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,

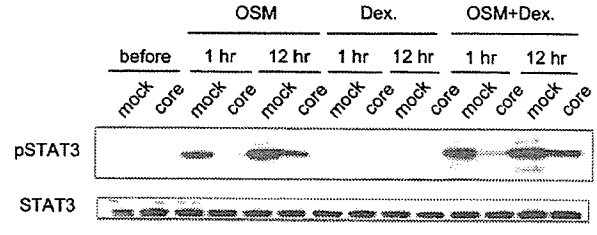

Fig. 3. Early phase response of STAT3 phosphorylation in the CL2 mock and core cells under stimulation with OSM and/or dexamethasone. The levels of pSTAT3 and the whole STAT3 were examined by Western blot analyses.

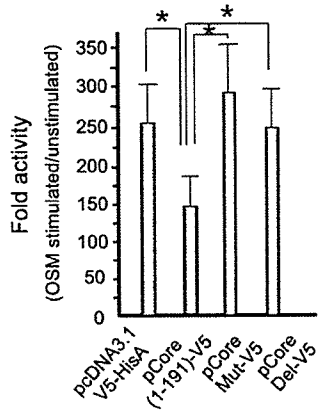

Fig. 4. Involvement of HCV core-JAK interaction in the HCV core-mediated inhibitory effect on the JAK-STAT signal transduction activated by OSM. The CL2 cells were cotransfected with various effector plasmids (pcDNA3.1/V5-HisA, pCore(1-191)-V5, pCoreMut-V5, and pCoreDel-V5) with pAPREluci and pRLtk.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

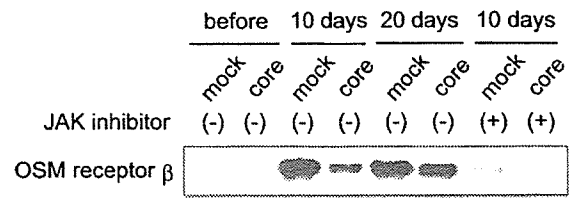

Fig. 5. Detection of OSMRβ in the CL2 mock and core cells under stimulation with OSM and dexamethasone in the presence or absence of JAK inhibitor. The cellular protein was harvested before, 10 days after, and 20 days after stimulation and used for Western blot analysis.

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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J Gastroenterol 2006; 41:862–872
DOI 10.1007/s00535-006-1858-2

Journal of Gastroenterology

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.

Received: March 30, 2006 / Accepted: June 12, 2006
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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.

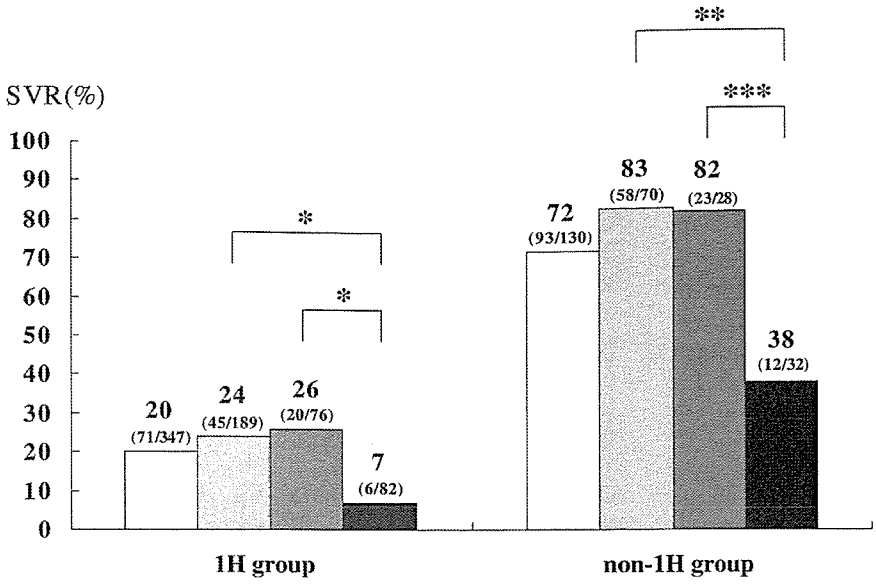

Fig. 1. Efficacy of combination therapy with dose reduction or discontinuance of ribavirin (intention-to-treat analysis). *1H group*, patients with genotype 1 and high viral load; *non-1H group*, patients not in the 1H group; *SVR*, sustained viral response. □ all patients; ▩ patients without dose reduction 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 10l/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

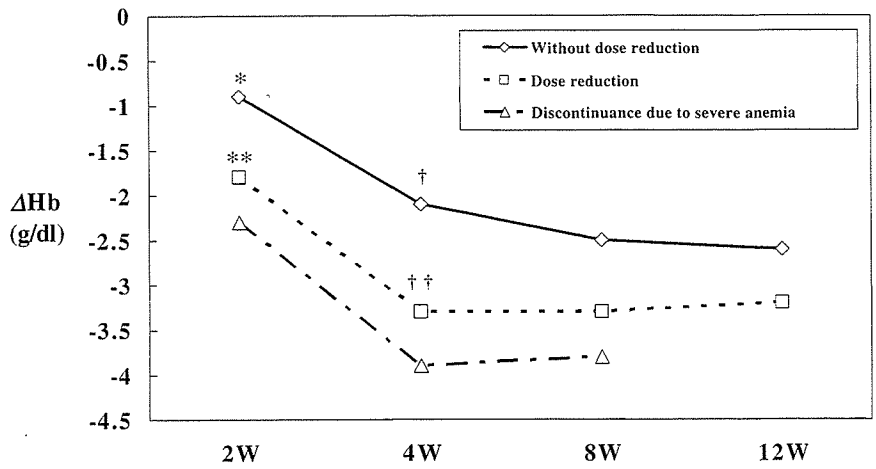

Fig. 2. Decline of hemoglobin according to dose reduction or discontinuance of ribavirin. *Significantly different from patients with dose reduction (P < 0.0001) and patients with discontinuance (P < 0.0001); **significantly different from patients with discontinuance (P < 0.02); †significantly different 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	37%	29%	34%	25%*
(n = 142)	(53/142)	(41/142)	(48/142)	(36/142)
ΔHb < 2.0	61%	19%	20%	10%
(n = 338)	(209/338)	(65/338)	(64/338)	(32/338)

*P < 0.0001

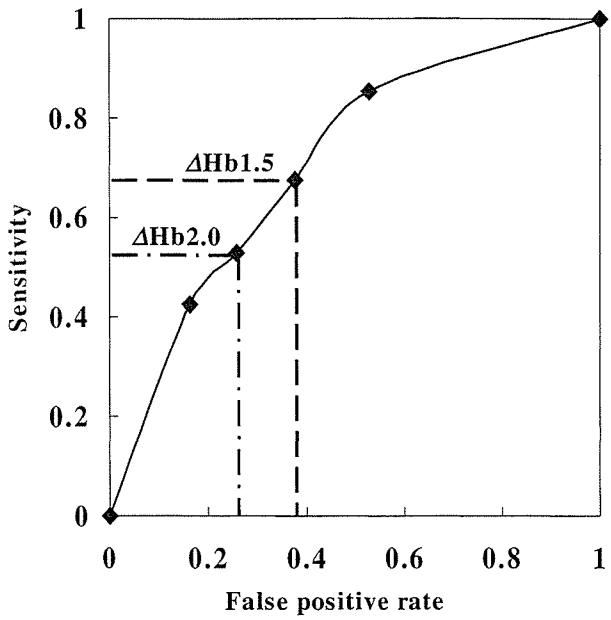

Fig. 3. Receiver-operating characteristic curve for ΔHb at the end of 2 weeks for discontinuance of ribavirin due to severe anemia

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	6MU/10MU	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.

Acknowledgments. Other institutions and participants in the Osaka Liver Disease Study Group (Digestive Disease Study Group of Osaka Renaissance) were the National Hospital Organization Osaka National Hospital, Y. Izumi; Osaka Rousai Hospital, K. Noda and M. Satoh; Osaka Kouseinenkin Hospital, M. Kurokawa; Kansai Rousai Hospital, M. Yamamoto; Osaka General Medical Center, T. Inoue; National Hospital Organization Osaka Minami Medical Center, Y. Inoue and M. Shigekawa; Osaka Police Hospital, J. Kondo; Kaizuka City Hospital, O. Nishiyama; and Osaka University Graduate School of Medicine, S. Shinzaki, I. Itose, S. Egawa, and T. Nishida.

This work was supported by a Grants-in-Aid for Research on Hepatitis and BSE from Health, Labour and Welfare Ministry of Japan, and for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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Int. J. Cancer: **120,** 1252–1260 (2007)

Natural killer cell-mediated ablation of metastatic liver tumors by hydrodynamic injection of IFNα gene to mice

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Interferon (IFN) α is a pleiotropic cytokine acting as an antiviral substance, cell growth inhibitor and immunomodulator. To evaluate the therapeutic efficacy and mechanisms of IFNα on hepatic metastasis of tumor cells, we hydrodynamically injected naked plasmid DNA encoding IFNα1 (pCMV-IFNa1) into Balb/cA mice having 2 days hepatic metastasis of CT-26 cells. Single injection of pCMV-IFNa1 efficiently enhanced the natural killer (NK) activity of hepatic mononuclear cells, induced production of IFNγ in serum and led to complete rejection of tumors in the liver. Mice protected from hepatic metastasis by IFNα therapy displayed a tumor-specific cytotoxic T cell response and were resistant to subcutaneous challenge of CT-26 cells. NK cells were critically required for IFNα-mediated rejection of hepatic metastasis, because their depletion by injecting anti-asialo GM1 antibody completely abolished the antimetastatic effect. To find whether NK cells are directly activated by IFNα and are sufficient for the antimetastatic effect, the responses to IFNα were examined in SCID mice lacking T cells, B cells and NKT cells. IFNα completely rejected hepatic metastasis in SCID mice and efficiently activated SCID mononuclear cells, as evidenced by activation of STAT1 and a variety of genes, such as MHC class I, granzyme B, tumor necrosis factor-related apoptosis-inducing ligand and IFNγ, and also enhanced Yac1 lytic ability. Study of IFNγ knockout mice revealed that IFNγ was not necessary for IFNα-mediated NK cell activation and metastasis protection. In conclusion, IFNα efficiently activates both innate and adaptive immune responses, but NK cells are critically required and sufficient for IFNα-mediated initial rejection of hepatic metastasis of microdisseminated tumors.

Key words: DNA; innate; adaptive; immunity NK

The liver is the most common site of metastatic malignancy and the status of this organ is an important determinant of survival in patients with advanced disease. The risk of hepatic metastasis remains high in many patients after potentially curative surgery at primary sites.[1] This suggests that the spread of tumor cells can occur in the liver even when they cannot be detected by current diagnostic modalities. To suppress the incidence of liver metastasis, whole liver therapy against microdisseminated tumors should be considered.[2] Since the liver contains an abundance of immune cells, the cytokine-meditated activation of these cells may be a promising approach toward this end.[3,4]

Interferon (IFN) α is a pleiotropic cytokine acting as an antiviral substance, cell growth inhibitor and immunomodulator. IFNα as well as IFNγ are primarily induced in response to viral infection of cells and ligate a cognate receptor for the Type 1 IFN expressed on target cells.[5] On the other hand, Type 2 IFN, IFNγ, is produced predominantly by T lymphocytes, natural killer (NK) cells and NKT cells and uses a distinct receptor. IFNα-mediated antiviral activity includes induction of 2′-5′ oligoadenylate synthetases, double-stranded RNA-activated protein kinase (PKR) and Mx proteins. IFNα can exert direct effects on tumor cells by inhibiting proliferation, inducing apoptosis and inhibiting the release of proangiogenic factors such as vascular endothelial growth factor.[6] IFNα-mediated immunomodulation includes dendritic cell maturation, NK cell activation, MHC Class I induction and cytokine production.[7] Most, if not all, of these actions are mediated by the Jak-STAT signaling pathway downstream of the Type 1 IFN receptor.[8–10] Type 1 IFN receptor upon ligand ligation phosphorylates Jack1 and then phosphorylates STAT1, which activates a variety of IFN-regulated genes. IFNα and IFNβ have been shown to elicit antitumor effects in various murine models of cancer.[11–14] IFNβ was also shown to be effective for retarding metastatic tumor growth in murine liver, but the underlying mechanisms have not been elucidated.[15]

In the present study, we investigated the efficacy of hydrodynamics-based expression of IFNα in the liver against a murine model of hepatic metastasis of CT-26 colon cancer cells and the mechanisms of an IFNα-mediated therapeutic effect of hepatic metastasis. Mice treated with IFNα completely rejected hepatic metastasis and became resistant to rechallenge by CT-26 cells. Although IFNα induced a variety of host responses including increased NK activity, increased IFNγ production and tumor-specific T cell responses, the initial rejection of hepatic metastasis was solely dependent on NK cells. Our study has shed light on NK cell activation as an important mechanism by which IFNα ablates microdisseminated tumors in the liver.

Material and methods

Mice

Specific pathogen-free female Balb/cA mice, SCID mice and their wild-type control mice were purchased from Clea Japan, Inc. (Tokyo, Japan). Rag2 knockout (Rag2 KO) mice were purchased from Taconic (Germantown, NY). IFNγ knockout (GKO) mice with a Balb/cA background was kindly provided by Dr. Yoichiro Iwakura (Institute of Medical Science, University of Tokyo).[16] All mice were used at the age of 5 to 8 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 the study protocol complied with the institution's guidelines.

Tumor models

Intrasplenic injection of tumor cells was used to establish microdisseminated liver tumors in mice.[17] CT-26 colon cancer cells originating from Balb/cA mice were maintained in DMEM supplemented with 10% FCS. Syngeneic mice were anesthetized with pentobarbital and given a cut on the left side flank. CT-26 cells (1×10^5) were suspended in 150 μl of PBS and injected into the spleen. For subcutaneous tumor models, CT-26 cells (5×10^5) were injected into the back of the mice under light anesthesia.

NK cell depletion

For depletion of NK cells *in vivo*, anti-asialo GM1 antibody (Wako, Osaka, Japan) was intraperitoneally administered.[17] We

Grant sponsor: The Ministry of Education, Culture, Sports, Science and Technology, Japan; Grant sponsor: The Ministry of Health, Labor and Welfare of Japan.
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Received 5 April 2006; Accepted after revision 22 May 2006
DOI 10.1002/ijc.22152
Published online 12 December 2006 in Wiley InterScience (www.interscience.wiley.com).

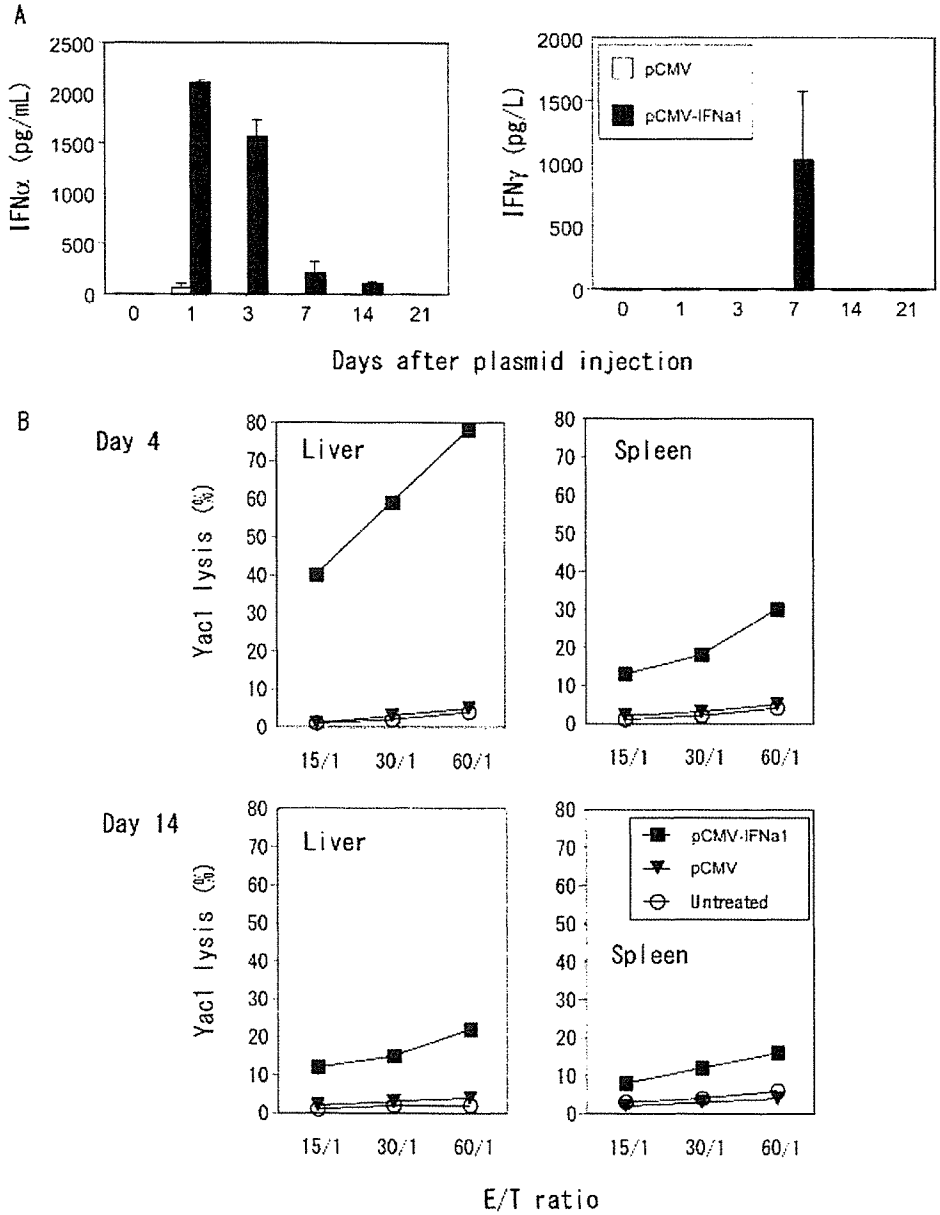

FIGURE 1 – Effects of hydrodynamic injection of IFNα-expressing plasmid. (*a*) Serum IFNα and IFNγ concentration. Balb/cA mice were hydrodynamically injected with either pCMV-IFNa1 (closed bars) or pCMV (open bars) and bled at indicated time points to measure the levels of serum IFNα and IFNγ. The results are indicated as mean and SD (n = 3/group). Shown are representative data for 2 independent experiments. (*b*) Yac1 lytic ability. Hepatic or splenic mononuclear cells were isolated from naive Balb/cA mice (open circles) and those injected with either pCMV-IFNa1 (closed squares) or pCMV (closed triangles). Yac1 lytic ability was measured by a standard chromium-release assay at indicated effector and target ratios (E/T ratio). All experiments were performed at least 3 times and representative data are shown.

determined the appropriate dosing to be 500 μg/mouse (50 μl when dissolved according to the manufacturer's instructions) based on FACS analysis of hepatic mononuclear cells. Injection of this dose of anti-asialo GM1 antibody depleted more than 95% of DX-5 positive, TCRβ-negative cells (NK cells) in the liver. NKT cells were less affected than NK cells, because 40% of Cd1d-tetramer positive cells, which are invariant NKT cells, still remained in the liver after the treatment. Anti-asialo GM1 antibody was injected 1 day after tumor inoculation and then every 5 days. For the control, the same amount of normal rabbit immunoglobulin (DAKO, Copenhagen, Denmark) was intraperitoneally administered.

Injection of naked plasmid DNA

A plasmid coding the murine IFNa1 gene, pCMV-IFNa1, was generously provided by Dr. Daniel J. J. Carr (University of Oklahoma, Health Science Center).[18] 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 as previously described.[19] 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 sec.

ELISA

Blood samples were serially obtained from the venous plexus in the retro-orbita under light anesthesia. The levels of serum IFNα and IFNγ were measured using commercially available ELISA kits (Biomedical Laboratories for murine IFNα; Endogen for murine IFNγ).

Mononuclear cells

Mononuclear cells were isolated from the liver or spleen as previously described.[20] The NK activity of mononuclear cells was assessed with standard 4-hr ^{51}Cr-releasing assay using Yac1 cells as targets. To examine CT-26-specific responses, splenocytes were stimulated with CT-26 cells for 5 days in the presence of 30 U/ml

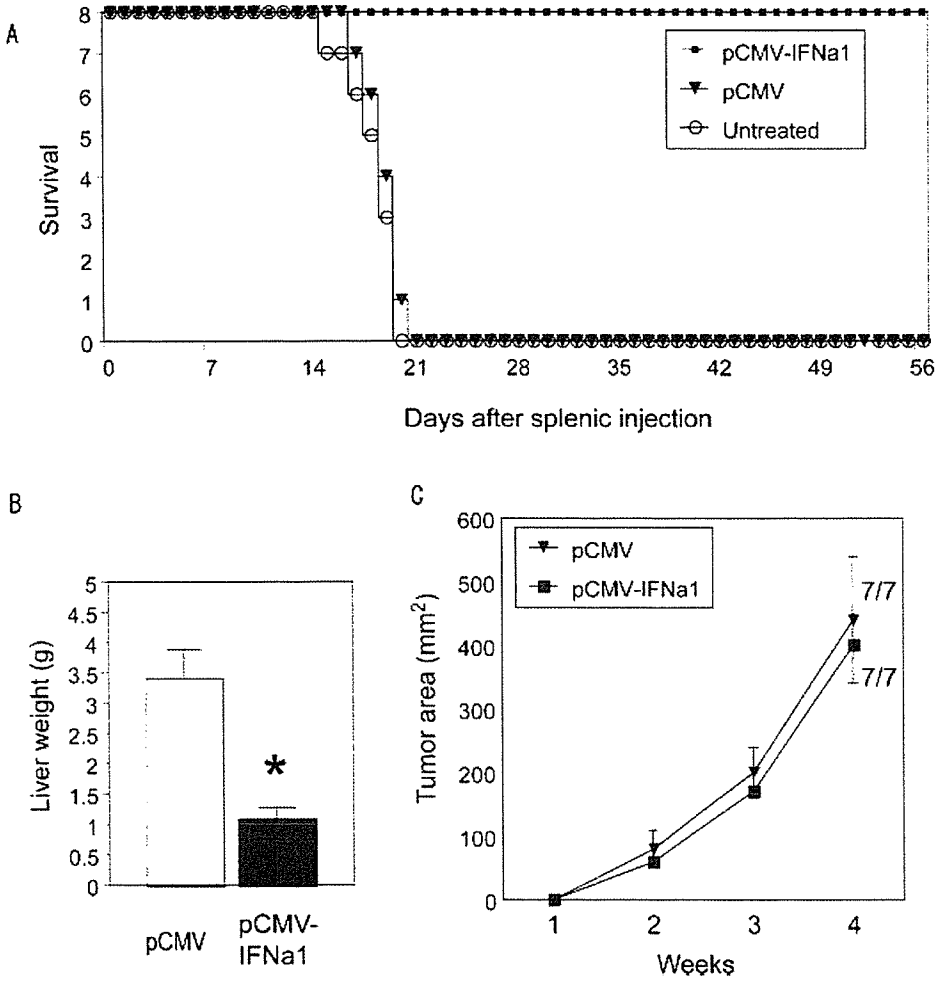

FIGURE 2 – Anti-tumor effects of IFNα therapy. (*a*) Survival. Balb/cA mice were intrasplenically injected with CT-26 cells. Two days later, the mice were randomly assigned to 3 groups and received hydrodynamic injection of either pCMV-IFNa1 (closed squares) or pCMV (closed triangles) or untreated (open circles). The number of survivors in each group was monitored. (*b*) Anti-metastatic effects. Balb/cA mice were intrasplenically injected with CT-26 cells and hydrodynamically injected with either pCMV-IFNa1 (closed bars) or pCMV (open bars) 2 days later. At 14 days after the splenic injection, the mice were sacrificed to examine liver tumor development by measuring liver weight. All experiments were performed at least 3 times and representative data are shown. *, $p < 0.05$ *vs.* pCMV injection group. (*c*) Anti-tumor effects on subcutaneous tumors. Balb/cA mice were subcutaneously injected with CT-26 cells and hydrodynamically injected with either pCMV-IFNa1 (closed squares) or pCMV (closed triangles) 2 days later. Tumor growth was examined every week. Tumor size was expressed as the mean tumor size of only those mice bearing tumors. Each data point represents the mean tumor size and SD. The ratio of the number of mice bearing tumor/the number challenged for each treatment group at 4 weeks is shown in the figure.

of murine IL-2 and subjected to analysis for lytic activity against CT-26 cells or BNL A.7 murine hepatoma cells by 4-hr ^{51}Cr-releasing assay. In some experiments, mononuclear cells were separated into CD90-positive cells (T cells) and CD90-negative cells (non-T cells) using the MACS system (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).

Western blotting

Mouse recombinant IFNα was generously provided by Fujisaki Institute, Hayashibara Biochemical Laboratories (Okayama, Japan). Mononuclear cells were treated with or without IFNα. Whole cell lysate was prepared from mononuclear cells from mice, and 20 μg of protein was separated by SDS-PAGE and transferred to PVDF membrane. The membrane was stained with anti-STAT1 antibody (Upstate Biotechnology, Lake Placid, NY) or antiphospho-sepcific STAT1 (Y701) antibody (Upstate Biotechnology) and visualized by chemiluminescence. The specificities of STAT1 and phosphorylated STAT1 signals were confirmed by siRNA experiment using BNL A.7 cells in the presence or absence of IFNα treatment (data not shown). Anti-STAT antibody recognizes STAT1α, whereas antiphospho-STAT1 antibody recognizes phosphorylated form of both STAT1α and STAT1β.

Microarray analysis

Total RNA was isolated from cultured SCID splenocytes in the presence or absence of IFNα by ISOGEN. RNA was analyzed using the GeneChip Mouse Genome Array 430 2.0 (Affymetrix, Santa Clara, CA). Analysis of difference expression was performed by GeneChip Operating Software Ver. 1.1. Genes were considered to be significantly upregulated according to the following criteria: (*i*) the mean fold increase was more than 4-fold; (*ii*) the expression of a gene was significant in NK cells after IFNα treatment; (*iii*) a significant increase was registered based on the algorithm of the software.

Statistics

Data are represented as mean ± SD. Comparisons between groups were analyzed by unpaired *t*-test with Welch's correction or ANOVA for experiments with more than two subgroups. *Post hoc* tests were done using the Bonferroni's *t*-test. $p < 0.05$ was considered statistically significant.

Results

Single intravenous injection of IFNa1 gene enhances NK activity and completely rejects hepatic metastasis of CT-26 cells

Hydrodynamics-based gene delivery establishes efficient foreign gene expression predominantly in the liver, especially in hepatocytes.[21,22] Serial measurement of serum IFNα demonstrated that pCMV-IFNa1 injection led to substantial IFNα production on Day 1. The levels of serum IFNα then declined but were still detectable at Day 14 (Fig. 1*a*). To examine biological effects of the produced IFNα, we evaluated the NK activity of mononuclear cells from the liver and spleen. pCMV-IFNa1 injection, but not

control pCMV injection, increased Yac1 lytic activity of hepatic mononuclear cells and, to a lesser extent, splenic mononuclear cells at 4 days. The levels of Yac1 lytic activity declined but were still higher at 14 days after the injection (Fig. 1*b*). We also measured IFNγ production in serum, since IFNα is known to activate IFNγ production.[23,24] pCMV-IFNa1 injection, but not pCMV injection, increased serum IFNγ at 7 days (Fig. 1*a*). Since serum IFNγ increased relatively at a later time point, it may be an indirect effect rather than a direct effect of IFNα. These data indicated that hydrodynamic injection of pCMV-IFNa1 efficiently produced biologically active IFNα for a while in mice.

To evaluate the therapeutic effects of IFNα, Balb/cA mice were intrasplenically injected with CT-26 cells. Two days later, the mice were randomized into 3 groups and intravenously injected with either pCMV-IFNa1 or pCMV or were not treated. All pCMV-injected mice or untreated mice died within 3 weeks (Fig. 2*a*). They exhibited massive liver tumor in the liver. In contrast, all mice receiving pCMV-IFNa1 survived more than 2 months. To evaluate tumor metastasis, we sacrificed another cohort of mice at 2 weeks after tumor inoculation. There were no macroscopic or microscopic liver tumors in the pCMV-IFNa1-injected mice. In contrast, livers from pCMV-injected mice had massive tumors and were significantly heavier than those from pCMV-injected mice (Fig. 2*b*).

These results clearly indicated the striking therapeutic effects of IFNα on hepatic metastasis of CT-26 cells. To examine this therapeutic effect at a site other than the liver, Balb/cA mice were subcutaneously injected on the back with CT-26 cells and hydrodynamically injected 2 days later with pCMV-IFNa1 or pCMV. No difference in tumor growth was noted between pCMV-IFNa1-injected mice and pCMV-injected mice (Fig. 2*c*).

Mice protected from hepatic metastasis by IFNα gene therapy were resistant to subcutaneous challenge of CT-26 cells and exhibited a tumor-specific T cell response

We next investigated the possibility of IFNα-mediated rejection of hepatic metastasis being followed by induction of an adaptive immune response to the original tumor. To this end, we subcutaneously injected CT-26 cells into the mice that had been protected from CT-26 hepatic metastasis by IFNα therapy. The mice were rechallenged with CT-26 cells 1 month after the initial splenic injection. The controls were naïve Balb/cA mice as well as those receiving pCMV-IFNa1 but not CT-26 splenic inoculation. The incidence of tumor formation was lower in mice that had rejected hepatic metastasis by IFNα therapy than in the control mice. Even if they developed subcutaneous tumors, tumor size was significantly smaller than in the control mice (Fig. 3*a*).

To examine the tumor-specific response, splenocytes were isolated 3 weeks after tumor inoculation and restimulated *in vitro* with CT-26 cells. Splenocytes isolated from CT-26 bearing mice treated with IFNα showed significant levels of killing ability against CT-26 cells, but not against BNL A.7 cells (Fig. 3*b*). When mice were intrasplenically injected with UV-irradiated CT-26 cells, the splenocytes did not show significant killing activity regardless of the subsequent IFNα therapy (Fig. 3*c*). Separation of effector cells into T cells and non-T cells based on CD90 expression revealed that this killing ability was mediated by T cells, but not by non-T cells (data not shown). Thus, a tumor-specific cytotoxic T cell response was established in mice that had rejected hepatic metastasis of CT-26 cells by IFNα therapy.

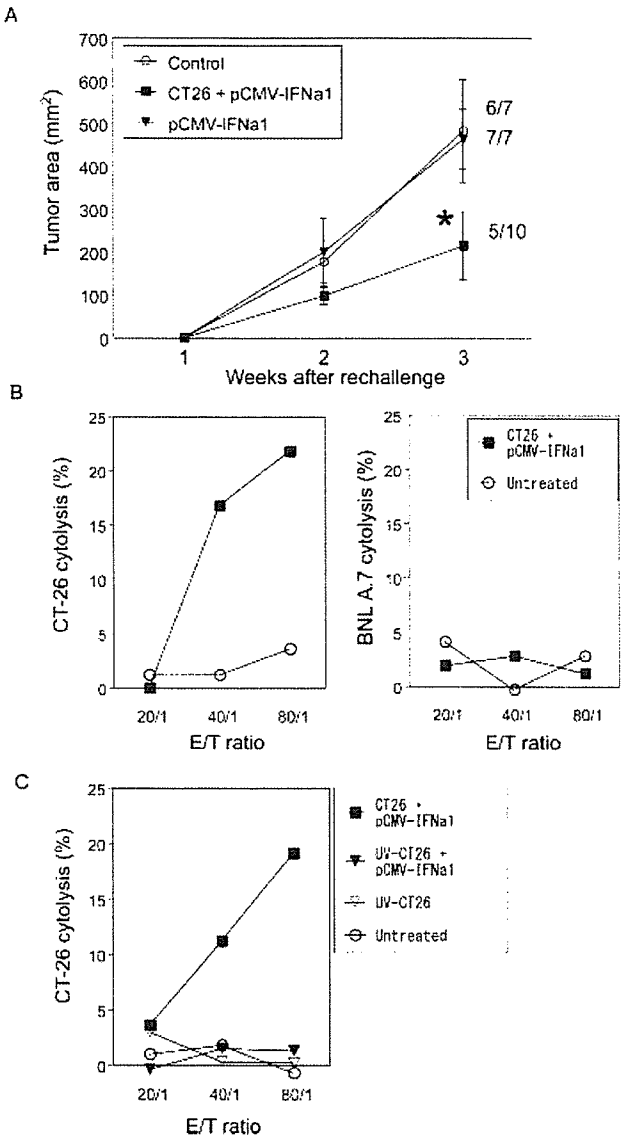

FIGURE 3 – Systemic immunity and tumor-specific T cell response. (*a*) Anti-tumor effects on rechallenged tumors. Balb/cA mice that had rejected hepatic metastasis of CT-26 cell by IFNα (closed squares), those treated with IFNα alone (closed triangles), and naïve mice (open circles) were challenged with subcutaneous injection of CT-26 cells 1 month after the previous treatment. Subcutaneous tumor growth was examined every week by measuring tumor area. Tumor size was expressed as the mean tumor size of only those mice bearing tumors. Each data point represents the mean tumor size and SD. The ratio of the number of mice bearing tumor/the number challenged for each treatment group at 3 weeks is shown in the figure. *, $p < 0.05$ *vs.* control or pCMV-IFNa1 injection only group. (*b*) *In vitro* tumor-specific killing ability. Balb/cA mice were intrasplenically injected with CT-26 cells and then treated with pCMV-IFNa1 2 days later. Splenocytes were isolated from CT-26 plus pCMV-IFNa1-injected mice at 3 weeks (closed squares) or naïve mice (open circles), restimulated with CT-26 cells for 5 days and then subjected to analysis for the lytic ability against CT-26 cells (left) or BNL A.7 cells (right). Shown are representative data for 3 independent experiments. (*c*) Requirement of CT-26 cells and IFNα on induction of tumor-specific killing ability. Balb/cA mice were intrasplenically injected with live CT-26 cells (squares) or UV-irradiated CT-26 cells (triangles) and then treated with (closed symbols) or without (open symbols) pCMV-IFNa1 2 days later. Splenocytes were isolated from mice at 3 weeks, restimulated with CT-26 cells for 5 days and then subjected to the analysis for the lytic ability against CT-26 cells. Mice injected with live CT-26 cells without following injection of pCMV-IFNa1 did not survive for 3 weeks naïve mice were included as controls (open circles). Shown are representative data for 3 independent experiments.

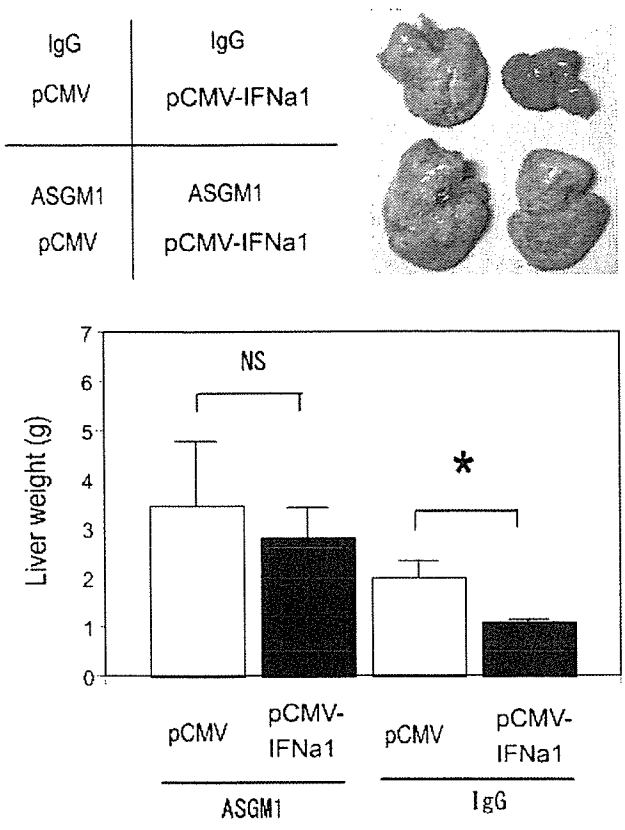

FIGURE 4 – Requirement of NK cells on IFNα-mediated anti-metastatic effects. Balb/cA mice were intrasplenically injected with CT-26 cells, intraperitoneally injected with either anti-ASGM1 or control IgG at 1 day, and hydrodynamically injected with either pCMV-IFNa1 (closed bars, n = 8/group) or pCMV (open bars, n = 7/group). Mice were sacrificed at 14 days to examine tumor growth in the liver. Top, a representative picture of the liver in each group. Bottom, comparison of liver weight among treatment groups. Experiments were performed at least 3 times and representative data are shown. *, $p < 0.05$. NS, not significant.

NK cells are required for IFNa-mediated initial rejection of hepatic metastasis

To examine whether the observed increase in NK activity of hepatic mononuclear cells is involved in the complete rejection of hepatic metastasis, we induced depletion of NK cells by injecting anti-asialo GM1 antibody. pCMV-IFNa1 injection completely abrogated hepatic tumor formation in control immunoglobulin-injected mice. In sharp contrast, pCMV-IFNa1 injection did not offer antimetastatic effects in anti-aialo GM1 antibody-injected mice, suggesting the critical contribution of NK cells to the anti-metastatic effects of IFNα (Fig. 4). We examined the possibility that hepatic mononuclear cells can serve as direct effectors cells for CT-26 eradication. Although CT-26 cells were more resistant to hepatic mononuclear cells than Yac1 cells, pCMV-IFNa1 injection clearly enhanced the killing ability of hepatic mononuclear cells against CT-26 cells (data not shown). This result indicated that CT-26 is potentially susceptible to hepatic mononuclear cells upon IFNα therapy.

IFNα directly activates NK cells

IFNα is known to be able to activate a variety of immune cells. To examine whether NK cells can be directly activated by IFNα, we analyzed SCID mice that lack T cells, B cell and NKT cells due to spontaneous DNA-dependent protein kinase point mutation.[25] SCID or wild-type splenocytes were cultured with IFNα and examined for STAT1 phosphorylation, which peaked at 30 min and declined at 6 hr after IFNα stimulation in both mice (Fig. 5*a*). However, the signals of STAT1 phosphorylation were weaker in SCID splenocytes than in wild-type cells. Of interest is the finding that STAT1 expression was reduced in SCID cells compared to wild-type cells. Similar data were also obtained from experiments on Rag2 KO mice, another model of deficiency for T cells, B cells and NKT cells. To examine the reasons for SCID or Rag2 KO cells expressing low levels of STAT1, we separated wild-type splenocytes into T cells and non-T cells based on CD90 expression. The levels of STAT1 expression were weaker in non-T cells than in T cells (Fig. 5*b*). Taken together, the difference in the levels of STAT1 expression among lymphocyte subsets could explain the reduced phosphorylation signals after IFNα treatment in SCID or Rag2 KO cells.

To examine the gene profiles activated by IFNα in NK cells, we used Affymetrix DNA array analysis on SCID hepatic mononuclear cells. Six hours treatment of IFNα (1,000 U/ml) upregulated 243 of 45,101 genes in SCID cells by more than 4-fold. They included well known IFNα-regulated genes such as H2, 2′-5′ oligoadenylate synthetases, Mx1, IRF and suppressor of cytokine signaling (SOCS). Among the effector molecules for cytotoxicity, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and granzyme B were activated. Various cytokines such as IL-15 and IFNγ were also upregulated. These data revealed that NK cells upon IFNα stimulation produced well-characterized IFN-inducible genes and others that are relatively specific to killer cells or immune cells.

pCMV-IFNa1 injection completely suppressed tumor formation in the liver in SCID mice

We examined the *in vivo* effects of IFNα in SCID mice. In agreement with SCID cell activation *in vitro*, pCMV-IFNa1 injection enhanced the Yac1 lytic ability of hepatic mononuclear cells in SCID mice (Fig. 5*c*). To examine whether NK cells are sufficient for IFNα-mediated rejection of hepatic metastasis, we injected pCMV-IFNa1 or pCMV into mice that had been intrasplenically injected with CT-26 cells 2 days earlier. pCMV-IFNa1 completely suppressed tumor formation in the liver (Fig. 5*d*). As described in the *Material and methods* section, anti-asialo GM1 injection reduces the number of NKT cells. However, this SCID experiment clearly showed that NKT cells are not required for NK cell activation by IFNα and its antimetastatic effects.

pCMV-IFNa1 injection completely suppressed tumor formation in the liver in GKO mice

IFNγ has been established as an endogenous inhibitor of tumor development and progression.[26] Exogenous administration of IFNγ suppresses tumor formation in a variety of models.[15,27] To examine the possibility of IFNγ being involved in antimetastatic effects on IFNα, we injected pCMV-IFNa1 or pCMV plasmid into GKO mice exposed to 2 days of metastasis of CT-26 cells. IFNα treatment led to complete rejection of CT-26 cells in GKO mice (Fig. 6*a*). pCMV-IFNa1 injection, but not pCMV injection, augmented the Yac1 lytic ability of mononuclear cells (Fig. 6*b*).

Discussion

Here we report that a single injection of pCMV-IFNa1 could lead to complete rejection of preexisting hepatic metastasis of colon cancer cells. This partly agrees with a previous report by Kobayashi et al.,[15] who hydrodynamically injected IFNβ- or IFNγ-expressing plasmid into CT-26 bearing mice and reported the antimetastatic effects of IFNβ or IFNγ. In contrast to our study, all mice died within 45 days due to metastasized tumor growth even if plasmid injection was began one day after tumor inoculation and repeated every other day. The complete protection against hepatic metastasis observed in the present study allowed

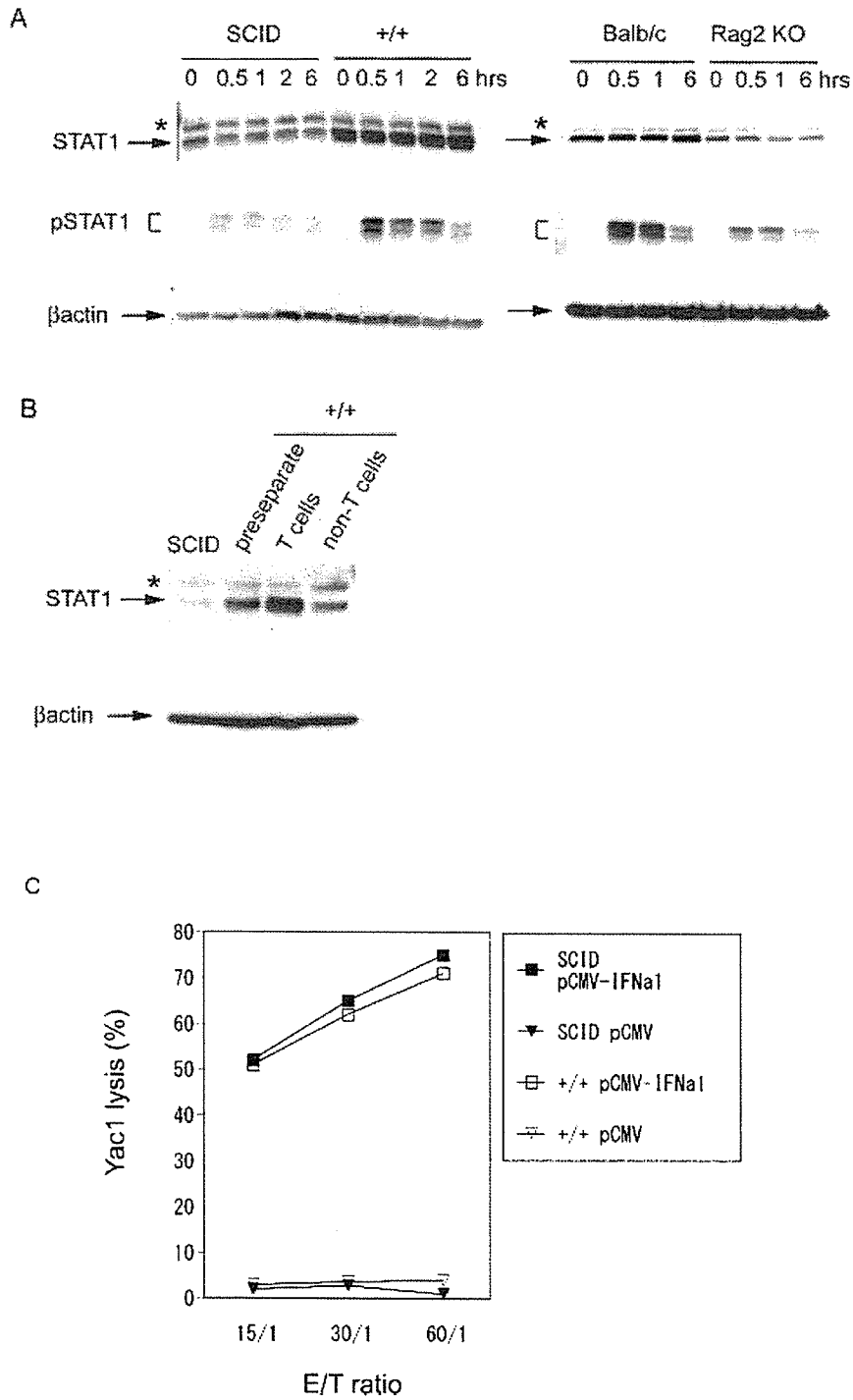

FIGURE 5 – IFNα-mediated NK cell activation and anti-metastatic effects in SCID mice. (*a*) STAT1 and phospho-STAT1 expression of splenocytes from SCID or Rag2 KO mice. Splenocytes were treated with 1,000 U/ml of IFNα and lysed at indicated time points (0 to 6 hr). Expression of STAT1 and phospho-STAT1 (pSTAT1) was analyzed by Western blot. +/+ and Balb/c indicate wild-type controls for SCID and Rag2 KO mice, respectively. *, non-specific band (see the *Material and methods* section). (*b*) STAT1 expression in T cells and non-T cells. Splenocytes from wild-type mice (+/+) were separated into T cells and non-T cells based on expression of CD90. Expression of STAT1 was analyzed by Western blot. SCID and preseparated wild-type splenocytes were included as controls. *, non-specific band (see the Material and methods section). (*c*) Yac1 lytic ability. SCID mice (closed symbols) or wild-type mice (open symbols) were hydrodynamically injected with either pCMV-IFNa1 (squares) or pCMV (triangles). Four days later, splenocytes isolated form the mice were examined for the lytic ability for Yac1 cells. Experiments were done at least 3 times and representative data are shown. (*d*) Anti-metastaic effects. SCID mice or wild-type mice were intrasplenically injected with CT-26 cells and hydrodynamically injected with either pCMV-IFNa1 or pCMV 2 days later. After 14 days, mice were sacrificed to examine tumor development in the liver. The numbers of hepatic tumors were compared among the groups. Experiments were performed 3 times and representative data are shown. *, $p < 0.05$.

us to investigate the adaptive response after antimetastatic effects. Mice that had rejected CT-26 cells by IFNα showed a tumor-specific T cell response and suppressed tumor growth of rechallenged skin tumor. Therefore, pCMV-IFNa1 injection not only caused initial rejection of metastasized tumors but also induced durable and systemic adaptive immunity. Interestingly, splenic injection of UV-irradiated CT-26 cells, even if followed by pCMV-IFNa1 injection, did not elicit significant tumor-specific T cell responses. Therefore, the efficient induction of adaptive T cell responses requires IFNα-mediated rejection of live tumor cells and cannot be recapitulated by simple injection of dead tumor cells and IFNα.

NK cells are present in a high percentage in the liver.[28] In the present study, we focused on NK cells which were rapidly activated by IFNα to examine the cellular mechanisms of protection against hepatic metastasis. Critical requirement of NK cells was demonstrated by anti-asialo GM1 antibody-injected mice which did not show protection against CT-26 metastasis. In contrast, T cells, B cells or NKT cells were dispensable for IFNα-mediated antimetastatic effects since IFNα therapy did show antimetastatic