFN-β mRNA levels were calculated relative to the level in the O cells treated with T-pIC, which was set at 1.0. (D) Quantitative RT-PCR analysis of IFN-β mRNA in various cells treated with M-pIC. Poly(I-C) was added to the medium (50 $\mu g \cdot mL^{-1}$) for 6 h before the sampling for RNA preparation. Quantitative RT-PCR analysis for IFN-β mRNA was performed as described in (C). The IFN-β mRNA level was calculated relative to the level in the O cells treated with M-pIC, which was set at 1.0.

treatment (T-pIC and M-pIC). In T-pIC treatment, RIG-I and MDA5 mRNAs were clearly induced in PH5CH8 and HuH-7 cells, and TLR3 mRNA was induced only in PH5CH8 cells. Moreover, there was no such induction in the other cell lines examined (supplementary Table S1). In M-pIC treatment, TLR3, RIG-I, and MDA5 were induced only in PH5CH8 cells (supplementary Table S1). The fact that these mRNAs were induced at substantial levels only in PH5CH8 cells treated with T-pIC or M-pIC suggests that the elevation of these mRNAs is mediated by the IFN-β induced by poly(I-C) treatment. In summary, these results revealed that PH5CH8 cells retain both the Cardif- and TRIF-mediated pathways for IFN-β production, whereas HuH-7 cells retain only the Cardif-mediated pathway, and that the HuH-7-derived cells lines used are lacking in both pathways for IFN-β production.

Parental PH5CH and PH5CH clones other than PH5CH8 also exhibit IFN-β response toward poly(I-C) treatment

PH5CH8 is one of eight cell lines that were previously cloned from parental PH5CH cells to examine HCV susceptibility *in vitro* [45]. Therefore, we used a dual luciferase assay to examine the effects of poly(I-C) treatment on the IFN-β gene promoter in PH5CH cells and these cloned cell lines. When T-pIC treatment was employed, the parental cells and all the cloned cell lines exhibited good IFN-β response, and the activation level in PH5CH2 and PH5CH6 cells was higher than that in PH5CH8 cells (Fig. 2A). However, when M-pIC treatment was used, the IFN-β response in the cloned cells and the parental cells was less than 50% of that in PH5CH8 cells (Fig. 2B). From these results, we concluded that PH5CH8 is the best cell line for the study of the dsRNA-induced antiviral signaling pathways.

M-pIC treatment activates IRF-3 through the TLR3/TRIF signaling pathway

To confirm that the TRIF-mediated pathway is activated in M-pIC treatment, and to determine if its activation is mediated by the TLR3 but not the TLR4 signaling pathway, we examined whether or not activation of IRF-3 by M-pIC treatment is specifically mediated by the TLR3 signaling pathway using TLR3-, TLR4-, and TRIF-specific small interfering RNA (siRNAs) [46,47]. Quantitative RT-PCR analysis revealed that the TLR3, TLR4, and TRIF mRNAs were drastically decreased (more than 70% reduction) in the

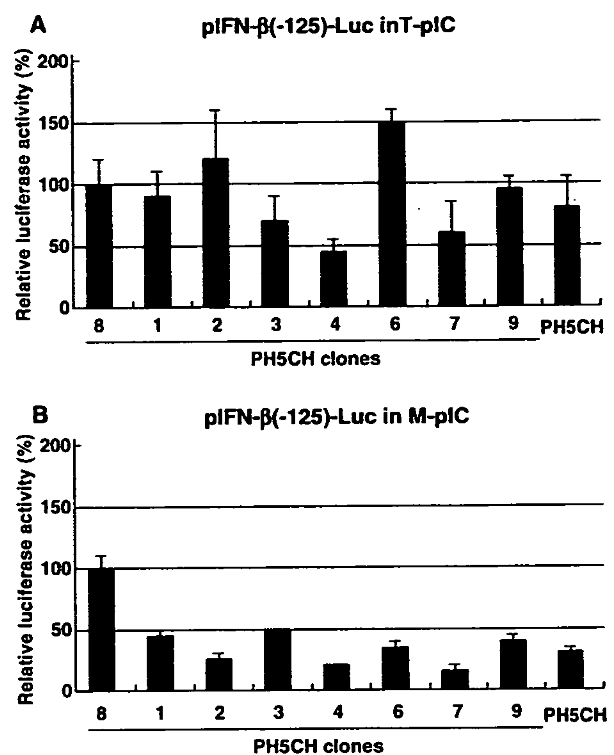

Fig. 2. IFN-β responses of parental PH5CH and PH5CH cloned cells by dsRNA treatment. (A) Dual luciferase reporter assay of the IFN-β gene promoter using parental PH5CH and PH5CH cloned cells treated with T-pIC. The T-pIC treatment and the dual luciferase reporter assay were performed as described in Fig. 1A. The IFN-β gene promoter activity level was calculated relative to the level in the PH5CH8 cells, which was set at 100. (B) Dual luciferase reporter assay of the IFN-β gene promoter using parental PH5CH and PH5CH cloned cells treated with M-pIC. The M-pIC treatment and the dual luciferase reporter assay were performed as described in Fig. 1B. The relative level of the IFN-β gene promoter activity was calculated as described in (A).

PH5CH8 cells transfected with TLR3, TLR4, and TRIF siRNAs, respectively, but not in the PH5CH8 cells transfected with the GL2 siRNA used as a control (Fig. 3A). We also confirmed that IRF-3 mRNA was not decreased in PH5CH8 cells transfected with any of these siRNAs (Fig. 3A). Under this condition, we performed a luciferase reporter assay using an IFN-β gene promoter in PH5CH8 cells treated with M-pIC. The activation of the IFN-β gene promoter was greatly suppressed (by more than 80%) in PH5CH8 cells transfected with TLR3 or TRIF siRNA, but not in the PH5CH8 cells transfected with GL2 or TLR4 siRNA (Fig. 3B). This result suggests that the activation of IRF-3 by M-pIC treatment is mediated by the TLR3/TRIF signaling pathway. We obtained further evidence by examining the status of the phosphorylation and dimerization of IRF-3. The results