rapid virological responders had a lower SVR rate. Eight (38%) patients showed SVR, 11 (52%) patients developed relapse after discontinuation of IFN, and two (10%) patients had no virological response.

Discussion

In Japan, pegIFN-α-2a monotherapy has been covered by public health insurance since December 2003. The standard duration of treatment with pegIFN-α-2a is 24 weeks for patients with low viral loads of genotype HCV-1 and any viral loads of genotype HCV-2 infection. Recent studies have reported that a treatment duration of more than 24 weeks in such cases does not increase the SVR rate.[11,13,21,22] Moreover, patients with early virological response seem to have a high rate of SVR.[23–25] In those patients, to reduce unnecessary exposure to treatment and its potential side-effects and to reduce costs, short-term IFN therapy has been used by several groups.[17–21] However, details of the IFN regimen differ from those of others and there are no studies that use short-term of pegIFN-α-2a treatment. We therefore conducted a prospective pilot study on the efficacies of an 8-week and 24-week pegIFN-α-2a regimen for patients with low viral titers of genotype HCV-1 and any viral titers of genotype HCV-2 who exhibited a virological response at 2 weeks after the initiation of IFN. In our study, patients with a relatively low viral load before the start of the IFN therapy tended to have a very early virological response.

Our results demonstrated that the virological response to the 8-week treatment (89.5% [17/19]) was excellent and was similar to the 24-week course (100% [15/15]). This high SVR rate of 8-week pegIFN-α-2a monotherapy seems as high as that reported in another short course study of 14-week pegIFN plus ribavirin combination treatment for patients with HCV genotype HCV-2 or HCV-3.[26] This high SVR rate of the 8-week course of pegIFN-α-2a may be associated with a rapid viral disappearance. Several studies have indicated that negative HCV-RNA at week 2 after the commencement of IFN is a predictor of SVR.[19,20,27,28] Therefore, for patients with a low HCV-1 viral load or those with HCV-2 infection with any viral load, we recommend switching to an 8-week course of pegIFN-α-2a monotherapy once they show an ultra rapid virological response, that is, negative HCV-RNA at week 2 from the start of IFN therapy. Furthermore, a longer course of IFN therapy with or without ribavirin can be prescribed when HCV-RNA becomes positive after discontinuation of the 8-week course of IFN therapy.

Although in our study all of the patients in the 24-week course showed SVR, it seems that 24 weeks is a long treatment period for those patients who become negative for HCV-RNA by week 2 of treatment to ascertain SVR. Our results showed that all patients in the 8-week course completed the course to the end of treatment. However, 33% of the patients of the 24-week course did not continue their treatment to the end of the course. Because patients tend to adhere to shorter regimens, which are also better tolerated than longer treatment regimens, a shorter exposure will probably translate into a better benefit–risk ratio in patients with early virological response.

Our study identified two relapsers among patients of the 8-week course after discontinuation of pegIFN-α-2a therapy. These two patients had a negative history of exposure to new HCV infection. One patient who had genotype HCV-2b and a high pretreatment viral load (HCV-RNA: 230KIU/mL) relapsed at 8 weeks after the discontinuation of IFN therapy, while the other who had genotype HCV-2a and a low pretreatment viral load (HCV-RNA: 50KIU/mL) relapsed at 12 weeks after the discontinuation of IFN therapy. These two patients could have SVR after additional IFN therapy for 24 weeks (one patient; pegIFN-α-2a monotherapy, one patient; pegIFN-α-2b and ribavirin combination therapy). We could not identify a definite factor associated with SVR or relapse. Although pretreatment factors, like genotype, viral load, and grade of fibrosis can be used to predict the mean treatment outcome for study cohorts, they are often of limited value in individual patients.[29,30]

As mentioned earlier, a short course of pegIFN-α-2a therapy for 8 weeks could be recommended in those patients who show an ultra rapid virological response at week 2 after the initiation of IFN therapy. Although the study by Shiffman *et al.*[31] demonstrates the inferiority of a shorter regimen in a large-scale, randomized, controlled study, the characteristics of their patients were largely different from those of our study, including racial difference (mostly Caucasian patients versus Japanese, a heavier body weight for the Caucasians versus Japanese patients) and differences in pretreatment viral load (variable and higher HCV-RNA level versus relatively low viral load in our patients). Patients' selection was also different between the two studies; our study was carried out only in cases negative for HCV-RNA at 2 weeks after the start of IFN compared to their randomized study, irrespective of a rapid virological response.

In conclusion, patients chronically infected with low titers of HCV-1 and those with HCV-2, regardless of their viral loads, who achieve an ultra rapid virological response, that is, HCV-RNA negativity at week 2, can receive only 8 weeks of pegIFN-α-2a monotherapy without compromising the chance of SVR. The results of our prospective study are encouraging, although the study population was small and was based on non-randomized methodology. The data of the present study are not conclusive for patients with very high pretreatment viremia who might achieve a rapid virological response or for those patients who do not achieve a rapid virological response. Further clinical trials are required to optimize the treatment duration in these patients.

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Eicosapentaenoic Acid Could Permit Maintenance of the Original Ribavirin Dose in Chronic Hepatitis C Virus Patients during the First 12 Weeks of Combination Therapy with Pegylated Interferon-α and Ribavirin

A Prospective Randomized Controlled Trial

Shintaro Takaki[a] Yoshiiku Kawakami[a] Michio Imamura[a] Hiroshi Aikata[a] Shoichi Takahashi[a] Hiroto Ishihara[b] Keiji Tsuji[c] Shiomi Aimitsu[d] Hiroiku Kawakami[e] Toshio Nakanishi[f] Mikiya Kitamoto[g] Takashi Moriya[h] Kenichi Satoh[i] Kazuaki Chayama[a]

[a]Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, [b]Department of Internal Medicine, Chuden Hospital, [c]Department of Internal Medicine, Hiroshima City Asa Hospital, [d]Department of Hepatology, Hiroshima Red Cross Hospital and Atomic Bomb Survivors Hospital, and [e]Kawakami Gastroenterology Clinic, Hiroshima; [f]Department of Gastroenterology, Shobara Red Cross Hospital, Shobara; [g]Department of Gastroenterology, Hiroshima Prefectural Hospital, [h]Department of Gastroenterology, Chugoku Rousai Hospital, and [i]Department of Environmetrics and Biometrics, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima, Japan

Key Words

Chronic hepatitis C · Ribavirin · PEG interferon · Combination therapy · Anemia · Eicosapentaenoic acid

Abstract

Objective: To evaluate the efficacy of eicosapentaenoic acid (EPA) against ribavirin (RBV)-associated hemolytic anemia during the first 12 weeks in chronic hepatitis C virus (HCV) combination therapy. ***Methods:*** This study was a prospective open-label, randomized controlled trial. 100 HCV patients were randomized to either the EPA group (n = 49) or non-EPA group (n = 51) who received combination therapy with or without EPA. We compared the changes in hemoglobin level and RBV plasma concentrations at week 12 in each group with RBV dose reduction rate and performed multivariate analysis to identify independent variables associated with RBV dose reduction. ***Results:*** 8 patients (17%) in the EPA group and 20 patients (29%) in the non-EPA group required RBV dose reduction, respectively. The cumulative RBV reduction rate was significantly lower in the EPA group than in the non-EPA group (p = 0.017), while the decrease of hemoglobin and RBV plasma concentrations from baseline was not significantly different. However, in the multivariate analysis, treatment with EPA showed significant variables for the reduction of RBV dose (odds ratio 3.235, p = 0.023). ***Conclusion:*** EPA could prevent the RBV dose reduction during the first 12 weeks in combination therapy, although further large-scale double-blind randomized controlled trials are required.

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Introduction

Chronic hepatitis C virus (HCV) infection, the most common cause of chronic liver disease worldwide, can lead to cirrhosis and hepatocellular carcinoma. HCV infects an estimated 170 million individuals worldwide, and 2 million people in Japan are infected [1–4]. Pegylated in-

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Shintaro Takaki, Department of Medicine and Molecular Science
Division of Frontier Medical Science, Programs for Biomedical Research
Graduate School of Biomedical Sciences, Hiroshima University
1-2-3, Kasumi, Minami-ku, Hiroshima 734-8551 (Japan)
Tel. +81 82 257 5192, Fax +81 82 257 5194, E-Mail takakiss@hiroshima-u.ac.jp

terferon-α (PEG-IFN-α) plus ribavirin (RBV), which currently represent standard treatment for chronic HCV infection, can increase sustained virological response (SVR) [5–11]. Some studies have reported a decrease in virus level during the several weeks after starting treatment, which might correlate with the likelihood of SVR [12–16]. Davis et al. [16] reported that most patients who are able to complete the first 12 weeks of therapy achieve an early virological response and have a high probability of SVR.

Although high RBV doses offer the best chance of a SVR, they increase the risk of hemolytic anemia. RBV-associated hemolysis, which frequently results in anemia, may exacerbate symptoms such as fatigue and shortness of breath, resulting in reduced patient compliance [7–11]. This common side effect of RBV is dose-related and usually resolves when therapy is discontinued [7–19] but the mechanism causing this hemolytic anemia is unknown. Previous studies reported that none of the patients who discontinued therapy achieved SVR, and adherence to combination therapy enhanced SVR in genotype 1 HCV patients [20, 21].

Eicosapentaenoic acid (EPA), used widely to treat hyperlipidemia and atherosclerosis [23, 24], is readily assimilated into erythrocyte membrane phospholipids and correlates positively with erythrocyte deformability [25]. In an uncontrolled pilot study by Ide et al. [26], 6 patients given EPA after the development of anemia showed significant increases in mean hemoglobin (Hb) values. Although these prospective study data indicate that EPA may suppress RBV-induced anemia, the efficacy of EPA for combination therapy is uncertain. Furthermore, no systematic studies have been published.

In the present study, we assessed HCV-infected patients receiving PEG-IFN-α and RBV combination therapy to determine whether efficacy of EPA against RBV-associated hemolytic anemia was sufficient to avoid RBV dose reduction during the early period of combination treatment.

Method

For this prospective open-label randomized controlled trial, 102 patients chronically infected with HCV genotype 1b at a high viral load were enrolled between December 2004 and January 2005. This protocol was approved by the local ethics committees, and was conducted according to the principles of the Helsinki Declaration. Patients from 14 hospitals in the Hiroshima Liver Study Group were included after they gave informed written consent and were found to meet all of the following inclusion criteria: infection with HCV genotype 1b at a high viral load (>100 kIU/ml) according to seropositivity for anti-HCV antibodies, using a third-generation enzyme-linked immunosorbent assay, and serum HCV RNA determination using a quantitative polymerase chain reaction assay; Hb of at least 12.0 g/dl; compensated liver disease; age of at least 18 years; no hepatocellular carcinoma, and no bleeding tendency.

Exclusion criteria were decompensated liver disease; autoimmune disease; clinically significant cardiovascular, metabolic, renal, hematologic, neurologic, or psychiatric disease; systemic infections; neoplastic disease; systemic immunosuppressive treatment; active alcohol or drug abuse within the previous year, and pregnancy.

All patients received both PEG-IFN-α_{2b} (PegIntron; Schering Plough, Kenilworth, N.J., USA), 1.5 μg/kg/week, s.c., and oral RBV at 600 mg/day (<60 kg body weight (BW)), 800 mg/day (between 60 and 80 kg BW), or 1,000 mg/day (>80 kg BW), with the manufacturer's drug information for RBV. The lower doses of RBV rather than the higher doses were chosen because of the difference in weight between the Japanese and American population. All patients were assigned randomly to either the EPA group or non-EPA group. Randomization was carried out using sealed envelopes and a computer-generated randomization list. EPA patients received oral EPA (Epadel S900; Mochida Pharmaceutical, Tokyo, Japan) at 900 mg/day divided into two daily doses throughout treatment.

All patients were evaluated for treatment efficacy and safety by each attending a doctor based on the World Health Organization classification of adverse events. Hematologic, biochemical, and virological parameters were determined by the local laboratories at each study center.

For anemia, the daily RBV dose was reduced by 200 mg when Hb fell to <10 g/dl, acute decrease and remains of Hb concentrations >3 g/dl from baseline, or clinical symptoms of anemia associated with a decrease of Hb >2 g/dl from the start of treatment, for example, palpitation, dyspnea on efforts, and fatigue. Once lowered, the RBV dose was used throughout the rest of the study. When patients complained of symptom fatigue or pallor, RBV was discontinued when Hb fell to <8.5 g/dl or when patients manifested more severe anemia including orthostatic hypotension. These RBV dose reduction criteria were adhered to in each center.

The primary efficacy endpoint of this study was comparison of the RBV dose reduction rate in each group to determine whether EPA maintains RBV dosage. The second endpoint was changes in Hb level between treatment groups and change in RBV dosage and plasma concentrations at week 12; all patients who were randomized and received this therapy were included in this analysis. The RBV plasma concentrations were determined by a validated high-performance liquid chromatography tandem mass spectrometric assay using ^{13}C-RBV as an internal standard [27, 28]. However, for patients who discontinued before week 12, the last available data were used in this study. Since pharmacokinetic studies showed that serum RBV concentrations take 4–8 weeks to reach a plateau, we chose week 12 as the endpoint in order to evaluate the influence of RBV-induced anemia.

Statistical Analysis

To detect a difference in Hb levels of 2 g/dl, using a two-sided test with a significance level of 0.05 and a power of 90%, 35 patients were required for each group. To compensate for non-evaluable patients, we planned to enroll 50 patients per group.

The χ^2 test or the Mann-Whitney U test was used for statistical comparisons between groups, as appropriate. Treatment outcomes

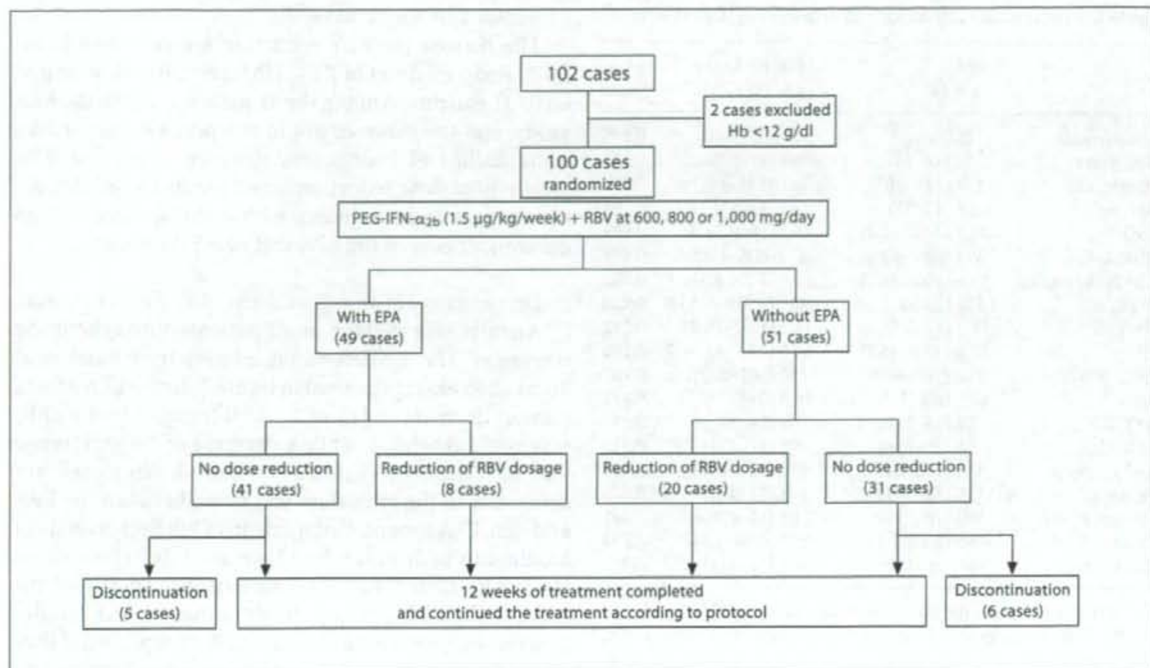

Fig. 1. Flow chart showing progression of participants throughout the protocol.

were analyzed on an intention-to-treat basis. Cumulative reduction rates of RBV dose were calculated using the Kaplan-Meier method and compared by the log-rank test. Multivariate analysis (multivariate logistic regression) was used to identify factors independently associated with RBV dose reduction. The influence of 19 variables on RBV dose reduction required by RBV-induced anemia was examined. Variables considered included: gender, age, height, pretreatment body weight (BW), body mass index (BMI), dose of RBV (absolute and per kg BW), pretreatment Hb, hematocrit (Ht), pretreatment red blood cell count (RBC), white blood cell count (WBC), platelet counts (PLT), pretreatment serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol (T-cho), iron (Fe), ferritin, creatinine (Cr), creatinine clearance (Ccr), and treatment with EPA.

All p values <0.05 were considered to indicate statistical significance. Calculations were performed with the SPSS Statistical Package, Version 11.0 (SPSS, Chicago, Ill., USA).

Results

Among 102 patients with HCV genotype 1b at a viral load of at least 100 kIU/ml, 2 were excluded because of an Hb value <12 g/dl. The remaining 100 patients were randomized to receive or not receive EPA. The baseline characteristics are summarized in table 1, and the flow chart in figure 1 shows the progression of participants from screening to 12 weeks of treatment.

EPA Group

Of 49 patients randomized to receive combination therapy with EPA, 41 needed no RBV reduction, while 8 patients required RBV dose reduction within 12 weeks of beginning treatment. Among them, 5 withdrew from the study before completing 12 weeks because of neutropenia depression and were lost to follow-up, all of them stopped the treatment without RBV dose reduction. Therefore, 44 patients completed the initial 12 weeks and continued according to the protocol. Patient compliance was not affected by EPA. No untoward side effects were attributed to EPA in this trial.

Non-EPA Group

Of 51 patients randomized to receive combination therapy without EPA, 31 needed no RBV reduction, while 20 required RBV dose reduction within 12 weeks of be-

Table 1. Baseline characteristics and valuables of the patients

	EPA (n = 49)	Non-EPA (n = 51)	P value
Male/female	25/24	24/27	0.699
Age, years	57 (18–74)	58 (21–72)	0.833
Height, cm	157 (141–167)	161 (144–179)	0.672
BW, kg	58 (42–91)	62 (42–85)	0.194
BMI	24.3 (20.0–32.0)	23.2 (18.8–29.9)	0.072
RBV, mg	694 (600–800)	670 (600–1,000)	0.465
RBV/BW, mg/kg	11.6 (10.2–14.5)	11.6 (7.7–14.3)	0.302
WBC, μ/l	4,520 (2,690–7,400)	4,480 (2,410–7,400)	0.220
Hb, g/dl	14.2 (12.1–16.3)	14.3 (12.0–16.8)	0.532
Ht, %	37.5 (30.8–44.2)	39.0 (33.1–47.0)	0.180
RBC, $\times 10^4/\mu l$	410 (317–490)	430 (362–522)	0.103
Plt, μ/l	14.9 (8.5–27)	14.5 (7–57)	0.171
AST, IU/l	58 (18–213)	62 (24–187)	0.343
ALT, IU/l	65 (18–256)	78 (21–217)	0.315
T-cho, mg/dl	133 (102–249)	144 (121–235)	0.933
Fe, μg/dl	116 (65–294)	105 (51–301)	0.872
Ferittin, ng/ml	190 (16–724)	203 (13–1,024)	0.081
Cr, mg/dl	0.68 (0.40–1.1)	0.67 (0.50–1.1)	0.753
Ccr, ml/min	98.1 (56–179)	94.1 (51–191)	0.351

Values are given as median (minimum–maximum).

BW = Body weight; BMI = body mass index; RBV = ribavirin; WBC = white blood cell count; Hb = hemoglobin; Ht = hematocrit; RBC = red blood cell count; PLT = platelet counts; AST = aspartate aminotransferase; ALT = alanine aminotransferase; T-cho = total cholesterol; Fe = iron; Cr = creatinine; Ccr = creatinine clearance.

Table 2. Reason for RBV dose reduction during 12 weeks after starting combination treatment

	EPA (n = 8)	Non-EPA (n = 20)	Total (n = 28)
(1) Hb levels <10 g/dl	1	10	11
(2) Acute decrease of Hb levels >3 g/dl	0	2	2
(3) Clinical symptoms of anemia associated with a decrease of Hb levels >2 g/dl	7	8	15

ginning treatment. Among them, 6 withdrew from the study before 12 weeks because of adverse effects including depression, thrombopenia, pneumonia, and were lost to follow-up. 45 patients completed the initial 12 weeks and continued according to the protocol. No patient required a blood transfusion and no other adverse effects were noted in either of the groups.

Reasons for Reduction of RBV

The reasons for RBV reduction are described in table 2. Reduced doses of RBV, Hb level <10 g/dl, were given to 11 patients. Among the 11 patients, 1 is in the EPA group and the other 10 are in the non-EPA group. An acute decline of anemia was observed in the non-EPA group. RBV dose reduction was done in 15 patients because of clinical symptoms with anemia; there was no difference between the EPA and non-EPA group.

Decreases in Hb and Cumulative RBV Reduction Rate

Anemia was present in all patients throughout the treatment. The median decrease in Hb from baseline at 4 and 12 weeks is presented in figure 2. In the EPA group, a mean decrease in Hb of 2.1 g/dl (range 0 to 4.6 g/dl) was noted at week 4, while a decrease of 2.4 g/dl (range –0.1 to 6.8 g/dl) was noted at week 12. No significant difference in the Hb values was noted between the EPA and non-EPA groups. Comparison of Hb decreases from baseline in each group by 12 weeks is described in table 3. Although there is no significance in any of the groups, the EPA group is likely to have lower Hb decreases compared with the non-EPA group, even if they did not reduce RBV. The initial Hb level distributions are described in figure 3. No difference was observed between the EPA and non-EPA groups after distribution of initial Hb levels.

However, the cumulative RBV dose reduction rate during 12 weeks of treatment (EPA 17%, non-EPA 29%) was significantly lower in the EPA group than the non-EPA group (fig. 4). In the non-EPA group, respective mean RBV plasma concentrations at weeks 4 and 12 were 2,349 ± 897 and 2,249 ± 874 mg/ml. In the EPA group, respective mean RBV plasma concentrations at weeks 4 and 12 were 2,201 ± 887 and 2,508 ± 705 mg/ml. Although the EPA group had a higher RBV concentration at 12 weeks, differences in RBV concentrations between the EPA group and non-EPA group were not significant (table 4).

Uni- and Multivariate Analysis

Variables were analyzed for the relationship to RBV dose reduction by both uni- and multivariate methods. Gender, age, height, BW, BMI, dose of RBV (both absolute and by patient BW), pretreatment Ht concentration, RBC, WBC, PLT, AST, ALT, T-cho, Fe, ferritin, Cr, and Ccr were not associated with development of anemia by univariate analysis, while higher pretreatment Hb and treatment with EPA were related to maintenance RBV dose. By multivariate analysis, only treatment with EPA

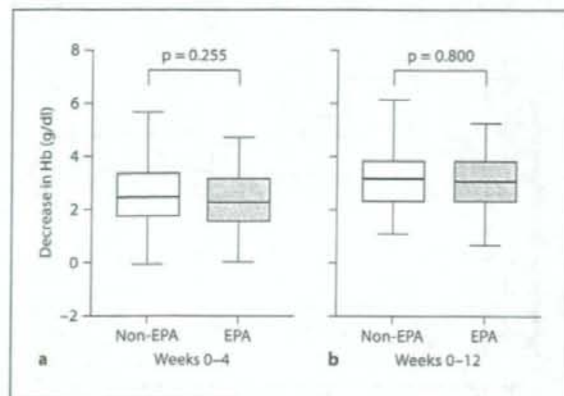

Fig. 2. Box-and-whisker plot comparing the decrease of Hb from baseline at week 4 (**a**) and at week 12 (**b**). No significant difference was seen between the EPA and non-EPA groups (week 4, p = 0.255, week 12, p = 0.800).

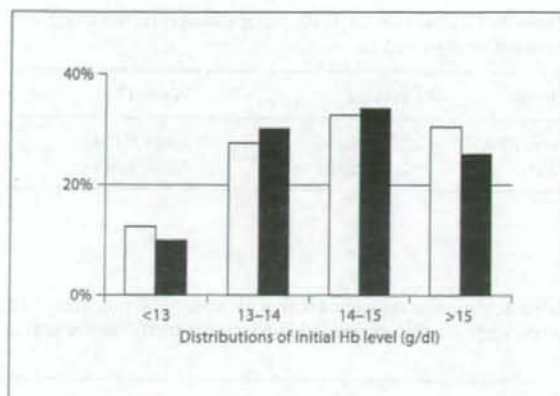

Fig. 3. No difference was observed between the EPA and non-EPA groups after distributing initial Hb levels. □ = EPA; ■ = non-EPA.

Table 3. Comparison of Hb decrease from baseline in each group by 12 weeks

	EPA (n = 44)			Non-EPA (n = 45)		
	RBV reduction (n = 8)	no reduction (n = 36)	p	RBV reduction (n = 20)	no reduction (n = 25)	p
4 weeks	2.4 (1.1)	1.7 (1.4)	0.08	2.8 (0.9)	2.3 (1.7)	0.31
8 weeks	2.5 (1.2)	2.1 (1.6)	0.46	3.3 (1.2)	2.5 (1.1)	0.09
12 weeks	2.9 (1.5)	2.4 (1.2)	0.30	3.1 (0.9)	2.8 (1.2)	0.35

Values are given as mean (SD).

significantly and independently reduced the likely need for reduction of RBV dose (odds ratio 3.235, p = 0.023) (table 5).

Virological Response

In the EPA group, RVR (rapid virological response), EVR (early virological response) and ETR (end of treatment response) were achieved in 8 of 49 (16%), 27 of 49 (55%), and 30 of 49 patients (61%), whereas in the non-EPA group, RVR, EVR and ETR were achieved in 16 of 51 (31%), 33 of 51 (65%), and 35 of 51 patients (68%), respectively. Since after the end of the treatment some patients in each group continued the combination therapy, the number of patients with SVR decreased. SVR was achieved by 15 of 36 (42%) in the EPA group and by 19 of 40 (45%) in the non-EPA group, respectively. In each viral response, no significant differences were achieved in either of the groups.

Discussion

The results of this study demonstrated that with EPA treatment anemic HCV-infected patients undergoing combination therapy more often could avoid RBV dose reduction in the first 12 weeks of therapy, although a lower pretreatment increased the likelihood of RBV dose reduction. To our knowledge this is the first reported randomized clinical trial of EPA in combination therapy for chronic hepatitis C.

Table 4. Comparison of RBV plasma concentrations (mg/ml) between weeks 4 and 12

Group	Week 4	p	Week 12	p
Non-EPA EPA	2,349 ± 897 2,201 ± 887	0.899	2,249 ± 874 2,508 ± 705	0.101

Table 5. Variables associated with RBV dose reduction during 12 weeks after starting combination treatment (multivariate analysis)

Variable	Category	OR	95% CI	p
Hb	(1) >13 g/dl (2) <13 g/dl	1 2.480	0.620–9.919	0.199
EPA	(1) With EPA (2) Without EPA	1 3.235	1.173–8.920	0.023

OR = Odds ratio; 95% CI = 95% confidence interval.

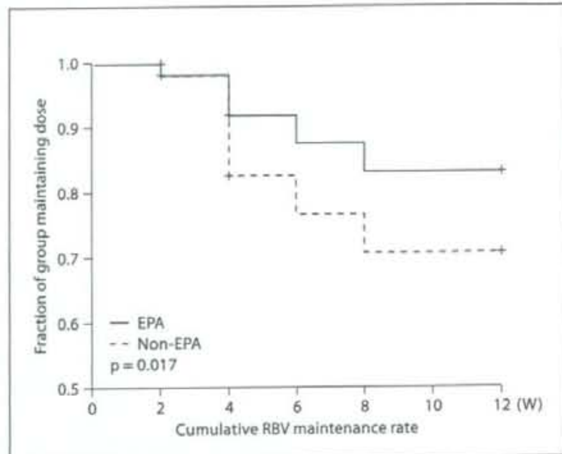

Fig. 4. Cumulative RBV dose maintenance rates during 12 weeks of treatment were 83% for the EPA and 71% for the non-EPA (RBV dose reduction rate: 17% for the EPA and 29% for the non-EPA), respectively. The original dose could be maintained in significantly more EPA than non-EPA patients (p = 0.017).

Many previous studies reported combination therapy with PEG-IFN and RBV for 24–48 weeks to be effective in treating chronic hepatitis C [1–5]. The most frequent toxic side effect of RBV is a reversible hemolytic anemia, which requires some patients to reduce or discontinue combination therapy. The mechanism underlying the hemolytic anemia is unclear, but is thought to involve accumulation of RBV triphosphate in erythrocytes [18, 19]. The effect of RBV accumulation on cellular respiration reduces the half-life of erythrocytes through extravascular hemolysis. EPA, the principal fatty acid in fish oils, has a wide variety of pharmacologic actions including an increase in deformability of erythrocytes [22]. EPA has been shown to improve erythrocyte deformability by incorporation into erythrocyte membrane phospholipid [23]. While Hino et al. [29] show a decrease of EPA content in erythrocyte membrane phospholipids in HCV patients with IFN and RBV therapy, it is thought that EPA supplementation would be useful for preventing RBV-induced anemia. Ide et al. [26] reported that EPA increased Hb in patients with RBV-related anemia. However, clinical trials have been limited to a pilot study, and a randomized trial was needed. In performing such a trial we focused on whether EPA administration diminished RBV-associated hemolysis and improved patient tolerance sufficiently to avoid RBV dose reductions in patients with chronic hepatitis C treated with a combination therapy. However, in our results there was no difference of decrease in Hb from baseline at 4 and 12 weeks. It is considered that the anemia symptom led to reduce the RBV dose before 10 g/dl for all anemia patients, therefore in the present studies no significant difference in Hb values was noted between the EPA and non-EPA groups. It is thought that EPA could be useful to decrease the symptom of RBV-induced anemia.

Previous studies have found full-dose RBV to be important in the early phase after initiating treatment. McHutchinson et al. [30] reported that patients who were unable to adhere to full-dose therapy during the first 12 weeks after initiating treatment had a lower rate of SVR than patients who did not require dose reduction until after week 12. Shiffman et al. [31] reported that reducing the RBV dose to <80 or 60% of the starting dose during the first 20 weeks of treatment was associated with a decline in SVR rate from 21 to 11% at 72 weeks. Furthermore, previous studies suggested that high RBV concentrations could contribute to the improvement in viral response in this group of patients [31–34].

Since the RBV concentrations remained stable after 4–8 weeks of treatment [5, 6], it is thought that RBV does not contribute to the antiviral effect during 4 weeks after the beginning of combination treatment. However, Arase

et al. [34] showed that a higher RBV concentration contributes to SVR and also showed that in order to raise the RBV concentration it is important to maintain a RBV dose in the early phase after the beginning of combination treatment. In our study, although RVR was lower in the EPA group, EVR, ETR, and SVR showed almost equal results between the EPA group and non-EPA group. We consider it is one of the reasons why RBV dose maintenance during the early phase would be more effective in the EPA group than in the non-EPA group in spite of other factors (e.g. IFN sensitivity, LDL-C, G-GTP, ICG-R15, and amino acid substitutions in the core region [35–38]). However, in our study, patients had combination treatment during the initial 12 weeks, but the effect of combination therapy with EPA on HCV was unknown. It is thought that further investigation is needed.

In summary, giving EPA could permit maintenance of the original RBV dose in HCV patients during the first 12 weeks of PEG-IFN-α and RBV combination therapy. However, since this study was of only intermediate size and an open-label study, which might introduce a bias to reduce the dose of RBV, further large-scale double-blind randomized controlled trials are required to investigate whether EPA could be useful for RBV hemolytic anemia.

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Susceptibility of Chimeric Mice with Livers Repopulated by Serially Subcultured Human Hepatocytes to Hepatitis B Virus

Rie Utoh,[1] Chise Tateno,[1,2] Chihiro Yamasaki,[1] Nobuhiko Hiraga,[3] Miho Kataoka,[1] Takashi Shimada,[4] Kazuaki Chayama,[2,3] and Katsutoshi Yoshizato[1,2,5]

We previously identified a small population of replicative hepatocytes in long-term cultures of human adult parenchymal hepatocytes (PHs) at a frequency of 0.01%-0.09%. These hepatocytes were able to grow continuously through serial subcultures as colony-forming parenchymal hepatocytes (CFPHs). In the present study, we generated gene expression profiles for cultured CFPHs and found that they expressed cytokeratin 19, CD90 (Thy-1), and CD44, but not mature hepatocyte markers such as tryptophan-2,3-dioxygenase (TO) and glucose-6-phosphatase (G6P), confirming that these cells are hepatic progenitor-like cells. The cultured CFPHs were resistant to infection with human hepatitis B virus (HBV). To examine the growth and differentiation capacity of the cells *in vivo*, serially subcultured CFPHs were transplanted into the progeny of a cross between albumin promoter/enhancer-driven urokinase plasminogen activator-transgenic mice and severe combined immunodeficient (SCID) mice. The cells were engrafted into the liver and were able to grow for at least 10 weeks, ultimately reaching a maximum occupancy rate of 27%. The CFPHs in the host liver expressed differentiation markers such as TO, G6P, and cytochrome P450 subtypes and could be infected with HBV. CFPH-chimeric mice with a relatively high replacement rate exhibited viremia and had high serum levels of hepatitis B surface antigen. *Conclusion:* Serially subcultured human hepatic progenitor-like cells from postnatal livers successfully repopulated injured livers and exhibited several phenotypes of mature hepatocytes, including susceptibility to HBV. *In vitro*–expanded CFPHs can be used to characterize the differentiation state of human hepatic progenitor-like cells. (HEPATOLOGY 2008;47:435-446.)

Abbreviations: 9MM, 9-month-old Caucasian male; 10YF, 10-year-old Caucasian female; 12YM, 12-year-old Asian male; 16YF, 16-year-old Asian female; AAT, α1-antitrypsin; AFP, α-fetoprotein; ALB, albumin; BGP, biliary glycoprotein; BrdU, 5-bromo-2'-deoxyuridine; CFPH, colony-forming parenchymal hepatocyte; CK, cytokeratin; G6P, glucose-6-phosphatase; h, human; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; CYP, cytochrome P450; m, mouse; MDR, multidrug resistance protein; MRP, multidrug resistance-associated protein; PH, parenchymal hepatocyte; RI, replacement index; RT-PCR, reverse-transcription polymerase chain reaction; SH, small hepatocyte; TO, tryptophan-2,3-dioxygenase; uPA, urokinase plasminogen activator.

From the [1]Yoshizato Project, Cooperative Link of Unique Science and Technology for Economy Revitalization (CLUSTER), Hiroshima Prefectural Institute of Industrial Science and Technology, Hiroshima, Japan; the [2]Hiroshima University Liver Project Research Center, Hiroshima, Japan; the [3]Division of Frontier Medical Science, Department of Medicine and Molecular Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan; [4]PhoenixBio Co., Ltd., Hiroshima, Japan; and the [5]Developmental Biology Laboratory and Hiroshima University 21st Century COE Program for Advanced Radiation Casualty Medicine, Department of Biological Science, Graduate School of Science, Hiroshima University, Hiroshima, Japan.

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Present address for Rie Utoh: Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, Tokyo, Japan.

Present address for Chise Tateno, Chihiro Yamasaki, and Katsutoshi Yoshizato: PhoenixBio Co., Ltd., Hiroshima, Japan.

Address reprint requests to: Katsutoshi Yoshizato, Ph.D., PhoenixBio Co., Ltd., 3-4-1 Kagamiyama, Higashihiroshima, Hiroshima 739-0046, Japan. E-mail: katsutoshi.yoshizato@phoenixbio.co.jp; fax: (81)-82-431-0017.

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Studies using rodents with damaged livers have shown that parenchymal hepatocytes (PHs) have great growth potential. When mouse (*m*) hepatocytes were transplanted into the livers of albumin promoter/enhancer-driven urokinase plasminogen activator (uPA)-transgenic mice,[1] they engrafted and repopulated the host liver. Serial transplantation experiments using *m*-hepatocytes in mice with tyrosinemia showed their enormous growth capacity.[2] The replicative potential of rat hepatocytes has also been demonstrated by transplanting them into the partially hepatectomized liver of a retrorsine-treated rat,[3] and uPA-transgenic mice crossed with severely immunodeficient mice, such as severe combined immunodeficient (SCID)/beige mice,[4] SCID mice,[5,6] or recombination activation gene 2 knockout mice[7] have been used to show the growth potential of human (*h*)-hepatocytes. When transplanted into uPA/SCID mice, PHs from a human juvenile male grew in the host liver to a level at which the proportion (replacement index) of the area of repopulated *h*-hepatocytes to the total number (host and donor) of hepatocytes reached 96% at 64 days posttransplantation.[5] Such *h*-hepatocyte–chimeric mice have been used to study the pharmacological responses of *h*-hepatocytes[5] and to investigate *h*-hepatitis viral infections.[4,6-8]

In contrast, normal hepatocytes have limited replicative capacity *in vitro* and acquire an abnormal phenotype if they are cultured for extended periods.[9,10] Studies on hepatocytes cultured in a newly devised medium (hepatocyte clonal growth medium[11,12]) revealed a subpopulation of highly replicative PHs, known as small hepatocytes (SHs), in both rats[12] and humans.[13] Their occupancy rate in *h*-liver ranged from 0.01% to 0.09% and was dependent on donor age.[13] The *h*-SHs formed colonies and grew continuously through several subcultures, which led us to name them colony-forming PHs (CFPHs).[13] Replication of the CFPHs was donor age–dependent up to passage 7 (p = 7),[13] and the cells did not exhibit a normal hepatocytic phenotype. Instead, they exhibited the traits of hepatocytes or biliary cells depending on the culture conditions. In addition, the CFPHs were not susceptible to infection with hepatitis B virus (HBV) (unpublished data).

In this study, we generated gene expression profiles of CFPHs and transplanted serially subcultured CFPHs into homozygous uPA/SCID mice to examine their growth and differentiation capacity. Our results indicate that the cells were engrafted onto the liver parenchyma and repopulated the tissue, ultimately differentiating into mature hepatocytes. Importantly, the *in vitro*–propagated CFPHs became susceptible to infection with HBV. This study supports our previous suggestion that CFPHs from *h*-postnatal liver are hepatic progenitor-like cells with the potential to assume a normal hepatocytic phenotype.[13]

Materials and Methods

h-*Hepatocytes.* This study was performed with the approval of the Hiroshima Prefectural Institute of Industrial Science and Technology Ethics Board. PHs were isolated as described[13,14] from livers donated by a 12-year-old Asian male (12YM) and a 16-year-old Asian female (16YF) according to the guidelines of the 1975 Declaration of Helsinki. Cryopreserved PHs from a 9-month-old Caucasian male (9MM) and a 10-year-old Caucasian female (10YF) were obtained from In Vitro Technologies (Baltimore, MD) and BD Biosciences (San Jose, CA), respectively.

Culture of CFPHs. Cryopreserved PHs from the 9MM, 12YM, and 16YF were thawed[5] and serially subcultured to obtain *in vitro*–expanded CFPHs.[13] Commercial 9MM PHs and freshly isolated 12YM and 16YF PHs were each subcultured to p = 3. The expanded cells were then cryopreserved, thawed upon use, and cultured on collagen-coated plates for 14-20 days as described.[13]

Flow Cytometry. We detached 12YM CFPHs (p = 4 or 5) from culture plates by treatment with 0.25% Trypsin-EDTA (Invitrogen, Carlsbad, CA), suspended, incubated on ice for 30 minutes with *m*-monoclonal antibodies against *h*Thy-1 (clone F15-42-1; Chemicon, Temecula, CA), and incubated with antibodies against *m*-immunoglobulin G Alexa-488 (Molecular Probes, Eugene, OR). We used *m*-immunoglobulin G_1 as a negative control. The cells were then analyzed and separated using a fluorescence-activated cell sorter (Becton Dickinson, Franklin Lakes, NJ) as reported.[12]

Transplantation of PHs and CFPHs. We detached 9MM and 12YM CFPHs (p = 4) from their culture plates and treated for 1 hour with DMEM containing 10% fetal bovine serum and 3 μg/mL anti–*h*-integrin $\alpha 1$ monoclonal antibodies (clone FB12, Chemicon).[15] This procedure improved engraftment of the CFPHs in uPA/SCID *m*-liver and reduced host mortality.

Transplantation of PHs and CFPHs was performed as described previously.[5] Homozygous uPA/SCID mice were injected with 0.75×10^6 9MM and 12YM PHs or $0.75\text{-}1.0 \times 10^6$ *in vitro*–expanded 9MM and 12YM CFPHs into the inferior splenic pole. When necessary, 10 mM 5-bromo-2′-deoxyuridine (BrdU) (Sigma, St. Louis, MO) and 1.2 mM 5-fluoro-2′-deoxyuridine (Wako, Osaka, Japan) in saline were injected intraperitoneally into the mice at 10 μL/g body weight 1 hour prior to death. The animals were treated according to the guidelines of our local committee on animal experiments.

Table 1. Summary of CFPH and PH Transplantation Experiments in uPA/SCID Mice

Group	Donor Cells	Time of Sacrifice (Weeks After Transplantation)	No. of Transplanted Mice	No. of Mice with Engraftment* [RE (%)]	RI† [Mean ± SD (n)]
A	12YM CFPHs (p = 4)	3	9	3 (33)	0.06-0.19% [0.14 ± 0.07% (n = 3)]
B	9MM CFPHs (p = 4)	3	6	4 (67)	0.03-0.05% [0.04 ± 0.01% (n = 4)]
C	9MM PHs	3	3	3 (100)	5.1-19.4% [6.4 ± 2.9% (n = 3)]
D	12YM CFPHs (p = 4)	9-10	27	14 (52)	0.2-27.0% [6.6 ± 8.3% (n = 14)]
E	9MM PHs	10-11	23‡	23 (100)‡	32.6-82.2% [57.4% (n = 2)]
F	12YM PHs	10	6	4 (67)	31.0-77.0% [62.3 ± 23.8% (n = 4)]
G§	12YM CFPHs (p = 4)	17-20	4	ND	ND

Abbreviation: ND, not determined.

*Number of mice whose livers were engrafted with transplanted PHs or CFPHs. The RE was determined via *h*ALB immunohistochemistry on sections prepared from 5 lobes of a liver.

†Ranges of RI of chimeric mice used in each group.

‡Data from Tateno et al.[5]

§Mice from group G were used for HBV infection studies.

We transplanted 9MM and 12YM CFPHs into 6 and 40 uPA/SCID mice, respectively. The mice were then killed 3, 9, or 10 weeks later, depending on the experimental purpose. In a previous report, we used 9MM and 12YM PHs as donor cells.[5] In this study, we used some of the preserved livers from these mice for histological examinations and as sources of RNA for reverse-transcription polymerase chain reaction (RT-PCR) analysis. The mice used in our transplantation experiments were separated into 7 groups (A-G) as shown in Table 1, which includes the rates of engraftment and replacement indices (RIs) of the chimeric mice.

Blood samples (5 μL) were collected periodically after transplantation from the tail veins of the hosts, and the level of *h*-albumin (ALB) in each was determined using a Human Albumin ELISA Quantitation Kit (Bethyl Laboratories, Montgomery, TX) to monitor the growth of the transplanted CFPHs.

RT-PCR. An RNeasy Tissue Kit (Qiagen, Valencia, CA) was used to isolate total RNA from freeze-thawed 9MM and 10YF PHs, cells of the *h*-hepatoma cell line HepG2, and 12YM and 16YF CFPHs (p = 4). RNA was also isolated with Isogen (Nippon Gene, Tokyo, Japan) from the livers of homozygous uPA/SCID mice and mice chimeric for 12YM PHs or 12YM CFPHs. Each RNA sample was treated with deoxyribonuclease (Takara Bio, Kyoto, Japan) and used as the template for RT-PCR. The RNA (1 μg) was reverse-transcribed with random hexamers using PowerScript Reverse Transcriptase (Clontech, Kyoto, Japan). All reactions were performed with Ex Taq (Takara Bio). Semiquantitative PCR was performed to allow linear amplification of the targets. The following *h*-specific or *m* and *h* cross-reactive genes were subjected to RT-PCR under the conditions shown in Supplementary Table 1: ALB, α1-antitrypsin (AAT), tryptophan-2,3-dioxygenase (TO), glucose-6-phosphatase (G6P), α-fetoprotein (AFP), cytokeratin 19 (CK19), biliary glycoprotein (BGP), Thy-1, CD44, multidrug resistance protein 1 (MDR1), multidrug resistance-associated protein 1 (MRP1), MRP2, and glyceraldehyde-3-phosphate dehydrogenase.

In Situ *Hybridization.* Cryosections (7 μm thick) were fixed with 4% paraformaldehyde, then incubated with 100 ng/mL proteinase K for 10 minutes at 37°C. The sections were then treated at 90°C for 6 minutes and hybridized for 2 hours at 37°C with biotinylated h-DNA probes (Dako, Glostrup, Denmark). The sections were also used to detect whole *h*-genomic DNA using the GenPoint System (Dako) according to the manufacturer's instructions. Finally, they were stained with hematoxylin-eosin.

Immunohistochemistry and Histochemistry. Formalin-fixed livers were embedded in paraffin and sectioned 5 μm thick. The sections were heated in a microwave oven for 5 minutes in Target Retrieval Solution (Dako), then placed at room temperature for 20 minutes. The livers used to generate frozen sections were embedded in OCT compound (Sakura Finechemicals, Tokyo, Japan), frozen in liquid nitrogen, and sectioned 5 μm thick. The cultured cells were fixed in cold ethanol for 10 minutes. The primary antibodies and conditions used for immunohistochemistry are listed in Supplementary Table 2. For bright-field immunohistochemistry, the antibodies were visualized using a Vectastain ABC Kit (Vector Laboratories, Burlingame, CA) using DAB substrates. Fluorescense immunohistochemistry was performed using Alexa 488–conjugated or Alexa 594–conjugated secondary antibodies (Molecular Probes). The nuclei were stained with Hoechst 33258. Glycogens were visualized using a periodic acid-Schiff (PAS) staining kit (Muto Pure Chemicals, Tokyo, Japan). RIs were determined using

*h*ALB-immunostained sections of chimeric *m*-livers as reported previously.[5]

HBV Infection. We obtained *h*-serum containing high-titer HBV DNA (8.1 $\log_{10}$ genome equivalents/mL serum) from an HBV genotype C carrier after obtaining informed consent. The serum was kept at −80°C until use. Four CFPH-chimeric mice were intravenously injected with 100 μL of the HBV-positive serum 9-12 weeks after transplantation.

HBV Marker Analysis. Hepatitis B surface antigen (HBsAg) was measured using an Architect Analyzer (Abbott, Osaka, Japan). Serum DNA was extracted using a SMITEST EX-R&D Nucleic Acid Extraction Kit (Genome Science Laboratories, Fukushima, Japan). Small amounts of HBV DNA (<300 copies/mL) were detected via nested PCR.[8] If HBV DNA was detected during the initial round of PCR, the copy number was determined via real-time PCR as reported.[8]

Results

***Phenotypes of CFPHs* In Vitro.** We seeded 9MM and 12YM PHs on culture dishes and confirmed that the CFPHs from the 2 donors were similar in morphology and replicative capacity. A small number of the CFPHs (0.01%-0.09% of the seeded PHs) began to replicate after 5 days, and the number of replicating cells gradually increased until colonies appeared at 17 days (Fig. 1A); after 21 days, the cells covered the surface of the dish (Fig. 1B). Most of the seeded PHs were not replicative, and they gradually flattened, acquiring a senescent morphology within 20 days of seeding (Fig. 1A). The CFPHs showed an epithelial cell–like morphology with scant cytoplasm (Fig. 1B), and they retained this appearance during subculture (Fig. 1C). The population doubling time (PDT) of the CFPHs gradually increased as the passage number increased. Up to p = 4, the CFPHs from the young donors replicated with a population doubling time of 170-220 hours; subsequently, the population doubling time increased until the cells finally became senescent.[13]

The expression of several marker genes was compared among PHs, HepG2 cells, and CFPHs (Fig. 1D). In our experience, no significant differences exist in the marker gene expression profiles of PHs among different donors, and the same trend applies to subcultured CFPHs.[13] At p = 4, the CFPHs expressed less ALB and AAT messenger RNA compared with the PHs. The PHs expressed TO and G6P, both of which are markers of mature hepatocytes, whereas the CFPHs did not. CK19, a hepatic progenitor/biliary cell marker, was expressed in both the CFPHs and HepG2 cells, but not in the PHs. BGP, a cell–cell adhesion molecule in epithelium, endothelium, and myeloid cells,[16] was expressed in the PHs and HepG2 cells, but only faintly in the CFPHs. The CFPHs, but not the PHs or HepG2 cells, expressed Thy-1, a hematopoietic/hepatic progenitor cell marker. AFP, a hepatic progenitor/carcinoma cell marker, was only detectable in HepG2 cells. CD44, an SH[17] or oval cell marker,[18] was strongly expressed in CFPHs, but only faintly in PHs and HepG2 cells. PHs and CFPHs faintly expressed MDR1. PHs expressed MRP2, but not MRP1. In contrast, CFPHs expressed MRP1, but not MRP2. A change from MRP2 to MRP1 expression during culture has been reported in rat hepatocytes.[19]

Thy-1 and CD44 expression in CFPHs was assessed via immunocytochemistry (Fig. 1E-F). A few CFPHs were positive for Thy-1 (Fig. 1E), whereas the majority was strongly positive for CD44 (Fig. 1F). Fluorescence-activated cell sorting indicated that a minor population of the CFPHs expressed Thy-1 (Fig. 1G-H), with an occupancy rate of 1%-3% (Fig. 1H). The CFPHs expressed CK7, CK8, CK18, and CK19 in the preconfluent state and became CK7- and CK19-negative in condensed regions postconfluence (data not shown), which is in agreement with our previous findings.[13] Other hepatic stem cell markers such as CD34 and c-kit were undetectable in our CFPHs (data not shown).

Repopulation of CFPHs in uPA/SCID Mouse Liver. We transplanted 12YM CFPHs (p = 4) into 27 homozygous uPA/SCID mice. The serum concentration of *h*ALB was monitored posttransplantation as a measure of the RI of CFPHs (Fig. 2A). Approximately half of the hosts had no or only a small increase in the level of *h*ALB throughout the experimental period. The remaining mice showed a continuous increase in the concentration of *h*ALB, which reached >10 μg/mL after 9 to 10 weeks. Animal 27 showed the greatest increase, reaching 0.7 mg/mL after 10 weeks. The RI of each of the 14 mice in which blood *h*ALB concentration was >8 μg/mL after 9 to 10 weeks was determined by dividing the *h*ALB-positive areas by the entire area measured,[5] and the data were plotted against the corresponding blood *h*ALB concentrations (Fig. 2B). RIs between 0.2% and 27.0% were well correlated with blood *h*ALB concentrations in the 9-728 μg/mL range.

Livers of mice engrafted with the CFPHs were subjected to immunohistochemical staining for *h*ALB (Fig. 3A-D,H) and *in situ* hybridization using *h*-genomic DNA probes (Fig. 3I). *h*ALB-positive cells were visible within 3 weeks posttransplantation as single cells or small clusters consisting of up to 25 cells (Fig. 3A-B). Larger clusters containing 20-450 *h*ALB-positive cells appeared after 9 to 10 weeks (Fig. 3C for animal 2 and Fig. 3D for animals 17 and 27). To detect replicating CFPHs, the mice were

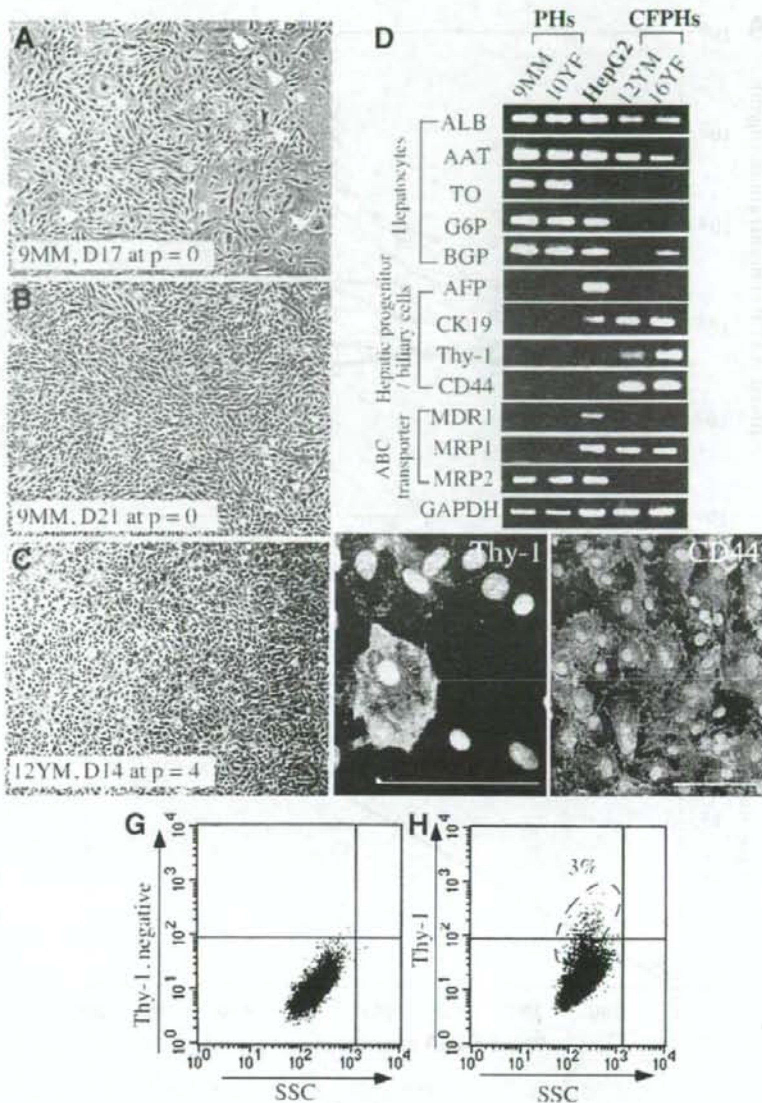

Fig. 1. CFPH growth and gene expression. (A-C) CFPH colony formation. We seeded 9MM PHs at 8×10^3 cells/cm^2 and cocultured with mitomycin C-treated Swiss 3T3 cells in *h*-hepatocyte clonal growth medium. A few CFPHs proliferated and formed colonies. CFPHs were cultured for (A) 17 and (B) 21 days. PHs were nonreplicative and were gradually expelled by replicative CFPHs. Arrowheads indicate the remaining flattened PHs, whose size increased. (C) Cryopreserved 12YM CFPHs (p = 3) were thawed and cultured in *h*-hepatocyte clonal growth medium with Swiss 3T3 cells for 14 days. (D) CFPH messenger RNA expression profiles. RNA was extracted from 9MM and 10YF PHs, HepG2 cells, and 12YM and 16YF CFPHs (p = 4). Semiquantitative RT-PCR was performed for ALB, AAT, TO, G6P, BGP, AFP, CK19, Thy-1, CD44, and the ABC transporters MDR1, MRP1, and MRP2. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. (E,F) Immunohistochemistry of Thy-1 and CD44. 12YM CFPHs (p = 4) were cultured for 14 days and stained for (E) Thy-1 and (F) CD44. The nuclei were stained with Hoechst 33258. Scale bar: 100 μm. (G,H) Flow cytometric analysis of CFPHs for Thy-1. Cells were suspended in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum with (G) *m*-immunoglobulin G_1 as a negative control or (H) anti-*h*Thy-1 antibodies. Living cells were analyzed via fluorescence-activated cell sorting. A small fraction (3% in this case) of the CFPHs was Thy-1$^+$. Three independent analyses were performed with similar results.

given BrdU after 9 weeks. BrdU-positive CFPHs were observed at the edges of the colonies (Fig. 3E-G). Serial liver sections were prepared from CFPH-chimeric mice 9 to 10 weeks after transplantation for *h*ALB immuohistochemistry (Fig. 3H) and for *in situ* hybridization with an *h*-DNA probe (Fig. 3I). The regions identified as containing *h*-hepatocytes by the 2 methods were identical.

Comparison of Repopulation by CFPHs and PHs. PHs and CFPHs (p = 4) were prepared from the livers of 9MM and 12YM donors and transplanted into uPA/SCID mice, and the mice were killed 3 and 10 weeks posttransplantation. The transplanted cells were identified as *h*ALB-positive from histological sections. The number of PH- and CFPH-derived clusters was 125.0 ± 28.2 (n = 3) and 3.3 ± 7.5 (n = 7), respectively, per cross-section of the left lobe of the livers 3 weeks after transplantation, suggesting that the rate of engraftment of the CFPHs was much lower than that of the PHs.

The CFPHs were smaller in size compared with the PHs after 3 weeks (Fig. 4A-B). The cytoplasm of the

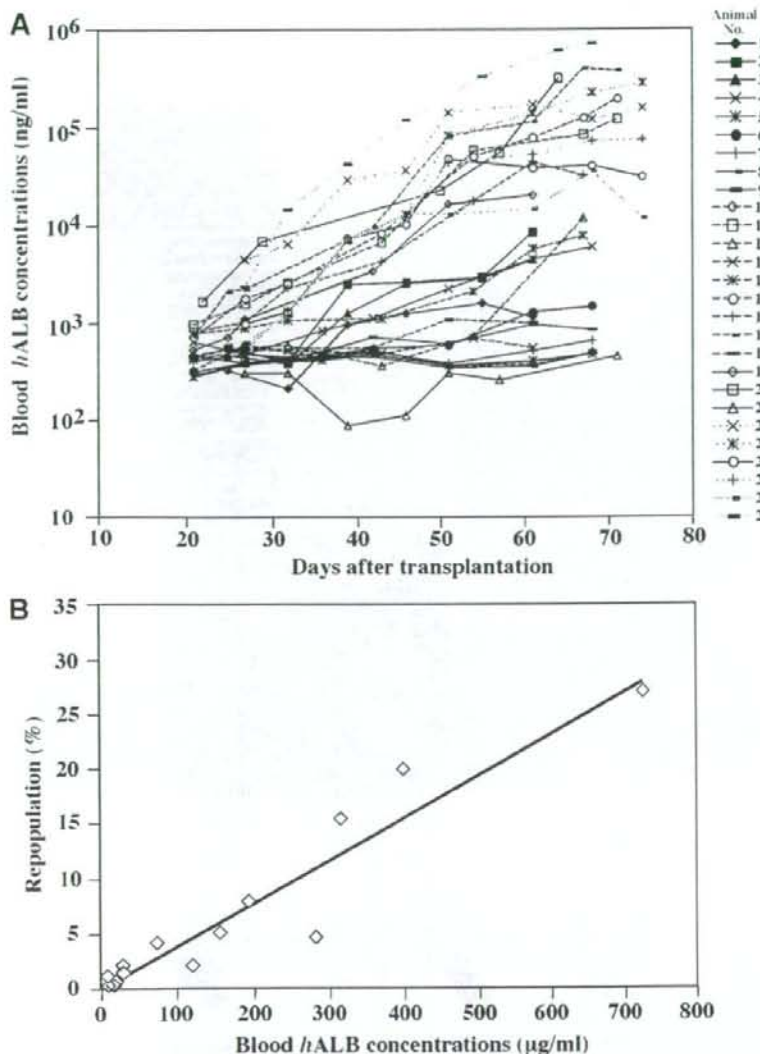

Fig. 2. Transplantation of CFPHs into uPA/SCID mice. The chimeric mice in this experiment are included in group D in Table 1. (A) We transplanted 12YM CFPHs (p = 4) into 27 mice and the serum level of *h*ALB was monitored individually. Ten hosts (animals 1, 5, 6, 7, 8, 9, 10, 13, 18, and 21) did not show significantly elevated *h*ALB levels during the experimental period. Four hosts (2, 3, 4, and 14) showed slight elevation. The *h*ALB concentration of 13 mice (11, 12, 15, 16, 17, 19, 20, 22, 23, 24, 25, 26, and 27) reached >10 μg/mL at 9 to 10 weeks after transplantation. (B) Correlation between the blood *h*ALB level and RI. Fourteen CFPH-chimeric mice (animals 2, 11, 12, 15, 16, 17, 19, 20, 22, 23, 24, 25, 26, and 27) were selected from the mice shown in panel A for RI determination. Their liver sections were immunostained for *h*ALB. RIs were determined for each animal and plotted against the *h*ALB concentration. The correlation coefficient (r^2) between the 2 parameters was 0.91.

former was less abundant and more strongly stained for *h*ALB than that of the latter. We observed *h*CD44 in the plasma membrane of the CFPH-derived cells (Fig. 4E), but not in that of the PH-derived cells (data not shown). At 10 weeks posttransplantation, the CFPHs had increased in size to match those of the PHs, whose sizes were unchanged (Fig. 4C-D), and *h*CD44 expression disappeared from the CFPH-derived cells (Fig. 4F). The diameter of each CFPH and PH was quantified as follows: 18.3 ± 5.1 μm (mean ± SD, n = 65) versus 25.8 ± 6.4 μm (n = 124) at 3 weeks and 27.0 ± 5.5 μm (n = 185) versus 25.8 ± 4.8 μm (n = 187) at 10 weeks. We found no significant differences in this parameter between the 12YM and 9MM samples. Thus, it appears that the CFPHs replicated without changing their original small size until 3 weeks posttransplantation, when they became larger.

Liver sections from the chimeric mice were stained with hematoxylin-eosin to compare the morphological features of PHs and CFPHs at 10 weeks. The repopulated CFPHs (Fig. 4G) showed no significant difference in morphology compared with the repopulated PHs (Fig. 4H). As reported previously,[5,6] the PHs in the chimeric livers were enlarged and had less eosinophilic cytoplasm

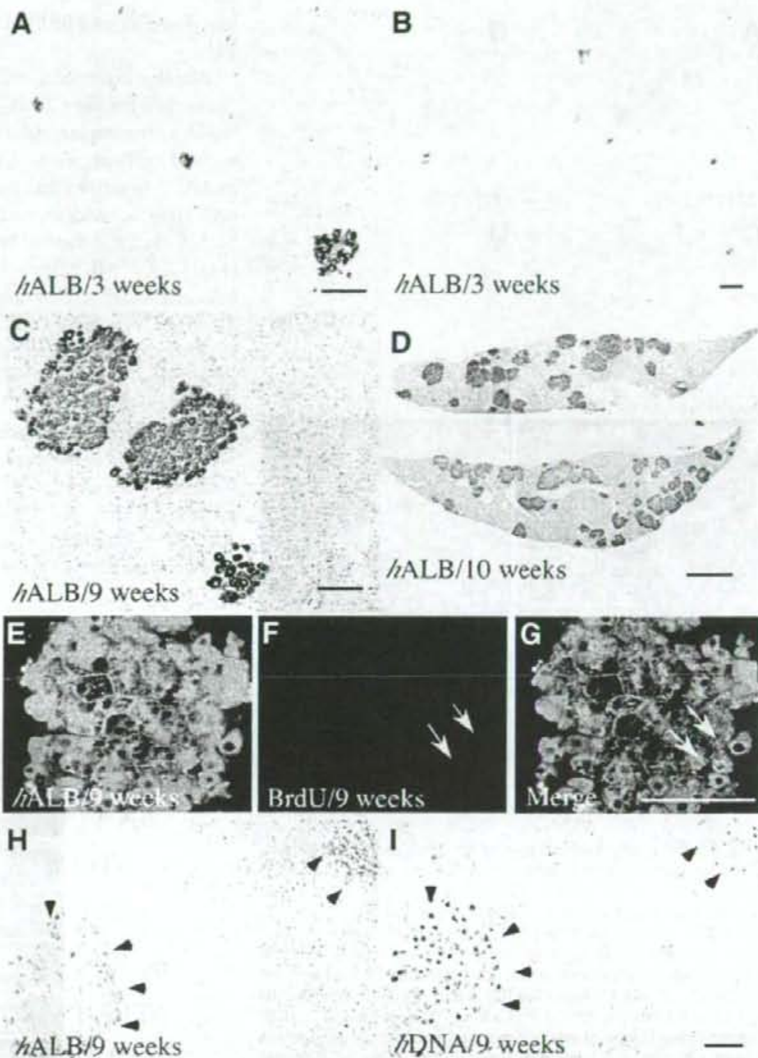

Fig. 3. Engraftment and repopulation of CFPHs in chimeric mouse liver. The chimeric mice in this experiment are included in groups A and D in Table 1. We performed *h*ALB immunohistochemistry using liver sections from CFPH-chimeric mice (A,B) 3, (C) 9, and (D) 10 weeks after transplantation. (A,B) Small clusters composed of 1-25 cells were scattered throughout the liver at 3 weeks in 3 of 9 mice. (C,D) The clusters became larger at 9 to 10 weeks. The liver sections in panel C were prepared from animal 2 in Fig. 2A (RI = 1.1%). The liver sections in panel D were prepared from animals 17 (RI = 20.0%; upper section) and 27 (RI = 27.0%; lower section). Three mice were randomly selected for the BrdU incorporation experiments (animals 2, 19, and 20 in Fig. 2A). They were given BrdU 1 hour before death at 9 weeks posttransplantation. Serial liver sections were subjected to (E) *h*ALB- and (F) BrdU immunohistochemical staining. The image in panel G is panel E and panel F merged. Similar results were obtained from these experiments, and the result from animal 19 (RI = 0.6%) is shown in panels E-G. Serial liver sections were prepared from CFPH-chimeric mice (animals 2, 15, and 17 in Fig. 2A) 9 to 10 weeks after transplantation for *h*ALB immunohistochemistry (H) and for *in situ* hybridization with an *h*-genomic probe (I). Similar results were obtained from the 3 mice. The results shown in panels H and I were obtained from animal 2 (positive cells are indicated by arrowheads). Scale bars in panels A-C, G, and I: 100 μm. Scale bar in panel D: 1 cm.

than the PHs in *h*-livers. The livers of the mice that had low *h*ALB levels at 10 weeks posttransplantation were mostly occupied by red nodules, which have been reported to be formed by the transgene-deleted hepatocytes of the host.[20]

Gene and Protein Expression Profiles of CFPHs in Chimeric Mice Compared with Those of PHs. Three 12YM CFPH-chimeric mice (11, 15, and 17) were randomly selected from the mice in Fig. 2A and killed 10 weeks after transplantation. RNA was extracted from each liver to generate gene expression profiles via RT-PCR. RT-PCR was also performed on 2 12YM PH-chimeric mice that were included in a previous study.[5] The CFPH livers expressed *h*ALB, *h*AAT, *h*TO, *h*G6P, and *h*MRP2, but not *h*CK19, *h*Thy-1, or *h*MRP1, just as in the PH-livers (Fig. 5). Previously, we showed that the PHs in chimeric mice expressed various *h*-cytochrome P450 (*h*CYP) subtypes in a manner similar to the donor liver.[5] In this study, we found that the expression of *h*CYPs 1A2, 2C8, 2C9, 2D6, and 2E1, but not 3A4, in the CFPH-chimeric mice was similar to that in the PH-chimeric mice (data not shown). Expression of *h*CYP3A4 was very

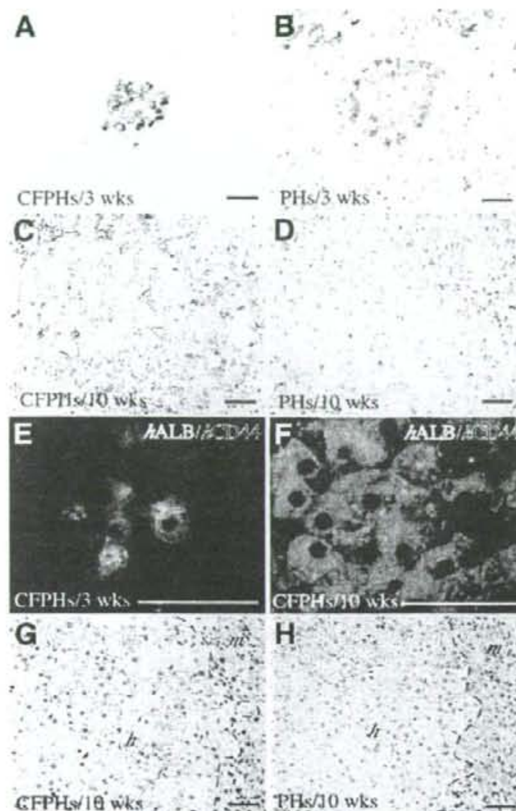

Fig. 4. Immunohistochemical staining for CFPHs and PHs in chimeric mice. Immunohistological analysis with antibodies against (A-D) *h*ALB and (E-F) *h*CD44. We produced 3 12YM CFPH-chimeric mice and 4 9MM CFPH-chimeric mice [(A) and (E), included in groups A and B in Table 1] and 3 9MM PH-chimeric mice [(B), group C], which were killed at 3 weeks posttransplantation. At 10 weeks posttransplantation, 3 12YM CFPH-chimeric mice that were randomly selected from the mice shown in Fig. 2A (15, 16, and 17) were killed [(C) and (F), group D), as were 9MM and 12YM PH-chimeric mice, 2 mice each [(D), groups E and F]. (A-D) Representative images of liver sections prepared from the animals and stained with anti-*h*ALB antibodies. The diameters of the *h*ALB-positive cells were measured in 10-15 randomly selected fields. (E,F) Double-fluorescence immunostaining. Green and red stains depict *h*ALB and *h*CD44, respectively. (G,H) Hematoxylin-eosin staining. (G) Eight CFPH mice were randomly selected from the mice shown in Fig. 2A and killed at 10 weeks posttransplantation. Their liver tissues were then subjected to hematoxylin-eosin staining. (H) Three 12YM PH-chimeric mice were killed at 10 weeks posttransplantation for hematoxylin-eosin staining as above. Similar results were obtained for the 8 CFPH-chimeric mice and 3 PH-chimeric mice. (E-F) Sections from (E) a CFPH-chimeric mouse (RI = 20.0%) and (F) a PH-chimeric mouse (RI = 57%). *h*, *h*-hepatocyte region; *m*, *m*-hepatocyte region. Dashed lines show the boundary between the 2 regions. Scale bars: 50 μm.

low (less than one-fifth) in CFPHs compared with that in PHs.

Protein expression was investigated immunohistochemically for the CFPH-chimeric livers at 3, 9, and 10 weeks posttransplantation. All of the examined CFPHs were Thy-1–negative, CK7-negative, CK19-negative, and AFP-negative (data not shown). The *h*ALB-positive cells were coincident with the *h*CK18-positive cells at both 3 (data not shown) and 9 weeks posttransplantation (Fig. 6A-C). MRP2-positive signals were present on the bile canalicular membranes of the transplanted CFPHs at 10 weeks (Fig. 6D-F). CYP3A4-expressing CFPHs were localized in the pericentral zone (Fig. 6G-I) as reported previously,[21] but their distributions were unique. Although some of the CFPHs were positive for CYP3A4, approximately 70% of them were negative. In contrast, all of the CFPHs in the pericentral zone strongly expressed CYP1A2 (Fig. 6J-L), which is known to be expressed in postnatal liver.[22] The CFPHs in the chimeric mice were strongly PAS-positive (Fig. 6N), whereas the *in vitro* CFPHs were faintly PAS-positive (data not shown). From

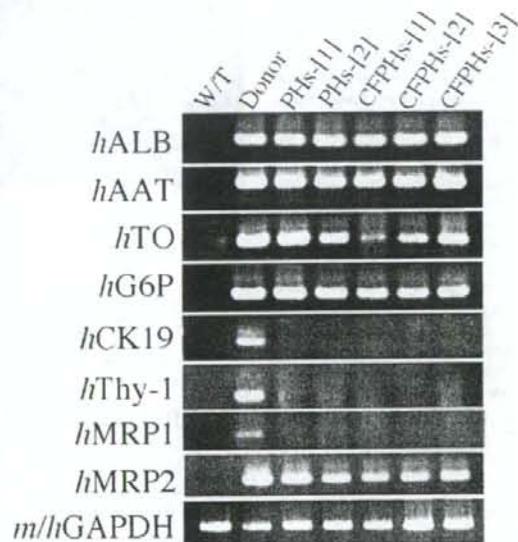

Fig. 5. Gene expression profiles of CFPHs in chimeric mice. Two uPA/SCID mice were transplanted with 12YM PHs ([1] and [2]); 3 uPA/SCID mice were transplanted with 12YM CFPHs ([1], [2], and [3]). The chimeric mice in this experiment are included in groups D and F in Table 1. After 10 weeks, the livers were removed for RT-PCR analysis. At the time of death, the PH-[1]-, PH-[2]-, CFPH-[1]-, CFPH-[2]-, and CFPH-[3]-chimeric mice had RIs of 41.0%, 57.0%, 2.1%, 7.9%, and 20.0%, respectively. The analysis was repeated using liver tissues from donor and uPA/SCID mice without transplantation (W/T). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplification was used as an internal control.

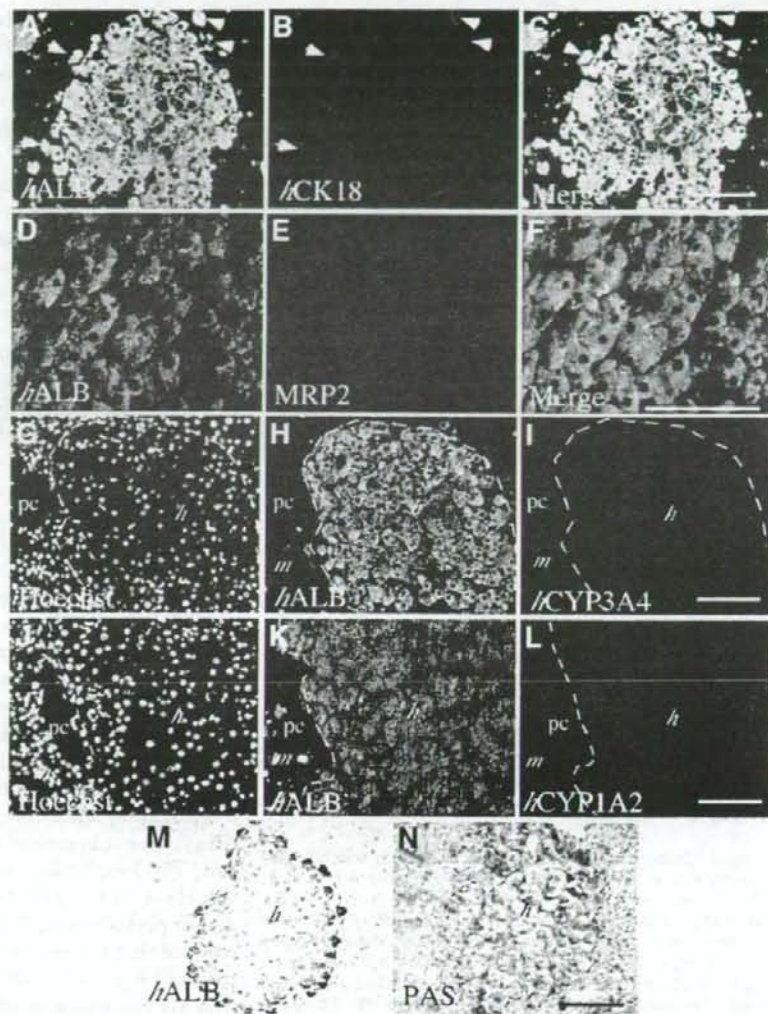

Fig. 6. Protein expression profiles of the CFPHs in chimeric livers. Mice were transplanted with 12YM CFPHs, and their livers were removed 9 to 10 weeks after transplantation for immunohistochemical analysis of (A,D,H,K) *h*ALB, (B) *h*CK18, (E) MRP2, (I) CYP3A4, and (L) CYP1A2. The chimeric mice in this experiment are included in group D in Table 1. Representative images are shown. (A-F) Double-fluorescence immunostaining. (A,D) *h*ALB is stained green. (B) *h*CK18 and (E) *h*MRP2 are stained red. Panels A and B were merged to create panel C; panels D and E were merged to create panel F. The arrowheads in panels A-C show macrophages engulfing such wastes as lipids. Serial sections of liver tissues subjected to 2 series of immunohistochemical examinations, one for (G-I) *h*CYP3A4 and the other for (J-L) *h*CYP1A2. The sections were stained with (G,J) Hoechst 33258, and for (H,K) *h*ALB, (I) *h*CYP3A4, and (L) *h*CYP1A2. Serial sections of liver tissues at 9 weeks posttransplantation were subjected to *h*ALB-immunostaining (M) and PAS staining (N). The positive cells appear brown in (M) and red in (N). *h*, *h*-hepatocyte region; *m*, *m*-hepatocyte region; pc, pericentral zone. Dashed lines show the boundary between the *h*-hepatocyte and *m*-hepatocyte regions. Scale bars: 100 μm.

these results, we conclude that the transplanted CFPHs differentiated into functionally mature hepatocytes. No *h*-cell tumors were formed during any of our experiments in the uPA/SCID mice.

Infection of CFPH-Chimeric Mice with HBV. To further examine whether CFPHs had exhibited normal differentiated phenotypes in chimeric mice, we tested their susceptibility to HBV infection. Four CFPH-chimeric mice with various serum *h*ALB levels (0.2, 1.6, 7.3, and 222.0 μg/mL) were inoculated with 100 μL of HBV-positive *h*-serum at 9-12 weeks posttransplantation. The animals were then tested every 2 weeks for HBV viremia and serum *h*ALB levels (Fig. 7A). The amount of HBV DNA in the animals increased between 2 and 8 weeks after inoculation, and all 4 mice developed measurable viremia within 8 weeks. However, a correlation was observed between the HBV DNA and/or HBsAg level and the *h*ALB level: the former appeared to be high when the latter was high (Fig. 7A). HBsAg was detectable in the serum of the chimeric mice when they showed elevated virus titers: the HBsAg levels of chimeric mice with HBV DNA levels of 2×10^3, 5.2×10^5, 5.9×10^7, and 7.7×10^8 copies/mL 8 weeks after inoculation were <0.05, <0.05, 3.2, and 124.0 IU/mL, respectively. HBV was infectious to CFPH-chimeric mice with very low levels of *h*ALB (<10^4 ng/mL), and all mice showed quantitatively

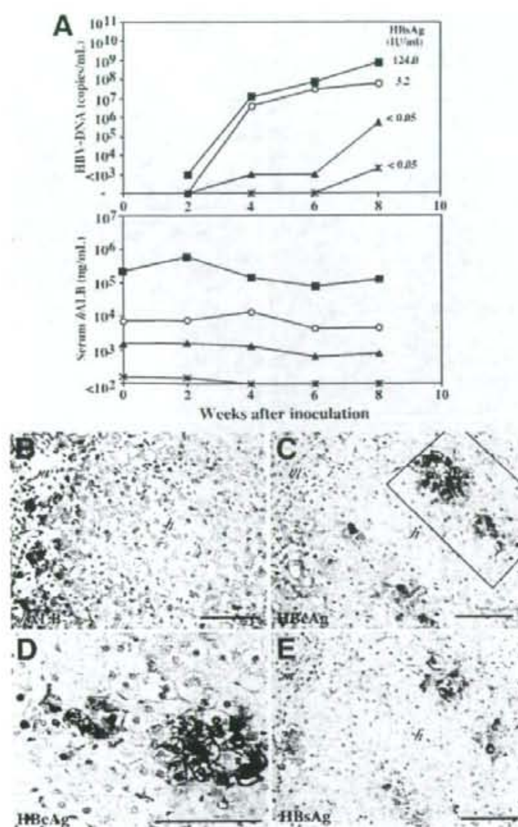

Fig. 7. Susceptibility of chimeric mice to infection with HBV. The chimeric mice in this experiment are included in group G in Table 1. uPA/SCID mice were transplanted with 12YM CFPH (p = 4). (A) The serum *h*ALB concentration of each mouse was determined 9-12 weeks posttransplantation just before the mouse was intravenously injected with 100 μL of HBV-positive *h*-serum (0.2 μg/mL at 12 weeks, 1.6 μg/mL at 10 weeks, 7.3 μg/mL at 11 weeks, and 222.0 μg/mL at 9 weeks). The animals were examined every 2 weeks for HBV viremia and serum *h*ALB level. The upper and lower graphs show the HBV DNA levels (copies/mL) and serum *h*ALB concentrations (ng/mL), respectively. The amount of HBV DNA (<10^3 copies/mL) was semiquantitatively measured via nested PCR. The values in the upper graph represent the HBsAg levels at 8 weeks. (B-E) Immunohistochemical analysis of chimeric livers infected with HBV. Serial sections of liver tissues at 8 weeks after inoculation were stained for (B) *h*ALB, (C,D) hepatitis B core antigen, and (E) HBsAg. The region enclosed by a square in panel C is magnified in panel D. Scale bars: 100 μm.

measurable viremia (>10^3 copies/mL) up to 8 weeks after inoculation. In contrast, most PH-chimeric mice with <10^4 ng/mL *h*ALB did not show quantitatively measurable levels of viremia up to 12 weeks after inoculation (data not shown) as reported previously.[8] In this study, we confirmed that CFPHs were not susceptible to infection with HBV prior to transplantation. The presence of hepatitis B core antigen and HBsAg in the CFPHs from HBV-infected chimeric livers was examined immunohistochemically (Fig. 7C,E). CFPHs were positive for both antigens that were sporadically distributed in the same regions among the CFPH colonies. Hepatitis B core antigen–positive cells accounted for 18.7 ± 8.3% of the total number of CFPHs (n = 3; total cell count = 1,215) (Fig. 7C), and both the nucleus and cytoplasm of the cells showed signals (Fig. 7D).

Discussion

This study supports our previous conclusion that CFPHs are *h*-hepatic progenitor-like cells.[13] Cultured CFPHs expressed such hepatic progenitor cell markers as CK19, Thy-1, and CD44, but not mature hepatocyte markers such as TO and G6P. We also found that *in vitro*–expanded CFPHs in uPA/SCID mice were able to repopulate the parenchyma, in which they differentiated into mature hepatocytes. FISH (fluorescence *in situ* hybridization) using mouse X chromosome probes showed that the engrafted and propagated CFPHs did not fuse to the mouse cells (data not shown). Thus, replicative CFPHs isolated from postnatal liver are normal, functional hepatocyte progenitor-like cells.

The existence of stem/progenitor cells in the adult liver is controversial.[23-25] In the present study, we showed that the CFPHs expressed CK19, Thy-1, and CD44, but not AFP, in serial culture. Thy-1 antigens are expressed in *h*-hepatic progenitor cells in fetal liver[26] and in rat oval cells,[27] but not in normal adult hepatocytes. We showed that Thy-1-expressing cells were present among the CFPHs at an occupancy of 1%-3%. SHs show greater growth potential than PHs in rats.[12] Other studies have reported that CD44 is a specific marker for rat SHs *in vitro* and *in vivo*, and that its expression level decreases with SH maturation *in vitro*.[17] Moreover, a recent study demonstrated that CD44 was strongly expressed by oval cells in a 2-acetylaminofluorene/partial hepatectomy, a D-galactosamine, and a retrorsine/partial hepatectomy rat model, but not by small hepatocyte-like progenitor cells (SHPCs)[18] that appeared in a retrorsine/partial hepatectomy model.[28] We detected CD44 expression in CFPHs at the plasma membrane. These results suggest that Thy-1 and CD44 may be common markers for both rat and *h*-hepatic progenitor cells.

Mouse embryonic liver stem cell lines differentiate into both hepatocytes and bile ducts in uPA/SCID mice.[29] Like PHs, our CFPHs differentiated into mature hepatocytes, but not into biliary epithelial cells, in uPA/SCID mice. CFPHs are considered to be hepatic progenitor-like cells, like rat SHs[12,30-33] and SHPCs.[28,34] SHPCs are