Table 1. Characteristics of HCC Cases and Controls

Study Variables	HCC Cases		Controls	
	Number with Complete Data	n (%)	Number with Complete Data	n (%)
Matched variables				
Gender	224		644	
Male		136 (60.7)		387 (60.1)
Female		88 (39.3)		257 (39.9)
Age at HCC diagnosis (yr)	224	67.6 (10.1)*	–	–
City	224		644	
Hiroshima		155 (69,2)		444 (68.9)
Nagasaki		69 (30.8)		200 (31.1)
Age at serum storage (yr)	224	66.4 (10.2)*	644	63.7 (9.8)*
Unmatched variables				
Viral etiology	211		640	
HBV−/HCV −		45 (21.3)		579 (90.5)
HBV+/HCV −		29 (13.7)		18 (2.8)
HBV−/HCV +		132 (62.6)		41 (6.4)
HBV +/HCV +		5 (2.4)		2 (0.3)
Alcohol consumption (g ethanol/day)	199		577	
None		97 (48.7)		315 (54.6)
0< <20		37 (18.6)		130 (22.5)
20≤ <40		20 (10.1)		64 (11.1)
≥40		45 (22.6)		68 (11.8)
BMI (kg/m^2)	210		633	
10 yrs before diagnosis				
≤19.5		38 (18.1)		122 (19.3)
19.6 - 21.2		33 (15.7)		136 (21.5)
21.3 - 22.9		36 (17.2)		142 (22.4)
23.0 - 25.0		49 (23.3)		124 (19.6)
>25.0		54 (25.7)		109 (17.2)
Smoking habit	199		578	
Never		80 (40.2)		283 (49.0)
Current smoker		107 (53.8)		262 (45.3)
Former smoker		12 (6.0)		33 (5.7)
Radiation dose to the liver (Gy)	204	0.46 (0.69)*	606	0.34 (0.56)*,†

*Mean (SD).
†Weighted mean radiation dose (among controls), calculated by weighting according to their countermatching selection probabilities.

BMI, and smoking habit based on all cases of HCC. The analysis was performed using 186 HCC cases and 600 controls, both separately (radiation only or hepatitis virus infection only) and jointly (radiation and hepatitis virus infection were fit simultaneously), based on subjects with known radiation dose and known HBV and HCV infection status. In analyses where effects of radiation and hepatitis virus infection were fitted separately, unadjusted RR at 1 Gy of HCC for radiation was 1.40 (95% confidence interval [CI], 1.07-1.89, $P = 0.013$), whereas unadjusted RRs of HCC for HBV+/HCV− status and HBV−/HCV+ status were 34 (95% CI, 13-106, $P < 0.001$) and 57 (95% CI, 27-140, $P < 0.001$), respectively. After adjustment for categorical alcohol consumption, BMI, and smoking habit, significant association was found between HCC and radiation dose or hepatitis virus infection, resulting in an RR at 1 Gy of 1.67 (95% CI, 1.22-2.35, $P < 0.001$) for radiation and RRs of 63 (95% CI, 20-241, $P < 0.001$) for HBV+/HCV− status and 83 (95% CI, 36-231, $P < 0.001$) for HBV−/HCV+ status. The above estimates changed little when radiation and hepatitis virus infection were fit simultaneously.

Risk of HCC for Radiation After Excluding Persons with Either or Both Hepatitis Virus Infections. After excluding subjects with either or both hepatitis virus infections, the RRs at 1 Gy of HCC for radiation were estimated as shown in Table 3. There were 161 cases including 119 HCV-infected individuals and 452 matched controls including 29 HCV-infected individuals without HBV infection only. There were 66 cases including 24 HBV-infected individuals and 176 matched controls including 5 HBV-infected individuals without HCV infection only. The adjusted analyses indicated that radiation exposure was significantly associated with increased risks for HCC,

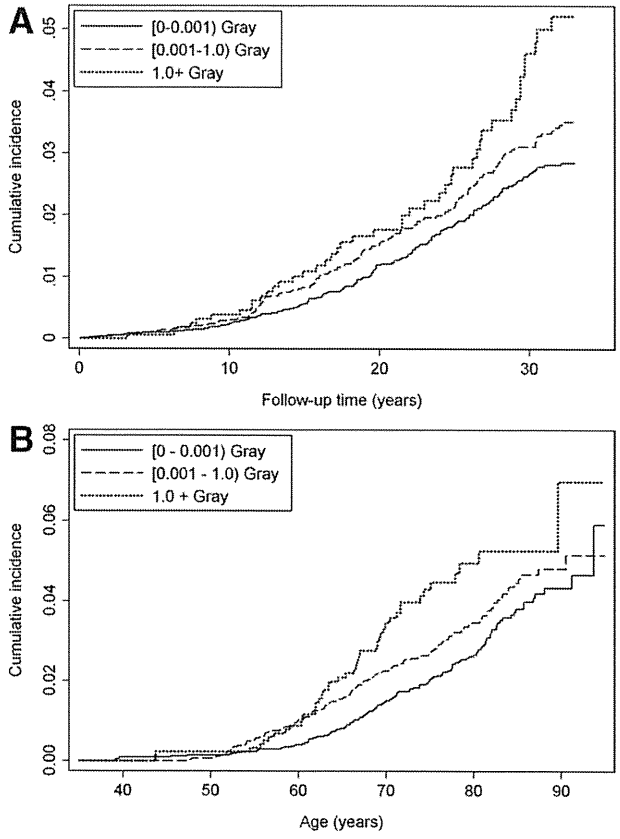

Fig. 1. Cumulative incidence of HCC (1970-2002) by radiation dose. Dotted line: radiation dose ≥1.0 Gy; dashed line: radiation dose 0.001 ≤ <1.0 Gy; solid line, radiation dose 0 ≤ <0.001 Gy. Cumulative HCC incidence by follow-up time (A) and age (B) increased significantly ($P = 0.028$, $P = 0.0003$, respectively) with radiation dose.

even after excluding HBV- or HCV-infected individuals. Furthermore, significant association was found between non-B, non-C HCC and radiation dose, resulting in an RR at 1 Gy of 1.90 (95% CI, 1.02-3.92, $P = 0.041$) for radiation without adjustment for categorical alcohol consumption, BMI, and smoking habit and 2.74 (95% CI, 1.26-7.04, $P = 0.007$) with such adjustment.

Risk of Non-B, Non-C HCC. Effects of alcohol consumption, BMI, and smoking habit on non-B, non-C HCC risk with or without adjustment for radiation dose were estimated using continuous and categorical covariates as shown in Table 4. RRs for continuous covariates are for a one-unit difference in the factor. Risk of non-B, non-C HCC for alcohol consumption per 20 g of ethanol per day was significant with a log-linear model (adjusted RR 1.64, 95% CI, 1.05-2.81, $P = 0.029$), but was limited to the category ≥40 g of ethanol per day (adjusted RR 5.49, 95% CI, 0.98-39.2, $P = 0.052$). Significant log-linear association was not found with continuous BMI, and even the category BMI >25.0 kg/m^2 (obese) 10 years before diagnosis did not evidence significant risk despite a rather large estimate of RR (adjusted RR 3.17, 95% CI, 0.92-12.3, $P = 0.068$). Current smoking evidenced significant risk (adjusted RR 5.95, 95% CI, 1.34-33.2, $P = 0.018$), but there were no continuous data on amount smoked. These results indicate that alcohol consumption per 20 g of ethanol per day, current smoking, and perhaps BMI of >25.0 kg/m^2 10 years before diagnosis are associated independently with increased risk for non-B, non-C HCC.

Discussion

The present study confirmed that radiation is associated with increased incidence of HCC among atomic bomb survivors. Additionally, the nested case-control study indicates that radiation and HBV and HCV infection are associated with increased risk for HCC, and that radiation remains an independent risk factor for HCC after taking into account hepatitis virus infection, alcohol consumption, BMI 10 years before HCC diagnosis, and smoking habit. Furthermore, significant association was observed between non-B, non-C HCC and radiation dose, alcohol consumption, and smoking, whereas obesity 10 years before diagnosis was marginally significantly associated with increased risk for non-B, non-C HCC.

In the analysis (Table 2) in which radiation dose and hepatitis virus infection were fitted separately, radiation was significantly associated with increased risk

Table 2. Risk of HCC for Radiation and HBV or HCV Infection Status

Variables	Number of Cases/Controls	Unadjusted RR (95% CI)		Adjusted* RR (95%CI)	
		Alone†	Joint‡	Alone†	Joint‡
Radiation (at 1Gy)	186/600	1.40 (1.07-1.89)	1.39 (0.93-2.26)	1.67 (1.22-2.35)	1.82 (1.09-3.34)
HBV+/HCV −	24/14	34 (13-106)	30 (11-91)	63 (20-241)	50 (16-184)
HBV−/HCV +	119/35	57 (27-140)	58 (28-147)	83 (36-231)	87 (37-251)

Abbreviations: CI, confidence interval; RR, relative risk.

*Adjusted for categorical alcohol consumption, BMI 10 yrs before diagnosis, and smoking habit.

†Radiation dose to the liver and hepatitis virus infection status were fit separately.

‡Radiation dose to the liver and hepatitis virus infection status were fit simultaneously.

Table 3. Risk of HCC for Radiation After Excluding Persons Infected with HBV and/or HCV

Subjects	Number of Cases/Controls	Unadjusted	Adjusted*
		RR at 1 Gy (95% CI)	RR at 1 Gy (95% CI)
Exclude HBV+ (no HCV adjustment)	161/452	1.48 (1.10-2.05)	1.91 (1.34-2.81)
(adjust for HCV)		1.60 (0.997-2.78)	2.32 (1.25-4.76)
Exclude HCV+ (no HBV adjustment)	66/176	1.61 (1.003-2.76)	1.91 (1.13-3.48)
(adjust for HBV)		1.68 (0.96-3.23)	2.16 (1.12-4.76)
Exclude both HBV+ and HCV+†	42/108	1.90 (1.02-3.92)	2.74 (1.26-7.04)

Abbreviations: CI, confidence interval; RR, relative risk.
*Adjusted for categorical alcohol consumption, BMI 10 yrs before diagnosis, and smoking habit.
†Non-B, non-C status.

for HCC with or without adjustment for alcohol consumption, BMI, and smoking habit. Although this finding is in agreement with our previous understanding that liver cancer risk is significantly associated with radiation without adjustment for hepatitis virus infection among atomic bomb survivors, it is difficult to compare the HCC risk estimates between the previous and current study results.[13-16] The difficulty is caused by the inclusion of hepatoblastoma and intrahepatic cholangiocarcinoma in addition to HCC as liver cancer cases in analyses of tumor registry-based liver cancer risk (ERR at 1 Sv = 0.49),[13] mortality study- and tumor registry-based[15,16] liver cancer mortality risk (male: ERR per Sv = 0.39, female: ERR per Sv = 0.35), and liver cancer risk (male: ERR per Gy = 0.32, female: ERR per Gy = 0.28), despite the fact that the majority of liver cancer cases were HCC. Because a relatively large fraction of liver cancer cases was included that were diagnosed only on the basis of death certificates,[13,16] complete exclusion of metastatic liver tumor cases from such cases may not have been possible. Metastatic liver tumor cases were excluded in an analysis of pathological review-based liver cancer risk (ERR per Gy = 0.81), but hepatoblastoma and intrahepatic cholangiocarcinoma were included with HCC.[14]

In the current analyses adjusted for alcohol consumption, BMI, and smoking habit, the RR estimates for radiation increased slightly and showed statistical significance with adjustment for HBV and HCV infection status. HBV infection may be considered an intermediate risk factor for HCC, because three of four previous HBV screenings demonstrated that HBsAg prevalence increases with radiation dose[17-19,38]; therefore, adjustment for HBV infection status might be expected to result in a decreased radiation risk estimate. However, such interpretation is difficult because

Table 4. Risk of Non-B, Non-C HCC for Alcohol Consumption, BMI, and Smoking Habit

Variables	Number of Cases/Controls	Unadjusted	Adjusted*
		RR (95% CI)†	RR (95% CI)†
Continuous			
Alcohol consumption (per 20 g ethanol per day)	37/96	1.51 (0.98-2.60)	1.64 (1.05-2.81)
BMI 10 yrs before diagnosis (per +1 kg/m^2 difference)	41/107	1.06 (0.95-1.18)	1.06 (0.95-1.19)
Categorical			
Alcohol consumption (g ethanol per day)			
None	22/58	1	1
0 < < 20	5/21	0.98 (0.24-3.60)	0.85 (0.18-3.48)
20 ≤ < 40	2/10	0.78 (0.09-4.49)	0.68 (0.08-4.07)
≥ 40	8/7	5.25 (1.04-33.5)	5.49 (0.98-39.2)
BMI (kg/m^2) 10 yrs before diagnosis			
≤ 19.5	8/18	1.64 (0.45-6.20)	1.66 (0.42-6.83)
19.6 - 21.2	3/22	0.74 (0.12-3.66)	0.80 (0.13-4.15)
21.3 - 22.9	6/25	1	1
23.0 - 25.0	10/24	1.76 (0.42-7.93)	2.37 (0.52-11.5)
> 25.0	14 /18	2.85 (0.86-10.5)	3.17 (0.92-12.3)
Smoking habit			
Never	17/58	1	1
Current smoker	19/38	3.78 (0.99-17.1)	5.95 (1.34-33.2)
Former smoker	1/3	2.83 (0.10-52.3)	4.67 (0.16-93.7)

Abbreviations: CI, confidence interval; RR, relative risk.
*Adjusted for radiation dose to the liver.
†Alcohol consumption, BMI, and smoking habit were fit simultaneously, either as continuous (alcohol and BMI only) or categorical factors.

the risk estimate was also adjusted for HCV infection status, although anti-HCV Ab prevalence is not significantly associated with radiation dose.[20] We therefore examined HBV and HCV infection status and concomitant radiation effects separately, excluding persons with one or the other viral infection.

RRs of HCC for radiation after excluding persons infected with HBV or HCV were generally higher than with the full data, but differed little depending on which virus was used for exclusion (Table 3). As with the full data, adjustment for HBV or HCV infection status reduced the statistical significance of the radiation effect but had little impact on the RR estimates themselves. The RR of HCC for radiation after excluding persons infected with HBV and HCV (i.e., the RR of non-B, non-C HCC for radiation) was significant with or without adjustment for alcohol consumption, BMI, and smoking habit. As there can be no viral mediation of the radiation risk in noninfected individuals, lower radiation risks estimated in infected individuals might be considered evidence of mediation, but mediation would imply that risk decreases with adjustment for viral infection status, which did not occur. The reduction in statistical significance with adjustment for HBV and HCV infection status might be due to loss of power when further parameters for the risks of HCC for hepatitis virus infection are estimated or the number of subjects is reduced by exclusion.

As with the results reported previously,[1] there is evidence that alcohol consumption of $\geq$40 g/day ethanol and BMI >25.0 kg/m^2 10 years before diagnosis are associated with non-B, non-C HCC risk (Table 4). However, the evidence is not as strong given the small amount of data after excluding persons infected with HBV and HCV. The current study demonstrates that smoking is significantly associated with non-B, non-C HCC risk, although lack of continuous data precluded estimation of the relationship to amount smoked. This finding is consistent with recent assessments by the International Agency for Research on Cancer (IARC) where HCC has been positioned as a smoking-related malignant disease.[39] Some studies have shown effects of smoking on risk of HCC, but few studies have incorporated, in a strict and in-depth manner, HBV and HCV infections.[11,40]

Cohort studies of atomic bomb survivors[13-16] and Mayak nuclear facility workers[22-24] have indicated beyond a doubt that radiation increases liver cancer risk, even though hepatitis virus infection was not taken into account. It is also well known that persistent long-term internal exposure to α particles from Thorotrast, a radioactive contrast agent, can induce hemangiosarcoma, cholangiocarcinoma, and HCC in humans.[41-43] Because a significant radiation effect is observed in a high proportion of HCC cases having a p53 mutation, it has been suggested that p53 is one of the intracellular targets of atomic bomb radiation and thus a cause of the increased HCC incidence among atomic bomb survivors.[44] A lifespan study in mice exposed to continuous low-dose-rate γ rays demonstrated that the incidence of HCC was significantly increased, especially in male mice.[25] Liver weights of irradiated mice were significantly greater than those of nonirradiated controls, and the lipid content was significantly increased in irradiated mouse livers.[45] It is considered that hepatic steatosis itself is a state conferring risk for high carcinogenicity, and that in steatohepatitis, oxidative stress due to fatty acid oxidation in hepatocytes may cause DNA injury and eventually lead to carcinogenesis.[46] There is a significant association of radiation dose with prevalence of fatty liver among Nagasaki AHS participants, although a significant association has not been found between obesity (BMI $\geq$26.0 kg/m^2) and radiation dose.[47] These findings may explain part of the mechanism of increased risks of HCC with radiation exposure.

The main strengths of our study include its prospective cohort-based, nested case-control design, which minimizes selection bias, the use of stored sera, and a wealth of epidemiological information obtained prior to HCC diagnosis. It is difficult and expensive to perform full cohort serum analyses, whereas the nested case-control design utilized here can provide substantial reductions in cost and effort with little loss of statistical efficiency.[36] Another major strength of our study is that it incorporated, in a strict and in-depth manner, hepatitis virus infection status and HCC cases were identified through the Hiroshima Tumor and Tissue Registry and Nagasaki Cancer Registry, supplemented by additional cases detected by way of pathological review of related diseases.[26]

A limitation of our study is that the joint effects of radiation and hepatitis virus infection could not be estimated from the standpoint of causality. As discussed previously, HBV and possibly HCV infection may act as intermediate risk factors in radiation-associated HCC. Previous studies have consistently demonstrated that prevalence of HBsAg increases with radiation dose within the AHS,[17-19] although no dose response for anti-HCV Ab has been detected.[20] Therefore, when the risk of HCC for radiation is estimated while controlling for HBV infection, some of the radiation risk may be absorbed in the coefficient for HBV infection. In other words, the radiation risk coefficient

does not represent the radiation effect independent of mediation by HBV infection and the HCC risk for HBV infection itself is not correctly estimated, because the actual causal pathway is not explicitly modeled. In addition, we cannot easily disentangle the joint effects of radiation and HBV infection using standard regression models, because HBV infection is not a true confounding risk factor but an intermediate risk factor. Nevertheless, that the radiation risk did not decrease with concomitant adjustment for viral infection suggests that the practical extent of mediation may be small. We are currently developing methods of statistical analysis that jointly consider the dose response for the intermediate viral factor as well as the joint risk of HCC for both hepatitis virus infection and radiation in the countermatched, nested case-control design.

In conclusion, radiation exposure was associated with increased risk of HCC, even after adjusting for HBV or HCV infection, alcohol consumption, BMI, and smoking habit. Moreover, radiation exposure was an independent risk factor for non-B, non-C HCC with no apparent confounding by alcohol consumption, BMI, or smoking habit. The mechanistic form of joint effects of radiation and HBV or HCV infection on HCC risk could not be estimated, but the development of new statistical methods that jointly consider the dose response for the intermediate viral factor will make such an analysis possible in the future. In particular, in-depth understanding of the mechanisms by which radiation exposure as well as obesity, alcohol drinking, and smoking contribute to development of non-B, non-C HCC may lead to prevention, early detection, and better therapeutic strategies.

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MECHANISMS OF GASTROINTESTINAL, PANCREATIC AND LIVER DISEASES

Animal model for study of human hepatitis viruses

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Abstract

Human hepatitis B virus (HBV) and hepatitis C virus (HCV) infect only chimpanzees and humans. Analysis of both viruses has long been hampered by the absence of a small animal model. The recent development of human hepatocyte chimeric mice has enabled us to carry out studies on viral replication and cellular changes induced by replication of human hepatitis viruses. Various therapeutic agents have also been tested using this model. In the present review, we summarize published studies using chimeric mice and discuss the merits and shortcomings of this model.

Introduction

Hepatitis B virus (HBV) and hepatitis C virus (HCV) are pathogens that cause chronic infection in humans. There are 360 million and 170 million people infected worldwide with HBV or HCV, respectively.[1,2] Infected individuals develop acute hepatitis, chronic hepatitis and liver cirrhosis. The viruses are also important causative agents of hepatocellular carcinoma, especially in the Asia–Pacific region.[3] Study of the biology and development of therapies for each virus has long been hampered by the lack of a small animal model that supports hepatitis virus infection. This is probably as a result of the lack of receptor molecules necessary for viral infection in animal liver cells.

Transgenic mice that express over-length HBV-DNA export viral particles into the serum,[4] and such animals can be used to evaluate antiviral agents,[5–7] as well as HBV-targeted siRNA[8]. However, the virus life cycle is not established in this model, and it is inappropriate for studying drug-resistant HBV strains. Accordingly, researchers attempted to transplant human hepatocytes into mice. The development of the trimera mouse was one such attempt, in which human hepatocytes were transplanted under the kidney capsule of immune-deficient mice after lethal irradiation.[9,10] However, the number of hepatocytes that could survive on the kidney capsule was small, and normal liver architecture was not present. Although 85% of HBV-inoculated animals developed HBV viremia, the titer was less than 10^5 virus particles or IU/mL.[9] Similarly, 85% of HCV-inoculated animals also developed viremia,[10] but the level of the viremia only reached 10^5/mL. Thus, the advent of human hepatocyte transplanted uPA/scid mice has provided the first really useful model for acute and chronic infections of human hepatitis virus.

Human liver cell transplanted uPA/scid mice

Transgenic mice in which the urokinase gene is driven by the human albumin promoter/enhancer were developed and shown to have accelerated hepatocyte death and consequent chronic stimulation of hepatocyte growth.[11] Transplanted rat hepatocytes proliferated and repopulated injured livers in immunodeficient uPA mice, which were produced by mating uPA transgenic mice with scid mice.[12] Human hepatocytes were then transplanted into uPA/scid mice; these cells proliferated and replaced the apoptotic mice liver cells (Fig. 1).

Such human hepatocyte chimeric mice have been shown to be susceptible to both HBV[16] and HCV[17] infections. Repopulation levels by human hepatocytes have been estimated by measuring human albumin levels in mouse serum. Replication levels of both HBV[13] and HCV[17] were higher in mice in which the repopulation index was higher. A unique attempt to remove mouse residual liver cells with the herpes simplex virus type-1 thymidine kinase (HSVtk)/ganciclovir (GCV) system failed to result in a higher repopulation rate as a result of damage to the transplanted human hepatocyte caused by bystander effects.[18] Despite this, mice with livers that have been highly repopulated with human hepatocytes

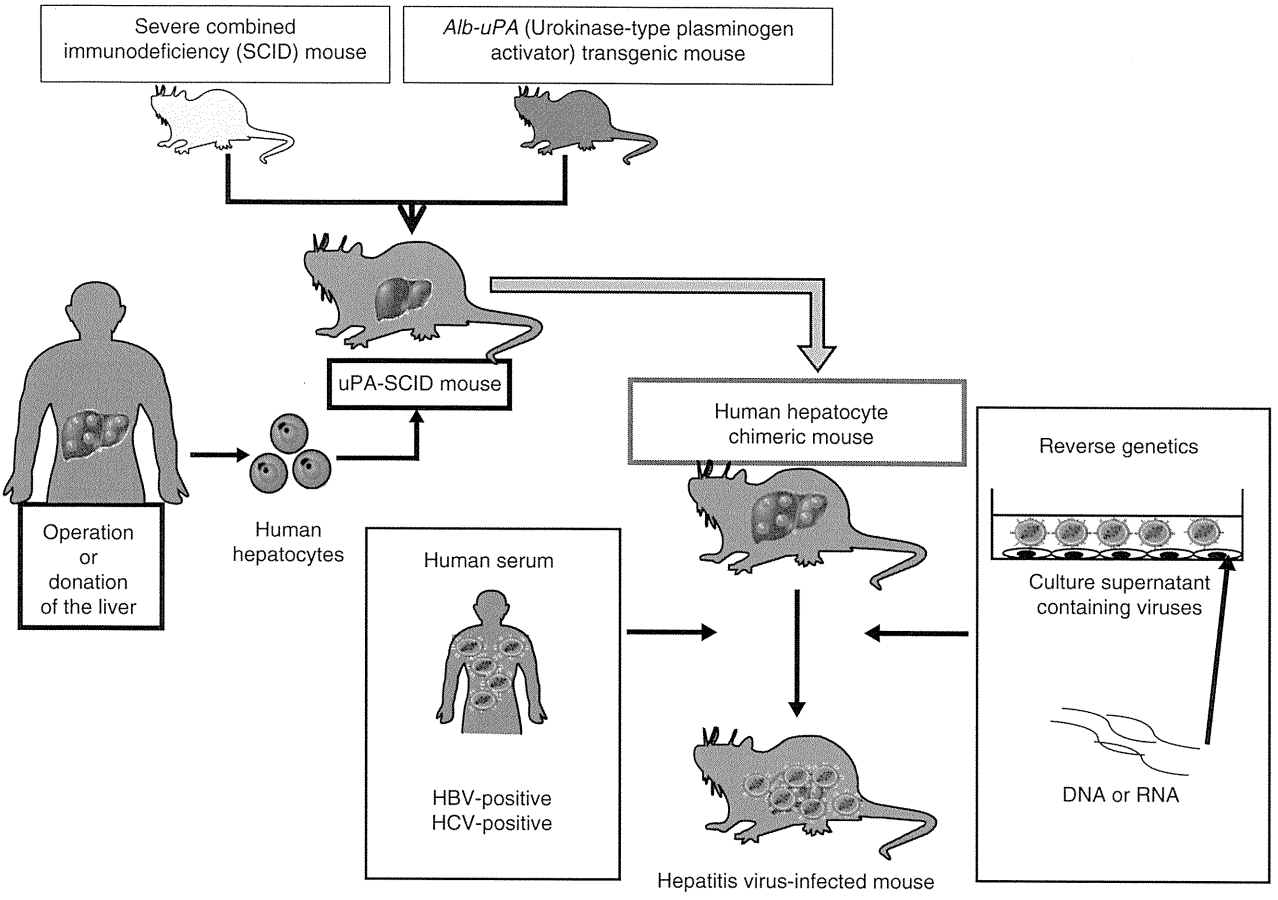

Figure 1 Generation of human hepatocyte chimeric mice and hepatitis virus infection model. A uPA/scid mouse was created by mating uPA transgenic mouse and scid mouse. Human hepatocytes obtained by surgical resection or donation were transplanted to newborn mice. The chimeric mice can be infected with hepatitis B virus (HBV) or hepatitis C (HBC) virus by injecting human serum containing these viruses. Alternatively, the mice can be infected by HBV[13] or HCV[14] created in cell culture or by injecting HCV RNA into the mouse liver.[15]

are susceptible to infection with both HBV and HCV, and as such comprised the most effective small animal model for chronic hepatitis so far developed.[19,20] An example of a highly repopulated mouse liver that we are using in experiments is shown in Figure 2.

Highly repopulated mice have been shown to be a valuable model for the study of drug metabolism.[21–29] Advances in technology for human hepatocyte transplantation have enabled serial passage of human hepatocytes in uPA/scid mice and have been shown to retain infectivity for HBV.[30]

This mouse model and other animal models for the study of hepatitis viruses have been summarized in reviews by Meuleman and Leroux-Roels,[31] Dandri *et al.*,[32,33] Barth *et al.*,[34] and Kneteman and Toso.[35] The present review will focus on key issues and updated information.

Study of hepatitis B virus infection using human hepatocyte chimeric mice

Since the initial reports of successful transmission of HBV to human hepatocyte chimeric mice in 2001 and 2004,[16,27] several researchers have reported transmission of HBV into similar mice.[13,36,37] In these studies, passage experiments studies show that HBV replicating in mice retain infectivity.[13,36] Further, the presence of viral proteins has been shown immunohistochemically in human hepatocytes transplanted into mouse livers, but these are not present in mouse hepatocytes.[13,36,37] Formation of viral particles in infected mouse livers can be shown by electron microscopy.[36,37] Genetically engineered viruses lacking HBe-antigen have also been shown to infect chimeric mice, proving that e antigen is dispensable for viral infection and replication.[13] In contrast, HBx protein has been shown to be indispensable for viral replication.[38] Transcomplementation of HBx protein with hydrodynamic injection restored HBV infectivity in mice. Interestingly, all revertant viruses show a restored ability to express HBx.[38]

By infecting chimeric mice with genotype A, B and C, differing proliferative capacity has been shown between HBV genotypes.[37] In mice infected for a relatively short time, there are no morphological changes in HBV infected mice livers in studies.[13,36] In contrast, the occurrence of liver cell damage has been reported after long-term infection of chimeric mice with HBV[39] or with specific strains of HBV;[40] these findings are consistent with direct cytopathic effects of HBV under certain conditions.

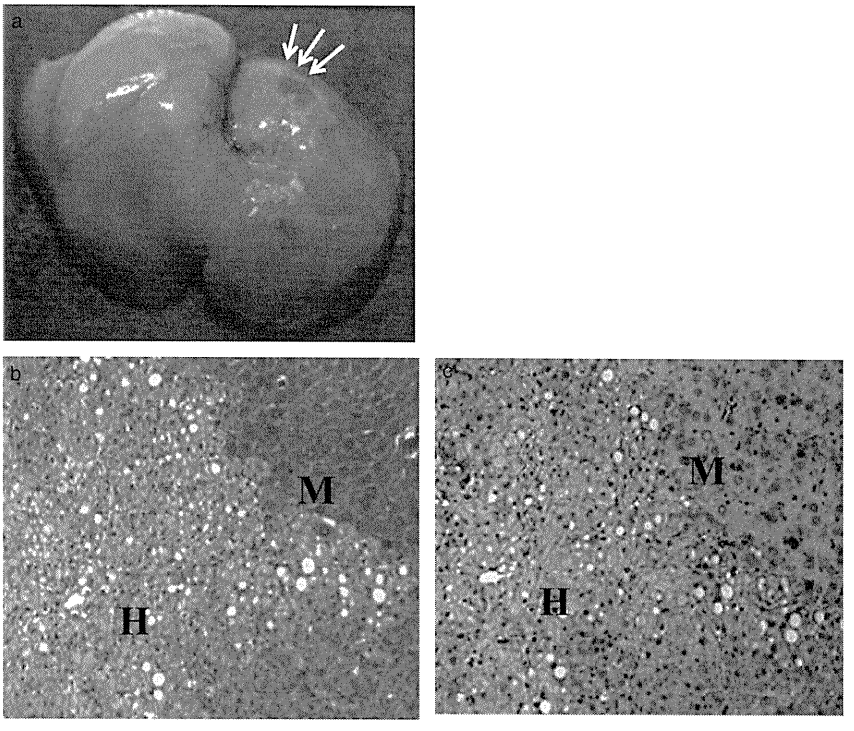

Figure 2 Representative uPA/scid mouse livers repopulated by human hepatocytes. (a) Mouse liver almost completely repopulated by human hepatocytes. Only a small portion of mouse hepatocytes are shown by arrows. (b) Microscopic figure of the mouse liver. M and H indicate regions consisting of mouse and human hepatocytes, respectively (Hematoxylin–eosin staining, magnification: ×100). (c) Microscopic figure of the mouse liver stained with antibody directed against human serum albumin.

The biological properties of a newly identified unique strain of HBV, genotype G, which replicates only in the presence of another genotype, were confirmed using the chimeric mouse.[41] Infectivity of another novel HBV strain, identified from a Japanese patient, that is divergent from known human and ape HBV has also been confirmed.[42] Titration of HBV infectivity, which previously could only be carried out using chimpanzees, can be carried out effectively using chimeric mice.[43]

Taking advantage of the absence of human immune cells in the chimeric mice, Noguchi *et al.*[44] showed that hypermutation of HBV increases in human hepatocytes under interferon treatment. Dandri *et al.* measured viral half-life in human and chimeric mice repopulated with wooly monkey hepatocytes.[45] The results clearly showed that viral half-life is shortened by immunological mechanisms in humans with low viral levels, but not in chimeric mice where functional immunity is absent. Hiraga *et al.*[46] showed an absence of interference between HBV and HCV.

Evaluation of therapeutic agents is the most important role for this mouse model. Tsuge *et al.*[13] assessed the effect of interferon and lamivudine using chimeric mice. Similarly, Dandri *et al.*[47] showed the effects of adefovir using uPA/scid mice repopulated with tupaia hepatocytes, which also support replication of human HBV. Oga *et al.*[48] identified a novel lamivudine-resistant variant that has an amino acid substitution outside of the YMDD motif. They showed that lamivudine was ineffective against the novel mutant strain. It is thus apparent that this mouse/human liver chimeric model is ideal to study the susceptibility of mutant strains to various drugs, because mutant viruses can easily be made and infected into chimeric mice.[13] The model has also been utilized to evaluate viral entry inhibitors derived from the large envelope protein.[49]

Study of hepatitis C virus using human hepatocyte chimeric mice

As observed in studies on HBV, HCV infection efficiency was poor and levels of viremia were low in mice where the repopulation rate of the mouse liver with human hepatocyte was low.[17,50] As shown in Figure 3, human albumin levels in mouse serum were significantly higher in mice in which measurable viremia developed (Hiraga *et al.* unpublished data). Recent studies have therefore been carried out using highly repopulated mice. The usefulness of a newly developed HCV assay,[51] and infectivity of a newly identified intergenotypic recombinant strain,[52] have been reported using the chimeric mice.

Using the remarkable replication ability of the JFH1 genotype 2a strain,[53] infectivity of JFH1 or intergenotypic chimeric viral particles, previously shown in cell culture, has now been shown to be infectious in chimeric mice.[54–56] Infectivity of viruses that were replicated in chimeric mice in cell culture has also been shown, and virus fitness has been studied.[55,56] The role of the HCV core+1 open reading frame and core *cis*-acting RNA elements has also been examined using the chimeric virus.[57] These elegant studies have the limitation that the non-structural part of the virus is limited to that of JFH1. Hiraga *et al.*[14] have shown that infectious clones of genotype 1a and JFH1 can be infected with direct injection of *in vitro* transcribed RNA into the mouse liver.[14] Similarly, Kimura *et al.*[15] reported the establishment of infectious clones of genotype 1b and ablation of RNA polymerase by site-directed mutagenesis abolish infectivity. These infectious clones will be useful for the study of drug-resistant strains.

The model of HCV infection has also been used to show that infection of the virus can be prevented by antibodies against

Table 1 New therapeutic strategies tested by human hepatocyte chimeric mice

n	Drug or cell	Strategy	Reference
1	Interferon alpha 2b	Activation of antiviral genes	Kneteman *et al.*[65]
	BILN-2061	NS3-4A protease inhibition	
	HCV371	NS5B polymerase inhibition	
2	Modified BID	Induction of apoptosis	Hsu *et al.*[66]
3	Serine palmitoyltransferase inhibitor	Disruption of lipid raft	Umehara *et al.*[67]
4	Lymphoblastoid interferon alpha	Activation of antiviral genes	Hiraga *et al.*[14]
5	Amphipathic DNA polymers	Blocking viral entry	Matsumura *et al.*[60]
6	Sec-butyl-analogue of HCV-371	NS5B polymerase inhibition	LaPorte *et al.*[68]
7	HCV796	NS5B polymerase inhibition	Kneteman *et al.*[69]
8	Liver allograft-derived lymphocyte	Adoptive immunotherapy	Ohira *et al.*[70]
9	Telaprevir	NS3-4A protease inhibition	Kamiya *et al.*[71]

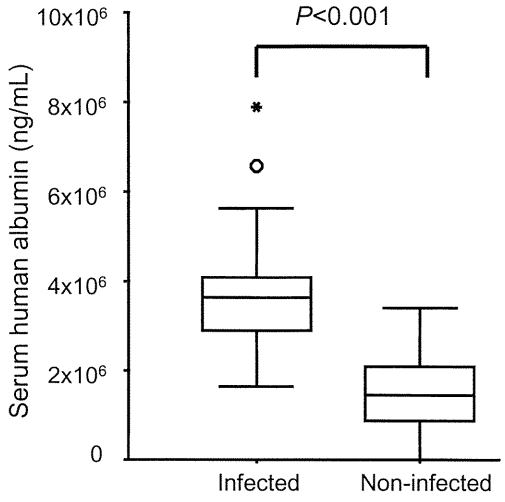

Figure 3 Human albumin levels in mice used in the hepatitis C virus (HCV) infection experiments. A total of 54 mice were injected with HCV positive serum samples containing 5×10^5 virus particles. A total of 24 mice became persistently positive for HCV-RNA, but 30 mice did not. Serum human albumin levels 2 weeks after human hepatocyte transplantation were compared between infected and non-infected mice.

CD81,[58] polyclonal human immunoglobulin directed to a similar strain,[59] and amphipathic DNA polymers.[60] Notably, the presence of broadly neutralizing antibodies to HCV that protect against heterologous viral infection has been reported, suggesting the possibility of a prophylactic vaccine against HCV.[61]

With respect to evasion of the virus against the innate immune response, altered intrahepatic expression profiles in the early phase of infection is of particular interest. The chimeric mice model is ideal for such studies; cross-hybridization of mouse and human can be avoided by careful experimental procedures.[62] Microarray analysis of livers of HCV infected and non-infected mice showed transcriptional activation of genes related to innate immune response, lipid metabolism, endoplasmic reticulum (ER) stress and apoptosis in HCV-infected mice.[63,64] The HCV infected mouse model is particularly useful for the study of newly developed HCV agents. The effect of recently developed chemicals and a unique therapy using intrahepatic lymphocytes have been shown using this model (Table 1). However, none of these therapies have yet been able to completely eradicate HCV from mice. It is noteworthy that ultra-rapid cardiotoxicity has been reported with the protease inhibitor BILN 2061 in the uPA/scid mice, but not in scid mice, implicating involvement of the uPA transgene.[72] Care should therefore be taken in interpreting the results obtained by this model.

Conclusion

Development of a small animal model using human hepatocyte chimeric mice has enabled us to study key aspects of HBV and HCV biology. The characteristic feature of the absence of human immune cells is suitable for studying viral replication and observing changes occurring in liver cells during viral infection, such as the innate immune response and cellular stress and metabolic responses. The model is also useful for studying the effect of drugs without the influence of cytokines and cytotoxic T lymphocytes. Nonetheless, the model is insufficient to study carcinogenesis of hepatitis viruses, because non-parenchymal cells in mouse liver are of mouse origin and do not support inflammation and fibrosis, which are probably closely related to carcinogenesis. The lack of human immune cells also limits the study of inflammation and immunity. Furthermore, the availability of human hepatocytes is limited. Despite these limitations, the current model shows great potential as a mouse model for the study of hepatitis viruses. Development of a small animal model with or without human immunity using stem cells or iPS cells would be an ideal model in the future.

Acknowledgments

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BRIEF REPORT

Effects of Hepatitis B Virus Infection on the Interferon Response in Immunodeficient Human Hepatocyte Chimeric Mice

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Complementary DNA microarray analysis of human livers cannot exclude the influence of the immunological response. In this study, complementary DNA microarray analysis was performed under immunodeficient conditions with human hepatocyte chimeric mice, and gene expression profiles were analyzed by hepatitis B virus (HBV) infection and/or interferon treatment. The expression levels of 183 of 525 genes upregulated by interferon treatment were significantly suppressed in response to HBV infection. Suppressed genes were statistically significantly associated with the interferon signaling pathway and pattern recognition receptors in the bacteria/virus recognition pathway ($P = 1.0 \times 10^{-8}$ and $P = 1.2 \times 10^{-8}$, respectively). HBV infection attenuated virus recognition and interferon response in hepatocytes, which facilitated HBV escape from innate immunity.

Chronic hepatitis B virus (HBV) infection is associated with the development of virus-related liver diseases, including chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. Interferon α (IFN-α) has been used for the treatment of chronic hepatitis B, and many large clinical trials and meta-analyses have demonstrated the effectiveness of interferon [1–3]. However, the effect of IFN-α therapy is unsatisfactory, and the molecular basis for tolerance to IFN-α is not clearly defined.

DNA microarray technology has enabled genome-wide analysis of gene transcript levels with the use of clinical tissues and animal models, which has yielded insights into the molecular features of several liver diseases [4–6]. However, it has been difficult to determine whether the changes in gene expression were caused by viral interference or by the human immune response, because all of these studies that used clinical and experimental samples were analyzed under the influence of adaptive immune responses. Recently, Mercer and colleagues developed a human hepatocyte chimeric mouse model [7]. These mice were derived from severe combined immunodeficiency (SCID) mice, which are severely immunocompromised, and the mouse liver cells were extensively replaced with human hepatocytes [7, 8]. With the use of this chimeric mouse model, in which HBV can continuously infect human hepatocytes, the effect of drugs and the response of viral infection can be analyzed in human hepatocytes under immunodeficient conditions [9]. In this study, we performed microarray analysis with human hepatocyte chimeric mouse livers to assess the direct impacts of HBV infection and IFN treatments on gene expression profiles. We successfully demonstrated that HBV infection attenuated the expression of IFN-stimulating genes under immunodeficient conditions, which suggests that HBV proteins might afford escape mechanisms from cellular innate immunity.

METHODS

A serum sample was obtained from a HBV carrier after obtaining written informed consent for the donation and evaluation of the blood sample. The inoculum was positive for Hepatitis B surface and Hepatitis B e antigens, with slightly elevated levels of serum alanine aminotransferase and high-level viremia (HBV DNA load, 7.1 log copies/mL). The studied patient was infected with HBV genotype C. The experimental protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the Hiroshima University Hospital ethical committee (approval ID: D08-9).

The uPA$^{+/+}$/SCID$^{+/+}$ mice were prepared and the human hepatocytes were transplanted as described elsewhere [8]. The experiments were performed in accordance with the guidelines of the local committee for animal experiments at Hiroshima University.

Sixteen chimeric mice, in which >90% of the liver tissue was replaced with human hepatocytes, were divided into

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4 experimental groups. Group A contained 4 mice that were neither infected with HBV nor treated with IFN. Group B consisted of 3 mice that were treated with IFN-α for 6 h (7,000 IU per gram of body weight) just before being humanely killed but were not infected with HBV. Mice in groups C and D were inoculated via the mouse tail vein with human serum containing 6×10^6 copies of HBV. After inoculation, we collected mouse serum samples every 2 weeks and analyzed HBV DNA titers by real-time polymerase chain reaction (PCR) and human albumin levels by means of a human albumin enzyme-linked immunosorbent assay quantitation kit (Bethyl Laboratories), as described elsewhere [9]. Virus and human albumin titer levels are shown in Supplementary data 1. All 9 mice developed measurable viremia 4 weeks after inoculation. Eight weeks after inoculation, 4 of the 9 infected mice (group C) were humanely killed without IFN treatment and the remaining 5 mice (group D) were humanely killed after 6 h of IFN-α treatment (7,000 IU per gram of body weight). The mice were infected, had serum samples extracted, and were killed humanely under ether anesthesia, as described elsewhere [8].

All 16 chimeric mice were killed humanely, and human hepatocytes were finely dissected from the mouse livers and stored in liquid nitrogen after submerging in RNA later solution (Applied Biosystems). Total RNA was extracted with TRIzol reagent (Invitrogen) and labeled with cyanine 3 by use of a low RNA input linear amplification kit (Agilent Technologies) after amplification. Cyanine-3–labeled complimentary RNA was hybridized to a 44-K whole human genome oligo microarray (Agilent). Detailed protocols are described in Supplementary data 2.

Gene expression profiles were analyzed using GeneSpring GX software (version 10.0.2; Tomy Digital Biology). The detailed protocol is described in Supplementary data table 3. Complete linkage hierarchical clustering analysis was applied using Euclidean distance, and differentially expressed genes were annotated using information from the Gene Ontology (GO) Consortium. Global molecular networks and comparisons of canonical pathways were generated using Ingenuity Pathway Analysis (IPA) software (version 8.6; Ingenuity Systems).

Total RNA was extracted from the implanted human hepatocytes in the mouse livers by use of an RNeasy mini kit (Qiagen) and was reverse transcribed. The selected messenger RNA (mRNA) was quantified by real-time PCR using the 7300 real-time PCR system (Applied Biosystems), and the expression of glyceraldehyde-3-phosphate dehydrogenase served as a control. The amplification protocol and primer sequences are described in Supplementary data 4 and 5.

RESULTS

To analyze the direct effects of IFN in human hepatocytes, we compared the gene expression profiles between groups A (mice without IFN treatment) and B (mice with IFN treatment). Of the 1403 genes that remained after screening with the Welch *T* test, 685 genes showed a >3.0-fold change between groups. Of these 685 genes, 525 genes were up-regulated and the other 160 genes down-regulated by IFN. The top 20 IFN-regulated genes are listed in Supplementary data table 6. GO analysis revealed that 8 (40%) of the top 20 genes that were upregulated with IFN treatment were related to immune response.

To analyze the effect of HBV infection in human hepatocytes, we compared the gene expression profiles between groups A (mice without HBV infection) and C (mice with HBV infection). Among the 1,714 genes hat remained after screening, 373 genes showed a >3.0-fold change between groups. Of these 373 genes, 159 genes were up-regulated and the other 214 genes down-regulated by HBV. The top 20 HBV-regulated genes are listed in Supplementary data table 7. Several oncogenic genes such as growth differentiation factor 15 and glial cell derived neurotrophic factor were included in the top group. Most of the top 20 genes that were downregulated with HBV infection were associated with transcriptional regulation.

To examine whether HBV infection may alter the effect of IFN response in human hepatocytes, we compared gene expression profiles among all groups. As mentioned above, 525 genes were upregulated by >3.0-fold by IFN in the absence of HBV infection. A comparison of groups C (mice with HBV infection but no IFN treatment) and D (mice with both HBV infection and IFN treatment) revealed that 183 (34.9%) of the 525 genes showed statistically significantly reduced IFN response with HBV infection ($P < .01$) (Supplementary data 8A). The top 20 genes in which IFN response was significantly changed by HBV infection are shown in Table 1. The mRNA expression levels of 11 selected genes among the 183 genes with reduced IFN response were also analyzed by real-time PCR, and the reductions in IFN response by HBV infection were verified (Supplementary data 8B). Additionally, we used IPA software to analyze the influence of HBV infection on the IFN response of these 183 genes by means of a pathway-oriented approach. Pathway analysis revealed that several pathways were affected by HBV infection (Table 2). The IFN response was statistically significantly attenuated by HBV infection in the pathways related to IFN signaling and pattern recognition of bacteria and viruses ($P = 1.0 \times 10^{-8}$ and $P = 1.2 \times 10^{-8}$, respectively).

DISCUSSION

Elsewhere we have demonstrated a human hepatocyte chimeric mouse model that can be chronically infected with hepatitis B and C viruses [9–11]. This mouse model facilitates analysis of the effect of viral infection and the response to medication under immunodeficient conditions. In this study, we performed complementary DNA microarray analysis using the chimeric mouse model and obtained gene expression profiles to analyze

Table 1. Genes With Interferon Responsiveness Downregulated by Hepatitis B Virus (HBV) Infection

Gene symbol	GenBank accession no.	Function	Fold change in expression level		*P*
			Without HBV infection	With HBV infection	
ENST00000322831	None	Unknown	4.52	−1.45	4.15×10^{-7}
AA593970	AA593970	EST	9.70	1.61	5.58×10^{-7}
THC2533996	None	Unknown	3.74	−2.50	6.97×10^{-7}
LOC388532	None	Unknown	3.11	−2.48	1.61×10^{-6}
ZNF267	NM_003414	Transcription regulator	7.66	1.79	2.30×10^{-6}
ZNF217	NM_006526	Transcription regulator	3.69	1.03	3.62×10^{-6}
CRSP3	NM_015979	Transcription regulator	7.50	−1.02	4.06×10^{-6}
MGC39372	BC025340	Hypothetical protein	30.92	7.03	5.74×10^{-6}
BF972140	BF972140	EST	16.91	4.71	5.78×10^{-6}
LOC731599	XR_015536	Hypothetical protein	3.17	−4.18	8.58×10^{-6}
LOC645676	AK126559	Hypothetical protein	3.76	1.35	9.13×10^{-6}
THC2650457	None	Unknown	78.07	6.28	1.29×10^{-5}
ZNF24	NM_006965	Transcription regulator	3.69	1.36	1.64×10^{-5}
CCDC68	NM_025214	Unknown	5.88	−2.83	1.89×10^{-5}
SP110	NM_004510	Transcription regulator	5.00	10.77	2.00×10^{-5}
FLJ21272	AK024925	Hypothetical protein	14.70	2.49	3.18×10^{-5}
PLEKHF1	NM_024310	Unknown	6.65	1.84	4.70×10^{-5}
AK026418	AK026418	Unknown	9.50	2.58	5.02×10^{-5}
hCG_1790262	XM_001133847	Unknown	3.13	−2.94	6.25×10^{-5}
CEBPD	NM_005195	Transcription regulator	8.16	1.56	7.03×10^{-5}
FLJ20273	NM_019027	RNA binding	3.37	1.11	7.11×10^{-5}

NOTE. *P* values were analyzed by the Welch *T* test. *CEBPD,* CCAAT/enhancer binding protein (C/EBP) delta; *CCDC68,* coiled-coil domain containing 68; *CRSP3,* mediator complex subunit 23 (*MED23*); EST, expressed sequence tag; *FLJ20273,* RNA binding motif protein 47 (*RBM47*); *PLEKHF1,* pleckstrin homology domain containing, family F (with FYVE domain) member 1; *SP110,* SP110 nuclear body protein; *ZNF24,* zinc finger protein 24; *ZNF217,* zinc finger protein 217; *ZNF267,* zinc finger protein 267.

the direct influence of HBV infection and IFN-α treatment on human hepatocytes.

To avoid contamination with mouse tissue, human hepatocyte chimeric mice, in which liver tissue is largely (>90%) replaced by human hepatocytes, were used in the present study. However, a small amount of mouse-derived cells, such as interstitial cells, bile duct cells, and vascular cells, still remain in the chimeric mouse livers. Because of high homology between the human and mouse genomes, the signals from microarray analyses may be influenced by cross-hybridization with mouse mRNA. It is difficult to produce $uPA^{+/+}/SCID^{+/+}$ mice >10 weeks old without hepatocyte transplantation, and a previous study demonstrated that it is feasible to use microarray analysis in a functional genomics analysis of chimeric mice [12]. Therefore, to compensate for the contamination, the mice in group A, which were neither infected with HBV nor treated with IFN, were used as negative controls.

To analyze the effect of IFN treatment, we compared gene expression profiles between groups A (mice without IFN treatment) and B (mice with IFN treatment); 525 genes with >3.0-fold upregulation following IFN treatment were observed. Among them, chemokine (C-X-C motif) ligand 9, chemokine (C-X-C motif) ligand 10, and chemokine (C-X-C motif) ligand 11, which promote T cell adhesion, were remarkably highly induced with IFN treatment (Supplementary data table 6) [13]. These results suggest that the antiviral effects of IFN might involve not only direct activation of IFN-stimulated proteins such as myxovirus resistance protein A and double strand RNA-dependent protein kinase but also activation of immunity via chemokines.

Second, we compared the profiles between groups A (mice without HBV infection) and C (mice with HBV infection). As shown in Supplementary data table 7, more than half (12) of the top 20 genes upregulated by HBV infection localized to the cell membrane or the extracellular region, but 14 (70%) of the 20 downregulated genes localized to the nucleus. In addition, GO analysis demonstrated that genes related to cell cycle and DNA modification were affected by HBV infection. We speculate that HBV infection promotes cell growth and DNA damage in the hepatocyte nucleus and activates the immune response in the cytoplasm. From the clinical standpoint, some healthy HBV carriers develop hepatocellular carcinoma without chronic hepatitis or cirrhosis. The present results strongly support this observation, showing that most of the affected genes are known to be associated with carcinogenesis.

Clinically, HBV is known to develop tolerance to IFN treatment in patients with chronic hepatitis B, although the mechanism is not clear. We analyzed the IFN response with and

Table 2. Pathway Analysis of 183 Interferon-Induced Genes With Interferon Responsiveness Downregulated by Hepatitis B Virus Infection

Canonical pathways	*P*	Genes
Interferon signaling	1.00×10^{-8}	*IFIT3, SOCS1, IFIT1, MX1, IFNGR1, JAK2, STAT1, TAP1, IRF1*
Role of pattern recognition receptors in recognition of bacteria and viruses	1.20×10^{-8}	*IL12A, OAS2, OAS3*(includes EG:4940), *IFIH1, PIK3R3, TLR4, NOD2, TICAM1, DDX58, CASP1, NOD1, TLR3, RIPK2*
Type 1 diabetes mellitus signaling	2.00×10^{-4}	*SOCS1, IL12A, RIPK1, GAD1, SOCS6, SOCS2, IFNGR1, JAK2, STAT1, IRF1*
Prolactin signaling	2.70×10^{-4}	*PIK3R3, SOCS1, SOCS6, SOCS2, NMI, JAK2, STAT1, IRF1*
TREM1 signaling	3.50×10^{-4}	*TLR4, NOD2, ICAM1, CASP1, JAK2, TLR3, CASP5*
Production of nitric oxide and reactive oxygen species in macrophages	3.90×10^{-4}	*PIK3R3, TLR4, RND3, PPP2R2A, PPM1J, RHOU, IFNGR1, MAP3K8, IRF8, JAK2, STAT1, IRF1*
Pathogenesis of multiple sclerosis	1.10×10^{-3}	*CXCL10, CXCL9, CXCL11*
Activation of IRF by cytosolic pattern recognition receptors	2.60×10^{-3}	*IFIH1, RIPK1, DDX58, STAT1, IFIT2, ISG15*
Dendritic cell maturation	2.60×10^{-3}	*B2M, PIK3R3, TLR4, ICAM1, IL12A, IL1RN, IRF8, JAK2, TLR3, STAT1*
Interleukin 12 signaling and production in macrophages	3.60×10^{-3}	*PIK3R3, TLR4, IL12A, IFNGR1, MAP3K8, IRF8, STAT1, IRF1*
Sphingosine-1-phosphate signaling	3.60×10^{-3}	*PIK3R3, S1PR2, RND3, CASP1, RHOU, CASP4, CASP7, CASP5*
JAK-STAT signaling	4.00×10^{-3}	*PIK3R3, SOCS1, SOCS6, SOCS2, JAK2, STAT1*
Growth hormone signaling	4.70×10^{-3}	*PIK3R3, SOCS1, SOCS6, SOCS2, JAK2, STAT1*
Retinoic acid mediated apoptosis signaling	8.50×10^{-3}	*TNFRSF10B, PARP8, TNFSF10, TIPARP, IRF1*

NOTE. *B2M,* beta-2-microglobulin; *CASP1,* caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase); *CASP4,* caspase 4, apoptosis-related cysteine peptidase; *CASP5,* caspase 5, apoptosis-related cysteine peptidase; *CASP7,* caspase 7, apoptosis-related cysteine peptidase; *CXCL9,* chemokine (C-X-C motif) ligand 9; *CXCL10,* chemokine (C-X-C motif) ligand 10; *CXCL11,* chemokine (C-X-C motif) ligand 11; *DDX58,* DEAD (Asp-Glu-Ala-Asp) box polypeptide 58; *GAD1,* glutamate decarboxylase 1 (brain, 67kDa); *ICAM1,* intercellular adhesion molecule 1; *IFIH1,* interferon induced with helicase C domain 1; *IFIT1,* interferon-induced protein with tetratricopeptide repeats 1; *IFIT2,* interferon-induced protein with tetratricopeptide repeats 2; *IFIT3,* interferon-induced protein with tetratricopeptide repeats 3; *IFNGR1,* interferon gamma receptor 1; *IL1RN,* interleukin 1 receptor antagonist; *IL12A,* interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35); IRF, interferon regulatory factor; *IRF1,* interferon regulatory factor 1; *IRF8,* interferon regulatory factor 8; *ISG15,* ISG15 ubiquitin-like modifier; *JAK2,* Janus kinase 2; *MAP3K8,* mitogen-activated protein kinase kinase kinase 8; *MX1,* myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse); *NMI,* N-myc (and STAT) interactor; *NOD1,* nucleotide-binding oligomerization domain containing 1; *NOD2,* nucleotide-binding oligomerization domain containing 2; *OAS2,* 2′-5′-oligoadenylate synthetase 2, 69/71kDa; *OAS3,* 2′-5′-oligoadenylate synthetase 3, 100kDa; *PARP8,* poly (ADP-ribose) polymerase family, member 8; *PIK3R3,* phosphoinositide-3-kinase, regulatory subunit 3 (gamma); *PPM1J,* protein phosphatase, Mg2+/Mn2+ dependent, 1J; *PPP2R2A,* protein phosphatase 2, regulatory subunit B, alpha; *RHOU,* ras homolog gene family, member U; *RIPK1,* receptor (TNFRSF)-interacting serine-threonine kinase 1; *RIPK2,* receptor-interacting serine-threonine kinase 2; *RND3,* Rho family GTPase 3; *S1PR2,* sphingosine-1-phosphate receptor 2; *SOCS1,* suppressor of cytokine signaling 1; *SOCS2,* suppressor of cytokine signaling 2; *SOCS6,* suppressor of cytokine signaling 6; *STAT1,* signal transducer and activator of transcription 1, 91kDa; *TAP1,* transporter 1, ATP-binding cassette, sub-family B (MDR/TAP); *TICAM1,* Toll-like receptor adaptor molecule 1; *TIPARP,* TCDD-inducible poly(ADP-ribose) polymerase; *TLR3,* Toll-like receptor 3; *TLR4,* Toll-like receptor 4; *TNFRSF10B,* tumor necrosis factor receptor superfamily, member 10b; *TNFSF10,* tumor necrosis factor (ligand) superfamily, member 10; *TREM1,* triggering receptor expressed on myeloid cells 1.

without HBV infection, focusing on the 525 upregulated genes with IFN treatment and using all obtained gene expression profiles. Interestingly, 61.3% of the extracted genes maintained an IFN response, but in 34.9% of those genes, IFN responses were attenuated by HBV infection (Supplementary data 8A). Genes corresponding to interferon signaling, including suppressor of cytokine signaling 1 (*SOCS1*) and interferon regulatory factor 1, and those corresponding to pattern recognition of bacteria and viruses, including nucleotide-binding oligomerization domain containing 1 (*NOD1*) and receptor-interacting serine-threonine kinase 2 (*RIPK2*), were statistically significantly associated with HBV-mediated attenuation to IFN response ($P = 1.0 \times 10^{-8}$ and $P = 1.2 \times 10^{-8}$, respectively). According to these results, HBV infection significantly up-regulated *SOCS1* expression and reduced the IFN responsiveness of *SOCS1*. Thus, *SOCS1* might support chronic infection of HBV in escaping the effects of innate immunity or IFN therapy. On the other hand, genes involved in recognition of viral infection were also inhibited following HBV infection. Both *NOD1* and *RIPK2* are related to innate and adaptive immune responses [14, 15]. We speculated that inhibition of *NOD1* or *RIPK2* expression facilitates HBV survival. Although further study is needed, these results may have important implications for the mechanisms of viral escape from innate immunity.

In conclusion, we performed complementary DNA microarray analysis using human hepatocyte chimeric mice. With this system, we could analyze the direct effects of IFN treatment and HBV infection without the confounding effects of the lymphocyte immunological response and obtained evidence that HBV infection attenuated the virus recognition and IFN response in

hepatocytes, by which means HBV could evade innate immune detection and response.

Supplementary Data

Supplementary data are available at *The Journal of Infectious Diseases* online.

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Original Article

Combination of hepatitis B viral antigens and DNA for prediction of relapse after discontinuation of nucleos(t)ide analogs in patients with chronic hepatitis B

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Aim: The factors associated with hepatitis recurrence after discontinuation of nucleos(t)ide analogs (NAs) in patients with chronic hepatitis B were analyzed to predict the risk of relapse more accurately.

Methods: A total of 126 patients who discontinued NA therapy were recruited retrospectively. The clinical conditions of a successful discontinuation were set as alanine aminotransferase (ALT) below 30 IU/L and serum hepatitis B virus (HBV) DNA below 4.0 log copies/mL.

Results: Relapse of hepatitis B were judged to occur when maximal serum ALT became higher than 79 IU/L or when maximal serum HBV DNA surpassed 5.7 log copies/mL following NA discontinuation since these values corresponded with mean values of ALT (30 IU/L) and HBV DNA (4.0 log copies/mL), respectively. At least 90% of patients with either detectable hepatitis B e antigen or serum HBV DNA higher than 3.0 log copies/mL at the time of NA discontinuation relapsed within one year. In the remaining patients, higher levels of both hepatitis B surface and core-related antigens at the time of discontinuation, as well as a shorter course of NA treatment, were significantly associated with relapse by multivariate analysis.

Conclusions: It appears that negative results for hepatitis B e antigen and serum HBV DNA lower than 3.0 log copies/mL are essential for successful NA discontinuation, which may be attained by a longer treatment period. Levels of hepatitis B surface and core-related antigens are also significant factors independently associated with relapse of hepatitis.

Key words: discontinuation, hepatitis B core-related antigen, hepatitis B surface antigen, nucleos(t)ide analogs, relapse of hepatitis

INTRODUCTION

HEPATITIS B VIRUS (HBV) infection is a major health concern that has an estimated 350 to 400 million carriers worldwide. Chronic infection with HBV can cause chronic hepatitis, and may eventually develop into liver cirrhosis and hepatocellular carcinoma.[1–3] Over the last decade, major advances in the treatment of chronic hepatitis B have been made with nucleos(t)ide

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analogs (NAs) such as lamivudine (LVD), adefovir dipivoxil (ADV), and entecavir (ETV).[4] NAs are orally administered and are associated with low rates of adverse effects. Treatment with NAs shows strong suppression of HBV replication and consequently rapid improvement of elevated ALT levels. Furthermore, these drugs have been reported to lower the risk of complicating cirrhosis and hepatocellular carcinoma,[5–7] and so NAs are becoming widely used to treat patients with chronic hepatitis B. On the other hand, NAs carry the risk of developing drug-resistance;[8] drug-resistant viruses emerging during treatment may be associated with hepatitis flare-ups. Hepatitis B patients are also required to undergo prolonged treatment with NAs because early discontinuance often leads to relapse of hepatitis and ensuing hepatic failure following rises in alanine aminotransferase (ALT) level.[9,10]

Serum HBV DNA is normally used to monitor the antiviral effect of NAs. HBV DNA decreases rapidly and becomes undetectable in the majority of patients who are treated with NAs,[11–13] but relapse after discontinuation is not rare.[14–17] Since it is also true that favorable virological and biochemical responses to NAs may continue indefinitely in some patients,[9,15] reliable markers that can predict relapse of hepatitis after NA discontinuation are needed. Such markers would benefit not only patients who are considering discontinuation of NA treatment, but also clinicians, hospitals, and the medical economy.

In the present study, we assessed several factors associated with relapse of hepatitis after discontinuation of NAs in patients with chronic hepatitis B, including hepatitis B viral antigens, which have been reported as new and promising markers for monitoring the effect of antiviral agents, such as interferon and NAs.

METHODS

Patients

A TOTAL OF 126 patients with chronic hepatitis B who underwent and completed NA treatment between 2000 and 2010 were enrolled in this study. Patients were recruited retrospectively from 11 hospitals across Japan (Toranomon Hospital, Hokkaido University Hospital, Nagoya City University Hospital, Shinshu University Hospital, Hiroshima University Hospital, National Hospital Organization Nagasaki Medical Center, Chiba University Hospital, The Hospital of Hyogo College of Medicine, Japanese Red Cross Nagoya Daini Hospital, and Tokyo Women's Medical University Hospital, Sapporo Kosei General Hospital) and met the following conditions: (i) serum ALT higher than 30 IU/L and serum HBV DNA higher than 4.0 log copies/mL were observed at least twice within the 6 months prior to administration of NAs; (ii) stored serum samples at initiation and discontinuation of NAs were available for measurements of viral markers; (iii) clinical outcomes were followed for at least 6 months after the discontinuation of NAs; and (iv) tests for hepatitis C and human immunodeficiency virus antibodies were negative. Hepatitis B surface antigen (HBsAg) was confirmed to be positive on at least two occasions at least 6 months apart in all patients before treatment. Patients complicated with hepatocellular carcinoma or signs of hepatic failure at treatment discontinuation were excluded from the study. Our cohort consisted of 83 men and 43 women with a median age of 46 (range, 19 to 79) years when NA administration was discontinued. Hepatitis B e antigen (HBeAg) was positive in 64 patients (51%) at the initiation of treatment and in 24 patients (19%) at its discontinuation. HBV genotype was A in two (2%) patients, B in five (4%), C in 102 (81%), and undetermined in 17 (13%). Thirty-five of the 126 patients in this study were younger than 35 years old. Although not recommended as the first line treatment for this group by Japanese guidelines,[18] NA treatment was commenced since chronic active hepatitis had been persisting in all cases irrespective of their HBeAg status (26 positive and nine negative) at the initiation of treatment.

The decision to discontinue NAs was made by individual physicians using similar, but not uniform, conditions. Four patients who halted NAs for financial reasons were included. No patient underwent interferon treatment during or after NA treatment. The decision to recommence NA administration was also made by individual physicians, essentially when relapse of hepatitis became obvious. With few exceptions, patients were seen at least once a month during the first year after discontinuation of NAs, and at least once every several months afterwards. Stored serum samples were kept frozen at −20 °C or below until assayed. This study was approved by the Ethics Committees of all participating institutions.

Hepatitis B viral markers

Serological markers for HBV, including HBsAg, HBeAg, and antibody to HBe (anti-HBe) were tested using commercially available enzyme immunoassay kits (Abbott Japan Co., Ltd, Tokyo, Japan; Fujirebio Inc., Tokyo, Japan; and/or Sysmex Co., Kobe, Japan) at each hospital. Quantitative measurement of HBsAg[19] was done using a chemiluminescence enzyme immunoassay

(CLEIA)-based HISCL HBsAg assay manufactured by Sysmex Corporation (Kobe, Japan). The assay had a quantitative range of −1.5 to 3.3 log IU/mL. End titer was determined by diluting samples with normal human serum when initial results exceeded the upper limit of the assay range.

Serum concentration of HBV DNA was determined using an Amplicor HBV monitor kit (Roche, Tokyo, Japan),[20] which had a quantitative range of 2.6 to 7.6 log copies/mL. Serum HBV DNA was also determined using a COBAS TaqMan HBV kit (Roche, Tokyo, Japan)[21] with a quantitative range of 2.1 to 9.0 log copies/mL in 43 patients whose serum samples were available at the time of NA discontinuation. According to the manufacturer's instructions, detection of a positive signal below the quantitative range was described as a positive signal, and no signal detection was described as a negative signal. Six HBV genotypes (A–F) were evaluated according to the restriction patterns of DNA fragments from the method reported by Mizokami *et al.*[22]

Serum hepatitis B core-related antigen (HBcrAg) levels were measured using a CLEIA HBcrAg assay kit with a fully automated Lumipulse System analyzer (Fujirebio Inc., Tokyo, Japan) as described previously.[23,24] Briefly, 150 μL of serum was incubated with pretreatment solution and then added to a ferrite microparticle suspension in an assay cartridge. Ferrite particles were coated with a monoclonal antibody mixture against denatured HBcAg, HBeAg, and the 22 kDa precore protein. After incubation and washing, further incubation was carried out with alkaline phosphatase conjugated with two kinds of monoclonal antibodies against denatured HBcAg, HBeAg, and the 22 kDa precore protein. Following washing, a substrate solution was added to the test cartridge and then incubated. The relative chemiluminescence intensity was measured, and HBcrAg concentration was calculated by a standard curve generated using recombinant pro-HBeAg. The immunoreactivity of pro-HBeAg at 10 fg/mL was defined as 1 U/mL. We expressed HBcrAg in terms of log U/mL, with a quantitative range set at 3.0 to 6.8 log U/mL.

Statistical analyses

A linear regression model was used to examine for associations between mean and maximal values of both ALT and HBV DNA. Correlations between variables were calculated using the Spearman's rank correction correlation coefficient test. Each cut-off value was decided using receiver operating characteristic curve (ROC) analysis and results were evaluated by measuring the area under the curve (AUC). The Fisher's exact and Pearson's χ^2 tests were adopted to test for differences between subgroups of patients. To compare continuous data, the Mann–Whitney *U*-test was used. The Kaplan–Meier method was used to estimate rates of non-relapse observations, and the log-rank test was used to test hypotheses concerning differences in non-relapse observations between selected groups. Multivariate analyses were performed using the Cox regression model. Variables associated with a *P*-value < 0.2 in univariate analyses were included in a stepwise Cox regression analysis to identify independent factors associated with relapse of hepatitis after discontinuation of NAs. All tests were performed using the IBM SPSS Statistics Desktop for Japan ver. 19.0 (IBM Japan Inc., Tokyo, Japan). *P*-values of less than 0.05 were considered to be statistically significant.

RESULTS

Definition of hepatitis relapse after discontinuation of NAs

THE CLINICAL CONDITIONS of a successful discontinuation of NAs were set at serum HBV DNA below 4.0 log copies/mL and ALT below 30 IU/L according to the Japanese guidelines for the treatment of hepatitis B.[18] However, these criteria could not be directly applied to our cohort as post-therapy fluctuations in ALT and HBV DNA were difficult to evaluate consistently. In total, 26 (76%) of 34 patients with successful discontinuation of NAs showed transient abnormal levels of ALT and/or HBV DNA, especially during the early phase after cessation. We therefore used mean and maximal values of these markers to evaluate relapse of hepatitis B in this study; mean values were used to evaluate relapse of hepatitis as a whole, and maximal values were used to dynamically assess relapse during the follow-up period after NA discontinuation. Both ALT and HBV DNA were measured 11.0 times per year on average during the first year and 4.1 times per year on average thereafter.

The mean values of HBV DNA were significantly ($P < 0.001$) correlated with maximal values with a correlation coefficient of 0.853. Similarly, the mean values of ALT were significantly ($P < 0.001$) correlated with maximal values with a correlation coefficient of 0.940 (Fig. 1). The mean HBV DNA value of 4.0 log copies/mL corresponded to a maximal HBV DNA value of 5.7 by ROC analysis (AUC = 0.930, $P < 0.001$), and the mean ALT value of 30 IU/L corresponded to a maximal ALT value of 79 IU/L (AUC = 0.988, $P < 0.001$). These results suggested that patients having serum HBV DNA higher