However, serological markers including des-γ-carboxy prothrombin (DCP) and glycosylated AFP have shown limited success in detecting HCC in early stages [12–14]. Recent advances in imaging technologies allow the detection of early HCC, as reported in the guideline of the American Association for the Study of Liver Diseases [14]. Patients need to be surveyed for HCC, taking into consideration the incidence of HCC and cost-effectiveness. The discovery of hepatitis B virus (HBV) and hepatitis C virus (HCV), responsible for the majority of HCC cases [15], has enabled providers to identify the population at risk for HCC.

In Japan, HBV and HCV infections are associated with HCC in 15 and 80% of the cases, respectively [16, 17]. This retrospective study focused on HCV-associated HCC in Japan, and compared the efficacy of three methods for diagnosing HCC diagnosis. As the results show, regular repeated imaging was useful for early detection of HCC in patients infected with HCV.

Patients and methods

Patients

From April 2001 to March 2007, 338 consecutive patients were diagnosed with HCC in our institution. Among them, 240 patients infected with HCV were enrolled in this study. We retrospectively examined the procedure of diagnosis from clinical records and classified patients into one of three groups according to the method for diagnosing HCC. A total of 124 patients were diagnosed with HCC by regular imaging procedures such as ultrasonography, and they were categorized into the surveillance group (Group A). Hepatic damages such as rough surface pattern of the liver and dullness on the liver edge, as well as the detection of obvious varices on the first ultrasonography, led them to receive repeated imaging procedures. In 82% (102/124) of Group A patients, the interval between the latest imaging and diagnosis of HCC was within 6 months. The average interval between the latest imaging and diagnosis of HCC was 4.3 months [median, 3.6 months (range 2–11 months)]. They also received tests for HCC-related markers at least every 3 months. Group B comprised 79 patients who had been diagnosed with HCC during scheduled doctor visits for HCV-related liver disease or other diseases such as diabetes. These patients were not enrolled in a surveillance program at the time, and had not undergone any imaging procedures for at least 1 year before the diagnosis of HCC, while they received tests for HCC-related markers at least every 3 months. Among them, 26 patients received imaging due to elevated levels of HCC-related markers, such as AFP and DCP. In the remaining 53 patients in Group B, imaging was performed incidentally; they had not received imaging over the previous 1 year. The 37 patients who had not been screened for HCC were classified into Group C. They were diagnosed with HCC when symptoms developed (32 patients) or incidentally during a diagnostic workout for unrelated medical conditions such as traffic accident (5 patients). The study conformed to the ethical guidelines of the declaration of Helsinki, and was approved by the Institutional Review Board.

Surveillance strategy

Figure 1 outlines the surveillance program. Briefly, detection of any mass on ultrasonography instigated repeated imagings if the nodule diameter was up to 1 cm, or a dynamic study if the diameter exceeded 1 cm. HCC nodules are characterized by an intense contrast uptake during the arterial phase of dynamic computed tomography (CT) or magnetic resonance imaging (MRI), with the contrast washed away during the delayed or venous phase [12–14]. In the present study, the specific pattern of arterial uptake followed by venous washout was considered to represent HCC, since the value of "washout" in the venous phase has been recognized recently. If the vascular pattern on CT or MRI was not specific for HCC in a nodule with a diameter >1.5 cm, angiographically assisted CT or biopsy was undertaken to establish the diagnosis. Patients with nodules <1.5 cm in diameter who did not reveal HCC by angiographically assisted CT or biopsy underwent repeated surveillance procedures, and subsequent enlargement of the nodule during follow-ups indicated shifting to a dynamic study.

Diagnosis of cirrhosis

The diagnosis of chronic liver disease was made at the time of HCC detection by the following procedures.

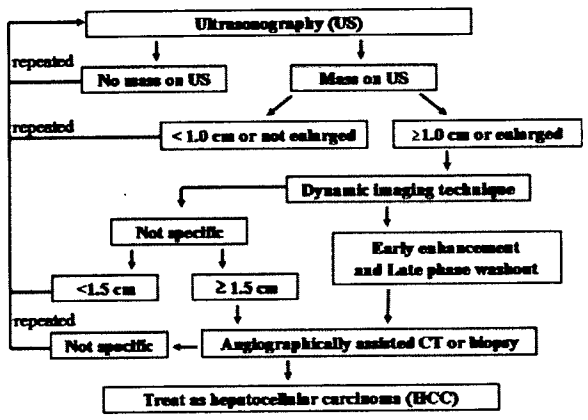

Fig. 1 Flow chart for the surveillance program including repeated imaging procedures

Histological findings were obtained in surgical specimens from 85 patients, and cirrhosis was diagnosed in 61 and chronic hepatitis or liver fibrosis in the remaining 24. Gastrointestinal varices in an additional 24 patients were considered diagnostic of cirrhosis. The remaining 131 patients were diagnosed to have cirrhosis according to the histologic scoring system [18].

Staging

Cancer stage was assessed by ultrasonography and dynamic CT or MRI. A total of 193 patients (80%) underwent angiography and/or angiographically assisted CT to obtain further details prior to resection, ablation or transarterial chemoembolization. In those patients, staging was also assessed by imaging on angiography and/or angiographically assisted CT. All patients underwent a chest X-ray, while additional investigations to detect metastases were performed only when extrahepatic involvement was suspected. Staging was not assessed by histologic findings on surgically resected specimens, even when they were available. Staging was determined according to the Liver Cancer Study Group of Japan classifications [19]. Staging was made also by the Milan criteria [20].

Treatment selection

Hepatic resection was indicated particularly to the patients with localized HCC who had maintained hepatic reserve capacity. When resection was contraindicated or refused by patients, the most appropriate treatment was selected according to the tumor status and liver function preserved [21]. Percutaneous ablation by ethanol injection [22] or radiofrequency ablation (RFA) [23] was considered in patients who had 1–3 nodules <3 cm in diameter, and were without vascular invasion or extrahepatic metastases. Transarterial chemoembolization [24] was offered to patients with either a paucifocal nodule not treatable by percutaneous ablation or multiple tumors not accompanied by thrombosis in main portal veins or extrahepatic metastasis. For the patients in Child-Pugh class C, transarterial chemoembolization was not recommended. In this study, resection and ablation were considered curative procedures based on their high efficacy.

Statistical analysis

The following 11 parameters were analyzed: age, sex, AFP, DCP, prothrombin activity, serum albumin level, total bilirubin level, liver state, tumor stage, HCC treatment and survival. Efficacy of the imaging program was evaluated by comparing clinical manifestation and prognosis among patients in the three groups. Differences in the distributions of tumor stage, tumor markers, and HCC treatment were evaluated by chi-squared test or Student's t test. Survival was calculated from the time of treatment start in patients who received it, and from the time of cancer diagnosis in patients without treatment. Data were censored at the time of death or the last follow-up visit. Survival was calculated according to the Kaplan–Meier method, and survival curves were compared by log-rank test. P values less than 0.05 were considered statistically significant.

Results

Background characteristics

There is no difference between Groups A and B in background of the patients except the programs with or without imaging. Table 1 details the background characteristics of all patients. Although the prevalence of cirrhosis was similar among the three groups, patients in Group C had poorer hepatic reserve with respect to albumin and total bilirubin levels ($P < 0.001$). The prevalence of non-cirrhotic liver in patients under 74 years was 26% (42/161), and 42% (33/79) in patients over 75 years. These differences were statistically significant ($P < 0.01$).

Features of HCC

The majority of HCC nodules were diagnosed by dynamic study including angiographically assisted CT, while HCC nodules in only 4 (1.7%) were confirmed by fine needle biopsy. Table 2 compares characteristics of HCC among the three groups. The frequency of solitary tumors was 66% (82/124) in Group A, 48% (38/79) in Group B, and 24% (9/37) in Group C, with a significant difference among three groups ($P < 0.001$). Nodules measuring less than 2 cm were detected in 64% (80/124) of patients in Group A, 25% (20/79) of those in Group B, and only 5% (2/37) of those in Group C ($P < 0.001$). The frequency of non-advanced tumor state decreased from 88% (109/124) in Group A, to 52% (41/79) in Group B, and to 27% (10/37) in Group C ($P < 0.001$). Cut-off values were set at 200 ng/ml and 40 mAU/ml, respectively, on AFP and DCP. In Group A, 47% (58/124) of the cases were negative for both, 46% (57/124) were positive for either, and 7% (9/124) were positive for both. In Group B, 11% (9/79) of the patients were negative for both, while 65% (51/79) were positive for ether, and 24% (19/79) were positive for both. In Group C, 11% (4/37) of the patients were negative for both, 57% (21/37) were positive for either, and 32% (12/37) were positive for both. These differences were statistically significant ($P < 0.001$). Thus, most patients in Groups B and C were positive for

Table 1 Background characteristics of patients

	Group A (surveillance) (n = 124)	Group B (scheduled doctor visits) (n = 79)	Group C (non-screened) (n = 37)	P value
Age at diagnosis of HCC (years)				
Median (range)	69.7 (49–89)	72.8 (49–87)	69.6 (50–87)	<0.05[b]
Gender				
Men	79 (64%)	52 (66%)	28 (76%)	NS
Women	45 (36%)	27 (34%)	9 (24%)	
History of blood transfusion	28 (22%)	19 (24%)	6 (16%)	NS
Excessive alcohol intake[a]	25 (20%)	20 (25%)	15 (49%)	NS
Liver state				
Not cirrhotic	34 (27%)	31 (39%)	10 (27%)	NS
Cirrhosis	90 (73%)	48 (61%)	27 (73%)	
Prothrombin activity (%)				
Median (range)	86 (48–125)	88 (57–135)	83 (39–124)	NS
Albumin (g/dl)				
Median (range)	3.6 (2.1–4.6)	3.8 (2.8–5.1)	3.4 (2.5–4.5)	<0.001[c]
Total bilirubin (mg/dl)				
Median (range)	0.9 (0.3–2.7)	0.8 (0.2–6.8)	1.4 (0.3–6.8)	<0.001[c]

NS not significant

[a] Excessive alcohol intake was defined as consumption of more 86 g alcohol/day

[b] Significant difference between Group B and Group A or Group C

[c] Significant difference between Group C and Group A or Group B

Table 2 Characteristics of the HCC nodule

	Group A (surveillance) (n = 124)	Group B (scheduled doctor visits) (n = 79)	Group C (non-screened) (n = 37)	P value
Solitary	82 (66%)	38 (48%)	9 (24%)	<0.001[b]
Size of main nodule				
–2 cm	80 (64%)	20 (25%)	2 (5%)	<0.001[b]
2.1–3 cm	31 (25%)	14 (18%)	6 (16%)	
3.1–5 cm	12 (10%)	33 (42%)	8 (22%)	
5.1 cm–	1 (1%)	12 (15%)	21 (57%)	
Vascular thrombus	4 (3%)	9 (11%)	10 (27%)	<0.001[b]
Distant metastases	1 (1%)	1 (1%)	5 (14%)	<0.001[c]
Tumor marker[a]				
Both negative	58 (47%)	9 (11%)	4 (11%)	<0.001[d]
Either positive	57 (46%)	51 (65%)	21 (57%)	
Both positive	9 (7%)	19 (24%)	12 (32%)	
Within the Milan criteria	109 (88%)	41 (52%)	10 (27%)	<0.001[b]

[a] HCC related tumor marker: AFP, DCP. Arbitrary cutoff values of 200 ng/ml and 40 mAU/ml were used for AFP and DCP, respectively

[b] Significant difference among all three groups

[c] Significant difference between Group C and Group A or Group B

[d] Significant difference between Group A and Group B or Group C

either or both AFP and DCP. Most patients in Group C who were in early tumor stages were diagnosed with HCC incidentally.

Figure 2a shows the distribution of tumor stages according to the Liver Cancer Study Group of Japan [19]. Proportions of patients in stages I and II were highest in the

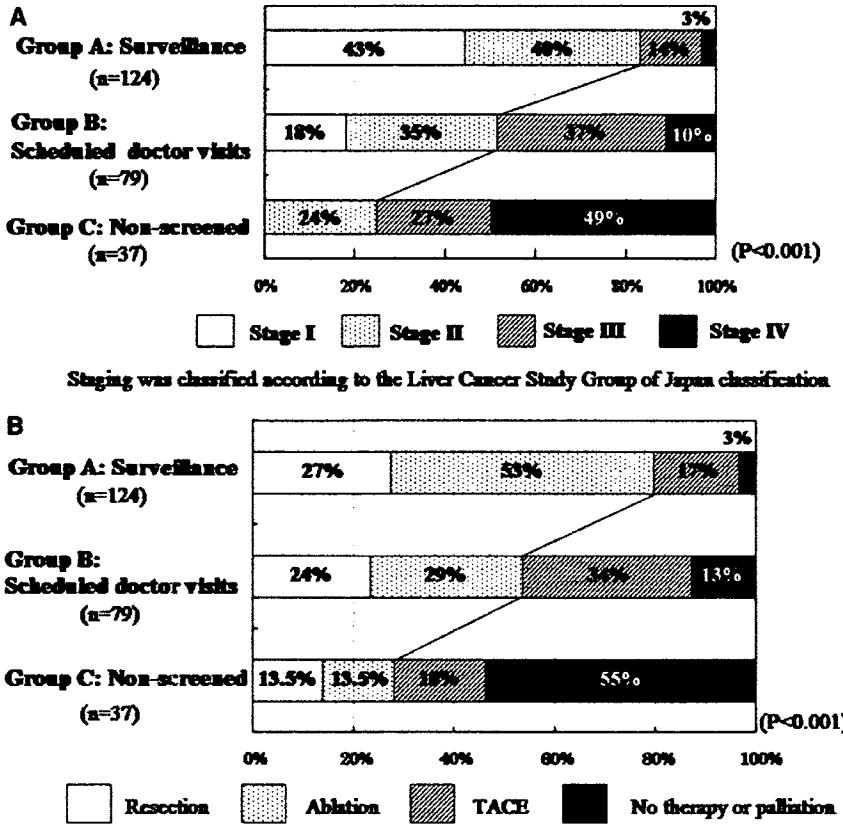

Fig. 2 a distribution of tumor stage according to the Liver Cancer Study Group of Japan [19]. b Distribution of treatment selected based on tumor stage and hepatic reserve

surveillance group (Group A); they decreased progressively through Group B to Group C ($P < 0.001$). The incidence of vascular thrombosis increased from 3% (4/124) in Group A to 11% (9/124) in Group B, and to 27% (10/37) in Group C ($P < 0.001$). Distant metastases were more frequent in Group C [14% (5/37)] than in Groups A and B [1% (1/124) and 1% (1/79), respectively] ($P < 0.001$). In Group A, the proportions of stages I and II was comparable between the patients with an interval between the latest imaging and diagnosis of HCC within 6 months and those with that of longer than 6 months [84% (86/102) vs. 77% (17/22)].

Treatment selection

Figure 2b shows the distribution of treatments selected based on the tumor stage and hepatic reserve. The proportion of patients treated with curative procedures, such as resection and ablation, was highest in Group A, and was lower in Groups B than C ($P < 0.001$). In Group C, the majority of patients received systemic chemotherapy or conservative care in hospice (palliation); most patients treated with curative procedures were diagnosed incidentally.

Survival

The median follow-up period was 35 months (range 3–94 months). During follow-ups, 148 patients died. Causes of death were cancer-related in 110 cases, liver failure in 6 (unrelated to treatment), gastrointestinal bleeding in 8, and others in the remaining 24. The distribution was similar between Groups A and B, while cancer-related causes were most prevalent (96%) in Group C. Figure 3a compares overall survival rates among the three groups. The cumulative survival rate was higher in group A than B ($P < 0.05$), and higher in group B than C ($P < 0.001$). Although survival rates of patients treated by curative procedures, such as resection and ablation, tended to be higher than the overall survival rate, there were no significant differences in the survival rates among patients in the three groups (Fig. 3b).

Discussion

For achieving better outcomes in patients with HCC, it is necessary to increase their eligibility for curative treatment. In the present study, 83% of patients under regular

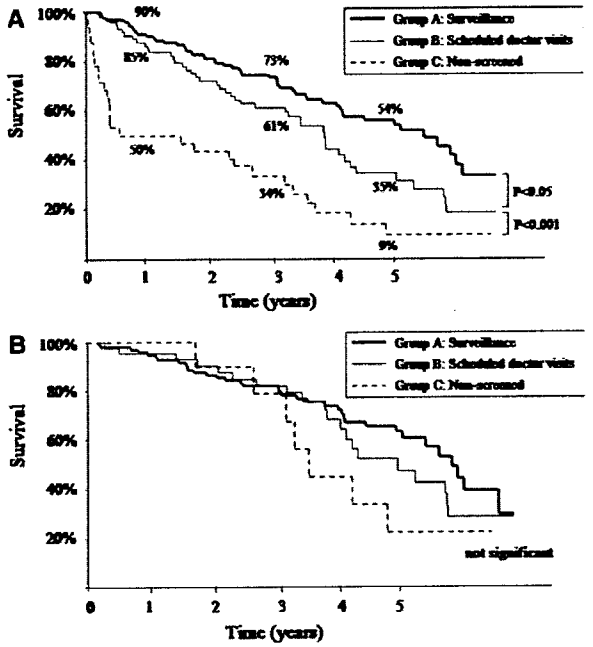

Fig. 3 **a** Survival rates in the three groups with different surveillance procedures. **b** Survival rates of the patients in three groups who had received curative treatments, such as resection and ablation

surveillance (Group A) were diagnosed with HCC at stage I or II, and the majority of them were indicated to curative treatments including surgical resection and RFA. As the results, patients in the surveillance group had a significantly better prognosis than those in the other groups without regular imaging screening (Group B) or none at all (Group C). Other reasons for the difference in prognosis among the three groups may include the following. Since the severity of underlying liver disease is a critical factor influencing the efficacy of surveillance programs, surveillance is reported to have few effects on improving the prognosis of patients with advanced cirrhosis [4, 10]. Although prevalence of cirrhosis was no different among the three groups, hepatic reserve was poorer in Group C than Group A or Group B. The dismal prognosis of patients in Group C, therefore, was attributed to either or both advanced tumor stage and poorer hepatic function. Indeed, analysis of only the patients who had received curative treatments, such as resection or ablation, revealed no significant differences in the survival among the three groups. However, the proportion of patients who had received curative treatment differed among the three groups with distinct diagnostic procedures.

Performance of surveillance would depend on the treatment selected and its efficacy. The 5-year survival of patients with a solitary HCC < 5 cm or up to 3 nodules <3 cm (Milan criteria [20]) exceeds 70% after transplantation, and that after resection surpasses 50% [12–14]. In general, transplantation offers the best long-term survival, and should be considered. In Japan, however, it is quite difficult for HCC patients to receive liver transplantation due to the shortage of donors [16], and liver resection is regarded as safe with less than 1% mortality [25]. Due to these background considerations, transplantation was not performed in the present study.

Should patients within the Milan criteria have undergone transplantation, differences in the outcome between Group A and Group B would have been reduced. In actuality, differences in the proportion of patients within the Milan criteria were lower that those in the distribution of stage I or II between them. The 5-year survival after resection was accomplished by 61% of patients with stage-II HCC and 73% of those with stage-I HCC; the staging was in accord with the definition of the Liver Cancer Study Group of Japan [16]. Thus, survival after resection in patients in Group A was comparable to that reported in transplanted patients within the Milan criteria. Indeed, the 5-year survival of patients in Group A who received curative treatments reached 63%. At present, the lack of sufficient liver donation is a worldwide problem in performing liver transplantation. Our results may indicate that surveillance by regular imaging can gain an excellent outcome where and when transplantation is hardly feasible, especially in patients with small HCC that can be treated by RFA or surgical resection.

With respect to HCC-related serological markers, most patients in Group A were negative for either AFP or DCP when they were diagnosed with HCC, in remarkable contrast to the majority of patients in Group B or C who were positive for either or both markers. In Group B, one-third of patients were tested for tumor markers during their scheduled doctor visits. However, the distribution of tumor stages was comparable between the patients with and without tumor-marker testing. Although yearly office visits would be helpful in early detection of HCC, periodical medical check-ups without screening by imaging may not necessarily detect early-stage disease, even if HCC-related markers such as AFP and DCP are tested for. This is the first report of poor performance of tumor markers including DCP in detecting early-stage HCC, and it suggests that various imaging procedures help detect HCC at a stage before levels of tumor markers elevate. Our results support the AASLD guideline that AFP alone should not be used for HCC screening when ultrasonography is not available [14]. On the other hand, it should be noted that 17% of patients in Group A in this study were diagnosed with HCC in stage III or IV, and 86% (18/21) of them were positive for either AFP or DCP. We therefore propose that HCC surveillance by regular imaging should be complemented with intermittent tests for tumor-markers, insofar as their elevated levels may reflect invisible nodules. As an

extension to this, repeated imaging with intermittent measurements of two different HCC-related tumor markers are included in the algorithm of the HCC surveillance program; it is described in Evidence-Based Clinical Practice Guidelines for HCC supported by the Japanese Ministry of Health, Labor and Welfare [26].

In a cirrhotic liver, small lesions detected by ultrasonography are likely to represent HCC. Even lesions not typical of cancer might transform into bona fide HCC during subsequent follow-ups. Generally, the incidence of HCC increases with the nodule size. In the present study, lesions >1 cm in diameter were examined by dynamic study, together with follow-ups by imaging at 3–6 month intervals, even when the appearance was atypical of HCC. Lesions >1.5 cm should be evaluated by dynamic study, preferably in combination with angiographically assisted CT or biopsy. Since the incidence of hypervascularity and moderately or poorly differentiated histology increases in HCC >1.5 cm [27–30], a 1.5-cm threshold in diameter may improve early diagnosis of HCC.

The AASLD guidelines recommend at-risk patients be screened by ultrasonography at 6–12-month intervals [14]. In our study, patients in Group B who had not undergone imaging for at least one year before the diagnosis often presented with advanced disease. A surveillance interval <12 months is therefore desirable. Although most patients in Group A were diagnosed with HCC within 6 months after the latest imaging, the proportion of stage I or II was similar between patients with the interval between the latest imaging and diagnosis of HCC below and above 6 months. However, optimal frequency of imaging was not determined in the present study. Further studies are required to determine the optimal screening interval.

Surveillance with imaging is feasible only in populations at risk for HCC, because radiological procedures are highly labor-intensive in comparison with serological testing. Major causes of cirrhosis in patients with HCC include HBV, HCV, alcoholic liver disease, exposure to aflatoxin, and possibly nonalcoholic steatohepatitis (NASH). Persistent infection with HBV or HCV is the most common cause of chronic liver disease including HCC, and increases the risk of HCC by approximately 20-fold. Heavy alcohol use and aflatoxin ingestion are environmental carcinogenic factors, and act synergistically with other risk factors [12–15]. In evaluating risks for HCC, geographic variations in incidence has to be taken into account. A recent study suggested an increased risk of HCC among patients with metabolic diseases such as diabetes or NASH [31–35]. However, the rate of HCC development in patients with NASH-related cirrhosis was significantly lower than that in those with HCV-related cirrhosis [33]. Thus, it remains uncertain how to assign surveillance programs to patients with metabolic disease.

In conclusion, surveillance programs including regular ultrasonography are useful for identifying HCC in early stages. HCC detected early is frequently indicated to curative treatments, such as resection and RFA, and is associated with better survival. Recently, several studies demonstrated that elderly patients infected with HCV developed HCC despite low-grade fibrosis stages [36, 37]. Elderly patients with HCV would be at high risk for the development of HCC, even though they do not show progression to cirrhosis. In the present study, most patients over 75 years were non-cirrhotic. Management of HCC should include early detection programs in all patients with HCV-related chronic liver disease including elderly patients in Japan.

References

1. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin. 2005;55:74–108.
2. Oka H, Kurioka N, Kim K, Kanno T, Kuroki T, Mizoguchi Y, et al. Prospective study of early detection of hepatocellular carcinoma in patients with cirrhosis. Hepatology. 1990;12:680–7.
3. Colombo M, de Franchis R, Del Ninno E, Sangiovanni A, De Fazio C, Tommasini M, et al. Hepatocellular carcinoma in Italian patients with cirrhosis. N Engl J Med. 1991;325:675–80.
4. Yuen MF, Cheng CC, Lauder IJ, Lam SK, Ooi CG, Lai CL. Early detection of hepatocellular carcinoma increases the chance of treatment: Hong Kong experience. Hepatology. 2000;31:330–5.
5. Tong MJ, Blatt LM, Kao VW. Surveillance for hepatocellular carcinoma in patients with chronic viral hepatitis in the United States of America. J Gastroenterol Hepatol. 2001;16:553–9.
6. Trevisani F, De NS, Rapaccini G, Farinati F, Benvegnù L, Zoli M, et al. Semiannual and annual surveillance of cirrhotic patients for hepatocellular carcinoma: effects on cancer stage and patient survival (Italian experience). Am J Gastroenterol. 2002;97:734–44.
7. Zhang BH, Yang BH, Tang ZY. Randomized controlled trial of screening for hepatocellular carcinoma. J Cancer Res Clin Oncol. 2004;130:417–22.
8. Yu EW, Chie WC, Chen TH. Does screening or surveillance for primary hepatocellular carcinoma with ultrasonography improve the prognosis of patients? Cancer J. 2004;10:317–25.
9. Mok TS, Yeo W, Yu S, Lai P, Chan HL, Chan AT, et al. An intensive surveillance program detected a high incidence of hepatocellular carcinoma among hepatitis B virus carriers with abnormal alpha-fetoprotein levels or abdominal ultrasonography results. J Clin Oncol. 2005;23:8041–7.
10. Trevisani F, Santi V, Gramenzi A, Di Nolfo MA, Del Poggio P, Benvegnù L, et al. Italian Liver Cancer group. Surveillance for early diagnosis of hepatocellular carcinoma: is it effective in intermediate/advanced cirrhosis? Am J Gastroenterol. 2007;102:2448–57.
11. Davila JA, Weston A, Smalley W, El-Serag HB. Utilization of screening for hepatocellular carcinoma in the United States. J Clin Gastroenterol. 2007;41:777–82.
12. Bruix J, Sherman M, Llovet JM, Beaugrand M, Lencioni R, Burroughs AK, et al. Clinical management of hepatocellular carcinoma. Conclusions of the Barcelona-2000 EASL conference. European Association for the Study of the Liver. J Hepatol. 2001;35:421–30.

13. Llovet JM, Beaugrand M. Hepatocellular carcinoma: present status and future prospects. J Hepatol. 2003;38:S136–49.
14. Bruix J, Sherman M, Practice Guidelines Committee, American Association for the Study of Liver Diseases. Management of hepatocellular carcinoma. Hepatology. 2005;42:1208–36.
15. El-Serag HB, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. Gastroenterology. 2007;132: 2557–76.
16. Ikai I, Arii S, Ichida T, Okita K, Omata M, Kojiro M, et al. Report of the 16th follow-up survey of primary liver cancer. Hepatol Res. 2005;32:163–72.
17. Ohishi W, Kitamoto M, Aikata H, Kamada K, Kawakami Y, Ishihara H, et al. Impact of aging on the development of hepatocellular carcinoma in patients with hepatitis C virus infection in Japan. Scand J Gastroenterol. 2003;38:894–900.
18. Ikeda K, Saitoh S, Kobayashi M, Suzuki Y, Tsubota A, Suzuki F, et al. Distinction between chronic hepatitis and liver cirrhosis in patients with hepatitis C virus infection. Practical discriminant function using common laboratory data. Hepatol Res. 2000;18: 252–66.
19. Liver Cancer Study Group of Japan. General rules for the clinical and pathological study of primary liver cancer. 2nd English ed. Tokyo: Kanehara; 2003.
20. Mazzaferro V, Regalia E, Doci R, Andreola S, Pulvirenti A, Bozzetti F, et al. Liver transplantation for the treatment of small hepatocellular carcinomas in patients with cirrhosis. N Engl J Med. 1996;334:693–9.
21. Schwartz M, Roayaie S, Konstadoulakis M. Strategies for the management of hepatocellular carcinoma. Nat Clin Pract Oncol. 2007;4:424–32. Review.
22. Kamada K, Kitamoto M, Aikata H, Kawakami Y, Kono H, Imamura M, et al. Combination of transcatheter arterial chemoembolization using cisplatin-lipiodol suspension and percutaneous ethanol injection for treatment of advanced small hepatocellular carcinoma. Am J Surg. 2002;184:284–90.
23. Kitamoto M, Imagawa M, Yamada H, Watanabe C, Sumioka M, Satoh O, et al. Radiofrequency ablation in the treatment of small hepatocellular carcinomas: comparison of the radiofrequency effect with and without chemoembolization. AJR Am J Roentgenol. 2003;181:997–1003.
24. Kamada K, Nakanishi T, Kitamoto M, Aikata H, Kawakami Y, Ito K, et al. Long-term prognosis of patients undergoing transcatheter arterial chemoembolization for unresectable hepatocellular carcinoma: comparison of cisplatin lipiodol suspension and doxorubicin hydrochloride emulsion. J Vasc Interv Radiol. 2001; 12:847–54.
25. Makuuchi M, Kokudo N, Arii S, Futagawa S, Kaneko S, Kawasaki S, et al. Development of evidence-based clinical guidelines for the diagnosis and treatment of hepatocellular carcinoma in Japan. Hepatol Res. 2008;38:37–51.
26. Group formed to establish "Guidelines for evidence-based clinical practice for the treatment of liver cancer". Clinical practice guidelines for hepatocellular carcinoma (in Japanese). Tokyo: Kanehara & Co., Ltd.; 2005.
27. Takayasu K. Hepatic angiography. In: Okuda K, Tabor E, editors. Liver cancer. New York: Churchill Livingstone; 1997. p. 347–59.
28. Nakashima Y, Nakashima O, Hsia CC, Kojiro M, Tabor E. Vascularization of small hepatocellular carcinomas: correlation with differentiation. Liver. 1999;19:12–8.
29. Kojiro M, Nakashima O. Histopathologic evaluation of hepatocellular carcinoma with special reference to small early stage tumors. Semin Liver Dis. 1999;19:287–96.
30. Forner A, Vilana R, Ayuso C, Bianchi L, Solé M, Ayuso JR, et al. Diagnosis of hepatic nodules 20 mm or smaller in cirrhosis: prospective validation of the noninvasive diagnostic criteria for hepatocellular carcinoma. Hepatology. 2008;47:97–104.
31. Adami HO, Chow WH, Nyrén O, Berne C, Linet MS, Ekbom A, et al. Excess risk of primary liver cancer in patients with diabetes mellitus. J Natl Cancer Inst. 1996;88:1472–7.
32. El-Serag HB, Tran T, Everhart JE. Diabetes increases the risk of chronic liver disease and hepatocellular carcinoma. Gastroenterology. 2004;126:460–8.
33. El-Serag HB, Hampel H, Javadi F. The association between diabetes and hepatocellular carcinoma: a systematic review of epidemiologic evidence. Clin Gastroenterol Hepatol. 2006;4:369–80.
34. Sanyal AJ, Banas C, Sargeant C, Luketic VA, Sterling RK, Stravitz RT, et al. Similarities and differences in outcomes of cirrhosis due to nonalcoholic steatohepatitis and hepatitis C. Hepatology. 2006;43:682–9.
35. Ohishi W, Fujiwara S, Cologne JB, Suzuki G, Akahoshi M, Nishi N, et al. Risk factors for hepatocellular carcinoma in Japanese population: a nested case-control study. Cancer Epidemiol Biomarkers Prev. 2008;17:846–54.
36. Miki D, Aikata H, Uka K, Saneto H, Kawaoka T, Azakami T, et al. Clinicopathological features of elderly patients with hepatitis C virus-related hepatocellular carcinoma. J Gastroenterol. 2008;43:550–7.
37. Saneto H, Kobayashi M, Kawamura Y, Yatsuji H, Sezaki H, Hosaka T, et al. Clinicopathological features, background liver disease, and survival analysis of HCV-positive patients with hepatocellular carcinoma: differences between young and elderly patients. J Gastroenterol. 2008;43:975–81.

ELSEVIER

Journal of Hepatology 51 (2009) 1046–1054

Journal of Hepatology

www.elsevier.com/locate/jhep

Absence of viral interference and different susceptibility to interferon between hepatitis B virus and hepatitis C virus in human hepatocyte chimeric mice☆

Nobuhiko Hiraga[1,2], Michio Imamura[1,2], Tsuyoshi Hatakeyama[1], Shosuke Kitamura[1], Fukiko Mitsui[1,b], Shinji Tanaka[1], Masataka Tsuge[1,2], Shoichi Takahashi[1,2], Hiromi Abe[2,3], Toshiro Maekawa[2,3], Hidenori Ochi[2,3], Chise Tateno[2,4], Katsutoshi Yoshizato[2,4,5], Takaji Wakita[6], Kazuaki Chayama[1,2,3,*]

[1]*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*
[2]*Liver Research Project Center, Hiroshima University, Hiroshima, Japan*
[3]*Laboratory for Liver Diseases, SNP Research Center, The Institute of Physical and Chemical Research (RIKEN), Yokohama, Japan*
[4]*PhoenixBio Co., Ltd., Higashihiroshima, Japan*
[5]*Osaka City University Graduate School of Medicine, Osaka, Japan*
[6]*Department of Virology II, National Institute of Infectious Diseases, Shinjuku-ku, Japan*

***Background/Aims*: Both hepatitis B virus (HBV) and hepatitis C virus (HCV) replicate in the liver and show resistance against innate immunity and interferon (IFN) treatment. Whether there is interference between these two viruses is still controversial. We investigated the interference between these two viruses and the mode of resistance against IFN.**

***Methods*: We performed infection experiments with either or both of the two hepatitis viruses in human hepatocyte chimeric mice. Huh7 cell lines with stable production of HBV were also established and transfected with HCV JFH1 clone. Mice and cell lines were treated with IFN. The viral levels in mice sera and culture supernatants and messenger RNA levels of IFN-stimulated genes were measured.**

***Results*: No apparent interference between the two viruses was seen *in vivo*. Only a small (0.3 log) reduction in serum HBV and a rapid reduction in HCV were observed after IFN treatment, regardless of infection with the other virus. In *in vitro* studies, no interference between the two viruses was observed. The effect of IFN on each virus was not affected by the presence of the other virus. IFN-induced reductions of viruses in culture supernatants were similar to those in *in vivo* study.**

***Conclusions*: No interference between the two hepatitis viruses exists in the liver in the absence of hepatitis. The mechanisms of IFN resistance of the two viruses target different areas of the IFN system.**

© 2009 European Association for the Study of the Liver. Published by Elsevier B.V. All rights reserved.

***Keywords*: Superinfection; JFH-1; IFN-stimulated genes**

Received 4 February 2009; received in revised form 14 July 2009; accepted 15 July 2009; available online 23 September 2009
Associate Editor: F. Zoulim

☆ C.T. and K.Y. are employees of PhoenixBio Co. Ltd., Higashihiroshima, Japan. The other authors who have taken part in this study declared that they do not have anything to disclose regarding funding from industry or conflict of interest with respect to this manuscript.

* Corresponding author. Tel.: +81 82 2575190; fax: +81 82 2556220.
E-mail address: chayama@hiroshima-u.ac.jp (K. Chayama).

Abbreviations: GAPDH, glyceraldehydes-3-phosphate dehydrogenase; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCV, hepatitis C virus; IFN, interferon; OAS, 2′,5′-oligoadenylate synthetase; PCR, polymerase chain reaction; SCID, severe combined immunodeficiency; uPA, urokinase-type plasminogen activator.

0168-8278/$36.00 © 2009 European Association for the Study of the Liver. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.jhep.2009.09.002

1. Introduction

Both hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are serious health problems worldwide. More than 350 million people are infected with HBV, and more than 170 million people are infected with HCV [1,2]. Both types of hepatitis viruses result in the development of chronic liver infection and lead to death due to liver failure and hepatocellular carcinoma [3]. To date, interferon (IFN) remains one of the most important drugs available for the treatment of both types of hepatitis viral infections. Although it is assumed that IFN suppresses viral replication through the effect of IFN-induced gene products such as mixovirus resistance protein A (MxA), RNA-dependent protein kinase (PKR), and 2′,5′-oligoadenylate synthetase (OAS) [4], the precise mechanism of action of these proteins on both hepatitis viruses are unknown.

Coinfection with both viruses leads to a rapid and severe progression of chronic liver disease [5], with a higher risk of hepatocellular carcinoma [6]. Currently, there is a debate about whether or not there is interference between the two hepatitis viruses, with some favoring such interference [7] and others arguing against such a concept [8]. A number of mechanisms can cause interference between viruses. A major mechanism of interference is induction of IFN by one virus to prevent replication of the second virus; however, viruses develop their own strategies to resist the effect of IFN. In clinical practice, practitioners often perceive that reduction of HBV in serum by IFN therapy is poorer compared with HCV. HCV levels in sera of IFN-treated patients decrease relatively rapidly, and a proportion of patients eventually show complete eradication of the virus. Furthermore, the recent use of pegylated IFN (PEG-IFN) in combination with ribavirin has improved the eradication rate [9]. Eradication of HBV by IFN, however, is usually difficult, even when using IFN combined with ribavirin [10].

The mechanisms developed by viruses to resist host innate immunity, including IFN signaling, are well established in some viruses. Such mechanisms involve interruption of IFN signaling by interacting molecules that transduce the signal from the IFN receptor through the Janus kinase (Jak) signal transducer and activator of transcription (STAT) pathway [4]. Viral proteins of paramixoviruses, for example, inhibit IFN signaling [11]. Several studies have also examined the mechanisms by which HCV resists the host immune system. These include degradation of Cardif adaptor protein by NS3A/4 protease [12]. Generally, expression of HCV protein is associated with inhibition of STAT1 function independent of STAT tyrosine phosphorylation [13]. Additionally, expression of the HCV core protein in cultured cells is associated with increased expression levels of the suppressor of cytokine signaling 3 (SOCS-3) [14]. The NS5A and E2 proteins are both inhibitors of PKR [15]. These strong actions of HCV against innate immunity are consistent with the high chronicity rate of the virus. IFN, however, effectively reduces HCV replicon in Huh7 cells [16], suggesting that the virus has little potential to disturb the actions of IFN.

In contrast to HCV, the mechanisms of IFN resistance by HBV are poorly understood. To date, only a few studies have reported the molecular mechanisms of HBV resistance against the actions of IFN. The HBV-related resistance to IFN, for example, involves upregulation of protein phosphatase 2A (PP2A) as the primary event, which subsequently leads to inhibition of protein arginine methyltransferase 1 (PRMT1) and reduced STAT1 methylation [17]. In addition to these molecular mechanisms, microarray analyses of serial liver biopsies of experimentally infected chimpanzees showed striking differences in the early immune responses to HBV and HCV. HCV, for example, induced early changes in the expression of many intrahepatic genes, including genes involved in type 1 IFN response [18], whereas HBV did not induce any detectable changes in the expression of intrahepatic genes in the first weeks of infection [19].

HBV–HCV double infection is a good model to use for assessment of the mechanism of IFN resistance by these two viruses because one can test the effect of IFN on one virus in the presence of the other virus. Recently, Bellecave et al. [20] established a novel *in vitro* model system in Huh7 cells that allowed the analysis of both viruses in a replicating context and reported the absence of direct viral interference. To this end, we used human hepatocyte chimeric mice and cell culture systems in the present study. The results showed that the presence of HBV does not affect the actions of IFN on HCV and vice versa. These results suggest the lack of interference between the two viruses in liver cells and indicate that the reported interference between the two viruses might be via inflammation including death of infected cells by cytotoxic T cells, cytokines including IFN-α and IFN-β, and interleukins produced by hepatocytes and infiltrating T cells.

2. Materials and methods

2.1. Transfection of Huh7 cells with HBV DNA and HCV RNA

Huh7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum at 37 °C and under 5% CO_2. Cloning of HBV DNA and the plasmid construction were performed as described previously [21]. For production of stably transfected cell lines, Huh7 cells were seeded onto 90-mm-diameter culture dishes. Twenty micrograms of the plasmid pTRE-HB-wt [21] was transfected by the calcium phosphate precipitation method. Twenty-four hours after transfection, the cells were split and cultured in Hygromycin B-DMEM selection medium (300 μg/ml; Invitrogen Japan K.K., Osaka, Japan), while 50 colonies were isolated and cultured for identification of virus-producing cell lines. Clones positive

for both hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) were selected and further analyzed for production of HBV particles. Finally, three cell lines that produced more than 10^5 copies per milliliter of HBV DNA in supernatant were selected and used for further experiments.

For transfection with HCV RNA, we used pJFH1, which contains the complementary DNA of full-length genotype 2a HCV clone JFH1 downstream of the T7 promoter [22]. *In vitro* synthesis of HCV RNA and electroporation into Huh7 cells were performed as described previously [22,23]. Briefly, cells were treated with trypsin, washed twice with ice-cold RNase-free phosphate-buffered saline, and resuspended in Opti-MEM I (Invitrogen, Carlsbad, CA, USA) at a final concentration of 7.5×10^6 cells per milliliter. Then, 10 μg of HCV RNA to be electroporated was mixed with 0.4 mL of cell suspension and subjected to an electric pulse (950 μF and 260 V) using the Gene Pulser II Electroporation System (Bio-Rad, Hercules, CA, USA). After electroporation, the cell suspension was left for 5 min at room temperature and then incubated under normal culture conditions in a 10-cm-diameter cell culture dish.

2.2. Generation of human hepatocyte chimeric mice

Generation of the urokinase-type plasminogen activator $(uPA)^{+/+}$ and severe combined immunodeficiency $(SCID)^{+/+}$ mice and transplantation of human hepatocytes were performed as described recently by our group [21,23,24]. All mice were transplanted with frozen human hepatocytes obtained from the same donor. Infection, extraction of serum samples, and euthanasia were performed under ether anesthesia. The concentration of serum human serum albumin, which correlates with the repopulation index [24], was measured in mice as described previously [21]. All animal protocols described in this study were performed in accordance with the guidelines of the local committee for animal experiments. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan.

2.3. Human serum samples

Human serum samples containing high titers of either HBV DNA (5.3×10^6 copies per milliliter) or genotype 1b HCV (2.2×10^6 copies per milliliter) were obtained from patients with chronic hepatitis with a written informed consent. The individual serum samples were divided into small aliquots and separately stored in liquid nitrogen until use. Chimeric mice were injected intravenously with 50 μL of either HBV- or HCV-positive human serum. Some mice were injected with HBV-positive human serum at 6 weeks after injection of HCV-positive human serum.

2.4. Analysis of HBV and HCV

HBsAg and HBeAg in culture supernatants were measured by commercially available enzyme-linked immunosorbent assay (ELISA) kits (Abbott Japan, Osaka, Japan). DNA was extracted from these samples by SMITEST (Genome Science Laboratories, Tokyo, Japan) and dissolved in 20 μL H_2O [21,25]. RNA was extracted from serum samples by Sepa Gene RV-R (Sankojunyaku, Tokyo), dissolved in 8.8 μL RNase-free H_2O, and reverse transcribed using random primer (Takara Bio Inc., Shiga, Japan) and M-MLV reverse transcriptase (ReverTra Ace, TOYOBO Co., Osaka, Japan) in a 20-μL reaction mixture according to the instructions provided by the manufacturer [23]. HCV core antigen in the culture medium was detected with HCV Ag assay (Ortho-Clinical Diagnostics, Rochester, NY, USA).

2.5. RNA extraction and measurement of mRNAs of interferon-induced genes by quantitative reverse transcription-polymerase chain reaction

Total RNA was extracted from cell lines using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). One nanogram of each RNA was reverse transcribed with ReverseTra Ace (TOYOBO Co.) and Random Primer (Takara Bio, Kyoto, Japan). We quantified the transcripts for MxA, OAS, and PKR. Amplification and detection were performed using ABI PRISM 7300 (Applied Biosystems, Foster City, CA, USA). Results were normalized to the transcript levels of glyceraldehydes-3-phosphate dehydrogenase (GAPDH).

2.6. Statistical analysis

Changes in HBV DNA and HCV RNA in mice sera were compared by Mann–Whitney test and unpaired *t* test. Differences in HBV DNA and HCV core antigen in mice sera and culture supernatants were analyzed by one-way analysis of variance followed by Scheffe's test. A P value of <0.05 was considered statistically significant.

3. Results

3.1. Infection of chimeric mouse with HBV and HCV and susceptibility to interferon

To investigate the interference between HBV and HCV and to examine the effect of IFN on both of these two viruses *in vivo*, we used six human chimeric mice. Each of six mice was inoculated intravenously with 50 μL of serum samples obtained from either HBV- or HCV-positive patients. The median HBV DNA level in HBV-positive serum-inoculated mice was 1.4×10^8 copies per milliliter (range: 5.3×10^6–3.6×10^9 copies per milliliter) at 6 weeks after inoculation (Fig. 1A), similar to our recent observation [21]. Similarly, the median HCV RNA level in HCV-positive human serum-inoculated mice was 1.0×10^7 copies per milliliter (range: 1.2×10^6–0.8×10^7 copies per milliliter) at 4 weeks after inoculation (Fig. 1B), as reported recently by our group [23]. Six weeks after inoculation, three of six HBV- or HCV-infected mice were treated daily with 7000 IU/g per day of intramuscular IFN-α for 2 weeks. Treatment resulted in a decrease of only 0.3 log in mice serum HBV DNA level compared to that in mice without treatment (Fig. 1A). In contrast, the same therapy resulted in a rapid decrease in HCV RNA to undetectable levels, as confirmed by quantitative polymerase chain reaction (PCR; Fig 1B).

To investigate the direct interference of the two viruses, we performed double-infection experiments. Ten chimeric mice were first inoculated intravenously with 50 μL of HCV-positive human serum samples. Six weeks after HCV infection when the mice developed HCV viremia, 50 μL of HBV-positive human serum samples were inoculated intravenously in 5 of 10 HCV-infected mice. All five mice became positive for both HBV and HCV at 2 weeks after HBV infection. No significant decrease in HCV RNA levels was observed in these superinfected mice before or after the development of HBV viremia (Fig. 2A). After HBV infection, there was no apparent decrease in HCV titer (Fig. 2B). Moreover, HBV DNA level in HBV–HCV-coinfected mice was comparable with that of only HBV-infected mice (Fig. 2B). These results sug-

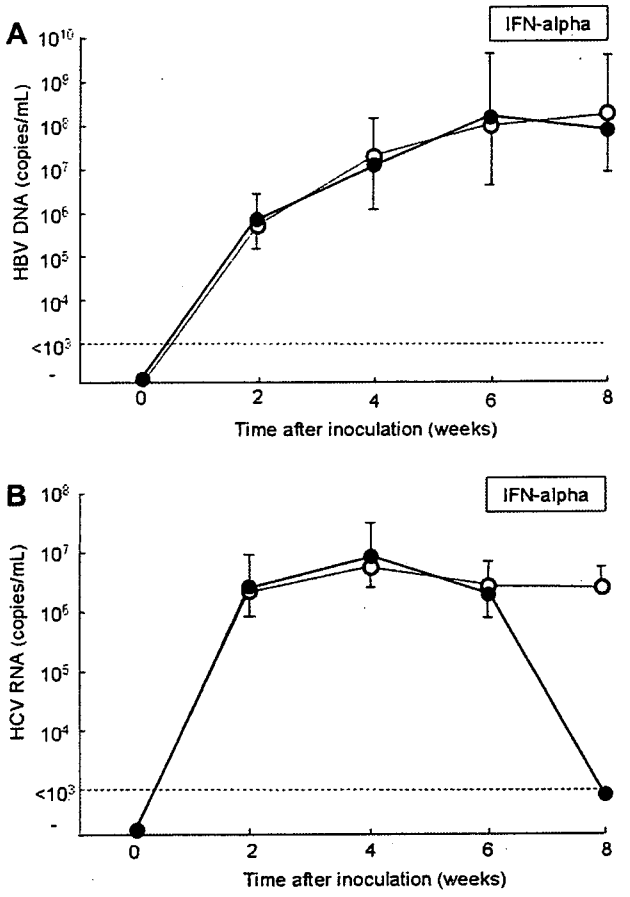

Fig. 1. Changes in serum virus titers in mice inoculated with hepatitis B virus (HBV) – positive or hepatitis C virus (HCV) – positive human serum samples. (A) HBV DNA levels in six mice inoculated with HBV-positive serum samples. (B) HCV RNA levels in six mice inoculated with HCV-positive serum samples. Six weeks after inoculation, three of six mice were treated daily with (closed circles) or without (open circles) 7000 IU/g per day of interferon-alpha intramuscularly for 2 weeks. Mice serum samples were extracted every 2 weeks after inoculation. Data are mean plus or minus standard deviation ($n = 3$). The horizontal dashed line represents the detection limit (10^3 copies per milliliter).

gest no interference between the two viruses in mice, which lack immunocytes known to cause hepatitis.

To further investigate if infection with either of the two hepatitis viruses alters the effect of IFN against the other virus, three HBV–HCV-coinfected mice were treated with IFN-α (Fig. 3A). Such treatment resulted in a rapid decrease in HCV RNA in all mice to undetectable levels as confirmed by quantitative PCR (Fig. 3B). In contrast, no significant decrease in HBV DNA titers was observed in these mice (Fig. 3B). These results are similar to the reduction of HCV RNA and HBV DNA in mice that were infected with either of these hepatitis viruses. These results indicate that HCV is more susceptible to IFN-α than HBV and that each virus does not alter the effect of IFN on the other virus. Because the effect of IFN on HCV was not disturbed by HBV, we assumed that HBV has no effect on the signal from IFN receptor to IFN-stimulated genes. It is possible, however, that HBV and HCV replicated in different cells in these mice. Because it was impossible to detect HCV protein and RNA in HCV-infected mouse liver by histologic examination, we performed *in vitro* experiments.

3.2. Production of both HBV- and HCV-producing cells and the effect of interferon

To investigate the effect of IFN on HBV and HCV *in vitro*, we created cell lines that produce both HBV and HCV. First, we established stable HBV-producing Huh7 cell lines. Three cell lines (Clone-39, -42, and -53) that produced HBsAg, HBeAg, and HBV DNA into the supernatant were selected (Table 1). These cell lines continuously produced HBV for more than 3 months (data not shown). Next, JFH1 RNA was transfected into these HBV-producing cell lines to produce both HBV DNA and HCV proteins into the supernatant. HBV DNA levels in the supernatants of these cell lines decreased in Clone-39, increased in Clone-42, and did not change in Clone-53 after JFH1 transfection (Fig. 4A). In contrast, HCV core antigen levels in the supernatants were higher in two of the three cell lines (Clone-39 and -42) than in Huh7 cells, and the level was not different in the remaining cell line (Clone-53) (Fig. 4B). These results indicate that the production of each of the two viruses does not disturb the replication of the other virus.

3.3. Effects of interferon on HBV and HCV in vitro

The effects of IFN on virus production in both HBV- and HCV-producing cell lines was examined by adding different amounts of IFN-α (0, 50, and 500 IU/mL) into the culture. The mRNA levels of intracellular IFN-stimulated genes such as MxA, OAS, and PKR increased in a dose-dependent manner in all three cell lines as well as in parental Huh7 cells (Fig. 5A). Following the addition of IFN, no apparent reduction of HBV was noted in the supernatant of HBV–HCV-cotransfected cell lines (Fig. 5B). In contrast, the levels of HCV core antigen in the supernatant decreased in all three cell lines treated with IFN, and the decrease was dose-dependent (Fig. 5C).

4. Discussion

Although IFN treatment for chronic HCV infection has improved with the advent of PEG-IFN, the rate of viral eradication remains unsatisfactory [9]. The mechanism responsible for failure of IFN to eradicate the virus completely must be clarified. To study the mechanism of viral resistance against IFN, analysis of viral interference may give us some hints because one of the major mechanisms of interference is through the action of IFN.

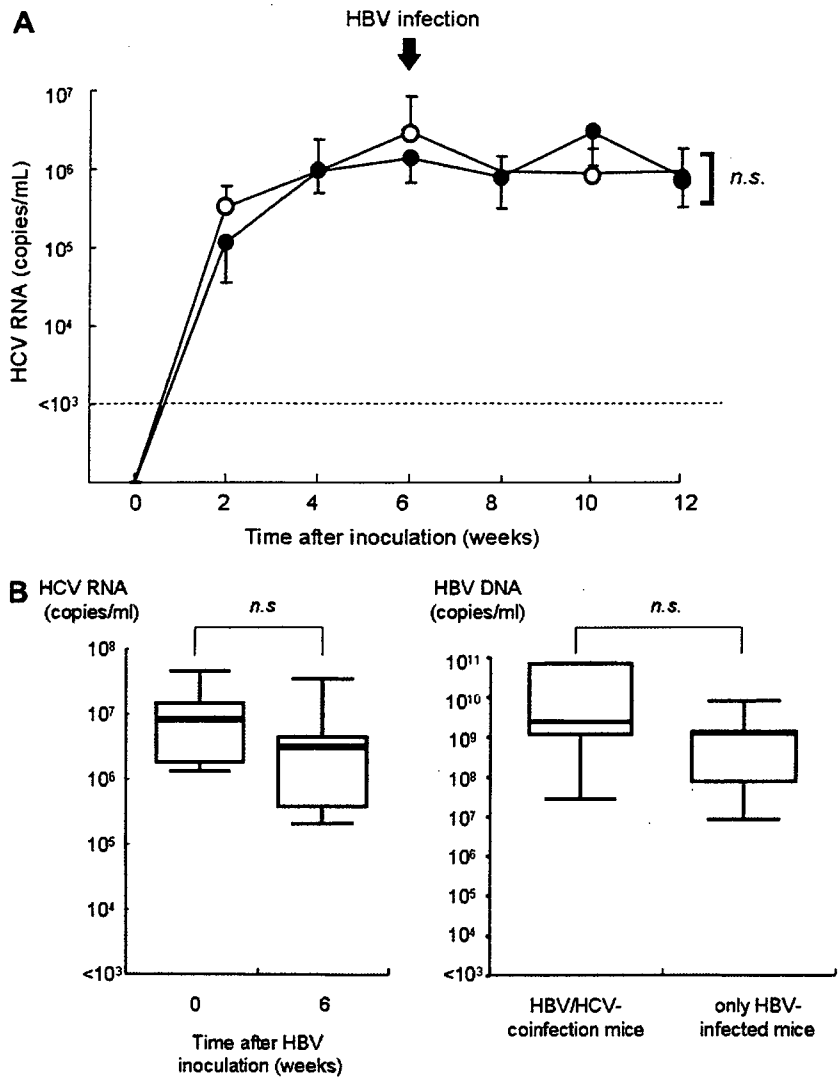

Fig. 2. Comparison of hepatitis C virus (HCV) and hepatitis B virus (HBV) titers in experimentally infected mice. (A) Ten mice were inoculated with HCV-positive serum samples. Six weeks after HCV infection, 5 of the 10 mice were inoculated with HBV-positive human serum samples (closed circles). The remaining five mice (open circles) did not receive HBV inoculation. Data are mean plus or minus standard deviation ($n = 3$). (B) Serum HCV RNA titers in five mice infected with HCV before and at 6 weeks after HBV superinfection (left panel). Serum HBV DNA titers in five mice coinfected with HBV and HCV were compared with those of five mice with HBV infection only (Fig. 1) at 12 weeks after HCV inoculation (right panel). In these box-and-whisker plots, lines within the boxes represent the median values; the upper and lower lines of the boxes represent the 25th and 75th percentiles, respectively.

Accumulation of mononuclear cells is usually seen in the livers of infected individuals, in association with the state of inflammation. It is thus difficult to examine the interference of hepatitis viruses in infection and replication in liver cells without taking into consideration the effect of these immune cells as well as the chemokines and cytokines produced by these cells. Instead, the present study was designed to examine the interference between HBV and HCV in an experimental setup lacking such inflammatory interferences. The SCID-based human hepatocyte chimeric mouse model is ideal for investigating such interaction. We expected either reduction of HCV after inoculation of HBV in HCV-infected mice or failure to develop HBV viremia or low-level HBV viremia in these mice due to viral interference; however, no reduction in HCV titers occurred in these mice, and HBV infection developed in a manner similar to that in naïve mice (Fig. 2). We thus confirmed that there is no interference between the two viruses in the absence of immune reaction via the infiltrating lymphocytes in the liver.

Wieland et al. reported that HBV did not induce any genes during entry or expansion in HBV-infected chimpanzee livers and suggested that HBV was a stealthy virus early in the infection [19]. Because no reduction in HCV was noted during and after the development of high-level HBV viremia, we assume that HBV escapes innate immunity via an excellent mechanism without

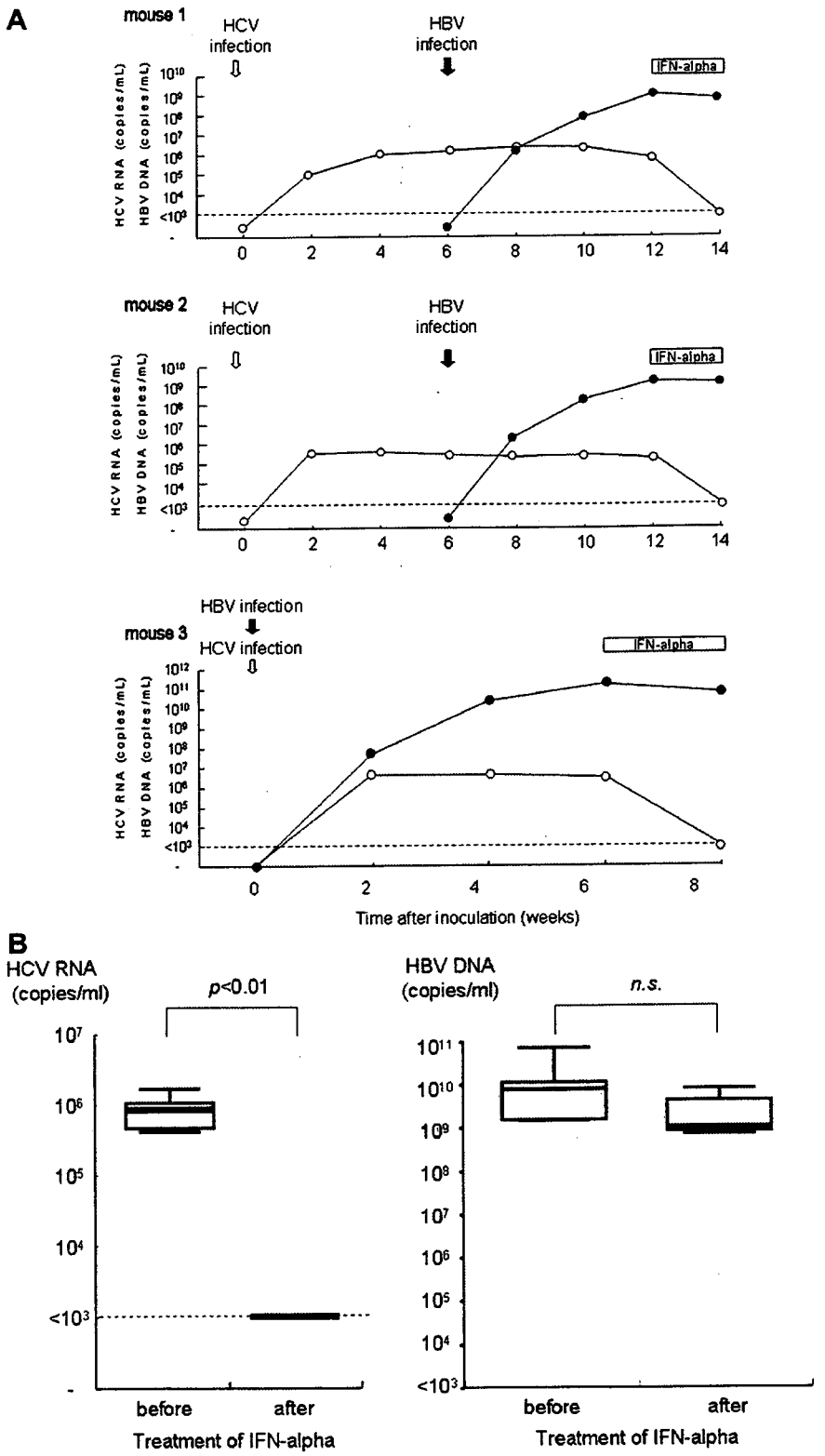

Fig. 3. Changes in serum hepatitis C virus (HCV) RNA and hepatitis B virus (HBV) DNA levels and effects of IFN on HBV–HCV-coinfected mice. Three mice (mouse 1, 2, and 3) were inoculated with both HBV- and HCV-positive human serum samples and treated daily with 7000 IU/g per day of interferon-alpha (IFN-α) intramuscularly for 2 weeks. Mice sera samples were obtained every 2 weeks after injection, and HCV RNA (open circles) and HBV DNA (close circles) were analyzed by quantitative polymerase chain reaction. (A) The horizontal dashed line represents the detectable limit (10^3 copies per milliliter). (B) Serum HCV RNA and HBV DNA titers in mice before and after 2-week IFN-α treatment. In these box-and-whisker plots, lines within the boxes represent median values; the upper and lower lines of the boxes represent the 25th and 75th percentiles, respectively.

Table 1
Hepatitis B virus (HBV) markers in supernatants of stable HBV-transfected cell lines.

Clone	HBsAg (IU/L)	HBeAg (IU/L)	HBV DNA (log copies per milliliter)
39	0.46	4.57	5.2
42	8.16	1.34	5.3
53	0.08	9.29	5.4

Abbreviations: HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen.

evoking the IFN production system in liver cells. Further study using double-infected mice treated with anti-HBV nucleotide analogs and anti-HCV protease inhibitors should be conducted to confirm the present findings.

With regard to the use of IFN as a treatment, we initially assumed that HBV infection would prevent the effect of IFN on HCV and possibly vice versa in double-infection mice. Unexpectedly, the reduction of HCV by IFN therapy was quite similar in mice infected with HCV only and in those coinfected with HBV and HCV (Figs. 1 and 3). This finding indicated that HBV does not disturb the effect of IFN through signal transduction from the IFN receptor through the Jak-STAT pathway. It was, however, considered possible that HBV and HCV infect different liver cells in mice and replicated without being affected by each other. It has been reported that the same liver cell could be infected with both HBV and HCV [20,26], but it was difficult in the present study to confirm that these two viruses replicate in the same liver cell of mice because it is difficult to visualize HCV antigen and RNA in pathologic sections of the mouse liver. To address this issue, we transfected HCV to stable HBV-producing cell lines (Fig. 4). We thought that both HCV and HBV were produced from successfully HCV RNA transfected cells because transfected cells were stable HBV-producing cells. Presence of the both hepatitis viruses in the same hepatocytes has also been shown by a recent report by Bellecave et al. [20]. We showed in our cell line experiments that only HBV-transfected cell lines produced HBV and that cells cotransfected with HBV and HCV did not show a clear effect of HCV replication on HBV production (Fig. 4A). Similarly, stable production of HBV did not alter the replication of HCV (Fig. 4B). These data are consistent with a recent report [20] that showed that HCV could infect cells producing HBV and suggest a lack of interference between the two viruses in liver cells.

Using HCV-transfected HBV-producing cell lines, we demonstrated that presence of HBV did not disturb the actions of IFN on HCV (Fig. 5C). HCV utilizes certain machinery to disrupt the innate immune system; however, once exposed a large concentration of IFN, the virus shows high sensitivity, as shown in the replicon system [16,27]. Thus, HCV seems to have a relatively weak ability to disturb the antiviral actions of IFN compared with HBV. In contrast, HBV showed strong resistance against IFN in cells with diminished HCV replication [28]. The fact that HBV does not disturb IFN signaling but resists the actions of IFN suggests that HBV counteracts the actions of IFN at IFN-induced antiviral product levels.

Although the culture environment is different from the replicon system, the JFH1 strain seems relatively resistant to IFN [29]. This suggests that the core and envelope proteins, which are absent in the replicon system, might play a role in IFN resistance; however, we could not show any effect for HCV infection on the actions of IFN on HBV replication. This finding sug-

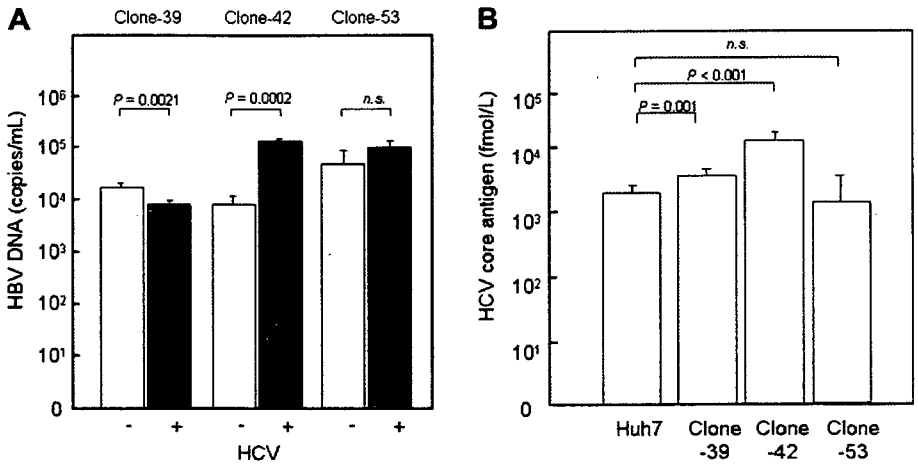

Fig. 4. Virus titers in supernatants of hepatitis B virus (HBV)–transfected or hepatitis C virus (HCV)–transfected cell lines. Huh7 cells were initially stably transfected with 1.4 genome-length HBV DNA. Three cell lines (Clone-39, -42, and -53) producing HBV DNA into the supernatant were selected. (A) HBV DNA levels in supernatants of HBV-producing cell lines 72 hours after transfection with JFH1 RNA (HCV positive) or control plasmid (HCV negative). (B) HCV core antigen levels in the supernatant of parental Huh7 cells and HBV-producing cell lines 72 h after transfection with JFH1 RNA. Data are mean plus or minus standard deviation ($n = 3$).

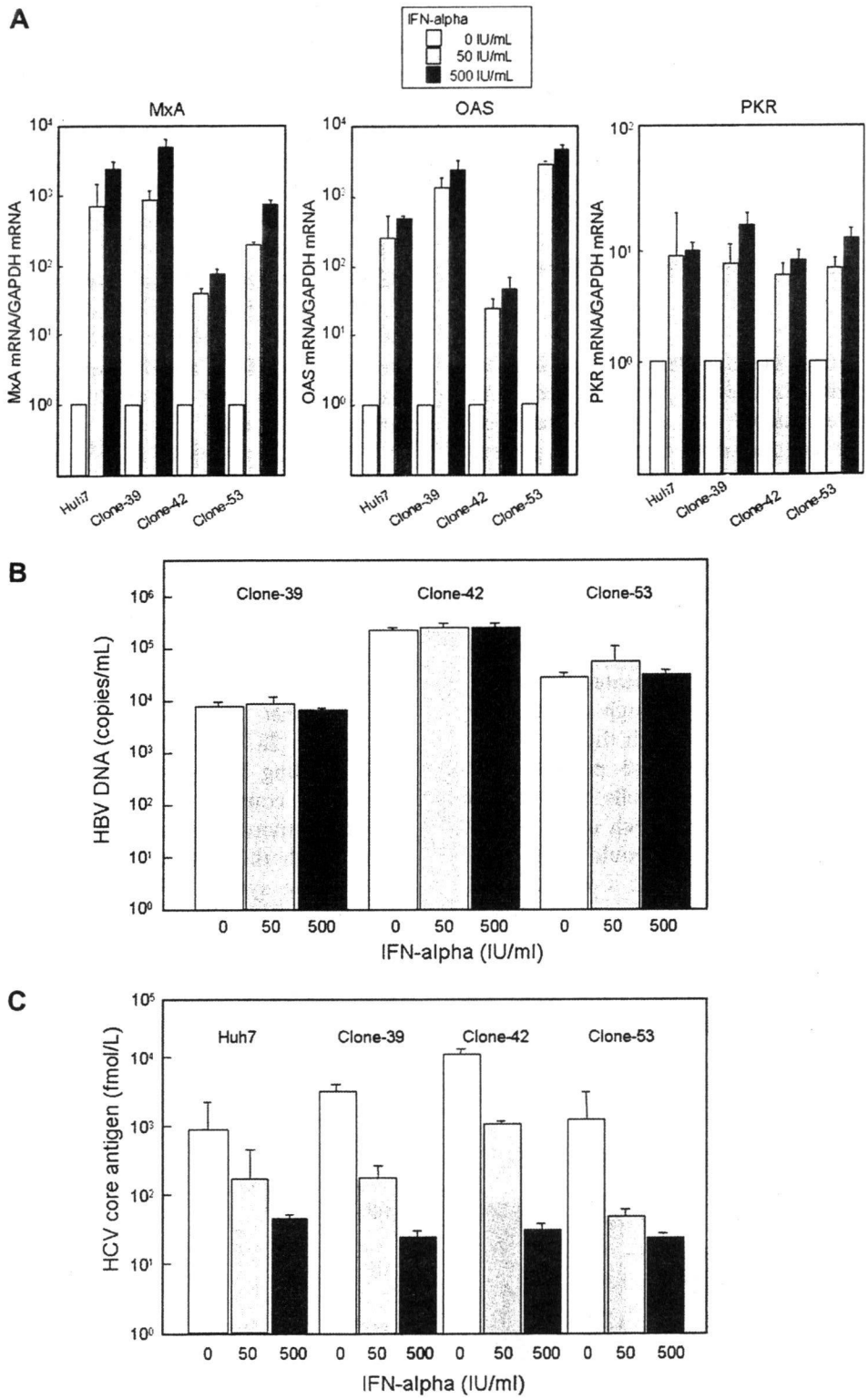

Fig. 5. Effects of interferon (IFN) treatment on hepatitis B virus (HBV) and hepatitis C virus (HCV) *in vitro*. Parental Huh7 cells and three HBV-transfected Huh7 cell lines (Clone-39, -42, and -53) were transfected with JFH1 RNA. Immediately after JFH1 transfection, the cell lines were treated with IFN-α (0, 50, and 500 IU/mL) for 72 h. (A) Intracellular gene expression levels of mixovirus resistance protein A (MxA), 2′,5′-oligoadenylate synthetase (OAS), and RNA-dependent protein kinase (PKR) were measured. RNA levels were expressed relative to glyceraldehydes-3-phosphate dehydrogenase (GAPDH) messenger RNA. (B) HBV DNA and (C) HCV core antigen in supernatants were measured. Data are mean plus or minus standard deviation ($n = 3$).

gests that the core and envelope proteins have only a weak effect on IFN resistance.

In clinical practice, HBV shows high resistance against IFN therapy. This is also the case in the cell culture system, as we showed in this study and has been reported in previous studies [20,28]. The mechanism by which hepatitis viruses resist IFN needs to be clarified in order to develop new and effective therapies for eradication of these viruses.

Acknowledgments

The authors thank Yoshie Yoshida, Kazuyo Hattori, and Rie Akiyama for their excellent technical help.

This study was supported in part by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Labor and Health and Welfare.

References

[1] Maddrey WC. Hepatitis B: an important public health issue. J Med Virol 2000;61:362–366.
[2] Global surveillance and control of hepatitis C. Report of a WHO Consultation organized in collaboration with the Viral Hepatitis Prevention Board, Antwerp, Belgium. J Viral Hepat 1999;6:35–47.
[3] Beasley RP. Hepatitis B virus. The major etiology of hepatocellular carcinoma. Cancer 1988;61:1942–1956.
[4] Samuel CE. Antiviral actions of interferons. Clin Microbiol Rev 2001;14:778–809.
[5] Sagnelli E, Coppola N, Scolastico C, Filippini P, Santantonio T, Stroffolini T, et al. Virologic and clinical expressions of reciprocal inhibitory effect of hepatitis B, C, and delta viruses in patients with chronic hepatitis. Hepatology 2000;32:1106–1110.
[6] Chiaramonte M, Stroffolini T, Vian A, Stazi MA, Floreani A, Lorenzoni U, et al. Rate of incidence of hepatocellular carcinoma in patients with compensated viral cirrhosis. Cancer 1999;85:2132–2137.
[7] Cacciola I, Pollicino T, Squadrito G, Cerenzia G, Orlando ME, Raimondo G. Occult hepatitis B virus infection in patients with chronic hepatitis C liver disease. N Engl J Med 1999;341:22–26.
[8] Raimondo G, Brunetto MR, Pontisso P, Smedile A, Maina AM, Saitta C, et al. Longitudinal evaluation reveals a complex spectrum of virological profiles in hepatitis B virus/hepatitis C virus-coinfected patients. Hepatology 2006;43:100–107.
[9] Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Gonçales Jr FL, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 2002;347:975–982.
[10] Liu CJ, Lai MY, Chao YC, Liao LY, Yang SS, Hsiao TJ, et al. Interferon alpha-2b with and without ribavirin in the treatment of hepatitis B e antigen-positive chronic hepatitis B: a randomized study. Hepatology 2006;43:742–749.
[11] Gotoh B, Komatsu T, Takeuchi K, Yokoo J. Paramyxovirus strategies for evading the interferon response. Rev Med Virol 2002;12:337–357.
[12] Evans JD, Seeger C. Cardif: a protein central to innate immunity is inactivated by the HCV NS3 serine protease. Hepatology 2006;43:615–617.
[13] Blindenbacher A, Duong FH, Hunziker L, Stutvoet ST, Wang X, Terracciano L, et al. Expression of hepatitis C virus proteins inhibits interferon alpha signaling in the liver of transgenic mice. Gastroenterology 2003;124:1465–1475.
[14] Bode JG, Ludwig S, Ehrhardt C, Albrecht U, Erhardt A, Schaper F, et al. IFN-alpha antagonistic activity of HCV core protein involves induction of suppressor of cytokine signaling-3. FASEB J 2003;17:488–490.
[15] Taylor DR, Shi ST, Romano PR, Barber GN, Lai MM. Inhibition of the interferon-inducible protein kinase PKR by HCV E2 protein. Science 1999;285:107–110.
[16] Frese M, Pietschmann T, Moradpour D, Haller O, Bartenschlager R. Interferon-alpha inhibits hepatitis C virus subgenomic RNA replication by an MxA-independent pathway. J Gen Virol 2001;82:723–733.
[17] Christen V, Duong F, Bernsmeier C, Sun D, Nassal M, Heim MH. Inhibition of alpha interferon signaling by hepatitis B virus. J Virol 2007;81:159–165.
[18] Su AI, Pezacki JP, Wodicka L, Brideau AD, Supekova L, Thimme R, et al. Genomic analysis of the host response to hepatitis C virus infection. Proc Natl Acad Sci USA 2002;99:15669–15674.
[19] Wieland S, Thimme R, Purcell RH, Chisari FV. Genomic analysis of the host response to hepatitis B virus infection. Proc Natl Acad Sci USA 2004;101:6669–6674.
[20] Bellecave P, Gouttenoire J, Gajer M, Brass V, Koutsoudakis G, Blum HE, et al. Hepatitis B and C virus coinfection: a novel model system reveals the absence of direct viral interference. Hepatology 2009;50:46–55.
[21] Tsuge M, Hiraga N, Takaishi H, Noguchi C, Oga H, Imamura M, et al. Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis B virus. Hepatology 2005;42:1046–1054.
[22] Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 2005;11:791–796.
[23] Hiraga N, Imamura M, Tsuge M, Noguchi C, Takahashi S, Iwao E, et al. Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis C virus and its susceptibility to interferon. FEBS Lett 2007;581:1983–1987.
[24] Tateno C, Yoshizane Y, Saito N, Kataoka M, Utoh R, Yamasaki C, et al. Near completely humanized liver in mice shows human-type metabolic responses to drugs. Am J Pathol 2004;165:901–912.
[25] Noguchi C, Ishino H, Tsuge M, Fujimoto Y, Imamura M, Takahashi S, et al. G to A hypermutation of hepatitis B virus. Hepatology 2005;41:626–633.
[26] Rodríguez-Iñigo E, Bartolomé J, Ortiz-Movilla N, Platero C, López-Alcorocho JM, Pardo M, et al. Hepatitis C virus (HCV) and hepatitis B virus (HBV) can coinfect the same hepatocyte in the liver of patients with chronic HCV and occult HBV infection. J Virol 2005;79:15578–15581.
[27] Blight KJ, Kolykhalov AA, Rice CM. Efficient initiation of HCV RNA replication in cell culture. Science 2000;290:1972–1974.
[28] Hayashi Y, Koike K. Interferon inhibits hepatitis B virus replication in a stable expression system of transfected viral DNA. J Virol 1989;63:2936–2940.
[29] Miyamoto M, Kato T, Date T, Mizokami M, Wakita T. Comparison between subgenomic replicons of hepatitis C virus genotypes 2a (JFH-1) and 1b (Con1 NK5.1). Intervirology 2006;49:37–43.

Adoptive immunotherapy with liver allograft–derived lymphocytes induces anti-HCV activity after liver transplantation in humans and humanized mice

Masahiro Ohira,[1,2] Kohei Ishiyama,[1,2] Yuka Tanaka,[1,2] Marlen Doskali,[1,2] Yuka Igarashi,[1,2] Hirotaka Tashiro,[1,2] Nobuhiko Hiraga,[2,3] Michio Imamura,[2,3] Naoya Sakamoto,[4] Toshimasa Asahara,[1,2] Kazuaki Chayama,[2,3] and Hideki Ohdan[1,2]

[1]Department of Surgery, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, [2]Liver Research Project Center, and [3]Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Minami-ku, Hiroshima, Japan. [4]Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo, Japan.

After liver transplantation in HCV-infected patients, the virus load inevitably exceeds pre-transplantation levels. This phenomenon reflects suppression of the host-effector immune responses that control HCV replication by the immunosuppressive drugs used to prevent rejection of the transplanted liver. Here, we describe an adoptive immunotherapy approach, using lymphocytes extracted from liver allograft perfusate (termed herein liver allograft–derived lymphocytes), which includes an abundance of NK/NKT cells that mounted an anti-HCV response in HCV-infected liver transplantation recipients, despite the immunosuppressive environment. This therapy involved intravenously injecting patients 3 days after liver transplantation with liver allograft–derived lymphocytes treated with IL-2 and the CD3-specific mAb OKT3. During the first month after liver transplantation, the HCV RNA titers in the sera of recipients who received immunotherapy were markedly lower than those in the sera of recipients who did not receive immunotherapy. We further explored these observations in human hepatocyte–chimeric mice, in which mouse hepatocytes were replaced by human hepatocytes. These mice unfailingly developed HCV infections after inoculation with HCV-infected human serum. However, injection of human liver–derived lymphocytes treated with IL-2/OKT3 completely prevented HCV infection. Furthermore, an in vitro study using genomic HCV replicon–containing hepatic cells revealed that IFN-γ–secreting cells played a pivotal role in such anti-HCV responses. Thus, our study presents what we believe to be a novel paradigm for the inhibition of HCV replication in HCV-infected liver transplantation recipients.

Introduction

Liver failure and hepatocellular carcinoma (HCC) due to chronic hepatitis C infection are the most common indications for liver transplantation (LT), and the incidences of both have been projected to increase further in the future. Recurrent HCV infection of the allograft is universal, occurs immediately after LT, and is associated with accelerated progression to cirrhosis, graft loss, and death (1, 2). This reflects the suppression of those host-effector immune responses that usually control HCV replication, suggesting that the immunosuppressive environment may play a major role in the rapid progression of recurrent HCV infection after LT (3, 4). Further, the immunosuppressive condition described above is considered to increase the incidence of cancer recurrence after LT in HCC patients. We recently proposed the novel strategy of adjuvant immunotherapy for preventing the recurrence of HCC after LT; this immunotherapy involves intravenously injecting LT recipients with activated liver allograft–derived NK cells (5, 6). Since the immunosuppressive regimen currently used after LT reduces the adaptive immune components but effectively maintains the innate components of cellular immunity (7–9), the augmentation of the NK cell response, which is thought to play a pivotal role in innate immunity, may be a promising immunotherapeutic approach (6). We confirmed that the IL-2/anti-CD3 mAb–treated (IL-2/OKT3–treated) liver allograft–derived NK cells expressed a significantly high level of the tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), which is a critical molecule for tumor cell killing. Further, these cells showed high cytotoxicity against HCC cells, with no such effect on normal cells (5). After obtaining approval from the ethical committee of our institute, we successfully administered adoptive immunotherapy with IL-2/OKT3–treated liver lymphocytes to liver cirrhosis patients with HCC in a phase I trial. Although the long-term benefits of this approach with regard to the control of HCC recurrence after LT remain to be elucidated, this trial provided a unique opportunity to study whether the adoptive administration of IL-2/OKT3–treated liver lymphocytes could also mount an anti-HCV response in HCV-infected LT recipients.

Previous studies have highlighted the important roles of innate lymphocytes in developing immunity against hepatotropic viruses, including HCV (10, 11). In this regard, it is known that patients with chronic HCV infection show diminished NK and NKT cell responses (12–14). In the case of an LT, it has recently been reported that the host $CD56^+$ innate lymphocyte population,

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: *J. Clin. Invest.* 119:3226–3235 (2009). doi:10.1172/JCI38374.

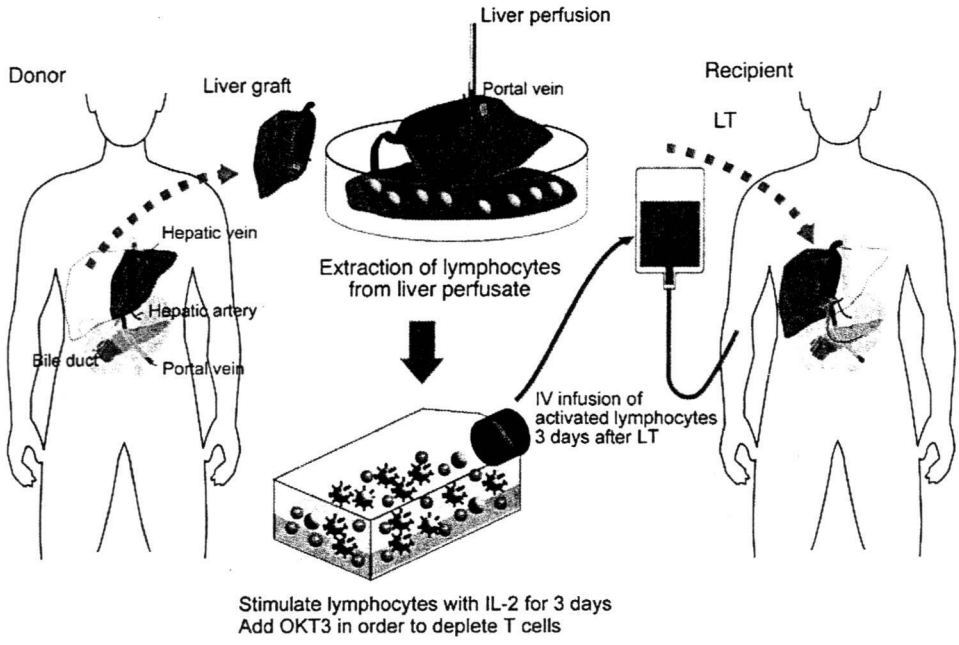

Figure 1
Schematic outline of adoptive immunotherapy with lymphocytes extracted from liver allograft perfusate. The therapy involved giving an intravenous injection of IL-2/OKT3–treated liver lymphocytes to LT recipients. The lymphocytes were extracted from the donor liver graft perfusate. After 3 days of culture with IL-2 (100 JRU/ml), the activated liver NK cell–enriched lymphocytes were administered to the LT recipients through venous circulation. OKT3 (1 μg/ml) was added to the culture medium 1 day before this administration in order to prevent GVHD.

consisting of NK and NKT cells, is appreciably associated with the severity of HCV recurrence after LT (15). These insights into the immunopathogenesis of HCV recurrence indicate that the innate immune components mentioned above are potential targets for therapeutic manipulation. In this study, we have demonstrated for the first time to our knowledge that adoptive immunotherapy with IL-2/OKT3–treated liver lymphocytes, including abundant NK and NKT cells, shows anti-HCV activity after LT, even in an immunosuppressive environment.

Results

Adoptive transfer of IL-2/OKT3–treated liver lymphocytes. The human liver contains a significant number of resident lymphocytes. These cells include abundant $CD56^+$ NK and NKT cells, many of which differ phenotypically and functionally from the circulating cells (14, 16). In our previous study, we performed ex vivo perfusion of the liver through the portal vein, which was necessary in order to flush blood from the liver graft before implantation. Liver-resident lymphocytes were then extracted from the perfusates (number of lymphocytes extracted from normal liver perfusates, 0.5 ± 0.1 cells per gram of liver weight; $n = 14$) (5). Proportions of $CD56^+CD3^-$ NK cells and $CD56^+CD3^+$ NKT cells among the lymphocytes extracted from the liver perfusates (NK cells, 46.4% ± 4.2%; NKT cells, 17.2% ± 2.3%; $n = 14$) were significantly ($P < 0.05$) higher than those among the lymphocytes derived from the peripheral blood of the same donors (NK cells, 21.9% ± 3.7%; NKT cells, 3.8% ± 0.9%; $n = 14$). Extensive preclinical studies have shown that liver allograft–derived resident NK cells mediate remarkably higher cytotoxic activity against HCC cells than do peripheral blood NK cells (5). On this basis, we undertook a clinical trial of adjuvant immunotherapy with IL-2/OKT3–treated liver lymphocytes for preventing the recurrence of HCC after LT in 14 recipients with HCC (Figure 1 and Tables 1 and 2). The therapy involved administering a single intravenous injection of IL-2/OKT3–treated liver lymphocytes to recipients 3 days after LT ($2–5 \times 10^8$ cells injected per subject). In order to prevent graft-versus-host disease (GVHD), i.e., to inactivate $CD3^+$ alloreactive T cells, we added an anti-CD3 mAb, OKT3, to the culture medium a day before the inoculation. During the follow-up period (mean, 23.4 months; range, 10.7–32.9 months), neither any remarkable adverse effects nor rejection episodes occurred. All 14 subjects who received the immunotherapy were alive without recurrence of HCC after LT (including 5 patients with HCC exceeding the Milan criteria; ref. 17). At our institute, the survival rate and recurrence rate of historical control patients with HCC exceeding the Milan criteria were 78% (30 of 37) and 10.8% (4 of 37), respectively. The lymphocytes in the peripheral blood of LT recipients who received immunotherapy in the early postoperative period showed significantly enhanced cytotoxicity against an HCC cell line (HepG2) as compared with those in the peripheral blood of LT recipients who did not receive the therapy in the same period (Figure 2A). Although the gross proportions of NK/NKT cells in the peripheral blood of patients treated with immunotherapy did not differ from those in the peripheral blood of untreated patients, the proportions of $TRAIL^+$ NK cells significantly increased after immunotherapy in the peripheral blood of the former patients. This increase in the $TRAIL^+$ NK cells in the peripheral blood lymphocytes was not observed in untreated patients (Figure 2B). Furthermore, there was a significant correlation between the frequency of $TRAIL^+$ NK cells in the peripheral blood lymphocytes and the NK cytolytic activity of the peripheral blood lymphocytes at 7 days after LT (Spearman rank-order correlation coefficient = 0.54, $P = 0.01$; Figure 2C), indicating the anti-HCC effect of adoptively injected $TRAIL^+$ NK cells. It would be pertinent to conduct additional clinical trials of this immunotherapy for preventing HCC recurrence after LT.

Anti-HCV activity after adoptive immunotherapy. Of the 14 LT recipients who received the immunotherapy, 7 had chronic HCV infection. During the period of this trial, 5 other HCV-infected LT recipients who did not agree to receive immunotherapy served as controls; the background of the controls, including HCV genotype, age, and immunosuppressive therapy, was similar to that of the immunotherapy recipients (Table 3). It has been reported

Table 1
Recipient and tumor characteristics

Patient no.	Age (yr)	Sex	MELD	Hepatitis virus infection	HLA A	HLA B	HLA C	Milan criteria	AFP (ng/ml)	PIVKA-II (AU/ml)	Tumor no.	Maximum tumor size (mm)	Path. vascular invasion	Path. stage	Postop. months	Outcome
1	67	M	19	B	24,-	13,40	03,-	OUT	–	2,584	5	35	–	III	32.9	Alive
2	53	M	16	B	2603,3303	4002,4403	0304,1403	IN	25.3	43	4	11	–	II	31.0	Alive
3	54	M	7	B	0206,3101	3501,5101	0303,1402	OUT	5.7	213	11	26	–	III	29.4	Alive
4	64	F	16	C	2601,2603	3501,4801	0303,-	IN	5.9	142	–	–	–	–	28.5	Alive
5	59	F	14	B	0206,2601	4002,5502	0102,0304	OUT	<5	65	1	13	b1	II	27.8	Alive
6	47	F	8	C	2402,2601	3501,5201	0303,1202	IN	18	46	3	12	–	II	26.2	Alive
7	57	M	29	B	2402,3101	5101,5201	1202,1402	IN	40.3	514	1	25	–	II	25.4	Alive
8	65	F	18	C	1101,2402	5401,5901	0102,-	IN	–	–	3	6	–	II	24.4	Alive
9	60	F	8	–	1101,3001	1302,4006	0602,0801	OUT	32.8	3,026	2	40	v1	IVA	22.7	Alive
10	56	M	8	C	2402,3303	5201,5801	0302,1202	OUT	–	304	11	22	–	III	19.1	Alive
11	56	M	9	C	0207,-	4601,-	0102,-	IN	47	20	3	25	–	III	17.5	Alive
12	58	M	22	C	1101,3101	1501,3501	0102,0415	IN	–	62	1	17	–	I	16.5	Alive
13	59	M	6	C	1101,2402	1507,1501	0303,0401	IN	202.9	19	3	16	–	II	15.8	Alive
14	51	M	16	B	1101,2601	4002,5401	0102,0304	IN	–	29	–	–	–	–	10.7	Alive

The Milan criteria specifies that liver cancer patients with a single tumor of 5 or fewer centimeters in diameter or 3 or fewer tumors, each no more than 3 cm in diameter, and with no macrovascular invasion, can expect an excellent outcome after LT, with only a 10% risk of cancer recurrence (31). AFP, alpha fetoprotein; F, female; M, male; MELD, model for end-stage liver disease; PIVKA-II, protein induced by vitamin K absence; Path., pathological; Postop., postoperative.

that HCV RNA concentrations sharply decrease a day after LT and increase rapidly thereafter (3). In some of the patients, who did not receive the immunotherapy, HCV RNA titers remained lower than that of the pretransplant titer 1 week after LT, suggesting the individual variation of increasing tempo. However, in almost all patients, HCV RNA titers exceeded the pretransplantation levels by 2 weeks after LT. Notably, HCV infection disappeared in 2 LT recipients after the immunotherapy, but this was not observed in the case of any HCV-infected LT recipients who did not receive the therapy. In one of these patients (who had the lowest HCV RNA levels before LT), HCV RNA has not been detected to date (20 months after LT), even with a qualitative assay. In the other patient, HCV RNA became detectable at 2 months after LT. On the other hand, the 2 patients with the highest HCV viral loads did not respond at all to the immunotherapy. Thus, the effects of immunotherapy were dependent on the HCV virus load before LT, probably because of the proportion of effectors and targets. All patients with HCV viremia are currently being treated with pegylated IFN-α2b and ribavirin. Nevertheless, during the first month after LT, the HCV RNA titers in the sera of LT recipients who received the immunotherapy were statistically lower than those in the sera of LT recipients who did not receive the therapy ($P < 0.05$) (Figure 3). Among the LT recipients who received the immunotherapy, at 2 weeks after LT, HCV RNA remained undetectable in 4 patients (responders), whereas it was detectable in the other 3 patients (nonresponders). The serum ALT levels did not differ between the responders and nonresponders (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI38374DS1), suggesting that the immunotherapy did not inhibit HCV RNA by injuring HCV-infected hepatocytes.

In vitro evidence to prove the anti-HCV activity of IL-2/OKT3–treated liver lymphocytes by using HCV replicon–containing hepatic cells. The liver allograft–derived lymphocytes were cultured in complete medium with and without IL-2 for 3 days. This was followed by adding OKT3 to the culture medium 1 day before coculturing the lymphocytes with HCV replicon–containing hepatic cells in a transwell system, at an indicated time. While the freshly isolated liver allograft–derived lymphocytes inhibited HCV replication in the HCV replicon–containing hepatic cells to some extent, the cultivation of these lymphocytes with IL-2/OKT3 markedly promoted anti-HCV activity. Absence of exposure to either IL-2 or OKT3 resulted in reduced anti-HCV activity of the lymphocytes (OKT3 had a more profound influence than IL-2) (Figure 4A). When the lymphocytes were treated with IL-2 alone, the $CD56^+$ fraction, including NK and NKT cells, that had been isolated by magnetic cell sorting inhibited HCV replication more strongly than the $CD56^-$ fraction; further, the $CD3^-CD56^+$ NK cell and $CD3^+CD56^+$ NKT cell subfractions showed equivalent anti-HCV activity (Figure 4, B and C). On the other hand, when the lymphocytes were treated with both IL-2 and OKT3, the $CD56^+$ and $CD56^-$ fractions showed similar levels of anti-HCV activity (Figure 4B). After the treatment with IL-2 and OKT3, IFN-γ was the predominant cytokine in the culture supernatant of the lymphocytes (Figure 5A), and intracellular IFN-γ expression was induced in the $CD3^-CD56^+$ NK, $CD3^+CD56^+$ NKT, and $CD3^+CD56^-$ T cells (Figure 5B). There was no difference between the proportions of $TRAIL^+$ and $TRAIL^-$ $CD3^-CD56^+$ NK cells producing IFN-γ (Supplemental Figure 2). Adding mAb against IFN-γ to the coculture of lymphocytes with HCV replicon cells markedly weakened the anti-HCV effects. The incomplete restoration of the anti-HCV effect by anti–IFN-γ treat-

Table 2
Donor and graft characteristics

Donor no.	Donor age (yr)	Donor sex	HLA A	HLA B	HLA C	Relationship	Graft	Graft weight (g)	No. of cells administered (× 10^6)
1	41	M	24,-	07,40	03,07	Offspring	Right	608	172
2	24	M	2402,2603	4002,2603	0304,5201	Offspring	Right	658	38
3	51	F	0201,2402	0702,3901	0702,-	Spouse	Right	670	129
4	34	M	2601,2603	4001,4801	0303,0401	Offspring	Left	414	143
5	31	M	0206,2402	4002,5401	0102,0304	Offspring	Posterior	702	135
6	53	F	2402,-	5201,5401	0102,1202	Sibling	Right	538	411
7	24	M	2601,3101	4006,5201	0801,1202	Offspring	Right	642	350
8	34	M	1101,-	4001,5401	0102,1502	Offspring	Right	846	229
9	37	M	0201,1101	1501,4006	0702,0801	Offspring	Left	402	811
10	28	M	1101,3303	5502,5801	0102,0302	Offspring	Right	686	517
11	28	M	0207,2402	4601,5201	0102,5201	Offspring	Right	558	414
12	27	M	0201,1101	1501,3501	0303,0415	Offspring	Right	628	509
13	54	F	1101,2402	1501,1507	0303,0401	Sibling	Right	650	460
14	21	F	2601,2603	1501,5401	0102,0303	Offspring	Right	436	382

ment suggests the possibility that other inflammatory cytokines may also be responsible for the anti-HCV effect, although we have not defined them at present (Figure 5C). Thus, the vigorous anti-HCV activity of IL-2/OKT3–treated liver lymphocytes was dependent, at least in part, on their IFN-γ–secreting activity.

IFN-γ–secreting activity in LT recipients after adoptive immunotherapy. At 14 days after LT, the number of IFN-γ–secreting cells in the peripheral blood of LT recipients who received adoptive immunotherapy was significantly higher than that in the peripheral blood of LT recipients who did not receive immunotherapy during the trial period (Figure 6). This result was consistent with the results of the in vitro studies showing the crucial role of IFN-γ produced in IL-2/OKT3–treated liver lymphocytes.

In vivo evidence to prove the anti-HCV activity of adoptive immunotherapy by using HCV-infected human hepatocyte–chimeric mice. HCV-infected mice have previously been developed by inoculating HCV-infected human serum into chimeric urokinase-type plasminogen activator–SCID (uPA-SCID) mice with engrafted human hepatocytes (18). This HCV-infected mouse model has been reported to be useful for evaluating anti-HCV drugs such as IFN-α and anti-NS3 protease (19). We also generated a human hepatocyte–chimeric mouse model, in which mouse hepatocytes were almost completely replaced by human hepatocytes (20). These mice consistently developed long-term HCV infections, showing high viral titers after inoculation with HCV genotype 1b-infected human serum (50 μl/mouse) (Supplemental Figure 3). Intraperitoneal injection of IL-2/OKT3–treated liver lymphocytes (20 × 10^6 cells/mouse), at 2 weeks after inoculation with the infected serum, consistently prevented the development of HCV infection in the human hepatocyte–chimeric mice (Figure 7A). Such anti-HCV effects were countered by anti–IFN-γ neutralizing antibodies in some chimeric mice, suggesting the potential role played by IFN-γ in the anti-HCV effects of the immunotherapy. The administration of recombinant human IFN-γ markedly and consistently prevented the development of HCV infection in the human hepatocyte–chimeric mice. Once the HCV RNA became undetectable in the sera of chimeric mice receiving either IL-2/OKT3–treated liver lymphocytes or recombinant IFN-γ, it could not be detected again. The constant levels of human serum albumin in the chimeric mice indicated that neither the immunotherapy nor recombinant IFN-γ administration had significant adverse effects on human hepatocytes in those mice (Figure 7B). Once HCV infection had developed in the human hepatocyte–chimeric mice, who showed high titers of HCV RNA in their sera (over 10^3 copies/ml) 4 weeks after the inoculation of HCV-infected serum, the preventive effects of the adoptive immunotherapy or recombinant IFN-γ on HCV infection were no longer observed (Figure 7C).

Table 3
Characteristics of HCV-infected LT recipients that received and did not receive immunotherapy

No.	Age	Sex	HCV genotype	MELD	Pre–HCV RNA (KIU/ml)	Postoperative months	Immunosuppressant
With immunotherapy							
4	64	F	1b	16	210	29	Basiliximab+FK506+MMF
6	47	F	1b	8	5,000	26	Basiliximab+CsA+MMF
8	65	F	1b	18	2,400	24	Basiliximab+CsA+MMF
10	56	M	1b	8	970	19	Basiliximab+FK506+MMF
11	56	M	1b	9	1,700	17	Basiliximab+FK506+MMF
12	58	M	1b	22	19	17	Basiliximab+FK506+MMF
13	59	M	1b	6	2,200	16	Basiliximab+FK506+MMF
Without immunotherapy							
A	51	M	1b	27	420	42	Basiliximab+FK506+MMF
B	44	M	1b	10	1,600	32	Basiliximab+FK506+MMF
C	54	M	1b	8	180	22	Basiliximab+CsA+MMF
D	56	M	2a	10	470	20	Basiliximab+FK506+MMF
E	57	M	1b	12	3,200	6	Basiliximab+FK506+MMF