differentiation into hepatocytes of murine hepatic progenitor cells [14]. Oncostatin M (OSM), a member of the interleukin (IL)-6 cytokine family, has been shown to induce development of hepatocytes from fetal hepatic cells in combination with dexamethasone [15]. Recently, OSM has also been reported to inhibit proliferation of rat hepatic progenitor cells, playing a pivotal role in differentiation into hepatocytes [16].

HCV is a plus-stranded RNA virus of approximately 9.5 kb in length [17]. From the HCV genome, at least 10 viral proteins (core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) are generated from the precursor protein [18,19]. Recent experimental evidence suggests that the HCV core protein, as well as other HCV proteins, affect various biological functions in the host cell, such as cellular growth, malignant transformation, apoptosis, and signal transduction [20–26]. However, it has not been clarified whether constitutive expression of the HCV core protein affects the differentiation process from hepatic progenitor cells to mature hepatocytes.

To more precisely evaluate this, we used the in vitro culture system of a murine normal liver epithelial cell line stimulated with OSM and dexamethasone to induce differentiation into hepatocytes. We investigated the influence of the HCV core protein on the process of hepatocyte differentiation by comparing the HCV core-expressing cells with negative control (mock) cells.

Materials and methods

Plasmid constructs. Plasmid pCore(1-191)-V5, an HCV core-expressing construct, was prepared from the plasmid pcDNA3.1/V5-HisA (Invitrogen, Co., Ltd.) [25]. Plasmids pCoreMut-V5 and pCoreDel-V5 were generated from pCore(1-191)-V5 by site-directed mutagenesis. These plasmids possessed the mutation (for pCoreMut-V5) or the deletion (for pCoreDel-V5) within a binding site for the Janus kinase (JAK) protein, which had been demonstrated to be located at amino acid positions 79–84 of the HCV core protein [25]. Both pCoreMut-V5 and pCoreDel-V5 encoded mutant types of the HCV core protein that did not allow binding to the JAK protein. Plasmid pAPRELuci contained the three repeats of the acute phase response element (APRE) upstream of the minimal promoter and luciferase gene, which was kindly provided by Dr. T. Hirano (Laboratory of Developmental Immunology, Graduate School of Frontier Biosciences, Osaka University). Plasmid pRLtk (Promega Co.), the seapansy luciferase-expressing plasmid, was used as a transfection efficiency control.

Cell culture and transfection. An embryonic murine liver cell line, BNL CL. 2 (CL2) (No. TIB 73, American Type Culture Collection), has been shown to possess the character of normal liver epithelial cells [27], which are regarded as possible hepatic progenitor cells. The cells were maintained in the Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 μg/ml of streptomycin sulfate, 100 U/ml of penicillin G sodium, and 0.25 μg/ml of amphotericin B in 5% CO_2 at 37 °C. Three independent clones of HCV-core expressing cells (designated CL2 core-I, -II, and -III) and the negative control cells (mock) were established from the CL2 cells, as described elsewhere [25,26]. For induction of hepatic differentiation, 5×10^5 of CL2 mock and core cells were seeded on a 6-cm-diameter culture dish and stimulated with 10 ng/ml of murine OSM (Sigma) and/or 10^{-7} M of dexamethasone (Sigma) every other day. The culture medium was also replaced with the same frequency. In some experiments, the CL2 mock and core cells were treated with 1 μM of Janus kinase (JAK)-specific inhibitor, 2-(1, 1-dimethylethyl)-9-fluoro-3, 6-dihydro-7H-benz[h]-imidaz [4,5-f] isoquinolin-7-one (CN biosciences) [28] every other day 1 h prior to the addition of OSM. Cells were harvested on days 10 or 20 after stimulation for Western blot and the RT-PCR analyses. In the present study, the CL2 core-I cells were mainly used for subsequent experiments. The results were also confirmed with CL2 core-II and -III cells in some experiments (corresponding to Figs. 1 and 2 in this study).

Reporter gene assay. For cotransfection analysis, 8.0×10^4 of the CL2 cells were seeded in a 6-well culture dish and cotransfected with 0.75 μg of the effector plasmid (pCore[1-191]-V5, pCoreMut-V5, pCoreDel-V5 or pcDNA3.1/V5-HisA) with 0.75 μg of the reporter plasmid (pAPRELuci) and 0.1 μg pRLtk. These cells were stimulated with 10 ng/ml of murine OSM or left unstimulated 1 day after transfection. Six hours later, they were lysed and subjected to the dual luciferase assay (Toyo Ink Co., Ltd.). The luciferase activity was normalized for transfection efficiency based on

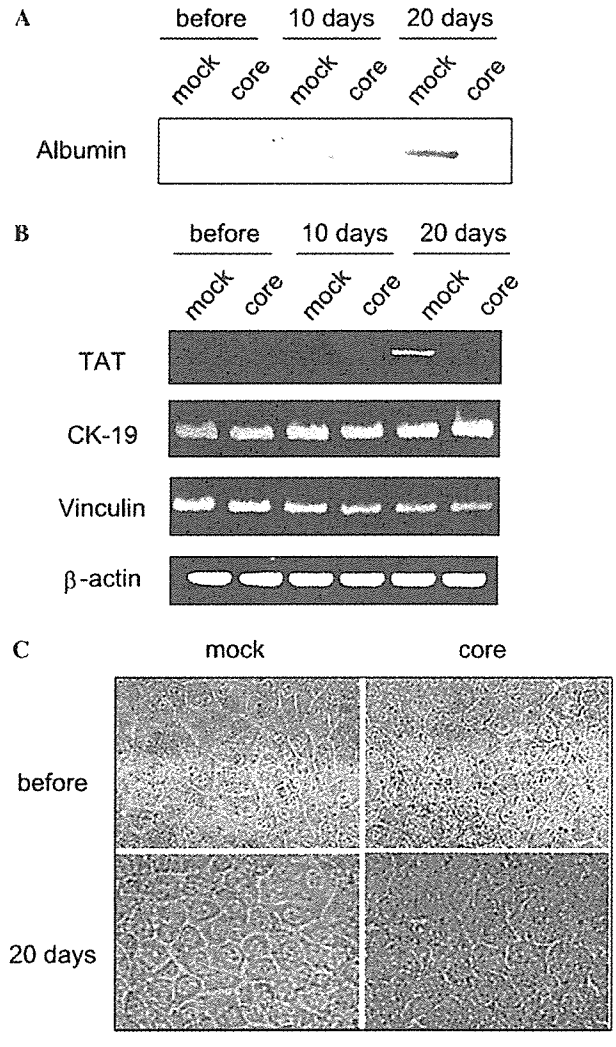

Fig. 1. (A) Detection of albumin in the CL2 mock and core cells under stimulation with OSM and dexamethasone. The cellular protein was harvested before, 10 days after, and 20 days after stimulation and used for Western blot analysis. (B) Detection of TAT, CK-19, and vinculin mRNAs in the CL2 mock and core cells under stimulation with OSM and dexamethasone. The total RNA was extracted before, 10 days after, and 20 days after stimulation and used for the RT-PCR assay. The β-actin mRNA was also measured as a loading control. (C) Microscopic observation of the CL2 mock and core cells under stimulation with OSM and dexamethasone. Phase contrast microscopy of 400 magnifications represents the CL2 mock and core cells before stimulation and 20 days after stimulation with OSM and dexamethasone.

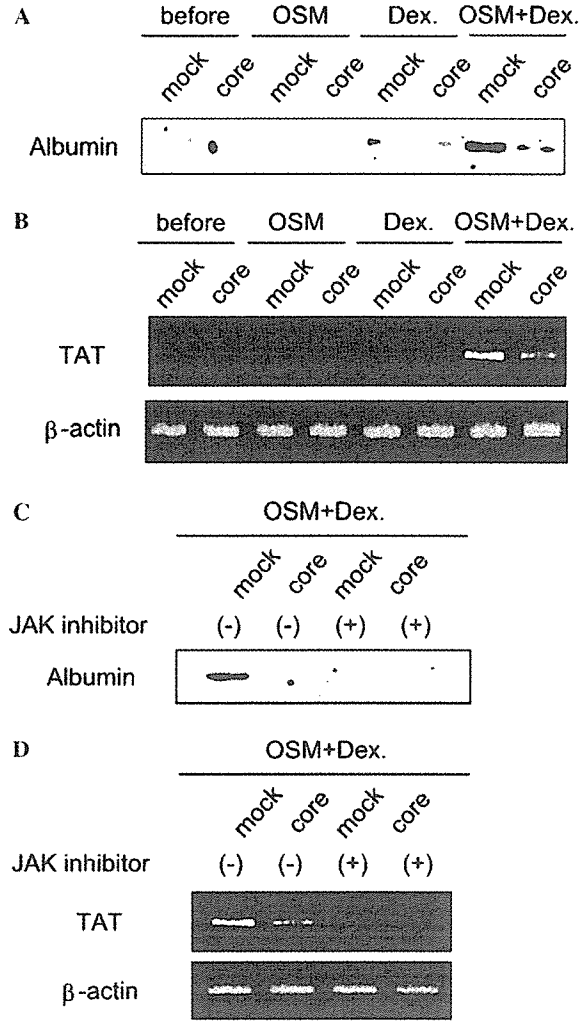

Fig. 2. (A) Detection of albumin in the CL2 mock and core cells under stimulation with OSM alone, dexamethasone alone, or both. The cellular protein was harvested before stimulation and 20 days after stimulation and used for Western blot analysis. (B) Detection of TAT mRNA in the CL2 mock and core cells under stimulation with OSM alone, dexamethasone alone, or both. The total RNA was extracted before stimulation and 20 days after stimulation and used for the RT-PCR assay. The β-actin mRNA was also measured as a loading control. (C) Measurement of albumin expression in the CL2 mock and core cells under stimulation with OSM and dexamethasone in the presence or absence of JAK inhibitor. The cellular protein was harvested 20 days after stimulation and used for Western blot analysis. (D) Measurement of TAT mRNA in the CL2 mock and core cells under stimulation with OSM and dexamethasone in the presence or absence of JAK inhibitor. The total RNA was extracted 20 days after stimulation and used for the RT-PCR assay. The β-actin mRNA was also measured as a loading control.

the result of the seapansy luciferase activity. The relative light unit of the unstimulated sample was regarded as 1, and the sample activities were calculated as multiples of this. All assays were done in triplicate, and the values were expressed as means ± SD.

Western blot analysis. The total cellular protein was extracted with the RIPA buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 100 μg/ml phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 50 mM sodium fluoride in phosphate-buffered saline (pH 7.4) [29]. Twenty micrograms of protein was separated with SDS–polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride membrane (Hybond P; Amersham Pharmacia Biotech Co., Ltd.). After blocking with milk, the membrane was incubated with a first antibody, followed by incubation with horseradish peroxidase-labeled immunoglobulin as a second antibody. The immune complex was detected by an enhanced chemiluminescent assay (Super Signal, Pierce). An antibody against signal transducer and activator transcription factor 3 (STAT3) was purchased from Santa Cruz Biotechnology, and an antibody against tyrosine phosphorylated STAT3 (pY^{705}STAT3; pSTAT3) came from Cell Signaling Technology. Antibodies against albumin and OSM receptor β (OSMRβ) were from Upstate Biotechnology.

PCR Analysis. The expression levels of tyrosine aminotransferase (TAT), cytokeratin (CK)-19, and vinculin mRNAs were analyzed by PCR assay. The total cellular RNA was extracted from the CL2 mock and core cells using an Isogen kit (Nippon Gene Co.) based on the guanidine–isothiocyanate method. Reverse transcription (RT) was performed with 1 μg of the RNA sample using the mutated Moloney murine leukemia virus reverse transcriptase (ReverTra Ace, Toyobo) and the oligo$(dT)_{20}$ primer (Toyobo). The cDNA was subsequently amplified with Taq/Pwo DNA polymerase (Expand High Fidelity PLUS PCR System, Roche Diagnostics). The specific primer sets are 5′-GGGGACCCTACTG TGTTTGG-3′ and 5′-GAGGCAGTGGACAGACTGCT-3′ for TAT, 5′-GTCCTACAGATTGACAATGC-3′ and 5′-CACGCTCTGGATCTG TGACAG-3′ for CK-19, and 5′-CGACTAACTGATGAGCTGGC-3′ and 5′-CACAGACTGCATGAGGTTCT-3′ for vinculin. Each cDNA was amplified by 35 PCR cycles involving denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s, and extension for 1 min, followed by final extension at 72 °C for 10 min. As an internal control, the β-actin mRNA was also amplified by 25 PCR cycles. The PCR products were subjected to the agarose gel electrophoresis and visualized by ethidium bromide staining. Under these assay conditions, the mRNA expression levels could be semiquantitated according to the band intensities.

Statistical analysis. Statistical analysis was performed using the non-paired *t* test as appropriate. *P* values less than 0.05 were considered to be statistically significant.

Results and discussion

In the present study, a murine normal liver epithelial cell line, CL2 [27], was stimulated with OSM and dexamethasone to induce differentiation into hepatocytes. To investigate the effect of the HCV core protein on the process of hepatocyte differentiation, the CL2 core cells, which constitutively expressed the HCV core protein [25,26], were compared with the negative control (mock) cells. We first examined the expression levels of hepatocyte-specific marker genes, albumin and TAT, and the bile duct epithelial cell-specific marker genes, CK-19 and vinculin, in the CL2 mock and core cells before and after stimulation with OSM and dexamethasone (Fig. 1A and B). Expression of albumin and TAT was considerably induced after the stimulation in both cells. CK-19 and vinculin were expressed before the stimulation, but their levels were not increased after the stimulation. Thus, the CL2 cells were found to express dual markers of hepatocyte and biliary lineages, which is a known phenotypic feature of hepatic progenitor cells. It was also shown that the stimulation with OSM and dexamethasone could induce differentiation into hepatocytes but not into bile duct cells in the CL2 mock and core cells. According to this, our system using the murine embryonic liver-derived "hepatic progenitor-like" CL2 cells may, to a certain extent, reproduce the process from the hepatic progenitor cells to mature hepatocytes.

As comparison of the CL2 mock and core cells in the expression levels of the marker genes, the degree of albumin expression after stimulation with OSM and dexamethasone was reduced in the CL2 core cells, compared with the mock cells (Fig. 1A). The induction level of TAT mRNA under the stimulation was also lower in the CL2 core cells than in the mock cells (Fig. 1B). On the other hand, the mRNA levels of CK-19 and vinculin were not different between the CL2 mock and core cells (Fig. 1B). Fig. 1C shows the morphological changes in the CL2 mock and core cells using phase contrast microscopy. The cells were grown as a monolayer with their morphology being epithelial-like before stimulation with OSM and dexamethasone. No substantial difference in the cellular appearance was observed between the CL2 mock and core cells before the stimulation. In the CL2 mock cells, the stimulation resulted in clear round-shaped nuclei with an increased nuclear/cytocellular ratio, which are known to be features of mature hepatocytes. In the CL2 core cells, such morphological changes appeared to be less apparent than those in the CL2 mock cells after the stimulation. These findings suggest that differentiation into hepatocytes may be substantially prevented by constitutive expression of the HCV core protein, as judged by the expression levels of the marker genes and cellular morphological features. By contrast, differentiation into bile duct epithelial cells may not be affected by the HCV core protein.

Next, the CL2 mock and core cells were stimulated with OSM alone, dexamethasone alone, or both, and the expression levels of the marker genes were examined (Fig. 2A and B). The stimulation with OSM alone did not induce expression of albumin and TAT, whereas the stimulation with dexamethasone alone resulted in weak expression of these marker genes. Their induction levels after stimulation with both OSM and dexamethasone were higher than those after stimulation with OSM or dexamethasone alone. Thus, the strongest induction of hepatocyte differentiation was seen under stimulation with both OSM and dexamethasone in the CL2 mock and core cells. Comparison of CL2 mock and core cells revealed apparent differences in the induction levels of albumin and TAT after stimulation with both OSM and dexamethasone, but not after stimulation with dexamethasone alone (Fig. 2A and B). This suggests that the HCV core may have an inhibitory effect on hepatocyte differentiation through an OSM-dependent process.

The interaction of OSM with its specific receptor on the cell surface leads to phosphorylation of JAK1/2, Tyk2, and STAT3, followed by nuclear translocation of the activated STAT3 homodimer. Next, the STAT3 dimer recognizes the specific DNA element, such as the acute phase response element (APRE), to regulate transcription of many STAT3-responsive genes [30,31]. We further examined the influence of the JAK inhibitor on expression of albumin and TAT in the CL2 mock and core cells stimulated with OSM and dexamethasone in order to validate involvement of the JAK-STAT pathway in the OSM-dependent hepatocyte differentiation. As shown in Fig. 2C and D, pretreatment of the JAK inhibitor blocked expression of albumin and TAT under stimulation, suggesting that activation of the JAK-STAT pathway may be responsible for hepatocyte differentiation induced by OSM and dexamethasone in the CL2 mock and core cells.

As the next step, the influence of the HCV core protein on the JAK-STAT signal transduction was studied in cells treated with OSM and/or dexamethasone. Fig. 3 shows the changes in the pSTAT3 level as a marker of STAT3 activation in the CL2 mock and core cells in the early phase (up to 12 h) after the stimulation. The induction level of the pSTAT3 after stimulation with OSM alone or both OSM and dexamethasone were weaker in the CL2 core cells than in the mock cells. As for changes in the whole STAT3 protein, its expression level was not affected by stimulation with OSM and/or dexamethasone in both cells. Thus, the HCV core protein was shown to prevent the OSM-dependent JAK-STAT signal transduction.

In our previous study, we demonstrated that the HCV core protein binds to the JAK protein, and that the interaction sites are located at amino acid positions 79–84 within the HCV core [25]. To clarify whether the inhibitory effect of the HCV core on the OSM-dependent JAK-STAT signal transduction was caused by the HCV core-JAK interaction, the reporter gene assay was conducted using CL2 cells by cotransfection of various effector plasmids with the reporter plasmid. pCoreMut-V5 and pCoreDel-V5 expressing the HCV core protein without the functional JAK-binding site, as well as pCore(1-191)-V5 and pcDNA3.1/V5-HisA (empty plasmid), were used as effector plasmids. As shown in Fig. 4, in the OSM-stimulated CL2 cells, the STAT3/APRE-dependent transcription activity was lower by transfection with the pCore(1-191)-V5 than by that with pcDNA3.1/V5-HisA. However, the STAT3/APRE-dependent transcription activity by transfection with pCoreMut-V5 or pCoreDel-V5 was restored to its original level by transfection with pcDNA3.1/V5-HisA. This indicates that the HCV core-JAK interaction may directly lead to inhibition of the JAK-STAT signal transduction and possibly to the inadequate differentiation into hepatocytes under OSM stimulation.

Expression of the OSM receptor subunits has been reported to be regulated by the OSM stimulation itself [32]. The expression levels of OSM receptor subunits,

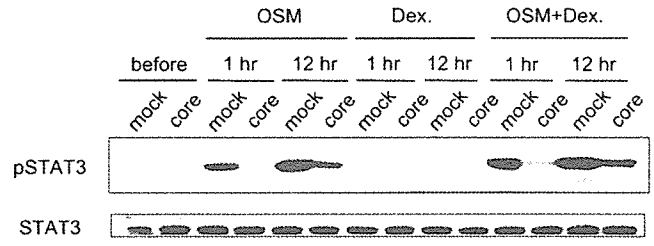

Fig. 3. Early phase response of STAT3 phosphorylation in the CL2 mock and core cells under stimulation with OSM and/or dexamethasone. The levels of pSTAT3 and the whole STAT3 were examined by Western blot analyses.

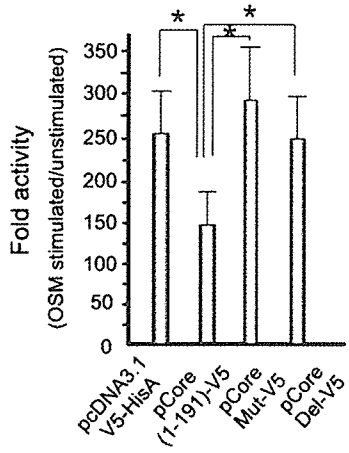

Fig. 4. Involvement of HCV core-JAK interaction in the HCV core-mediated inhibitory effect on the JAK-STAT signal transduction activated by OSM. The CL2 cells were cotransfected with various effector plasmids (pcDNA3.1/V5-HisA, pCore(1-191)-V5, pCoreMut-V5, and pCoreDel-V5) with pAPREluci and pRLtk. The cells were stimulated with OSM, or left unstimulated, and subjected to the dual luciferase assay. The firefly-luciferase activity was normalized for transfection efficiency based on the seapansy-luciferase activity. The relative light unit of the unstimulated sample was considered as 1, and the sample activities were calculated as multiples of it. The values were expressed as means ± SD. $*P < 0.05$ by the non-paired t test.

gp-130 and OSMRβ, were further examined. In the cytokine-untreated CL2 mock and core cells, the gp-130 was expressed with no substantial differences between the two cells, as described in our previous report [25]. The expression level of OSMRβ before stimulation was rather faint because it was below the detection limit of Western blot analysis and only detected by RT-PCR with no substantial differences between the CL2 mock and core cells (data not shown). As shown in Fig. 5, however, OSMRβ expression was clearly seen after stimulation with OSM and dexamethasone, and its induction level was lower in the CL2 core cells than in the mock cells. In addition, pretreatment of the JAK inhibitor blocked the induction of OSMRβ after the stimulation in both cells. This result indicates that the OSMRβ expression may depend on activation of the JAK-STAT pathway under OSM stimulation as a positive feedback loop. The HCV core protein may down-regulate OSMRβ expression as a secondary effect of the HCV core-mediated suppression of the JAK-STAT pathway.

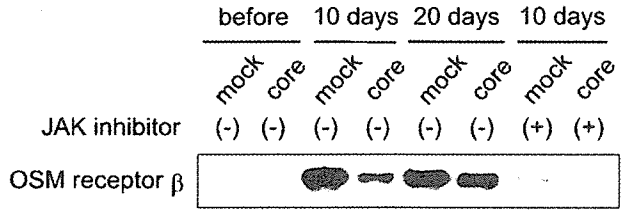

Fig. 5. Detection of OSMRβ in the CL2 mock and core cells under stimulation with OSM and dexamethasone in the presence or absence of JAK inhibitor. The cellular protein was harvested before, 10 days after, and 20 days after stimulation and used for Western blot analysis.

This may also account for the inhibitory effect on the OSM-dependent hepatocyte differentiation.

In this study, we suggested that constitutive expression of the HCV core protein may considerably inhibit the differentiation process from the progenitor cells to mature hepatocytes. The question arises of whether the hepatic progenitor cells can be infected with HCV. Recently, a system of HCV-vesicular stomatitis virus chimeric pseudotype virus has been established to easily assess the infectivity of HCV to cultured cells [33,34]. We examined the susceptibility to this HCV pseudotype virus of embryonic human hepatocytes and found that these immature hepatocytes could become infected (unpublished data). Therefore, we speculate that even the hepatic progenitor cells may be infected with HCV. The inhibitory effect of the HCV core on hepatocyte differentiation assessed in this study may be significant in a clinical setting of chronic HCV infection.

In conclusion, this is the first report focusing on the relationship between the HCV core protein and differentiation into hepatocytes. HCV core-mediated inhibition of hepatocyte differentiation may be exerted through the HCV core-JAK interaction and the subsequent inhibition of the OSM-dependent JAK-STAT signaling pathway. Down-regulation of OSMRβ expression as a secondary effect may also be a reason for HCV core-mediated inhibition of hepatocyte differentiation. The HCV core protein may play a crucial role in the pathogenesis of HCV-related liver diseases by affecting the differentiation process, as well as the proliferation, malignant transformation, and apoptosis, of the host cells.

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Review

Antiviral therapy for chronic hepatitis C: past, present, and future

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Antiviral therapy for chronic hepatitis C has dramatically advanced since the discovery of the hepatitis C virus (HCV) in 1989 and the introduction of interferon (IFN) monotherapy in the early 1990s. The current standard therapy uses a combination of pegylated IFN and ribavirin. The duration of therapy and response to therapy are HCV genotype-specific. Genotype 1 patients require 48 weeks of the combination therapy for 50% successful viral elimination, while genotype 2 patients require 24 weeks of therapy for 80% or 90% viral elimination. Early viral kinetics after the initiation of therapy is a useful predictor of the sustained virologic response (SVR), which is formally determined at 24 weeks after completion of the treatment. For example, an early virologic response, which is determined by a 2-log reduction of HCV RNA or viral elimination at 12 weeks after the initiation of therapy, is a strong negative predictor of SVR in genotype 1 patients. In contrast, a rapid virologic response of HCV RNA-negative at 4 weeks after the initiation of therapy identifies genotype 2 "super-responders," who may require a shorter period of therapy. Adherence to therapy is one of the most important factors for successful viral clearance. Hematopoietic growth factors such as epoetin and granulocyte-colony stimulating factor help reduce therapy-mediated cytopenia and improve patient compliance, thereby leading to better viral clearance. New types of anti-HCV agents such as HCV protease and polymerase inhibitors are needed for those patients that do not respond to combination therapy.

Introduction

In 1989, the hepatitis C virus (HCV) was discovered in the United States to be the causative agent of posttransfusion non-A, non-B hepatitis by Chiron Corporation (Emeryville, CA, USA).[1] By this discovery, HCV was revealed to be the cause of many hepatic diseases of previously unknown origin. HCV is closely associated with hepatocellular carcinogenesis and death due to chronic liver disease. Epidemiologically speaking, it is estimated that 1.7 million people in Japan and 170 million people worldwide are infected with HCV.[2] Many cases are asymptomatic and result in overt hepatic disease, manifested as hepatic cirrhosis or cancer, only following 20 to 30 years of persistent infection. Thus, HCV infection is of significant concern in terms of public health.

Spontaneous elimination of HCV occurs in approximately 30% of HCV-infected patients within 6 months after infection. However, after this period of time, viral elimination is very rare, with an annual rate of only about 0.2%. Persistent inflammation associated with HCV causes hepatic fibrosis, and as the stage of fibrosis progresses, the risk of cancer increases; annual rates of hepatocarcinogenesis are 0.5% for patients with modest fibrosis and 8% for those with liver cirrhosis.

HCV-associated, progressive hepatic disease can be directly inhibited by interferon (IFN), which is currently the only drug that can eradicate HCV. This review traces the progress of IFN-based therapy for hepatitis C since its introduction and provides a brief overview of the future of HCV treatment.

Introduction of IFN therapy

IFN therapy for hepatitis C dates from 1986, when Hoofnagle et al.[3] reported the normalization of serum alanine aminotransferase (ALT) levels following administration of recombinant human IFNα to patients with non-A, non-B hepatitis. In other words, IFN was shown to be biochemically effective as an anti-inflammatory agent before the discovery of HCV.[4,5]

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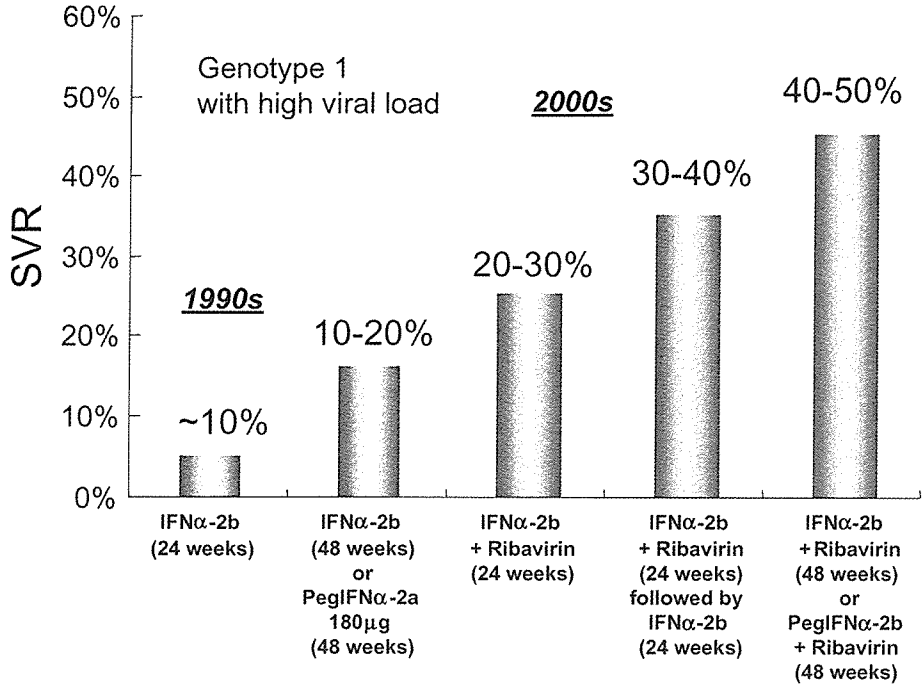

Fig. 1. Milestones of interferon (*IFN*)-based therapy for chronic hepatitis C. Progress in sustained viral clearance for a difficult to treat patient group, with genotype 1 and a high viral load, from the early 1990s. *SVR*, sustained virologic response; *PegIFN*, pegylated interferon

Later, the introduction of a virus identification method using a polymerase chain reaction (PCR) assay revealed cases in which patients became HCV-PCR negative following IFN administration.[6] The normalization of serum ALT levels is associated with viral eradication, with the exception of a few cases. The discovery of these biochemical and virological effects prompted approval of clinical use of IFN against hepatitis C in the United States in 1991 and in Japan in 1992.

The therapeutic responses to IFN can be classified as sustained virologic response (SVR), relapse, or nonresponse. SVR means complete elimination of HCV, which is defined as the loss of detectable HCV RNA during therapy and its continued absence for at least 6 months after the termination of therapy. Relapse is defined as being HCV-negative at the end of IFN treatment but HCV-positive within 6 months after the termination of therapy. Nonresponse is defined as the absence of a HCV-negative condition even with IFN administration. Initial studies showed that after 6 months of 6MU IFN administration to patients with chronic hepatitis C, SVR, relapse, and nonresponse were each observed in one-third of the patients. Subsequent studies revealed that the antiviral effect is determined mainly by viral factors, namely the viral load and the viral genotype.[7,8] Genotypes 1a and 1b are more resistant to IFN therapy than genotypes 2a and 2b, and patients with a high viral load are less likely to respond to IFN than those with a lower viral load. A subgroup analysis of patients treated with IFN monotherapy showed that the SVR rate in genotype 1 patients with a high viral load, accounting for approximately 60% of patients with hepatitis C in Japan, was only 5%. How to improve the therapeutic effect in these patients is the greatest problem for future research and development of IFN therapy (Fig. 1).

Progress of IFN-based therapy

For such resistant cases (patients with genotype 1 and high viral load), extended administration to optimize the total dose of IFN, the introduction of pegylated IFN (PEG-IFN) and coadministration with ribavirin have been used to substantially improve treatment over the past 10 years.

Optimization of the total dose of IFN: extended administration

Two means of increasing the total dose of IFN in resistant cases have been investigated: increasing the dose and extending the administration period. In Japan, patients had usually been given 6MU IFN three times a week for 6 months. Higher doses did not correlate with an increased SVR rate, partly because of the increased incidence of adverse effects and reduced patient compliance. However, extending the administration period proved effective for raising the SVR rate. Kasahara et al.[9] showed that 12 months of administration clearly

increased the SVR rate in genotype 1 patients, compared with 6 months of administration. However, in genotype 2 patients, there was no significant difference between 6 and 12 months of administration. The standard dose of IFN used in Europe and the United States, based on early clinical studies, has been 3 MU three times a week, with the result that European and U.S. therapeutic results after 6 months of IFN monotherapy are generally lower than those in Japan.[10,11] In Europe and the United States, the superiority of the 6-MU dose over a 3-MU dose has been shown by subsequent controlled studies, and many other clinical studies have shown the superiority of 12 months of therapy over 6 months.[12–14] The SVR rate for genotype 1 patients with a high viral load improved with IFN therapy of extended duration, shown first for IFN monotherapy and later for the combination of IFN with ribavirin.[15–17]

Based on these findings, administration of IFN for 12 months was approved early in Europe and the United States. In Japan, the 6-month limit for IFN therapy was removed in 2002, and self-injection of IFN was approved in 2005. These measures make it easier for patients to undergo long-term treatment.

Development of IFN preparations: introduction of PEG-IFN

The type I IFNs include IFNα, IFNβ, IFNω, and IFNλ, all of which share cell-surface receptor and intracellular pathways of action. IFN agents are used in various preparations. In the United States, recombinant IFNα-2b and IFNα-2a were initially approved. In Japan, in addition to these two preparations, natural IFNα and IFNβ can be used. These conventional preparations are considered to be of equal efficacy, although a few differences in the incidences of neutralizing antibodies and adverse effects have been noted.[18] Subsequently, a special agent, consensus IFN, was developed and put into clinical use.[19] It was designed by selecting the most frequently occurring amino acid at each site of the amino acid sequences of 13 known IFNα subtypes. Consensus IFN is considered to have a potent antiviral effect in genotype 1 patients with a high viral load, but it is still considered to be a conventional IFN agent.

Revolutionary progress in the development of IFN agents was recorded with the development of PEG-IFN and its introduction to clinical use. Pegylation is defined as modification of a drug by the addition of an artificial polymer, polyethylene glycol (PEG), for the purpose of delaying drug elimination, lowering its antigenicity, and modifying the drug's effect. Conventional IFN agents, with approximately 8-h elimination half-lives, require a dosing interval of 1 or 2 days to maintain an effective blood concentration.[20,21] The most beneficial effect of PEG-IFN is that it delays drug elimination, making it possible to maintain a stable blood concentration with once-weekly administration.[22] Currently, two PEG-IFN preparations are available: recombinant IFNα-2a and IFNα-2b, which are covalently bound to 40-kDa PEG and 12-kDa PEG, respectively. Both are thought to have about equal efficacy, but they have not been compared in clinical trials.

European and U.S. controlled studies have shown that PEG-IFN agents are generally more effective, both in monotherapy[23–25] and in combination with ribavirin, than conventional IFN agents.[26,27] In Japan, clinical studies have shown that PEG-IFN agents are not inferior to conventional IFN agents. However, no study has shown PEG-IFN agents to be significantly superior with respect to SVR, partly, perhaps, because the usual dose of control IFN agents used in Europe and the United States is 3 MU, which is less than that used in Japan. In sum, PEG-IFN is at least equivalent to conventional IFN in effectiveness, and it appears to be highly tolerable because it can be administered just once a week.

The adverse effects of IFN are classified into two types: those that occur soon after the start of administration, and those that manifest during long-term administration.[28] The former type includes flu-like symptoms, such as a high fever, headache, and myalgia, and abnormal blood test results such as thrombocytopenia and leukopenia. Effects seen with long-term administration include a wide variety of symptoms, such as pruritus, alopecia, fundal hemorrhage, depression, thyroid dysfunction, diabetes mellitus, pulmonary fibrosis, and cardiac arrhythmia. Adverse effects of PEG-IFN are similar to those of conventional IFN and are characterized by mild influenza-like symptoms during the early stage of administration and comparatively severe cytopenia. The occurrence of acute thrombocytopenia in the late stage of administration of PEG-IFNα-2a has also been noted. More caution is needed with respect to the occurrence of adverse effects of PEG-IFN owing to its delayed clearance.

Combination therapy: introduction of ribavirin

Ribavirin, developed in 1972, is a synthetic nucleic acid analog with a purine skeleton. It has antiviral activity in vitro to a wide variety of RNA and DNA viruses, and it is orally administered. Ribavirin has not been approved in Japan as an antiviral agent for monotherapy, but it has been approved in Europe and the United States for various viral diseases, such as severe respiratory syncytial virus infection in children. Its antiviral effect against HCV has not been proved by studies on monotherapy for hepatitis C.[29] In 1998, however, the combination of ribavirin with IFN was reported to have achieved a significantly higher SVR rate compared with IFN

monotherapy.[15,16,30,31] These reports were followed by large-scale clinical studies in Europe and United States[26,27] showing that a combination of PEG-IFN and ribavirin produces better results than one of IFN and ribavirin. With both combinations, 48 weeks of administration to genotype 1 patients achieved a significantly higher SVR rate than 24 weeks of administration.[15,16,32] For other patients, no significant difference was seen between groups receiving 24 or 48 weeks of therapy, and the 24-week administration period was reported to be sufficiently effective.

In Japan, a 48-week, multicenter, randomized, controlled study[33] was conducted on combinations of 6 MU IFNα-2b with ribavirin and 1.5 μg/kg PEG-IFNα-2b with ribavirin administered to genotype 1b patients with a high load of HCV-RNA, determined to be 100 KIU/ml or higher using Amplicor (by the original PCR method). Oral doses of ribavirin administered were 600 mg/day for patients weighing less than 60 kg, 800 mg/day for those weighing at least 60 kg but less than 80 kg, and 1000 mg/day for those weighing 80 kg or more. IFNα-2b was administered six times a week for the first 2 weeks and three times a week for the following 46 weeks, while PEG-IFNα-2b was administered once a week. The results for 506 patients indicated high rates of viral elimination by both therapies. The combination of PEG-IFNα-2b plus ribavirin and that of IFNα-2b and ribavirin achieved SVR in 121/254 patients (47.6%) and 113/252 patients (44.8%), respectively, a difference that was not significant. Based on this phase 3 study, 48 weeks of PEG-IFNα-2b and ribavirin combination therapy was approved for genotype 1 patients with a high viral load in Japan in 2004.

The adverse effects of ribavirin include hemolytic anemia and potential teratogenicity. Caution must be exercised with ribavirin administration when there is coexisting anemia or coronary heart disease. Contraception is also required during administration of ribavirin and up to 6 months after the end of its administration. Ribavirin is contraindicated for patients with renal failure, because it is excreted by the kidney and cannot be eliminated by dialysis.

Recent developments in PEG-IFN and ribavirin therapy

Coadministration of PEG-IFN and ribavirin has been established as the standard regimen of antiviral therapy for hepatitis C,[34] and the following questions next arise. How long should the dosing period be for this combination? How can its adverse effects be ameliorated and the treatment successfully completed? To what extent can this combination treatment be applied? Recent developments offer responses to these questions.

Exploring necessary and sufficient dosing periods: the impact of viral kinetics study

Usually the duration of coadministration of PEG-IFN and ribavirin is 48 weeks for difficult cases (e.g., genotype 1 patients with a high viral load) and 24 weeks for other cases, with expected SVR rates of approximately 50% and 80%, respectively.[32] Some studies have suggested that higher doses of ribavirin based on body weight are more effective for genotype 1, while a lower dose (fixed dose at 800 mg/day) is sufficient for viral genotypes other than genotype 1. To date, a variety of factors, both viral and host, that correlate with a sustained response to the combination therapy have been noted (Fig. 2). In contrast to viral factors, however, most host factors do not have a strong impact on the various treatment regimens. Recently, the viral kinetics after the start of therapy has been noted to be a useful early indicator of viral elimination, which is usually determined 24 weeks after the end of therapy.[20] To find out whether SVR is related to the rate of inhibition of viral replication after the start of PEG-IFN plus ribavirin combination therapy, Davis et al.[35] carried out a retrospective analysis of a controlled clinical study conducted by Manns et al.[26] In the clinical study, PEG-IFNα-2b (1.5 μg/kg per week) and ribavirin (800 mg/day) were coadministered to 511 patients with chronic hepatitis C for 48 weeks. If an early virologic response (EVR) is defined as a viral load decrease of 2 log or more or viral elimination after 12 weeks of treatment, then 71.8% of the patients who experienced EVR—74.4% of all patients—achieved SVR. Importantly, none of the patients who did not experience EVR achieved SVR. Similarly, with therapy with PEG-IFNα-2a (180 μg/week) plus ribavirin (1000 or 1200 mg/day, depending on body weight) for 48 weeks (n = 453), only 2 of 63 patients who did not experience EVR achieved SVR.[27] These findings show that EVR has negative predictive value, and therefore, if viral elimination is the aim of the treatment and if adverse effects cannot be negligible, the treatment should be discontinued in patients not displaying EVR. This "12-week rule" applies only to patients with viral genotype 1.[36]

The relationship between the time of becoming HCV-negative and SVR has also been examined in Japan in the above-mentioned clinical study[33] of PEG-IFNα-2b plus ribavirin. SVR rates for patients who became HCV-negative at 4, 12, or 24 weeks (23, 121, and 33 patients, respectively) were 100%, 71.1% and 36.4%, respectively. None of the 15 patients who experienced viral elimination after 24 weeks achieved SVR. Therefore, 24 weeks of additional administration to patients with no viral elimination within the initial 24 weeks produces no benefit.

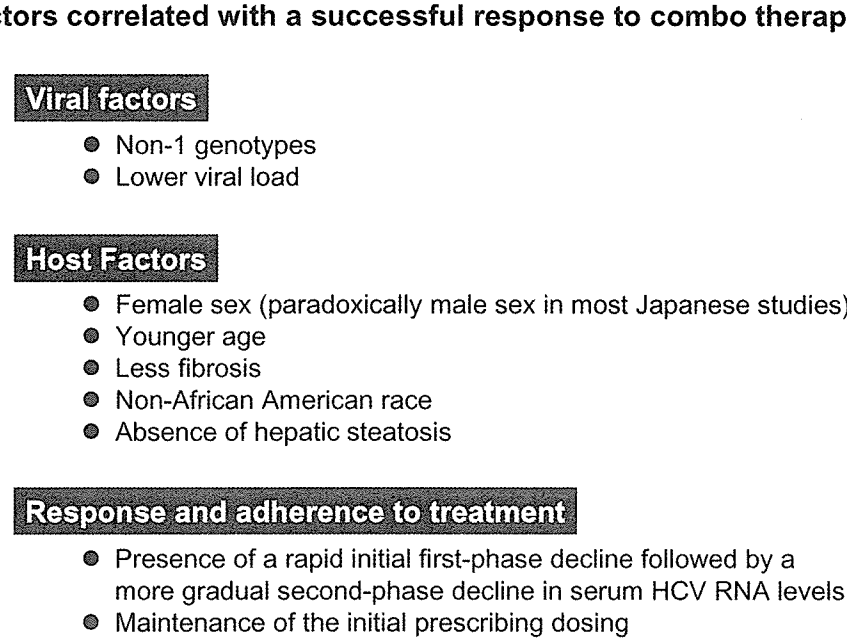

Fig. 2. Factors correlated with a successful response to combination therapy with pegylated interferon and ribavirin in chronic hepatitis C. HCV, hepatitis C virus

Genotype 1 patients who do not experience EVR are very intractable, as shown above. In other words, 48 weeks of therapy with PEG-IFN and ribavirin may be too short to maximize SVR in genotype 1 patients.[37] The usefulness of long-term administration for 48 weeks or longer is being investigated to improve the rate of achievement of SVR in such patients. Buti et al.[38] published a promising report on extending therapy with PEG-IFN plus ribavirin to 72 weeks for late virologic responders. They selected nine genotype 1 patients being treated with PEG-IFNα-2b (1.0μg/kg) plus ribavirin (800mg/day) who cleared HCV RNA between weeks 12 and 24 for therapy prolonged to 72 weeks. Eight patients completed therapy, and at week 24 of follow-up, seven maintained SVR and one had relapsed. A Spanish multicenter, randomized controlled study, in which patients with chronic hepatitis C who did not became HCV negative by 4 weeks of coadministration of PEG-IFNα-2a (180μg/week) and ribavirin (800mg/day) (about two-thirds of all patients) were randomized to groups receiving 48 weeks or 72 weeks of therapy, found that the group receiving 72 weeks of therapy achieved a significantly higher rate of SVR than the group receiving 48 weeks of therapy. On the other hand, a recent clinical trial showed that genotype 1 patients who were HCV RNA-negative after 4 weeks of coadministration of PEG-IFNα-2a (180μg/week) and ribavirin (1000 or 1200mg/day) achieved an SVR rate of 66% with a further 20 weeks of therapy.[39] Unfortunately, this study did not randomize the patients to compare 24 weeks of therapy with a 48-week therapy period. The study, however, does show that 24 weeks of therapy can achieve relatively high rates of viral elimination for these genotype 1 "super-responders."

For other, non-1 viral genotypes, studies are being done to identify a dosing period shorter than 24 weeks that can be used to achieve sufficient SVR. In one study, genotype 2 and 3 patients were given PEG-IFNα-2b (1.0μg/kg each week) and ribavirin (1000 or 1200mg/day, based on body weight), and those who experienced viral elimination after 4 weeks of therapy were assigned to 24-week or 12-week therapy groups. The results showed that the SVR rate for the 12-week group was the same as that for the 24-week group, indicating that 12 weeks of combination therapy is sufficient for these patients.[40] Similar data have also been reported for PEG-IFNα-2a (180μg/week) plus ribavirin (800 to 1200mg/day) therapy.[41]

As mentioned above, for treatment of non-1 viral genotypes and some genotype 1 patients, sufficient SVR rates can be achieved and unnecessary treatment avoided by adopting the dosing period by using the early viral inhibition effect as an indicator. The early viral kinetics can be also applied to identify more difficult to treat patients with viral genotype 1, who can then be given longer treatment to improve SVR rates (Fig. 3).

Reducing cytopenic effects and improving compliance: the use of hematopoietic growth factors

Patient compliance has been noted by many clinical studies to be the largest factor contributing to the therapeutic effect of PEG-IFN plus ribavirin combination

therapy (Fig. 2). Compliance can be divided into those factors related to patient adherence to the regimen and dose interruptions or modifications mandated by the physician in response to cytopenia, rash, gastrointestinal symptoms, or depression. McHutchison et al.[42] outlined an "80:80:80 rule" in genotype 1 patients; that is, the doses of PEG-IFN and ribavirin and the dosing period should exceed 80% of the initial plan to achieve a sufficient SVR rate. Early dose reduction within 12 weeks is more harmful than later dose reduction. To maximize viral clearance of the PEG-IFN and ribavirin combination therapy, countermeasures are needed against adverse effects to improve patient compliance.

Compared with IFN monotherapy, combination therapy is characterized by additional adverse effects represented by hemolytic anemia. If anemia occurs, the dose of ribavirin must be reduced or the administration of ribavirin must be discontinued. To help avoid this adverse effect, attention is being drawn to drug intervention with erythropoietin. An 8-week, double-blind study was conducted in which epoetin alpha 4000 U/week or a placebo was given to patients who experienced a decrease in hemoglobin (Hb) levels to 12 g/dl or less during coadministration of PEG-IFN and ribavirin in the United States, and the dose of ribavirin, Hb levels, and quality of life (QOL) were compared at the end of the study.[43] Compared with the placebo group, the reduction in Hb levels was significantly inhibited in the epoetin alpha group; thus, reduction of the ribavirin dose could be avoided. Inhibition of the reduction in Hb levels also improved QOL.[44] Similarly, granulocyte-colony stimulating factor (G-CSF) is expected to be useful for avoiding leukocytopenia induced by PEG-IFN and ribavirin combination therapy. Prevention of adverse effects with hematopoietic growth factors may be a promising measure to allow the maintenance of the therapy protocol and to improve therapeutic outcomes.

Challenging special patient groups: chronic hepatitis C with persistently normal ALT levels

Persistently normal ALT levels are observed in 20%–30% of chronic HCV-infected patients among the general public. Such patients are sometimes called asymptomatic HCV carriers. Most of them present a picture of histologically minimal or mild chronic hepatitis; it is rare for the liver to be normal. Progression of fibrosis is noted in fewer than 10% of the patients. For this reason, the expression "chronic hepatitis C patients with persistently normal ALT levels" is often preferred to "asymptomatic HCV carriers." There was strong resistance against using IFN therapy for such patients in the 1990s[45,46] for both active and passive reasons. The former included a lower viral elimination effect, or SVR, compared with general hepatitis C patients, and the report of abnormal ALT levels in a high percentage of patients due to IFN therapy in early studies of asymptomatic HCV carriers.[47,48] Recent studies have shown that IFN monotherapy[49] and IFN plus ribavirin combination therapy[50,51] can help patients with persistently normal ALT levels achieve the same level of SVR as patients with abnormal ALT levels. The percentage of patients who display an increase in the ALT level in response to IFN therapy is also lower than that in the early studies.[51] Therefore, the active reasons against using IFN therapy for patients with persistently normal ALT levels can no longer be supported. The passive reason, that there is no evidence of improved long-term prognosis in this patient group by IFN therapy, still remains.

HCV patients with normal ALT levels have been not eligible for large-scale clinical studies, causing there to be a deceptively low level of evidence regarding the efficacy of antiviral therapy in such patients. However, the potential importance of antiviral therapy for such patients has been gaining attention in recent years, and an international, multicenter, randomized, controlled study of PEF-IFNα-2a plus ribavirin combination therapy has been conducted.[52] Eligible participants were 491 HCV RNA-positive patients whose ALT levels measured three times or more at intervals of at least 4 weeks did not exceed the upper limit of the normal ALT range. The patients were randomized at the proportion of 3:3:1 into three groups: patients receiving 24 weeks of therapy with PEF-IFNα-2a (180 μg/week) and ribavirin (800 mg/day), those receiving 48 weeks of PEF-IFNα-2a (180 μg/week) and ribavirin (800 mg/day), and a control group that did not receive any treatment. Acute exacerbation of ALT levels that exceeded ten times the upper limit was observed in two patients (one in the 24-week therapy group and one in the control group). The results regarding treatment effectiveness were identical to those for chronic hepatitis C patients with high ALT levels previously published by Hadziyannis et al.[32] Thus, a dosing period based on the algorithm established for chronic hepatitis C patients with abnormal ALT levels can be recommended for PEG-IFN plus ribavirin combination therapy for HCV-infected patients with persistently normal ALT levels.

Such findings strongly suggest that HCV-infected persons with persistently normal ALT levels should be considered eligible for IFN therapy. The 2004 American Association for the Study of Liver Diseases (AASLD) best-practice guideline[36] recommended as follows: "Regardless of serum aminotransferase levels, the decision to initiate therapy with interferon and ribavirin should be individualized based on the severity of liver diseases by liver biopsy, the potential serious side effects, the likelihood of response, and the presence of comorbid

conditions." What is crucial is not the ALT level but whether to treat the patient if his/her liver disease is not severe.

Future antiviral therapy for hepatitis C

IFN plus ribavirin combination therapy brought about substantial improvement in comparison with the IFN therapy introduced in the 1990s. This combination may lead to high viral elimination primarily because it decreases the incidence of relapse in patients who have become HCV-negative during the therapy. According to an analysis of patient characteristics by the aforementioned Japanese clinical study[33] of PEG-IFNα-2b plus ribavirin combination therapy for genotype 1b patients with high viral load, SVR rates in treatment-naïve patients, relapsers, and nonresponders were 43.1% (59/137 patients) 62.6% (57/91 patients), and 19.2 (5/26 patients), respectively. The fact that relapsers achieved higher SVR rates than treatment-naïve patients suggests that PEG-IFN plus ribavirin combination therapy maximizes the therapeutic effect of IFN and encourages complete viral elimination in IFN-responding patients. On the other hand, the low SVR rate in nonresponders indicates that PEG-IFN plus ribavirin combination therapy is not always useful in patients who do not respond to IFN. To improve SVR rates in such patients, more-effective antiviral agents other than IFN must be developed. Furthermore, as described earlier, PEG-IFN plus ribavirin combination therapy induces a variety of adverse effects. Clearly, safer and better tolerated therapies are needed.

Promising agents for future anti-HCV therapies are classified as HCV-specific inhibitors targeting its protease and polymerase activities, IFN inducers, or less-toxic ribavirin-like agents. A number of drugs are in preclinical or clinical trials.

HCV protease inhibitors

HCV encodes at least four enzymes required for virus replication. They include NS2/3 autoprotease, NS3 helicase, NS3/4A serine protease, and NS5B RNA-dependent RNA polymerase. Intensive work on developing specific inhibitors has focused on the last two.

SCH 503034 is a novel, orally active HCV protease inhibitor that exhibits potent and specific antiviral activity in HCV replicon assays. Recently, a phase 1b clinical trial was conducted for both monotherapy[53] and combination therapy with PEG-IFNα-2b.[54] SCH 503034 exhibited dose-dependent HCV antiviral activity in genotype 1 patients in whom PEG-IFN therapy had previously been unsuccessful. In combination with PEG-IFNα-2b, SCH 503034 had at least an additive effect on HCV suppression. VX-950 is an orally administered highly selective peptidomimetic inhibitor of HCV NS3/4A protease. In a phase 1b clinical trial, VX-950 was well tolerated for 5 to 14 days in both healthy subjects and patients with viral genotype 1, with no serious adverse effects. VX-950 showed a 4.4-log reduction in median HCV RNA at the end of 14 days of therapy.[55]

In addition of its critical role in virus replication, the NS3/4A protease also plays a role in suppressing the cellular antiviral response. Active NS3/4A prevents the phosphorylation and activation of interferon regulatory factor (IRF)-3 and the triggering of downstream IFN-induced antiviral effector genes.[56,57] IRF-3 activity has been shown to be restored by a HCV protease inhibitor. Thus, an effective protease inhibitor may block not only RNA replication but also the ability of HCV to evade innate antiviral responses.

HCV polymerase inhibitors

Valopicitabine (NM283) is a 3′-valyl prodrug of a nucleoside analog that exhibits anti-HCV activity via inhibition of viral RNA polymerase. Valopicitabine is currently in phase 2 clinical development for the treatment of chronic hepatitis C. In a phase 2a trial, valopicitabine demonstrated potent anti-HCV activity when administered in combination with PEG-IFNα-2b, with 4.5-log serum HCV RNA reduction at 6 months and no obvious viral breakthroughs. In a phase 2b clinical trial, the combination therapy was also effective for patients previously unresponsive to PEG-IFN and ribavirin combination therapy.[58]

Since HCV has a higher intrinsic mutation rate than HIV, resistance is expected to be a problem with the use of any type of HCV-specific inhibitor targeting NS3/4A or NS5B proteins. To suppress the risk of a possible escape mutant, combination therapy with PEG-IFN may be better than monotherapy because the former can more efficiently suppress the levels of HCV replication. In the future, a combination of two or three different types of HCV inhibitors may offer a promising approach, similar to HIV cocktail therapy.

Immune modulators

Successful spontaneous clearance of HCV infection is thought to require both innate (e.g., direct antiviral activities by cytokines and natural killer cells) and adaptive (T cell-mediated) immune responses. Chronic HCV infection is characterized by an inadequate immune response that fails to clear the virus.[59] Immune modulators, alone or in combination with direct antiviral agents such as IFN and HCV inhibitors, represent a possible opportunity to improve HCV clearance.

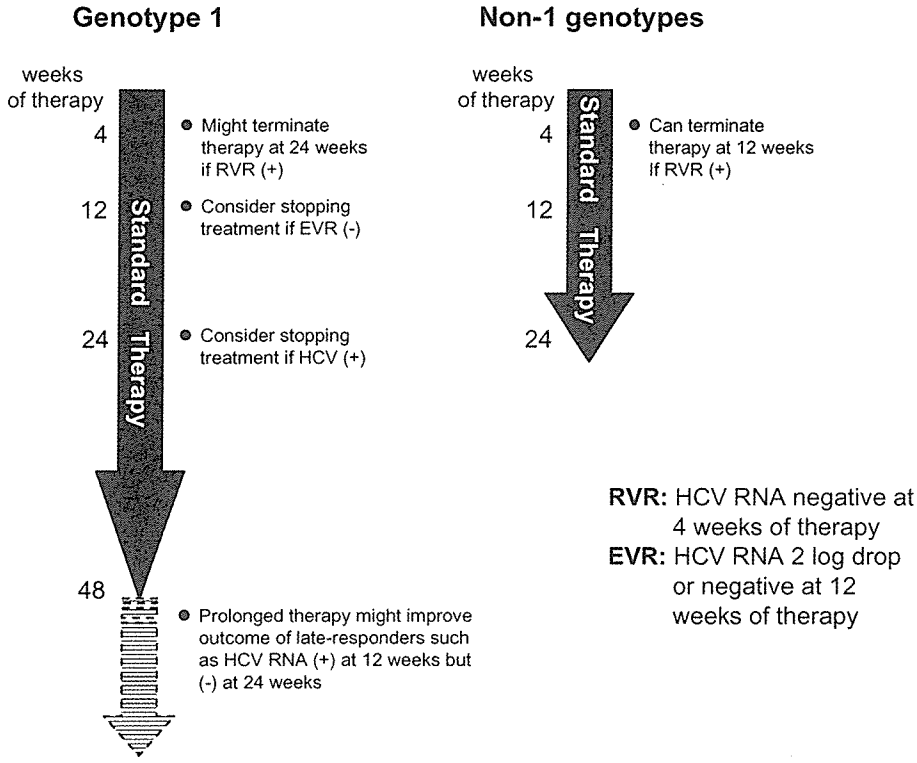

Fig. 3. Various treatment regimens of pegylated IFN and ribavirin combination therapy. *RVR*, rapid virologic response; *EVR*, early virologic response

CPG 10101 is a synthetic agonist of toll-like receptor (TLR) 9. HCV-infected patients receiving CPG 10101 subcutaneously had a more than 1-log reduction in HCV viral load while on therapy.[60] Further development of this agent will continue in conjunction with PEG-IFN and ribavirin.

Isatorbine is a TLR7 agonist. In a proof-of-concept clinical study, intravenous injection of isatorbine once daily for 7 days to patients chronically infected with HCV yielded a significant reduction of serum HCV RNA that correlated with induction of 2′,5′-oligoadenylate synthetase. Recently, the orally available prodrug of isatorbine, ANA975, was developed and studied in healthy phase 1 volunteers and showed promising pharmacokinetics and tolerability.[61]

Ribavirin-like agents

The addition of ribavirin to IFN therapy more than doubled the SVR rate, although its mechanism of action is unknown.[62] Furthermore, higher doses of ribavirin clearly improved response rates in genotype 1 patients.[32,63] However, ribavirin-induced hemolytic anemia is a major obstacle to implementation of a higher dosage regimen and limits its use in patients with comorbidities. To develop a better tolerated combination therapy, ribavirin-like agents lacking a hemolytic effect are needed. Viramidine is a ribavirin prodrug that is metabolized primarily in the liver. In a phase 2 study, fewer patients receiving viramidine developed anemia compared with those given ribavirin, but they also showed lower SVR rates.[64] Phase 3 trials have been undertaken of both PEG-IFNα-2a and PEG-IFNα-2b combined with viramidine in comparison with the combination with ribavirin.

Conclusion: viewpoints other than SVR

IFN treatment of patients with chronic hepatitis C were initially based on observations of its biochemical effects, before the discovery of HCV. Subsequently, evaluation of SVR at 6 months after stopping therapy as a clear end point made it possible to assess therapeutic results in a scientific manner. IFN therapy has been developing over the past decade, with the aim of improving the SVR rate, and higher rates are expected to be achieved with new, more specific antiviral agents.

The question arises as to what the ultimate purpose of hepatitis C treatment is. The answer is that it is the prevention of liver-related death of HCV-infected patients by suppressing progression to decompensated liver disease and liver carcinogenesis (Fig. 4), meaning that hepatitis C is not just an infectious disease, but a potentially serious liver disease. From this point of view, SVR is no more than a surrogate marker—albeit a very strong one—to improve the prognosis of HCV-infected patients. Hepatocellular cancer occurs even in patients

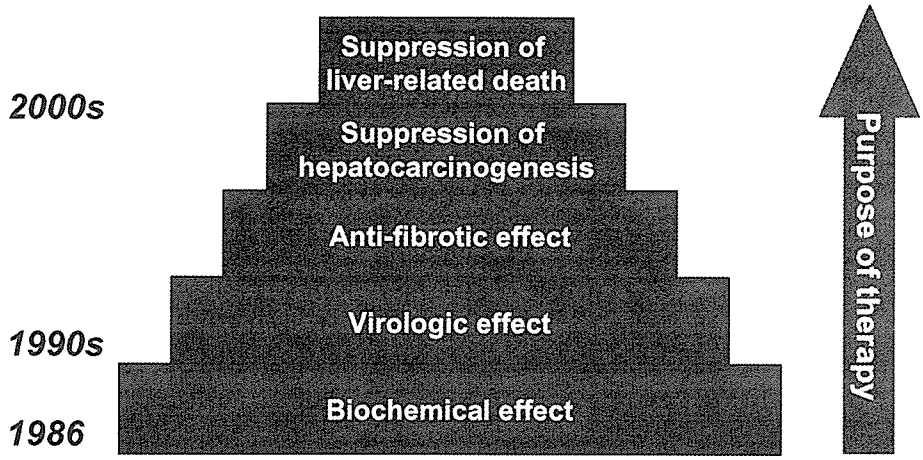

Fig. 4. Effects of IFN-based therapy in relation to the purpose of treatment for chronic hepatitis C. Retrospective analysis of rates of hepatocellular carcinoma and liver-related death after IFN monotherapy have shown a reduction in risk, especially in patients with moderate liver fibrosis[65–69]

who have experienced SVR, although its incidence is substantially lower in those patients than that in untreated patients or nonresponders. Thus, routine hepatocellular cancer screening is essential even after patients have experienced SVR, and early treatment is indispensable if it occurs. On the other hand, the cumulative incidence of hepatocellular carcinoma is clearly suppressed around half in even relapsers at least for 5 years after the termination of therapy compared with that in untreated patients.[65] Therefore, the therapeutic effect of IFN therapy should be evaluated not only on the basis of the SVR rate but also from the more important viewpoint of inhibition of hepatocellular cancer. In this context, repeated IFN therapy, for example every 5 years, for relapsers, and long-term, low-dose IFN therapy for nonresponders should also be considered until a new era dawns of treating hepatitis C with novel anti-HCV agents.

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Viral covalently closed circular DNA in a non-transgenic mouse model for chronic hepatitis B virus replication

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***Background/Aims*: The lack of small animal models supporting chronic hepatitis B virus (HBV) infection impedes the assessment of anti-viral drugs in the whole animal. Although transgenic mice have been used for this purpose, these models are clearly different from natural infection, because HBV is produced from the integrated HBV sequence harbored in all hepatocytes.**

***Methods*: Balb/cA nude mice were hydrodynamically injected with a plasmid having 1.5-fold over-length of HBV DNA and analyzed for HBV replication.**

***Results*: Hydrodynamically injected mice showed substantial levels of antigenemia and viremia for more than 1 year. Covalently closed circular DNA (cccDNA), the template of viral replication in natural infection, was produced in the livers and was critically involved in the long-term HBV production, because disruption of the *pol* gene of the inoculated DNA resulted in transient expression of HBV genes for less than 2 months. Administration of the IFNα gene transiently suppressed HBV DNA replication, but was not capable of eliminating HBV in this model.**

***Conclusions*: In vivo gene transfer of a plasmid encoding HBV DNA can establish chronic viral replication in mice, which involves, at least in part, new synthesis of the HBV cccDNA episome, thus recapitulating a part of human HBV infection.**

***Keywords*: HBV; Liver; DNA; Hydrodynamics; Naked; Gene; Transfer; Transfection; IFN; Mouse**

1. Introduction

Hepatitis B virus (HBV) causes both transient and persistent infection in the human liver [1,2] When healthy adults are exposed to this virus, they usually develop acute transient infection with various degrees of liver injury, and, in most cases, have favorable outcomes. In contrast, when immunocompromised hosts such as newborn babies, drug abusers, and patients receiving immunosuppressive drugs, are infected with HBV, they cannot eliminate it and often suffer from chronic liver injury and hepatocellular carcinoma. Chronic carriage of this virus is a major health problem in many countries. Patients with chronic HBV infection are currently treated with interferon (IFN) or nucleotide analogs such as lamivudine and adefovir. However, the limited success and frequent recurrence after cessation of therapy require new strategies for terminating this viral infection.

Study of HBV replication in vivo is hampered by the lack of suitable small and well-characterized animal models; thus far, only chimpanzees and the tree shrew (Tupaia), a relatively uncharacterized animal, appear to support HBV infection [3]. Several lines of transgenic mice have been established but HBV replication is generated from the integrated HBV sequence harbored in all hepatocytes, which is clearly different from the natural infection [4,5]. An alternative strategy is in vivo gene transfer of HBV DNA. Takahashi et al. [6] previously reported that

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intrahepatic injection of naked HBV DNA with cationic liposome can cross the species barrier and leads to HBV replication in rats. We and others have reported that hydrodynamics-based delivery of HBV DNA efficiently transduces murine livers and leads to HBV replication [7,8]. However, HBV replication in these models is terminated within a couple of weeks, presumably resulting from immunological elimination of HBV-expressing hepatocytes. Very recently, there have been reports of these models being applied for the assessment of anti-viral drugs [9–11]. However, the analysis may be hampered because this is a model of acute transient infection and would not allow observation of the long-term outcome.

In an attempt to develop a better long-term model, we hydrodynamically injected a plasmid encoding replication competent HBV DNA into immunocompromised mice and examined the kinetics of expression and replication of HBV. The mice produced HBV-related proteins for over 1 year, which appeared to be dependent on episomal HBV DNA replication in the liver, because the introduction of replication-incompetent HBV DNA led to transient expression of HBV genes. IFNα treatment of these mice showed transient repression of HBV replication but could not terminate it. These mice mimic a part of human HBV infection in terms of the template of viral replication and should be useful for analyzing the long-term outcome of anti-HBV therapy.

2. Materials and methods

2.1. Plasmids and mutagenesis

Plasmid pHBV1.5 containing an overlength (1.5-mer) copy of HBV DNA (GenBank accession no. AF305422) has been described previously [7]. A plasmid containing mutant HBV DNA carrying a stop codon instead of 54Trp of the *pol* gene was generated from pHBV1.5 by a GeneTailor Site-Directed Mutagenesis system (Invitrogen, Carlsbad, CA) and verified by sequencing. The site of the mutation was designed not to affect the expression of any HBV-related genes except for the *pol* gene. A plasmid coding the murine IFNa1 gene, pCMV-IFNa1, was generously provided by Dr Daniel J.J. Carr (University of Okulahoma, Health Science Center) [12].

2.2. Mice

Specific pathogen-free female Balb/cA nude mice were purchased from Clea Japan, Inc. (Tokyo, Japan) and were used at the age of 5 to 6 weeks. They were housed under conditions of controlled temperature and light with free access to food and water at the Institute of Experimental Animal Science, Osaka University Graduate School of Medicine. All animals received humane care and study protocol complied with the institution's guideline.

2.3. Injection of naked plasmid DNA

Plasmid DNA was prepared using an EndoFree plasmid system (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Hydrodynamic injection of plasmid DNA was performed according to previous reports [13,14]. In brief, 25 μg of plasmid DNA was diluted with 2.0 ml of lactated Ringer's solution and injected into the tail vein using a syringe with a 30-gauge needle. DNA injection was completed within 8 to 15 s.

2.4. Nothern blot

Total tissue RNA was isolated with Isogen (Nippon Gene, Toyama, Japan), and then 30 μg of total RNA was analyzed by Northern blotting with the HBV adw2 probe, as described previously [15].

2.5. Immunohistochemistry

For immunohistochemical detection of HBc protein, tissues were fixed with 10% neutral buffered formalin and embedded in paraffin. After being deparaffinized, sections 4 μm thick were incubated with anti-HBc antibody (Dako, Denmark), followed by immunoperoxidase staining using the ABC procedure (Vector Laboratories, Burlingame, CA) and counterstaining with hematoxylin.

2.6. Detection of hepatitis B antigens in serum

Under light anesthesia using sevoflurane, animals were bled from the retro-orbital vessels. Serum HBs antigen and HBe antigen were measured by chemiluminescent immunoassay (CLIA system, Abbott Laboratories, North Chicago, IL).

2.7. Real-time detection of HBV DNA in serum

Serum was treated with DNase I (Takara, Tokyo, Japan) and then proteinase K. DNA was extracted from the sera by a QIAamp DNA blood isolation system (Qiagen). HBV DNA was quantified by using real-time polymerase chain reaction (PCR) technology (Applied Biosystems, Foster City, CA) as described previously [16]. Primers and fluorescent probes are as follows: sense (nucleotides 168–188), 5′-CACATCAGGATTCCTAGGACC-3′; antisense (nucleotides 341–321), 5′-GGTGAGTGATTGGAGGTTGG-3′; probe (nucleotides 244–269), 5′-FAM-CAGAGTCTAGACTCGTGGTGGACTTC-3′.

2.8. Density analysis of HBV particles in serum

DNase I-treated serum was clarified by centrifugation at 15,000 rpm for 15 min using a 0.45 μm membrane filter. The clarified serum was layered on top of a 10–60% discontinuous sucrose gradient. Centrifugation was carried out at 141,000 *g* for 48 h. Fractions were collected from the bottom of the tube. After treatment with proteinase K, DNA was isolated from each fraction and applied for analysis of HBV DNA by PCR [7]. In an additional experiment, DNase I-treated serum was incubated with 1% Nonidet P-40 and 0.3% 2-mercaptoethanol for 16 h at 37 °C, and then used for density analysis.

2.9. Detection of HBV covalently closed circular DNA (cccDNA)

DNA was isolated from liver tissues by using a DNeasy Tissue kit (Qiagen). PCR detection of cccDNA was performed according to the procedure of Jun-Bin et al. [17] with some modification (Fig. 1). The PCR product was analyzed on a 1.2% agarose gel by electrophoresis. In some experiments, cccDNA was quantified using real-time PCR. To calculate the number of cccDNA per HBcAg-positeve hepatocyte, the total number of hepatocytes was estimated from the genomic DNA content in the murine liver under the assumption that the liver is about 70% hepatocytes. In addition, ampicillin resistance gene in the plasmids was amplified by using a sense primer (5′-TATGGCTTCATTCAGCTCCG-3′) and an antisense primer (5′-TCGAACTGGATCTCAACAGC-3′).

2.10. IFNα gene therapy

At 70 days after pHBV1.5 injection, nude mice were hydrodynamically injected with either pCMV-IFNa1 or pCMV mock plasmid and examined

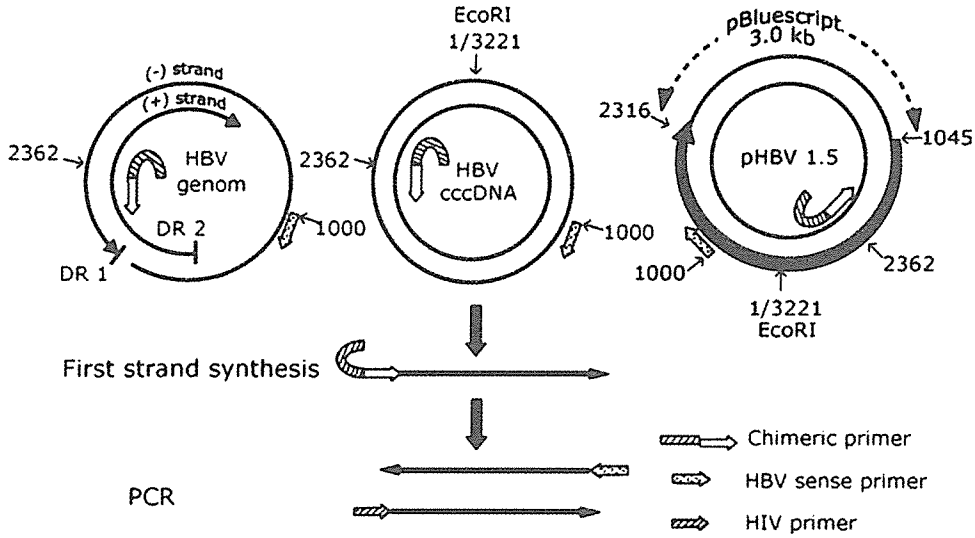

Fig. 1. Principle of PCR detection of HBV cccDNA. Three forms of HBV sequences which could have been present in our samples are shown: HBV genome, HBV cccDNA, and pHBV1.5. The number of nucleotides starts at the EcoRI site. A chimeric primer (5′-TCGCTTTCGGGTCCCTGGTCCCGTCGTC-3′) is composed of two segments: the segment A sequence near the 5′ end is HIV-specific and the segment B sequence near the 3′ end is complementary to the HBV DNA plus strand from nucleotide 2362 to 2351. With DNA polymerase activity, the chimeric primer extends and produces a new single DNA strand. Since the HBV plus strand has a gap, nucleotide extension will be stopped at the DR2 gap. On the other hand, an extremely long strand will be generated if pHBV1.5 acts as a template DNA. One-twentieth volume of the elongated strand was used as a template in the next PCR amplification in the presence of one primer, identical to the chimeric primer segment A (HIV primer; 5′-TCGCTTTCGGGTCCCT-3′) and another primer complementary to the HBV DNA minus strand from nucleotide 1000 to 1016 (HBV sense primer 5′-TTGTGGGTCTTTTGGG-3′), cycled 35 times through a program of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 1 min. The 1352 bp products will be amplified only from cccDNA. In real-time PCR, the DNA samples were digested with EcoRI and ScaI and subjected to the elongation reaction followed by PCR using a fluorescent probe (5′-FAM-GAGACCACCGTGAACGCCCATCAGAT-3′ (nucleotides 1444–1469)). ScaI site is located in the ampicillin resistance gene of the pBluescript but not in HBV DNA sequence.

for HBV replication. IFNα production was assessed using a commercially available mouse IFNα ELISA kit (PBL Biomedical Laboratories, Piscataway, NJ).

3. Results

3.1. Intravenous injection of pHBV1.5 leads to hepatitis B antigenemia as well as hepatic expression of HBcAg for more than 1 year

We injected 25 μg of pHBV1.5, which contains 1.5-fold overlength HBV DNA, into the tail veins of nude mice with acute circulatory overload. To investigate the expression of HBV, the presence of HBV transcripts was analyzed by Northern blot in various organs from mice sacrificed at 3 days after the injection (Fig. 2A). Two major bands corresponding to 3.5 and 2.4/2.1 kb transcripts were detected in the liver but not in other tissues including the kidney, spleen, thymus, lung, heart, and brain. The levels of HBsAg and HBeAg in the serum were serially determined by a quantitative CLIA method (Fig. 2B). Although the levels of HBsAg rapidly decreased 1.5 log within the first 2 weeks, all mice were persistently positive for HBsAg and HBeAg for more than 1 year. Immunohistochemical analysis revealed that around 4% of the hepatocytes were positive for HBc at 3 days after injection (Fig. 2C). HBcAg-positive cells gradually decreased in number but were still detected at one year after the injection (Fig. 2D). Although data are not shown, hepatic damage could not be detected, as evidenced by biochemical and histological analysis, throughout the course, except during the first week; it resulted from hemorrhagic destruction of the liver due to hydrodynamic pressure. Taken together, these results indicated that hydrodynamics-based delivery of a plasmid encoding replication-competent HBV DNA can establish specific expression of HBV genes in the liver and persistent expression without significant liver injury for a period of more than 1 year.

3.2. Long-term productive replication of HBV DNA

To examine if viral particles are produced into the circulation, sera obtained at 3 days after pHBV1.5 injection was treated with DNase I and fractionated by sucrose density gradient centrifugation. As shown in Fig. 3A, when each fraction was assayed in PCR for the presence of HBV DNA, the strongest signal was observed in the fraction with a density of 1.21 g/ml, corresponding to the density of HBV particles derived from human sera [18]. In addition, when serum was pre-treated with detergent before the centrifugation, the positive fraction shifted to a density of 1.28 g/ml, suggesting that detergent treatment releases core particles from HBV particles by removing the envelope.

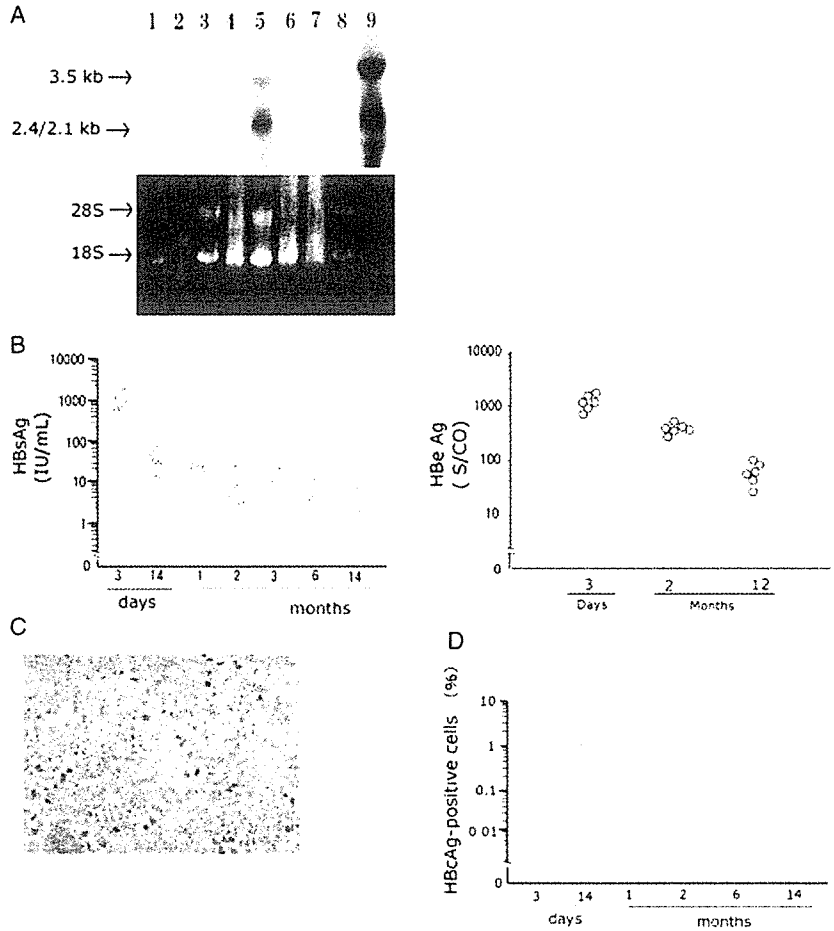

Fig. 2. Expression of HBV in hydrodynamically-transfected nude mice. (A) HBV RNA expression in various organs. Total RNA isolated from the indicated organs was analyzed for the presence of HBV-specific sequence 3 days after transfection (upper panel). Huh7 transfected with pHBV1.5 or pCMV were included as positive and negative controls, respectively. Arrows indicate 3.5- and 2.4/2.1-kb transcripts. A photograph of the ethidium bromide-stained gel is also shown in the lower panel. Lane 1, brain; lane 2, heart; lane 3, thymus; lane 4, lung; lane 5, liver; lane 6, kidney; lane 7, spleen; lane 8, pCMV-transfected Huh7; lane 9, pHBV1.5-transfected Huh7. (B) HBsAg and HBeAg in serum. The levels of HBsAg and HBeAg were serially determined in a cohort of mice hydrodynamically transfected with pHBV1.5. (C) Immunohistochemical detection of HBc Ag. Representative data for nude mice 3 days after pHBV1.5 injection. (D) Frequency of HBcAg-positive hepatocytes in the livers.

To examine the kinetics of viremia, we examined the levels of DNase I-resistant HBV DNA in serum by real-time PCR analysis (Fig. 3B). The levels of HBV DNA were as high as 1×10^7 copies/ml at 3 days after the injection and gradually decreased by 1.5 log over 1 year.

3.3. Long-term expression of HBV is dependent on HBV replication

The extremely long-term expression and carriage of HBV in this system led us to examine whether episomal replication could affect the kinetics of expression of HBV-related genes. Toward this goal, we introduced point mutation in the *pol* gene of pHBV1.5 which could produce the truncated form of the HBV polymerase without affecting the expression of any other HBV-related proteins. Mice hydrodynamically injected with mutant pHBV1.5 produced HBsAg as well as HBcAg at levels similar to those of wild-type pHBV1.5-injected mice 3 days after injection (Fig. 4A and B). However, mutant pHBV1.5-induced expression of HBsAg, HBeAg and HBcAg was terminated within 2 months, in striking contrast to wild-type pHBV1.5-induced gene expression (Fig. 1B and D). Northern blot analysis confirmed the transient expression of HBV genes after injection of mutant pHBV1.5 (Fig. 4C).

HBV DNA polymerase binds to the 5′ end of its own mRNA template, and the complex is then packaged into nucleocapsids, where viral DNA synthesis occurs [19]. HBV genomic DNA produced via the reverse transcription pathway predominantly consists of relaxed-circular DNA with a complete minus strand and a partially synthesized plus strand. In natural HBV infection in humans, part of the nucleocapsids migrates to the nucleus where relaxed-circular DNA is converted to cccDNA that serves as a template for transcription [19]. The finding in the present model of long-term expression of HBV involving HBV DNA replication suggested that viral cccDNA may be produced in murine livers and work as a transcriptional