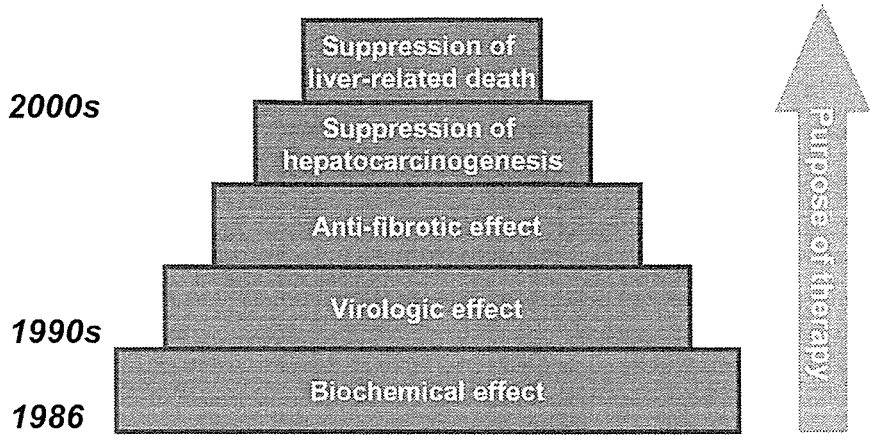

Fig. 4. Effects of IFN-based therapy in relation to the purpose of treatment for chronic hepatitis C. Retrospective analysis of rates of hepatocellular carcinoma and liver-related death after IFN monotherapy have shown a reduction in risk, especially in patients with moderate liver fibrosis[65–69]

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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Suppressive effect on hepatocyte differentiation of hepatitis C virus core protein

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Abstract

The influence of hepatitis C virus (HCV) protein(s) on cellular differentiation remains to be clarified. Using murine normal liver epithelial cells, we investigated whether HCV core protein affects differentiation into hepatocytes. Mock and HCV core-expressing cells were stimulated with oncostatin M (OSM) and dexamethasone, and the degree of differentiation was evaluated by measuring the expression of albumin and tyrosine aminotransferase (TAT). Lower amounts after stimulation were found in HCV core-expressing cells than in mock cells. Phosphorylation of the signal transducer and activator transcription factor 3 (STAT3) was prevented by the HCV core under OSM stimulation. Reporter gene assay revealed that the HCV core/Janus kinase (JAK) interaction directly suppressed the OSM-dependent JAK-STAT signal transduction. Furthermore, expression of OSM receptor β (OSMRβ) after stimulation was prevented by the HCV core. In conclusion, the HCV core may suppress differentiation into hepatocytes via inhibition of the JAK-STAT pathway and OSMRβ expression.

Keywords: Hepatitis C virus; Hepatitis C virus core protein; Hepatic progenitor cells; Differentiation into hepatocytes; Oncostatin M; Signal transducer and activator transcription factor 3 (STAT3); Janus kinase (JAK)-STAT signaling pathway

Hepatitis C virus (HCV) causes persistent infection and leads to chronic hepatitis, liver cirrhosis, and eventually hepatocellular carcinoma (HCC) [1,2]. Chronic liver inflammation induced by HCV brings about repeated hepatocyte apoptosis and liver regeneration, resulting in disease progression. During this process, apoptotic hepatocytes are known to be primarily compensated by replication of intact hepatocytes. However, the replicative activity of hepatocytes has been suggested to be impaired in chronic liver disorder in humans [3] and mice [4]. Proliferation of hepatic progenitor cells (also termed oval cells) and their differentiation into hepatocytes are possible alternative mechanisms for hepatocyte regeneration. Hepatic progenitor cells are a bipotential cellular population that can differentiate into both hepatocytes and bile duct cells. Hepatic progenitor cells have been suggested to play an important role in liver regeneration in many experimental models of the liver injury [5–8]. In addition, it has been reported that hepatic progenitor cells are frequently detected in liver tissues of chronic type C hepatitis patients, and that their numbers increase in parallel with disease severity [9–11]. This suggests that hepatic progenitor cells may be substantially involved in the pathogenesis of chronic type C liver disease.

Among various extracellular factors, dexamethasone, a synthetic glucocorticoid, has been shown to contribute to hepatic maturation of rodent fetal and adult hepatocytes [12,13]. Dexamethasone has been reported to promote

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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

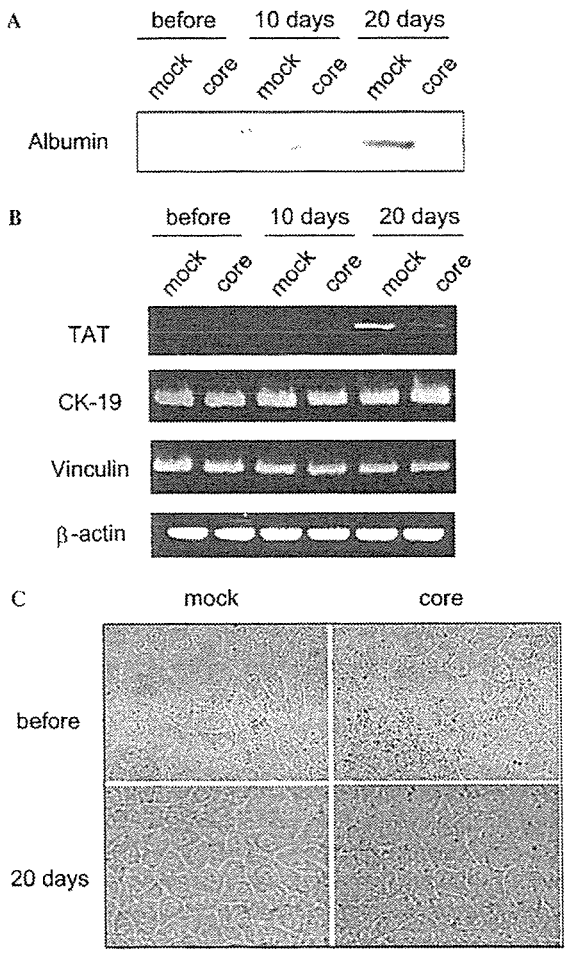

Fig. 1. (A) Detection of albumin in the CL2 mock and core cells under stimulation with OSM and dexamethasone. The cellular protein was harvested before, 10 days after, and 20 days after stimulation and used for Western blot analysis. (B) Detection of TAT, CK-19, and vinculin mRNAs in the CL2 mock and 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.

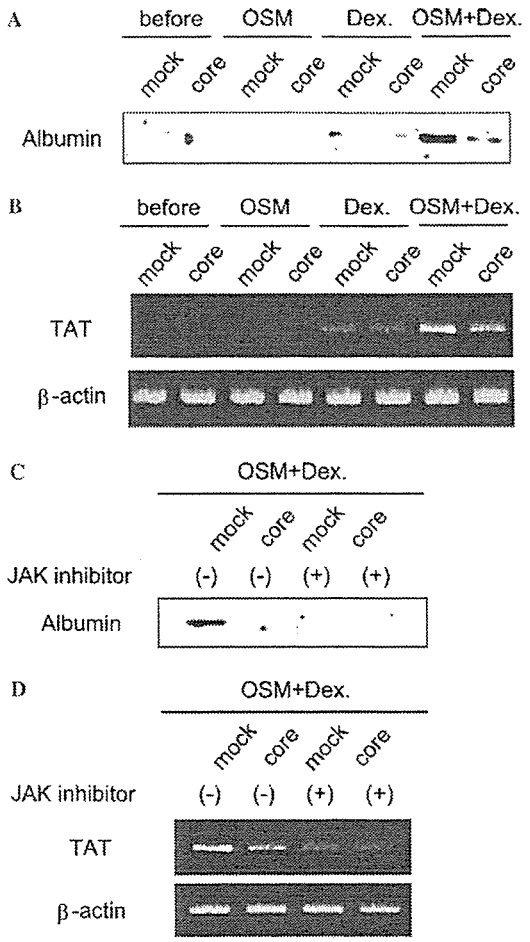

Fig. 2. (A) Detection of albumin in the CL2 mock and core cells under stimulation with OSM alone, dexamethasone alone, or both. The cellular protein was harvested before stimulation and 20 days after stimulation and used for Western blot analysis. (B) Detection of TAT mRNA in the CL2 mock and core cells 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,

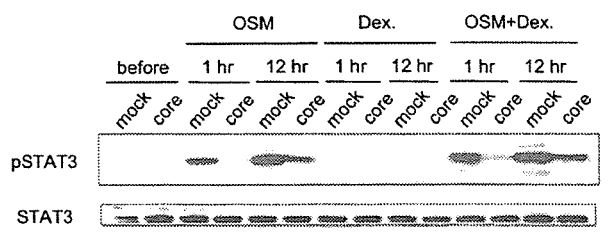

Fig. 3. Early phase response of STAT3 phosphorylation in the CL2 mock and core cells under stimulation with OSM and/or dexamethasone. The levels of pSTAT3 and the whole STAT3 were examined by Western blot analyses.

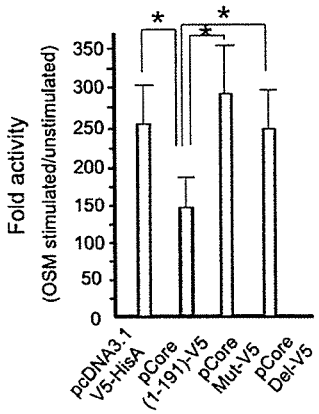

Fig. 4. Involvement of HCV core-JAK interaction in the HCV core-mediated inhibitory effect on the JAK-STAT signal transduction activated by OSM. The CL2 cells were cotransfected with various effector plasmids (pcDNA3.1/V5-HisA, pCore(1-191)-V5, pCoreMut-V5, and pCoreDel-V5) with pAPREluci and pRLtk. 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.

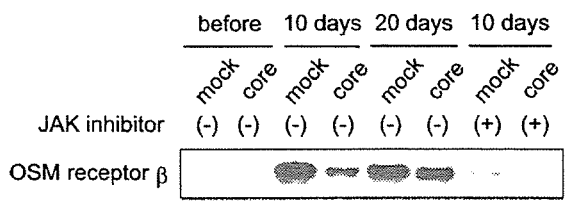

Fig. 5. Detection of OSMRβ in the CL2 mock and core cells under stimulation with OSM and dexamethasone in the presence or absence of JAK inhibitor. The cellular protein was harvested before, 10 days after, and 20 days after stimulation and used for Western blot analysis.

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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Early decline of hemoglobin correlates with progression of ribavirin-induced hemolytic anemia during interferon plus ribavirin combination therapy in patients with chronic hepatitis C

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Background. The aim of this study was to examine the factors correlated with the progression of ribavirin-induced hemolytic anemia in patients with chronic hepatitis C treated by interferon and ribavirin combination therapy. ***Methods.*** This study was conducted on 505 patients by the Osaka Liver Disease Study Group. A decline of hemoglobin (Hb) concentration by 2 g/dl at the end of 2 weeks from the start of the treatment ("2 by 2" standard) was adopted as a predictive factor for progression to severe anemia. The ribavirin apparent clearance (CL/F) was also examined. ***Results.*** Of 482 patients whose Hb value was more than 12 g/dl before the treatment, 68 patients (14%) had to discontinue ribavirin owing to severe anemia. Patients in the "2 by 2"-positive group (Hb decline over 2 g/dl) and the group with lower CL/F were significantly more likely to discontinue ribavirin owing to severe anemia. Discontinuation was more common among patients aged 60 years or older than for those under 60 years old (21% vs. 9%, $P < 0.001$). Among patients aged 60 years or older, only the "2 by 2" standard was significantly associated with the discontinuance of ribavirin owing to severe anemia in a multivariate analysis (odds ratio, 4.18; $P < 0.001$). ***Conclusions.*** The "2 by 2" standard of Hb decline can be used to identify patients likely to develop severe anemia. The early reduction of ribavirin can help prevent progression to severe anemia, thus allowing ribavirin therapy to be completed even in older patients.

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Key words: chronic hepatitis C, interferon and ribavirin combination therapy, progression of anemia, "2 by 2" standard

Introduction

Hepatitis C virus (HCV) is estimated to infect up to 170 million people worldwide,[1] and two million people in Japan. Long persistence of HCV infection can lead to progression of liver fibrosis, causing liver cirrhosis and ultimately hepatocellular carcinoma.[2,3] Past studies have made clear that interferon (IFN) therapy is effective for eliminating HCV,[4,5] but the sustained viral response (SVR) rate of IFN monotherapy is not sufficient. The addition of the nucleoside analog ribavirin to IFN in the treatment of patients with chronic hepatitis C can significantly improve the SVR rate, and combination therapy with IFN or pegylated-IFN (Peg-IFN) has been recommended as a standard regimen worldwide.[6–10] However, additional side effects of ribavirin have been reported, such as hemolytic anemia, which have not been found with IFN monotherapy, leading to discontinuance of the treatment.[11–14]

In previous studies, the discontinuance rate of IFN and ribavirin combination treatment due to severe side effects has been reported to be 6%–13%.[6,7] Ribavirin-induced hemolytic anemia has been suggested to depend on a high plasma concentration of ribavirin.[15] The ribavirin apparent clearance (CL/F), which reflects the plasma concentration of ribavirin at 4 weeks after the start of combination therapy, has been used as a

predictive factor for ribavirin-induced hemolytic anemia before the start of treatment.[16–18] Furthermore, in the manufacturer's drug information for ribavirin,[19] a dose reduction is recommended when hemoglobin (Hb) levels decrease to less than 10 g/dl, and discontinuance of ribavirin is recommended when Hb levels fall to less than 8.5 g/dl during combination therapy with IFN and ribavirin. However, according to this guideline, not a few patients are forced to discontinue ribavirin because the dose reduction to avoid severe anemia does not occur in time.

What is needed is a convenient guideline for avoiding ribavirin discontinuance due to severe anemia. In this study, we evaluated the correlation of Hb decline at 2 weeks after the start of combination therapy with the discontinuance of treatment due to progression of ribavirin-induced hemolytic anemia. We also assessed the utility of an early decline of Hb in comparison with the CL/F standard for predicting the progression to severe anemia.

Patients and methods

Patients

The current study was conducted at Osaka University Hospital and other institutions participating in the Osaka Liver Disease Study Group. The 505 patients with chronic hepatitis C included in this study were treated with a combination of interferon-α-2b and ribavirin between January 2001 and December 2005. All patients were anti-hepatitis C virus antibody positive, had HCV RNA detectable in their serum by the polymerase chain reaction method, and had elevated serum alanine transaminase (ALT) (above the upper limit of normal) within the 6 months prior to treatment. Excluded from this study were patients who were positive for hepatitis B surface antigen or anti-human immunodeficiency virus antibody or those with other forms of liver disease (alcoholic liver disease, hepatotoxic drugs, autoimmune hepatitis). Twenty-three patients whose Hb was under 12 g/dl before the treatment were also excluded because the aim of this study was to analyze the progression of anemia; patients with a low Hb level before treatment are known to have a tendency toward progression of anemia. The remaining 482 patients were followed in this study.

The baseline clinical features of the 482 patients are shown in Table 1. Their mean age was 55.2 ± 10.9 years, and 66% were men. Among the patients, 347 had HCV RNA with genotype 1 and high viral loads (1H group) and 130 had HCV RNA with genotype 2 or low viral loads (non-1H group). The mean ALT level was 100 ± 74 IU/l. In this study, a high viral load was defined as a serum HCV-RNA level of more than 10^6 equivalents/ml by branched DNA assay or more than 10^5 copies/ml serum by Amplicor-HCV monitor assay.

Treatment schedule

Of the 482 patients treated with a combination of interferon-α-2b and ribavirin, 273 were IFN naïve and 209 were undergoing retreatment. All patients were scheduled to receive interferon-α-2b (Intron-A, Schering-Plough, Kenilworth, NJ, USA) at a dose of 6 (n = 371) or 10 (n = 111) MU intramuscularly every day for the first 2 weeks and three times a week thereafter. Ribavirin (Rebetol; Schering-Plough) was given orally twice a day for a total dose of 800 mg (n = 261), 600 mg (n = 215), or 400 mg (n = 6) per day. The IFN dose was decreased from 10 to 6 MU or from 6 to 3 MU when the

Table 1. Baseline characteristics of patients

Number	482	
Age (y.o)	55.2 ± 10.9	(21–75)
Sex (male/female)	320/162	
Body weight (kg)	62.3 ± 9.9	(35–94)
HCV serotype (1/2/unknown)	364/111/7	
(1H/non-1H/unknown)	347/130/5	
Fibrosis (0/1/3/4/unknown)	19/192/202/13/56	
WBC (/mm^3)	5184 ± 1531	(2100–13200)
RBC (×10^4/mm^3)	449 ± 42	(329–617)
Hb (g/dl)	14.4 ± 1.2	(12.0–19.2)
Plt (×10^4/mm^3)	15.4 ± 5.4	(4.4–36.1)
ALT (IU/l)	100 ± 74	(17–736)
Serum creatinine (mg/dl)	0.8 ± 0.2	(0.3–1.7)
Ribavirin dosage/body weight (mg/kg)	11.4 ± 1.5	(4.6–17.8)

Data are shown as means ± SD
HCV, hepatitis C virus; 1H group, patients with genotype 1 and high viral load; non-1H group, patients not in the 1H group; Fibrosis, Knodell's histological score (category4); WBC, white blood cells; RBC, red blood cells; Hb, hemoglobin; Plt, platelets; ALT, alanine aminotransferase

white blood cell (WBC) count was below 1500/mm^3, the neutrocyte count below 750/mm^3, or the platelet (Plt) count below 5×10^4/mm^3. IFN was discontinued when the WBC count was below 1000/mm^3, the neutrocyte count below 500/mm^3, or the Plt count below 2.5×10^4/mm^3. The ribavirin dose of 200 mg was reduced when the Hb concentration decreased to less than 10 g/dl, and the ribavirin was discontinued when the Hb concentration decreased to less than 8.5 g/dl, in accordance with the manufacturer's drug information for ribavirin.[19] Ferric medicine or erythropoietin to prevent anemia was not administered. Ribavirin was scheduled to be administered for 24 weeks for all patients, and IFN for 24 weeks for 307 patients and for 48 weeks for 175 patients.

Patients with persistently undetectable HCV RNA 6 months after completion of treatment were considered to have achieved SVR.

Blood tests

All patients were examined for serum HCV-RNA level and underwent hematological and biochemical tests just before therapy, at the end of week 2, and every 4 weeks thereafter during treatment. When treatment was completed, the patients were assessed every 4 weeks until 24 weeks after the end of treatment.

Total ribavirin clearance

Using the method of Kamar et al.,[17] CL/F at the start of the treatment was calculated as follows:

$$\text{CL/F (l/h)} = 32.3 \times \text{BW} \times (1 - 0.0094 \times \text{Age}) \times (1 - 0.42 \times \text{Sex})/\text{Scr},$$

where BW = body weight; sex = 0 for male and 1 for female; and Scr = serum creatinine.

Definition of "severe anemia" leading to discontinuance of ribavirin

In this study, "discontinuance of ribavirin due to severe anemia" was defined as follows: discontinuance of ribavirin due to a decrease of Hb to less than 8.5 g/dl or clinical symptoms of anemia associated with a decrease of Hb of more than 3 g/dl from the start of combination therapy.

Liver histology

Hepatic fibrosis was assessed by Knodell's histological score (category 4).[20] Fibrosis stage was evaluated on a scale from 0 to 4: 0 = no fibrosis; 1 = fibrosis portal expansion; 3 = bridging fibrosis (portal–portal or portal–central linkage); 4 = cirrhosis.

Statistical analysis

Age, body weight, ribavirin dosage/body weight, WBC count, red blood cell (RBC) count, Hb concentration, Plt, serum ALT levels, and Scr are expressed as means ± SD. The SVR rate was evaluated using an intention-to-treat (ITT) analysis. The differences in proportions were tested by the χ-squared test. For univariate and multivariate analyses, a logistic regression analysis was used to predict ribavirin-induced severe anemia. A value of $P < 0.05$ (two-tailed) was considered to indicate significance.

Results

Efficacy of the combination therapy with dose reduction or discontinuance of ribavirin

The relationship between dose reduction or discontinuance of ribavirin and the SVR rate on ITT analysis is shown in Fig. 1. The SVR rate was 20% (71/347) for all 1H patients and 72% (93/130) for all non-1H patients. Among the 1H patients, SVR was achieved for 24% (45/189) without dose reduction of ribavirin and for 26% (20/76) with dose reduction. Significantly lower SVR rates were observed for patients who had to discontinue ribavirin treatment owing to adverse effects (7%, 6/82) in comparison with those with ($P < 0.01$) or without ($P < 0.01$) dose reduction. In the non-1H group, similar SVR rates were found with dose reduction of ribavirin [SVR rate without dose reduction, 83% (58/70), vs. SVR rate with dose reduction, 82% (23/28)], and the SVR rate of patients who had to discontinue ribavirin owing to adverse effects was significantly lower (38%, 12/32) than that for those with ($P < 0.001$) or without ($P < 0.0001$) dose reduction.

The same tendency was observed even in the 307 patients treated with IFN for 24 weeks. Among the 1H patients treated for 24 weeks, SVR was achieved for 19% (17/91) without dose reduction of ribavirin, 15% (6/41) with dose reduction, and 3% (2/75) with discontinuance. There were significant differences between the patients with discontinuance and those without ($P < 0.01$) or with ($P < 0.05$) dose reduction. Among the non-1H patients treated for 24 weeks, SVR rates were 85% (39/46) for the patients without dose reduction of ribavirin, 85% (17/20) for those with dose reduction, and 33% (10/30) for those with discontinuance. Significantly lower SVR rates were observed for patients who had to discontinue ribavirin than for those with ($P = 0.05$) or without ($P < 0.05$) dose reduction.

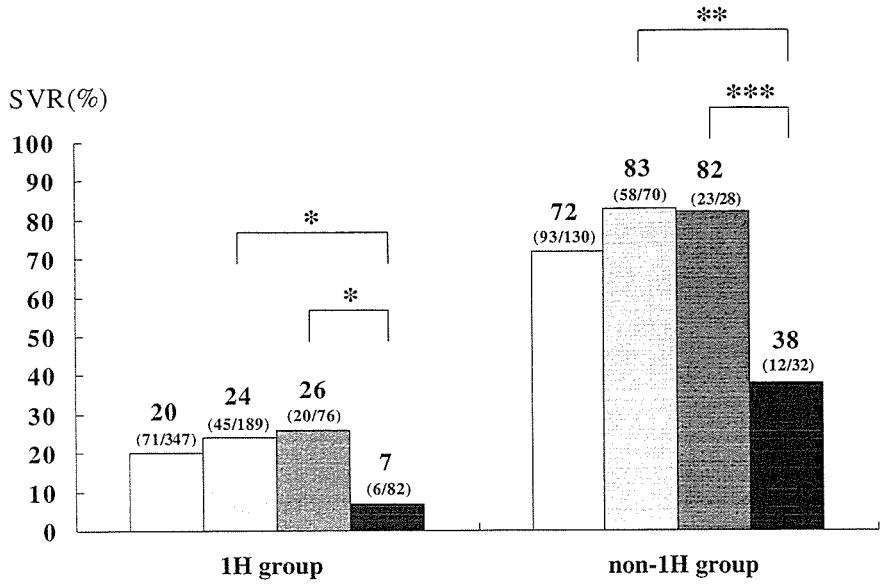

Fig. 1. Efficacy of combination therapy with dose reduction or discontinuance of ribavirin (intention-to-treat analysis). *1H group*, patients with genotype 1 and high viral load; *non-1H group*, patients not in the 1H group; *SVR*, sustained viral response. □ all patients; ▨ patients without dose reduction of ribavirin; ▩ patients with dose reduction of ribavirin; ■ patients with discontinuance of ribavirin. *, $P < 0.01$; **, $P < 0.0001$; ***, $P < 0.001$

Table 2. Rate of the ribavirin reduction or discontinuance due to adverse effects with different levels of CL/F

			Discontinuance	
	No reduction	Dose reduction	All cases	Cases due to severe anemia
20 ≤ CL/F	94%	2%	4%	0%
(n = 45)	(42/45)	(1/45)	(2/45)	(0/45)
15 ≤ CL/F < 20	66%	19%	15%	6%
(n = 100)	(66/100)	(19/100)	(15/100)	(6/100)
10 ≤ CL/F < 15	54%	24%	23%	14%
(n = 179)	(96/179)	(42/179)	(41/179)	(25/179)
CL/F < 10	37%	28%	35%	23%
(n = 158)	(58/158)	(44/158)	(56/158)	(37/158)

Frequency of and reasons for dose reduction or discontinuance of ribavirin during combination therapy

We examined the rate of discontinuance of therapy due to adverse effects up to the end of 24 weeks, because all cases of discontinuation occurred before the end of 24 weeks. Of the 482 patients, 401 patients completed 24 weeks of therapy, and 81 patients (17%) had to discontinue both IFN and ribavirin before the end of the 24 weeks. Of the 401 patients undergoing 24 weeks of therapy, the entire treatment schedule without reduction or discontinuance of either drug was completed by 262 patients (54%). The ribavirin dose was decreased for 106 patients (22%) and was stopped without discontinuance of IFN for 33 patients (7%). Overall, 114 patients (24%) discontinued ribavirin treatment. The reasons for dose reduction or discontinuance of ribavirin were anemia, general fatigue, digestive disorder, eczema, neutropenia, thrombocytopenia, or psychological disorder. Among the patients discontinuing ribavirin, the major reasons were anemia (14%), general fatigue (2%), or digestive disorder (2%).

CL/F and dose reduction or discontinuance of ribavirin

CL/F calculated for all patients was 4.6–32.5 l/h. The mean CL/F was 13.0 l/h, and the median was 11.9 l/h. At the start of treatment, CL/F was less than 10 l/h for 33% (158/482) of patients, 10–15 l/h for 37% (179/482), 15–20 l/h for 21% (100/482), and more 20 l/h for 9% (45/486).

Table 2 shows the rates of dose reduction or discontinuance of ribavirin in relation to different levels of CL/F. The rate of discontinuance of ribavirin among all patients was 4% (2/45) for patients with CL/F ≥ 20, 15% (15/100) for those with 15 ≤ CL/F < 20, 23% (41/179) for those with 10 ≤ CL/F < 15, and 35% (56/158) for those with CL/F < 10. The rate of discontinuance of ribavirin due to severe anemia was 14% (68/482) among all pa-

tients. There was no discontinuance of ribavirin due to severe anemia among patients with CL/F ≥ 20, but the rate of discontinuance was 6% (6/100) among those with 15 ≤ CL/F < 20, 14% (25/179) among those with 10 ≤ CL/F < 15, and 23% (37/158) among those with CL/F < 10. The rate of continuance of ribavirin without dose reduction decreased in proportion to the decline of CL/F. In this study, we adopted two categories of CL/F, below 15 l/h (CL/F < 15) and below 10 l/h (CL/F < 10), to assess CL/F as a factor for predicting anemia progression.

We also analyzed the predictive factor of anemia progression according to patient age, because CL/F varies widely with patient age and tends to be lower among older patients. Among patients under 60 years old (n = 288), 17% (48/288) had CL/F under 10 l/h, 38% (109/288) had CL/F 10–15 l/h, 30% (86/288) had CL/F 15–20 l/h, and 16% (45/288) had CL/F over 20 l/h. On the other hand, among those 60 years old or older (n = 194), 57% (110/194) had CL/F under 10 l/h, 36% (70/194) had CL/F 10–15 l/h, 7% (14/194) had CL/F 15–20 l/h, and none had CL/F over 20 l/h. Thus, the majority (93%) of the patients 60 years old or older had a low CL/F (<15), whereas only 55% of those under 60 years old had CL/F < 15.

Early decline of Hb and progression of anemia during combination therapy

Figure 2 shows the decline of Hb from the start of combination therapy. We conducted this analysis for the 433 patients: those who did not need a dose reduction of ribavirin (n = 262), those who needed a dose reduction owing to a decrease of Hb to less than 10 g/dl (n = 103), and those who discontinued ribavirin due to "severe anemia" (n = 68). We excluded 49 patients from this analysis: 46 patients stopped combination therapy for reasons other than anemia, such as general fatigue or digestive disorder, and the other three patients were not responding to antiviral treatment and stopped therapy before 24 weeks without a dose reduction of ribavirin. Following the initiation of combination therapy, Hb concentration decreased rapidly until the end of the 4th week. At the end of 2 weeks, Hb had decreased by 0.9 ± 1.2 g/dl among the patients without dose reduction of ribavirin, by 1.8 ± 1.3 g/dl among those with dose reduction, and by 2.3 ± 1.4 g/dl among those who discontinued ribavirin. At the end of 4 weeks, Hb had decreased by 2.1 ± 1.5 g/dl among the patients without dose reduction of ribavirin, by 3.2 ± 1.5 g/dl among those with dose reduction, and by 3.9 ± 1.5 g/dl among those discontinuing ribavirin.

ΔHb [ΔHb = (Hb value just before treatment) − (Hb value during treatment)] both at the end of 2 weeks and at the end of 4 weeks were significantly larger among the patients discontinuing ribavirin than among those without dose reduction of ribavirin (P < 0.0001, P < 0.0001, respectively). In this study, we adopted the category of ΔHb at the end of 2 weeks because it allowed the progression of anemia to be estimated at an earlier phase of treatment than did ΔHb at the end of 4 weeks.

To establish the cutoff value of ΔHb at the end of 2 weeks, we used two categories of ΔHb: a decrease in Hb concentration at 2 weeks to 2 g/dl below the baseline (ΔHb2.0) or to 1.5 g/dl below the baseline (ΔHb1.5). We conducted this analysis for 480 patients, because two patients stopped combination therapy before 2 weeks for reasons other than anemia. With the ΔHb2.0 standard, the rate of discontinuance of ribavirin due to severe anemia was 10% (32/338) in the ΔHb < 2.0 group and 25% (36/142) in the ΔHb ≥ 2.0 group, with the difference being significant (P < 0.0001) (Table 3). With the ΔHb1.5 standard, the rate of discontinuance of ribavirin due to severe anemia was significantly higher

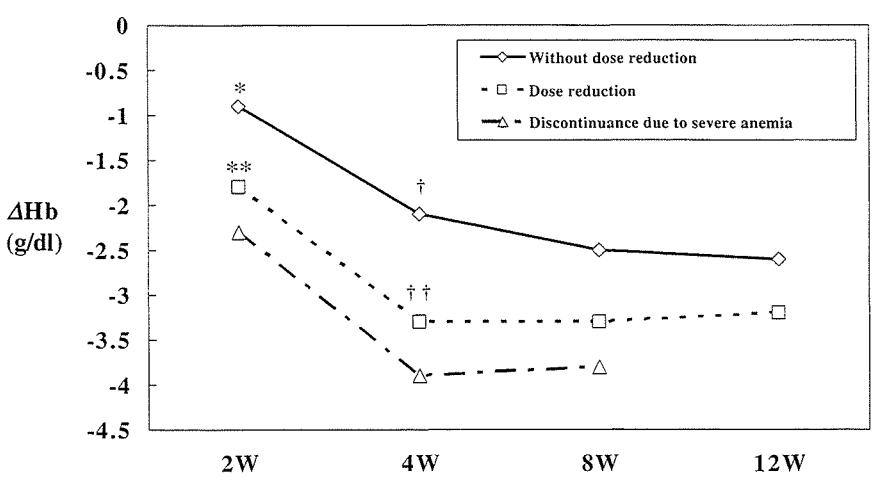

Fig. 2. Decline of hemoglobin according to dose reduction or discontinuance of ribavirin. *Significantly different from patients with dose reduction (P < 0.0001) and patients with discontinuance (P < 0.0001); **significantly different from patients with discontinuance (P < 0.02); †significantly different from patients with dose reduction (P < 0.0001) and patients with discontinuance (P < 0.0001); ††significantly different from patients with discontinuance (P < 0.01)

Table 3. Rate of the ribavirin reduction or discontinuance due to adverse effects with rate of anemia progression

			Discontinuance	
	No reduction	Dose reduction	All cases	Cases due to severe anemia
ΔHb ≥ 2.0 (n = 142)	37% (53/142)	29% (41/142)	34% (48/142)	25%* (36/142)
ΔHb < 2.0 (n = 338)	61% (209/338)	19% (65/338)	20% (64/338)	10% (32/338)

*P < 0.0001

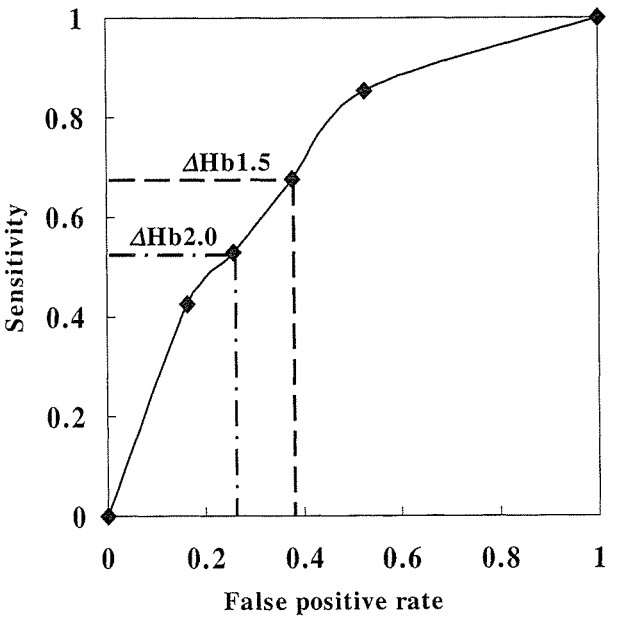

Fig. 3. Receiver-operating characteristic curve for ΔHb at the end of 2 weeks for discontinuance of ribavirin due to severe anemia

in the ΔHb ≥ 1.5 group than in the ΔHb < 1.5 group (8%, 22/279 vs. 23%, 46/201; P < 0.0001). Figure 3 shows the receiver-operating characteristic curve using ΔHb at the end of 2 weeks for the discontinuance of ribavirin due to severe anemia. Between the ΔHb2.0 and ΔHb1.5 standards, no significant difference was found in sensitivity (53%, 36/68, vs. 68%, 46/68; NS). On the other hand, the false positive rate was significantly lower with the ΔHb2.0 standard than with the ΔHb1.5 standard (26%, 93/360, vs. 38%, 136/360; P < 0.001), and accuracy was significantly higher with the ΔHb2.0 standard than with the ΔHb1.5 standard (71%, 303/428, vs. 63%, 270/428; P = 0.02). Therefore, we adopted ΔHb2.0 at the end of 2 weeks (the "2 by 2" standard) as a predictive factor for discontinuance of ribavirin due to severe anemia because of the higher specificity rate of ΔHb2.0 (lower false positive rate).

Logistic regression analysis for discontinuance of ribavirin in combination therapy

We assessed the factors correlated with the discontinuance of ribavirin due to severe anemia by logistic regression analysis. The following factors were evaluated: age, sex, body weight, ribavirin dosage/body weight, IFN dosage, Scr, Hb value at the start of the therapy, CL/F category, and early decline of Hb ("2 by 2" standard). Older age, lower body weight, lower Hb at the start of the therapy, lower CL/F (CL/F < 10 or CL/F < 15), and "2 by 2"-positive (the patients whose Hb had decreased by more than 2 g/dl at 2 weeks from the start of the treatment) were factors significantly associated with discontinuance of ribavirin due to severe anemia by univariate logistic regression analysis (Table 4). Next, we assessed the factors correlated with the discontinuance of ribavirin due to severe anemia by multivariate logistic regression analysis. Among the factors selected as significant by the univariate analysis, we omitted age and body weight from the multivariate analysis because they were included as parameters in the numerical formula for CL/F. Therefore, we evaluated the Hb value at the start of therapy, the CL/F category, and the "2 by 2" category by multivariate analysis. The CL/F borderline values of 10 l/h and 15 l/h were evaluated separately. In the multivariate logistic regression analysis, lower Hb at the start of therapy, lower CL/F (CL/F < 10 or CL/F < 15), and "2 by 2"-positive were significantly associated with discontinuance of ribavirin due to severe anemia (Table 5).

Useful predictive factors for discontinuance of ribavirin among older patients

Among the 288 patients under 60 years old, 50 (17%) had discontinued ribavirin by the end of 24 weeks for various reasons, including anemia, general fatigue, digestive disorder, and psychological disorders. Among the 194 patients aged 60 years and older, 64 (33%) had discontinued ribavirin, with severe anemia accounting for approximately 65% (41/64). More than twice as many patients aged 60 years and older discontinued ribavirin treatment compared with younger patients;

Table 4. Univariate analysis for the discontinuance of ribavirin due to severe anemia

Factor	Category	Odds ratio	95% CI	*P* value
Age			1.045–1.117	<0.0001
Sex	Male/Female	1/1.18	0.663–2.029	0.56
Body weight			0.928–0.981	<0.001
Serum creatinine			0.551–9.492	0.25
Ribavirin/Body weight			0.945–1.357	0.18
IFN dosage	6MU/10MU	1/1.03	0.557–1.893	0.93
Hb			0.480–0.780	<0.0001
CL/F	≥15/<15	1/5.56	0.076–0.427	0.0001
	≥10/<10	1/3.14	0.187–0.540	<0.0001
"2 by 2"	Negative/Positive	1/3.23	0.182–0.527	<0.0001

CI, confidence interval; IFN, interferon; CL/F, apparent clearance; "2 by 2", ΔHb2.0 at the end of 2 weeks; "2 by 2"-positive means ΔHb ≥ 2.0; "2 by 2"-negative means ΔHb < 2.0

Table 5. Multivariate analysis for the discontinuance of ribavirin due to severe anemia

Factor	Category	Odds ratio	95% CI	*P* value
Hb			0.446–0.785	0.0003
CL/F	≥15/<15	1/3.18	0.126–0.786	0.01
"2 by 2"	Negative/Positive	1/4.35	0.127–0.419	<0.0001
Hb			0.440–0.784	0.0003
CL/F	≥10/<10	1/1.98	0.278–0.923	0.03
"2 by 2"	Negative/Positive	1/4.63	0.119–0.393	<0.0001

this difference was significant (21%, 41/194, vs. 9%, 27/288; $P = 0.0003$) (Table 6).

We assessed the analysis for discontinuance of ribavirin due to severe anemia among the patients aged 60 years or older. Older age, lower CL/F (CL/F < 10), and "2 by 2"-positive were factors significantly associated with discontinuance of ribavirin due to severe anemia by univariate logistic regression analysis (Table 7A). Next, we assessed the factors correlated with the discontinuance of ribavirin due to severe anemia by multivariate logistic regression analysis. Among the three factors selected as significant by univariate analysis, we omitted the factor of age from the multivariate analysis as it was included as a parameter in the numerical formula for CL/F. In the multivariate logistic regression analysis of the CL/F category (CL/F < 10) and the "2 by 2" category, the latter was the only significant factor associated with the discontinuance of ribavirin due to severe anemia (Table 7B). Using the "2 by 2" standard, the rate of discontinuance of ribavirin due to severe anemia was 14% (18/133) in the "2 by 2"-negative (the patients whose Hb decreased by less than 2 g/dl from the start of treatment) group and 38% (23/60) in the "2 by 2"-positive group, with the difference being significant ($P < 0.0001$) (Table 8).

We next compared the sensitivity, specificity, and accuracy of the CL/F category with those of the "2 by 2" category as predictive factors for discontinuance of ribavirin due to severe anemia among patients aged 60 years or older. Table 9 shows the comparison between the CL/F < 15 category and the "2 by 2" category (Table 9A) and that between the CL/F < 10 category and the "2 by 2" category (Table 9B). Although sensitivity was higher for the lower CL/F category [CL/F < 15, 100% (41/41); CL/F < 10, 71% (29/41)] than for the "2 by 2" category (56%, 23/41), specificity and accuracy were significantly higher for the "2 by 2" category than for the CL/F category [specificity: "2 by 2," 77% (96/125) vs. CL/F < 15, 7% (9/125), $P < 0.0001$; "2 by 2" vs. CL/F < 10, 47% (59/125), $P < 0.0001$; accuracy: "2 by 2," 72% (119/166) vs. CL/F < 15, 30% (50/166), $P < 0.0001$; "2 by 2" vs. CL/F < 10, 53% (88/166), $P < 0.001$].

Table 6. Major causes of discontinuance of ribavirin

	Age < 60	Age ≥ 60
Severe anemia	27 (9%)	41 (21%)*
General fatigue	7	3
Digestive disorders	5	3
Neutropenia	1	1
Thrombocytopenia	2	4
Eruption with itching	2	4
Psychological disorders	3	3
Others	3	5
Total	50/288 (17%)	64/194 (33%)

*$P < 0.001$

Table 7. Univariate and multivariate analysis for the discontinuance of ribavirin due to severe anemia among the patients aged 60 years and older

A. Univariate analysis

Factor	Category	Odds ratio	95% CI	*P* value
Age			1.007–1.250	0.04
Sex	Male/Female	1/1.67	0.280–1.286	0.19
Body weight			0.947–1.021	0.37
Serum creatinine			0.865–33.586	0.07
Ribavirin/Body weight			0.775–1.205	0.76
IFN dosage	6 MU/10 MU	1/1.92	0.803–4.579	0.14
Hb			0.537–1.106	0.16
CL/F	≥15/<15	—	—	0.97
	≥10/<10	1/2.16	0.217–0.989	0.047
"2 by 2"	Negative/Positive	1/4.24	0.112–0.497	0.0001

B. Multivariate analysis

Factor	Category	Odds ratio	95% CI	*P* value
CL/F	≥10/<10	1/2.12	0.213–1.042	0.063
"2 by 2"	Negative/Positive	1/4.18	0.112–0.507	0.0002

Table 8. Rate of the ribavirin reduction or discontinuance due to adverse effects with the rate of anemia progression among the patients aged 60 years and older

			Discontinuance	
	No reduction	Dose reduction	All cases	Cases due to severe anemia
ΔHb ≥ 2.0	27%	23%	50%	38%*
("2 by 2"-positive) (n = 60)	(16/60)	(14/60)	(30/60)	(23/60)
ΔHb < 2.0	46%	29%	25%	14%
("2 by 2"-negative) (n = 133)	(61/133)	(39/133)	(33/133)	(18/133)

*P < 0.0001

Table 9. Comparison of "2 by 2" standard and CL/F standard for the discontinuance of ribavirin due to severe anemia among the patients aged 60 years and older

A.

	"2 by 2"-positive	CL/F < 15	*P* value
Sensitivity	56% (23/41)	100% (41/41)	<0.0001
Specificity	77% (96/125)	7% (9/125)	<0.0001
Accuracy	72% (119/166)	30% (50/166)	<0.0001

B.

	"2 by 2"-positive	CL/F < 10	*P* value
Sensitivity	56% (23/41)	71% (29/41)	0.17
Specificity	77% (96/125)	47% (59/125)	<0.0001
Accuracy	72% (119/166)	53% (88/166)	<0.001

Discussion

Ribavirin, developed in 1972, is a synthetic nucleic acid analog, which has antiviral activity in vitro against a wide variety of RNA and DNA viruses. Combination therapy of ribavirin with IFN or Peg-IFN led to remarkable progress in antiviral therapy for chronic hepatitis C. To raise the SVR rate for such combination therapy, it is very important to predict the discontinuance of the therapy due to an adverse effect and prevent it. In this study, we observed the incidence of hemolytic anemia, the major side effect of ribavirin. The factors correlated with the progression of anemia were analyzed to avert the need to discontinue ribavirin treatment of patients with chronic hepatitis C receiving combination therapy.

Several studies in the United States and European countries have reported that higher ribavirin dosage or a higher plasma concentration of ribavirin increases the SVR rate.[21,22] However, a higher ribavirin dose or higher plasma concentration of ribavirin entails the risk of having to discontinue ribavirin treatment. In Japan, analysis of the relationship between the SVR rate and a dose reduction or discontinuance of ribavirin, has shown that reducing the dose of ribavirin does not affect the SVR rate. In the present study, the SVR rate of the patients discontinuing ribavirin was also shown to be significantly lower than the patients who did not discontinue it

in both the 1H group and the non-1H group ($P < 0.01$ and $P < 0.01$, respectively). The SVR rate was almost the same between patients without a dose reduction of ribavirin and those with a dose reduction in both groups (1H, 24% vs. 26%; non-1H, 83% vs. 83%). Therefore, averting ribavirin discontinuance, even if its dose must be reduced, can lead to improvement of the SVR rate. This means that it is important to identify patients prone to develop severe anemia leading to ribavirin discontinuance while they are still in the early phase of treatment, and to consider ribavirin dose reduction before anemia progression.

CL/F relating to the plasma concentration of ribavirin at the end of 4 weeks after initiation of the combination therapy has been used as a predictive factor for the progression of anemia.[16–18] In this study, the patients with a lower CL/F value, which is thought to be correlated with a high plasma concentration of ribavirin, showed a higher rate of discontinuance of ribavirin due to severe anemia than those with a higher CL/F value. This indicates that prediction of anemia progression using the CL/F is useful before the initiation of combination therapy. We analyzed predictive factors for discontinuance of ribavirin due to severe anemia using two CL/F categories, CL/F < 10 and CL/F < 15, taking into account that the mean CL/F was 13.0 l/h and the median was 11.9 l/h, and compared the usefulness of those categories with that of the "2 by 2" standard.

We focused on the early decline of the Hb concentration after the initiation of combination therapy. Monitoring of the Hb decline allowed clear assignment of the patients into three groups: patients without dose reduction of ribavirin, those with dose reduction, and those who discontinued ribavirin. At the end of 2 weeks, a significant relationship was already observed among the three groups. Therefore, we examined the relationship between the beginning of a progression to severe anemia and the decrease in the Hb concentration at the end of 2 weeks (ΔHb). Since a standard value of ΔHb for dose reduction of ribavirin must be established, we compared ΔHb2.0 with ΔHb1.5, and found that the specificity and accuracy of ΔHb2.0 as a predictive factor for the discontinuance of ribavirin due to severe anemia was higher than those of ΔHb1.5. We therefore adopted ΔHb2.0 at the end of 2 weeks from the start of treatment (the "2 by 2" standard) as the predictive factor for discontinuance of ribavirin due to severe anemia, because an early reduction of ribavirin should be limited to those patients with a higher specificity rate for the progression of anemia. Furthermore, ΔHb2.0 is easier to calculate.

In the multivariate logistic regression analysis, both the CL/F category and the "2 by 2" category were useful for all patients as independent predictive factors for discontinuing ribavirin due to severe anemia (Table 5). Patients with lower CL/F (CL/F < 10 or CL/F < 15) and those who were "2 by 2" positive were significantly associated with the discontinuance of ribavirin due to severe anemia. Thus, the CL/F standard should be used as a predictive factor before combination therapy is begun, and the "2 by 2" standard should be used during the combination therapy. We also assessed which would be the more useful predictive factor for discontinuance of ribavirin due to severe anemia among older patients. Multivariate analysis showed that only the "2 by 2" standard was significantly related to the discontinuance of ribavirin due to severe anemia among older patients (Table 7B). Moreover, the "2 by 2" standard showed higher specificity (77%) and accuracy (72%) for the discontinuance of ribavirin due to severe anemia among older patients than either CL/F value (Table 9). The ribavirin dose of 200 mg should be reduced for aged patients whose Hb decreases over 2 g/dl from the start of combination therapy in order to avoid having to discontinue ribavirin administration altogether.

Hemolytic anemia has been reported to be induced by ribavirin administration, depending on the plasma ribavirin concentration[15] and the fragile membrane of RBC in which ribavirin accumulates.[23] Furthermore, the plasma clearance of ribavirin has been reported to depend on renal function.[24,25] The anemia associated with IFN and ribavirin therapy is a "mixed anemia," in which both hemolysis and bone marrow suppression occur simultaneously. In this study, many patients, especially older ones, had to discontinue ribavirin due to severe anemia, as previously reported.[26] A major reason for this was thought to be the tendency of the plasma concentration of ribavirin to rise due to lower renal function and impaired hematogenous function as the anemia progressed. In predicting the discontinuance of ribavirin due to severe anemia using the CL/F category, the lower CL/F implies that older patients and patients with low renal function are high-risk groups. However, CL/F does not account for the fragile membrane of RBC or the hematogenous function. Therefore, the CL/F standard cannot be a good marker for individual patients, because CL/F does not reflect in vivo phenomena triggered by ribavirin. CL/F is related simply to the plasma concentration of ribavirin at the end of 4 weeks after the initiation of combination therapy. On the other hand, the "2 by 2" standard can be useful as a predictive factor of ribavirin discontinuance forces by severe anemia for all patients, including older patients. It indicates that the "2 by 2" standard reflects plural factors, such as the occurrence of hemolysis and hematogenous functions. We suggest that the "2 by 2" standard is more useful than the CL/F category as a predictive factor for discontinuance of ribavirin due to severe anemia, especially among older patients.

In conclusion, it is important to monitor the early decline of the Hb concentration after initiation of combination therapy and to reduce the dose of ribavirin at the end of 2 weeks based on the magnitude of the Hb decline. An early reduction of ribavirin before progression to severe anemia can reduce the number of patients who are destined to discontinue ribavirin therapy. This should help improve the patients' quality of life by preventing the progression to severe anemia. Further prospective study is necessary to evaluate the antiviral outcome by ITT analysis using early reduction of ribavirin based on the "2 by 2" standard.

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