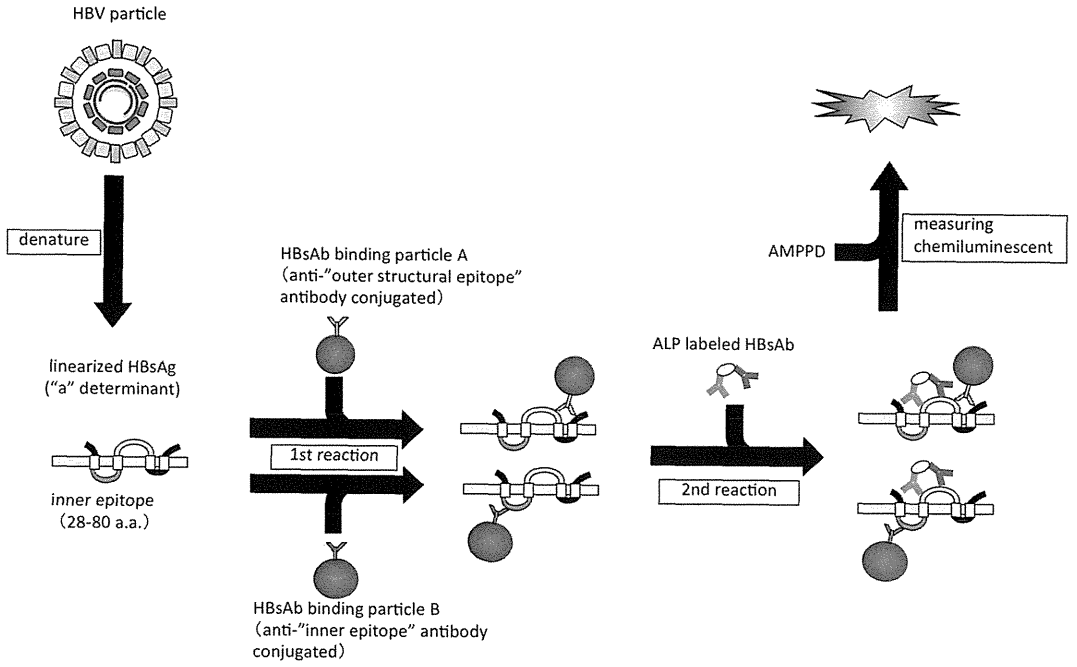


FIG 1 The principle of Lumipulse HBsAg-HQ.

patients with apparent HBsAg seroclearance as determined by the Abbott Architect assay.

MATERIALS AND METHODS

Samples. Four hundred seventy-one patients with chronic HBV infection visited our hospital from 2009 to 2012. One hundred eighty-one patients were asymptomatic carriers, 232 had chronic hepatitis B (CHB), and 58 had liver cirrhosis. Of these, 13 patients took lamivudine, one adefovir, 19 lamivudine plus adefovir, 140 entecavir, 8 entecavir plus adefovir, and 9 tenofovir. Thirty patients with acute HB (AH) infection (8 of whom developed chronic hepatitis) visited our hospital from January 2009 to 2012. We determined HBsAg seroclearance according to the Abbott Architect assay in 26 HBV-infected patients during the observation period. Of these, 10 were not treated with nucleotide analogues (spontaneous HBsAg loss group) and 8 were treated (NA-treated group). Of the 8 NA-treated patients, 2 on lamivudine therapy were HBsAg seronegative after stopping therapy, and the other 6 were HBsAg seronegative during entecavir therapy. Eight AH patients became HBsAg seronegative.

The study protocol conformed to the 1975 Declaration of Helsinki and was approved by the ethics committees of our institutions, and informed consent was obtained from each carrier. We rechecked HBsAg status of the patients by the Lumipulse HBsAg-HQ assay in their serial blood serum samples and compared the results with those of the Architect HBsAg-QT assay.

Methods. (i) Measurement of HBsAg by Lumipulse HBsAg-HQ assay. HBsAg was measured on the two-step sandwich assay principle with a fully automated chemiluminescent enzyme immunoassay system (Lumipulse G1200; Fujirebio, Inc.). The assay principle for this new reagent was based on that previously reported by Matsubara et al. (10). Briefly, samples were pretreated with a solution, including surfactant to disrupt HBV particles, to dissociate HBsAg from HBsAg–anti-HBs complexes and to denature epitopes to a linear form. Linearized HBsAg were then detected using two monoclonal antibodies against external structural regions as determinant "a" and the internal epitope as a capture reagent, with two monoclonal antibodies coupled to alkaline phosphatase as the detector. For the assay procedures, 100 μl blood serum and/or plasma samples together with 20 μl pretreatment solution were incubated with the monoclonal antibodies binding ferrite microparticles at 37°C for 10 min. After automatic washing, 250 μl of the alkaline phosphatase-labeled antibodies were added and further incubated at 37°C for 10 min. After the washing step, 200 μl substrate solution (AMPPD [3-(2′-spiroadamantane)-4-methoxy-4-(3″-phosphoryloxy)phenyl-1,2-dioxetane disodium salt]) (Applied Biosystems, Bedford, MA) was added and incubated at 37°C for 5 min. The relative intensity of chemiluminescence was measured and the HBsAg concentration was calculated by comparison with a standard curve. The range of HBsAg concentrations assayed was 5 to 150,000 mIU/ml, and retesting was accepted with a 200-fold dilution of samples that exceeded this range. In the present study, the cutoff value of HBsAg concentration was set at 5 mIU/ml. HBsAg in blood serum was also quantified at the same intervals using the Abbott Architect HBsAg-QT assay (cutoff value, 50 mIU/ml) (Fig. 1).

(ii) Quantification of HBV DNA. Serum HBV DNA was measured using the TaqMan PCR assay (Cobas TaqMan; Roche Molecular Systems [lower limit of detection, 2.1 log copies/ml]).

(iii) Quantification of HBcrAg. Serum HBcrAg was measured using CLEIA, as described previously (12, 13). Briefly, sodium dodecyl sulfate pretreated serum was incubated with monoclonal antibodies against denatured HBcAg and HBeAg. After washing and incubation with alkaline phosphatase-labeled secondary antibodies, the relative chemiluminescence intensity was measured, and the HBcAg concentration was calculated by comparison with a standard curve generated using a known concentration of recombinant HBeAg-containing peptide. The cutoff value of HBcrAg was 3 log U/ml.

(iv) Quantification of anti-HBs. Serum anti-HBs was measured using the Architect system's anti-HBs. A specimen was considered positive for anti-HBs when the concentration was ≥10.0 mIU/ml.

RESULTS

Table 1 shows clinical data at baseline for the three groups with HBsAg seroclearance according to data from the Abbott Architect assay. In four of 10 spontaneous HBsAg loss cases, HBsAg had already been <50 mIU/ml as measured by the Abbott Architect assay at the first visit. Table 1 shows the characteristics of all 26 patients in these 3 groups. The HBV DNA and HBcrAg levels at

TABLE 1 Clinical data at baseline of 3 groups with HBsAg seroclearance as determined by the Abbott Architect assay

Patient characteristic	Data for group (*n*): Spontaneous HBsAg loss (10)	NA treated (8)[a]	Acute hepatitis (8)
Age at first visit or medication (yr)	60.6 ± 12.6	46.8 ± 12.2	50.5 ± 10.8
Sex (no. of males/no. of females)	10/0	7/1	8/0
Route of infection (no. of vertical/no. of horizontal)	10/0	4/4	0/8
No. with genotype Aa/Ae/Ba/Bj/C	0/0/0/2/8	1/1/1/1/4	1/4/1/0/2
Clinical data			
ALT (median [range]) (IU/liter)	23.5 (8–51)	76 (11–220)	1,682 (455–3,622)
HBeAg (no. positive/no. negative)	0/10	5/3	8/0
HBV DNA (median [range]) (log copies/ml)	2.3 (<2.1 to 3.4)	7.4 (4.1 to >9.1)	6.5 (3.8–8.5)
HBcrAg (median [range]) (log IU/ml)	<3 (<3 to 3.3)	6.8 (4.2–8.6)	7.1 (6.6–8)
Abbott Architect HBsAg-QT detection (median [range]) (mIU/ml)	1,300 (<50 to 10,880)	2,676,800 (9,680–89,679,600)	362,500 (91,200–40,000,000)
NA therapy (no. with none/no. with LVD/no. with ETV)[b]	10/0/0	0/2/6	5/0/3

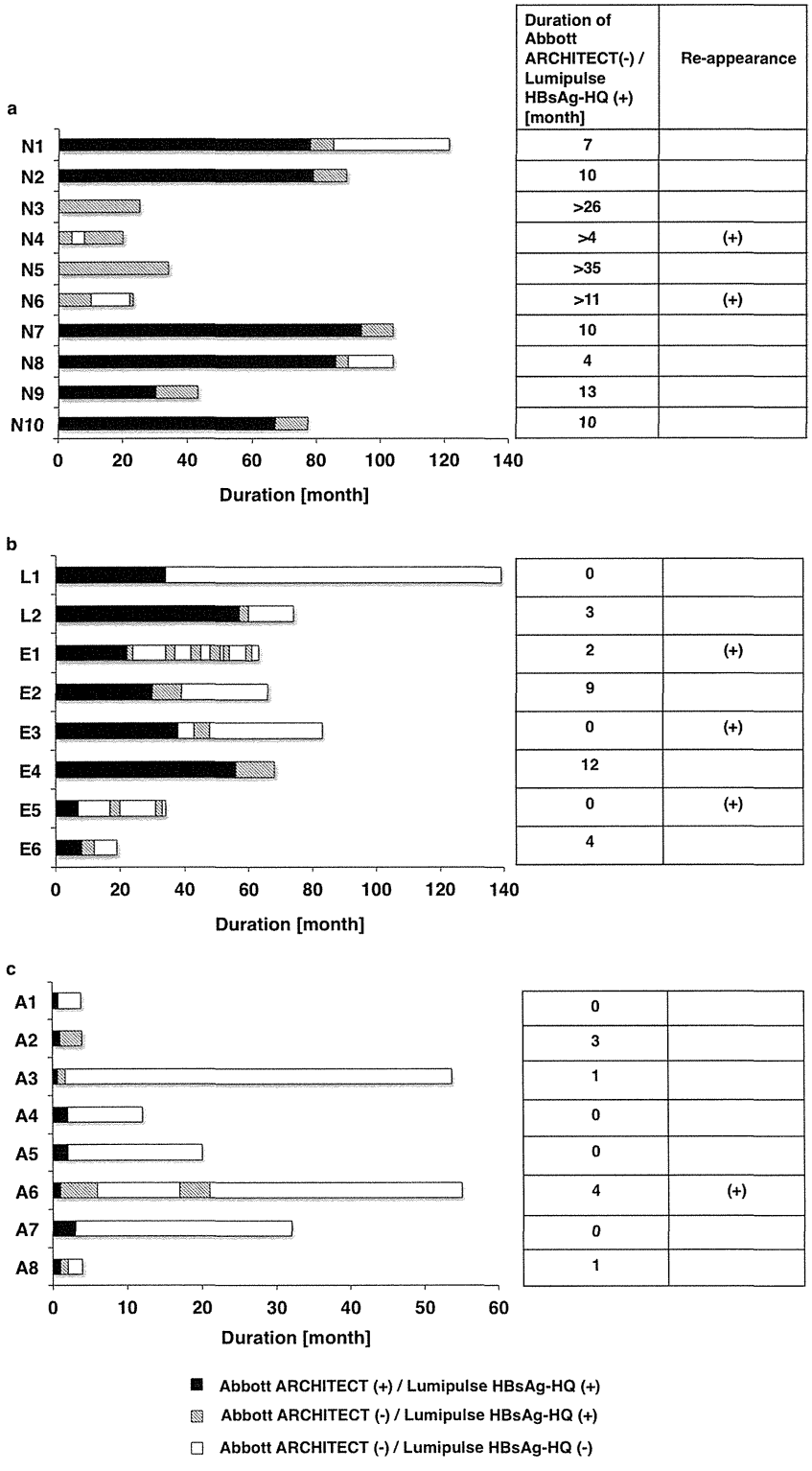
[a] NA, nucleotide analogue.
[b] LVD, lamivudine; ETV, entecavir.

baseline were significantly higher in the NA-treated and AH groups than in the spontaneous HBsAg loss group. The HBsAg levels at baseline were also significantly higher in the AH group and the NA-treated group than in the spontaneous HBsAg loss group. However, HBsAg became undetectable by the Abbott Architect assay immediately in the AH group (median, 1 month), compared with the NA-treated group (32 months) and the spontaneous HBsAg loss group (78.5 months [excluding 4 patients with HBsAg of ≤50 mIU/ml by the Abbott Architect assay at the first visit]). In 19 of the 26 cases, the HBsAg levels were still detectable by the Lumipulse HBsAg-HQ assay at the time point when they were undetectable by the Abbott Architect assay. At the last time point with detectable HBsAg by Lumipulse HBsAg-HQ assay, the Abbott Architect assay could not detect HBsAg in all 10 spontaneous HBsAg loss patients, but the Abbott Architect assay was also able to detect at the last time point in three (case no. L1, E3, and E5) of eight NA-treated group patients and four (case no. A1, A4, A5, and A7) of eight AH patients. In the spontaneous HBsAg loss group, the decline in HBsAg was slower than in the NA-treated and AH groups (Fig. 2a to 2c). Differences in the median duration between the Abbott Architect and Lumipulse HBsAg-HQ assays were seen at 10 months (excluding 4 patients with HBsAg of <50 mIU/ml by the Abbott Architect assay at the first visit), 2.5 months, and 0.5 months in the spontaneous HBsAg loss group, NA-treated group, and AH group, respectively. We observed the reappearance of HBsAg measured by Lumipulse HBsAg-HQ assay in 2 patients (case no. N4 and N6) in the spontaneous HBsAg loss group, 3 (case no. E1, E3, and E5) in the NA-treated group, and one (case no. A6) in the AH group (Fig. 2a to 2c). At the last time point with detectable HBsAg by the Lumipulse HBsAg-HQ assay, HBV DNA was undetectable by the Cobas TaqMan assay in 4 of 10 spontaneous HBsAg loss patients (40%), 4 of 8 NA-treated patients (50%), and one of 8 AH patients (12.5%). At the last time of detection by the Lumipulse HBsAg-HQ assay, HBcrAg was <3 log U/ml in 8 of 10 spontaneous HBsAg loss patients (80%), 2 of 8 NA-treated patients (25%), and none of the 10 AH patients (0%). At the last time point of detection by the Lumipulse HBsAg-HQ assay, anti-HBs was positive in one of 10 spontaneous HBsAg loss patients (10%), none of the 8 NA-treated patients (0%), and 2 of 10 AH patients (20%) (Tables 2 to 4). In case no. A1 and A7, HBsAg was relatively high at the last time point at which HBsAg was detectable by the Lumipulse HBsAg-HQ assay (Table 4). In case no. A1, however, HBsAg was undetectable by the Abbott Architect and Lumipulse HBsAg-HQ assays after 1 month. In case no. A7, HBsAg was undetectable by the Abbott Architect and Lumipulse HBsAg-HQ assays after 3 months.

To elucidate possible HBs escape mutants, we examined the S gene sequences of all 26 patients at the first visit. Patient N2 had an amino acid G145S mutation, L1 had an amino acid S143T mutation, and L2 had amino acid I126N and F134Y mutations. None had an amino acid G145R mutation. At the last time point that HBsAg was detected by the Abbott Architect assay, anti-HBs was positive in patient N2 (from the spontaneous HBsAg loss group) with an amino acid G145S mutation. We performed an inhibition assay for samples N1 and N2 at the time of Abbott Architect undetectability but Lumipulse HBsAg-HQ detectability to confirm whether the identification of HBsAg by the Lumipulse HBsAg-HQ assay was specific. HBsAg detection of these samples was inhibited, indicating that the Lumipulse HBsAg-HQ assay was indeed specific. The following are three representative cases.

(i) Case no. N7 was a 71-year-old male. His alanine transaminase (ALT) was 19 IU/liter, HBV DNA was 3.7 log copies/ml at his first visit, the HBV genotype was C, HBeAg was negative, and anti-HBe was positive. The HBsAg level as measured by the Abbott Architect assay was 162,000 mIU/ml. The patient was followed as an inactive HB carrier. The last time at which HBsAg was detectable by the Abbott Architect assay was 87 months after the first visit, and it became undetectable in 3 months. However, it was still detectable by the Lumipulse HBsAg-HQ assay (78 mIU/ml). HBV DNA by Cobas TaqMan assay decreased to <2.1 log copies/ml. The Lumipulse HBsAg-HQ assay was still positive even 10 months after the Abbott Architect assay became negative. The HBsAg level measured by the Lumipulse HBsAg-HQ assay was 5.8 mIU/ml at this time (Fig. 3a).

(ii) Case no. E1 was a 51-year-old male who had been infected



Duration of Abbott ARCHITECT(-) / Lumipulse HBsAg-HQ (+) [month]	Re-appearance
7	
10	
>26	
>4	(+)
>35	
>11	(+)
10	
4	
13	
10	

0	
3	
2	(+)
9	
0	(+)
12	
0	(+)
4	

0	
3	
1	
0	
0	
4	(+)
0	
1	

FIG 2 HBsAg dynamics by the Abbott Architect and Lumipulse HBsAg-HQ assays in the spontaneous HBsAg loss group (a), the NA-treated group (b), and the AH group (c).

with HBV by transfusion in adulthood and had developed chronic hepatitis B. His ALT was 57 IU/liter, HBV DNA was 8.6 copies/ml by the Cobas TaqMan assay, the HBV DNA genotype was Ba, HBeAg was positive, and anti-HBe was negative. The HBsAg level as measured by the Abbott Architect assay was 4,983,730 mIU/ml. The patient was treated with entecavir. After 24 months, HBsAg became undetectable by the Abbott Architect assay, and from this point to the last observation point, the Abbott Architect assay was

TABLE 2 Clinical data of spontaneous HBsAg loss patients at the last time point at which HBsAg was detectable by the Lumipulse HBsAg-HQ assay

Clinical data	Values for patient no.:									
	N1	N2[b]	N3[a,b]	N4[a,b]	N5[a,b]	N6[a,b]	N7[b]	N8	N9[b]	N10[b]
Nucleotide analogue therapy	None	None	None	None	None	None	None	None	None	None
Age (yr)	61	54	91	50	76	63	71	62	62	65
HBeAg (+/−)	−	−	−	−	−	−	−	−	−	−
Abbott Architect HBsAg-QT detection (mIU/ml)	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
Lumipulse HBsAg-HQ detection (mIU/ml)	8.0	51.0	12.0	8.9	10.4	5	5.8	20.4	11.7	30.3
HBV DNA (log copies/ml)	Not detected	Not detected	<2.1	<2.1	2.9	2.6	<2.1	Not detected	2.7	Not detected
HBcrAg (log IU/ml)	<3	3	<3	<3	3.2	<3	<3	<3	<3	<3
Anti-HBs (mIU/ml)	<10	973.8	<10	<10	<10	<10	<10	<10	<10	<10

[a] Abbott Architect HBsAg-QT assay (IU/ml) was already negative at first visit.
[b] Lumipulse HBsAg-HQ assay was still able to detect HBsAg at the last observation time.

continuously unable to detect HBsAg. The HBsAg level as measured by the Lumipulse HBsAg-HQ assay was 14.7 mIU/ml at the first point that was undetectable by the Abbott Architect assay, and it had been detectable for 3 months. After 3 months, HBsAg became undetectable by the Lumipulse HBsAg-HQ assay and anti-HBs reached >10 mIU/ml. From this point, anti-HBs was continually >10 mIU/ml. Interestingly, after 1 year, HBsAg measured by Lumipulse HBsAg-HQ assay became detectable again (25.2 mIU/ml), although HBV DNA by the Cobas TaqMan and HBsAg by the Abbott Architect assays remained undetectable. At some time points, HBsAg as determined by the Lumipulse HBsAg-HQ assay was detectable, and at the same time, anti-HBs was >10 mIU/ml (Fig. 3b).

(iii) Case no. A6 was a 38-year-old male diagnosed as having acute hepatitis B. After 1 month, HBeAg became seronegative and anti-HBe became seropositive. Three months after the first visit, HBV DNA was <2.1 log copies/ml, HBsAg became undetectable by the Abbott Architect assay, anti-HBs was 22.75 IU/ml, and the Lumipulse HBsAg-HQ assay detected HBsAg. After this time, anti-HBs was continually >10 mIU/ml. Thirteen months after the first visit, the Lumipulse HBsAg-HQ assay detected the reappearance of HBsAg (7.6 mIU/ml), although anti-HBs was still positive at 23.18 IU/ml (Fig. 3c).

DISCUSSION

The Lumipulse HBsAg-HQ assay showed improved sensitivity after disrupting HBV particles, dissociating HBsAg from HBsAg/anti-HBs complexes, and denaturing epitopes into linear forms. A major difference between the Abbott Architect and the Lumipulse HBsAg-HQ assays is that the latter detects HBsAg-anti-HBs complexes as well as small S proteins, which are present 10,000 to 1,000,000 times more frequently than Dane particles. The detection limit of the Lumipulse HBsAg-HQ assay (5 mIU/ml) was 10 times lower than that of the Abbott Architect assay, but there was otherwise a good correlation between the two. In clinical practice, more precise and broader HBsAg dynamics might therefore be followed by using the Lumipulse HBsAg-HQ assay. Differences between the two assays in detectable HBsAg persisted for a long time in the spontaneous HBsAg loss group (median, 10 months), followed by the NA-treated group (2.5 months) and the AH group (0.5 months).

In addition to the significant decrease or loss of all HBV replication in the blood serum, the long-term outcome after HBsAg seroclearance is good if there is no preexisting cirrhosis or viral superinfection. This view is supported by studies showing increased survival, a lower rate of hepatic decompensation, and a reduced frequency of hepatocellular carcinoma (HCC) in patients who have cleared HBsAg (14, 15). In carriers without cirrhosis and with no evidence of viral superinfection (hepatitis C virus [HCV] and/or hepatitis D virus [HDV]) at HBsAg seroclearance, liver function can improve or remain stable and hepatic decompensation rarely occurs; however, the incidence of HCC varies significantly, as was previously reported (16, 17). These discrepancies might depend on concurrent hepatitis, the severity of liver disease, age, and other factors. Yuen et al. (17) reported that HBsAg seroclearance of patients aged ≥50 years was associated with a higher risk of developing HCC than in patients of age <50 years, suggest-

TABLE 3 Clinical data of NA-treated patients at the last time point at which HBsAg was detectable by the Lumipulse HBsAg-HQ assay

Clinical data	Values for patient no.:							
	L1	L2	E1	E2	E3	E4[a]	E5	E6
Nucleotide analogue therapy	LVD	LVD	ETV	ETV	ETV	ETV	ETV	ETV
Age (yr)	62	49	53	40	44	44	67	39
HBeAg (+/−)	−	−	−	−	−	−	−	−
Abbott Architect HBsAg-QT detection (mIU/ml)	80[b]	<50	<50	<50	90[b]	<50	90[b]	<50
Lumipulse HBsAg-HQ detection (mIU/ml)	77.3	5	14.7	8	44.6	6.5	42.5	89
HBV DNA (log copies/ml)	<2.1	Not detected	Not detected	Not detected	3.3	2.2	<2.1	Not detected
HBcrAg (log IU/ml)	<3	3.3	4.3	4.1	3.2	<3	3.8	4.3
Anti-HBs (mIU/ml)	<10	<10	<10	<10	<10	<10	<10	<10

[a] The Lumipulse HBsAg-HQ assay was still able to detect HBsAg at the last observation time.
[b] HBsAg was detectable by both assays at this point, but HBsAg became undetectable at the next point.

TABLE 4 Clinical data of AH patients at the last time point at which HBsAg was detectable by Lumipulse HBsAg-HQassay

Clinical data	Values for patient no.:							
	A1	A2	A3	A4	A5	A6	A7	A8
Nucleotide analogue therapy	None	None	None	None	None	ETV	ETV	ETV
Age (yr)	62	34	53	50	39	39	53	54
HBeAg (+/−)	−	−	−	−	−	−	+	+
Abbott Architect HBsAg-QT detection (mIU/ml)	91,200[a]	<50	<50	240[a]	680[a]	<50	11,500[a]	<50
Lumipulse HBsAg-HQ detection (mIU/ml)	112,289.3	5.6	13.6	180.4	771.9	7.6	12,358.4	34.3
HBV DNA (copies/ml)	3.8	Not detected	2.3	2.2	3	<2.1	<2.1	<2.1
HBcrAg (log IU/ml)	6.8	4.0	5.4	4.9	3.2	3.1	3.7	4.3
Anti-HBs (mIU/ml)	<10	24.41	<10	<10	<10	23.18	<10	<10

[a] HBsAg was detectable by both assays at this point, but HBsAg became undetectable at the next point.

ing that we have to consider the age at which HBsAg becomes undetectable.

In most patients in our study (9 of 10 in the spontaneous HBsAg loss group and 7 of 8 in each of the NA-treated and AH groups), HBV DNA or HBcrAg was still detectable by the Abbott Architect assay at the time of HBsAg seroclearance (data not shown). Suzuki et al. (18) reported that HBcrAg correlates with intrahepatic covalently closed circular DNA in chronic hepatitis B patients. Hence, as the current CLEIA HBsAg quantification methods are inadequate for following some cases of HBV infection, the use of the Lumipulse HBsAg-HQ assay together with HBcrAg and HBV DNA testing might be valuable for evaluating patient response to treatment with interferon and NAs. Additionally, we reported that the measurement of HBcrAg is useful for predicting relapse after the cessation of lamivudine therapy for chronic hepatitis B; an HBcrAg level of <3.4 log U/ml at this time was the only independent predictive factor for the absence of post-treatment relapse (19). Thus, the combination of highly sensitive HBsAg detection by the Lumipulse HBsAg-HQ assay and HBcrAg might improve the accuracy of predicting response to treatment and relapse. Highly sensitive HBsAg detection by the Lumipulse HBsAg-HQ assay might be useful for several clinical applications. First, the Lumipulse HBsAg-HQ assay might replace HBV DNA monitoring by a PCR-based method for blood screening. As shown in Tables 2 to 4, at the last time point that HBsAg was detectable by the Lumipulse HBsAg-HQ assay, HBV DNA was undetectable in 9 of 26 patients (34%) by the Cobas TaqMan assay. This suggests that the sensitivity of the Lumipulse HBsAg-HQ assay for HBV detection was at least as high as that for the Cobas TaqMan assay at some time points. The Lumipulse HBsAg-HQ assay is simpler, more convenient, and less expensive than HBV DNA quantification by real-time PCR. At present in Japan, nucleic acid testing is used for detecting HBV in blood donors, but the Lumipulse HBsAg-HQ assay might substitute for nucleic acid testing for screening HBV if the sensitivity could be improved.

Second, the Lumipulse HBsAg-HQ assay may be useful for detecting occult HBV infection as well as HBV reactivation. Occult HBV infection is defined as infection with detectable HBV DNA but undetectable HBsAg with or without antibodies to HBV core antigen (anti-HBc) and/or anti-HBs (20–22). Recent interest in occult HBV infection has focused on the potential of donors with such infections to transmit the virus to susceptible recipients (23, 24). In this study, we detected HBsAg by the Lumipulse HBsAg-HQ assay in occult hepatitis B virus infection (OBI) patients, including those with HBsAg clearance as determined by the Architect assay (case no. N1, N3, N4, N5, N6, N7, N10, E3, E4, E5, E6, A3, A6, A8, and A9). In case no. N5, even >35 months after HBsAg became undetectable by the Abbott Architect assay, HBsAg was still detectable by the Lumipulse HBsAg-HQ assay. The Lumipulse HBsAg-HQ assay may change the diagnosis of patients defined as having current occult HBV infection. In case no. E1, HBsAg was detectable by the Lumipulse HBsAg-HQ assay at some time points, although HBV DNA by the Cobas TaqMan assay and HBsAg by Abbott Architect assay remained undetectable. In many cases (cases N1, N2, N4, N6, N8, N10, L2, E1, E2, E3, E5, E6, A2, A4, and A6), the HBV DNA and Lumipulse HBsAg-HQ results did not correlate. Interestingly, the original highly sensitive HBsAg assay reported by Matsubara et al. (10) had a similar sensitivity with HBV DNA detection during the acute phase of HBV infection. If the sensitivity of the Lumipulse HBsAg-HQ assay is improved, it would be sensitive enough to monitor HBV reactivation instead of needing to rely on HBV DNA monitoring. More importantly, there have been cases of HBV reactivation in patients with resolved infection (HBsAg-negative, anti-HBc, and/or anti-HBs positive) during the course of chemotherapy and/or immunotherapy (especially therapy with rituximab plus steroids), sometimes proving fatal (25–29). The Lumipulse HBsAg-HQ assay might be more convenient for such screening than TaqMan PCR.

Third, previous CLEIA HBsAg quantification methods, including the Abbott Architect assay, apply monoclonal/polyclonal antibodies against external structural regions within the determinant "a" loop. HBsAg escape mutations, such as G130D, T131N, M133T, and G145R, were found in patients who were positive for anti-HBs but negative for HBsAg (9, 30). Oon et al. (32) reported that HBV carriers, including HCC patients who were negative for HBsAg but positive for anti-HBc and anti-HBs, had the T126S, Q129D, M133L, T140I, and G145R mutations within the S region. Wu et al. (31) reported that amino acid residues at positions 122 and 145 of HBsAg had a major effect on antigenicity and immunogenicity. HBsAg mutants can escape current detection and persist in HBV-infected individuals after the loss of HBsAg (32). In the present study, we therefore determined the HBs amino acid sequences of all cases (with detectable HBV DNA), some of which had amino acid I126N, F134Y, S143T, and G145S (not G145R) mutations. It is possible that these HBsAg mutants escape detection by current HBsAg assays and the sensitivity becomes low (33). Based on the pretreatment, however, the Lumipulse HBsAg-HQ assay was able to detect HBsAg mutants because it uses two monoclonal antibodies against the external structural region as determinant "a" and the internal epitope as the capture target. Additionally, the Lumipulse HBsAg-HQ assay can detect HBsAg from samples with anti-HBs.

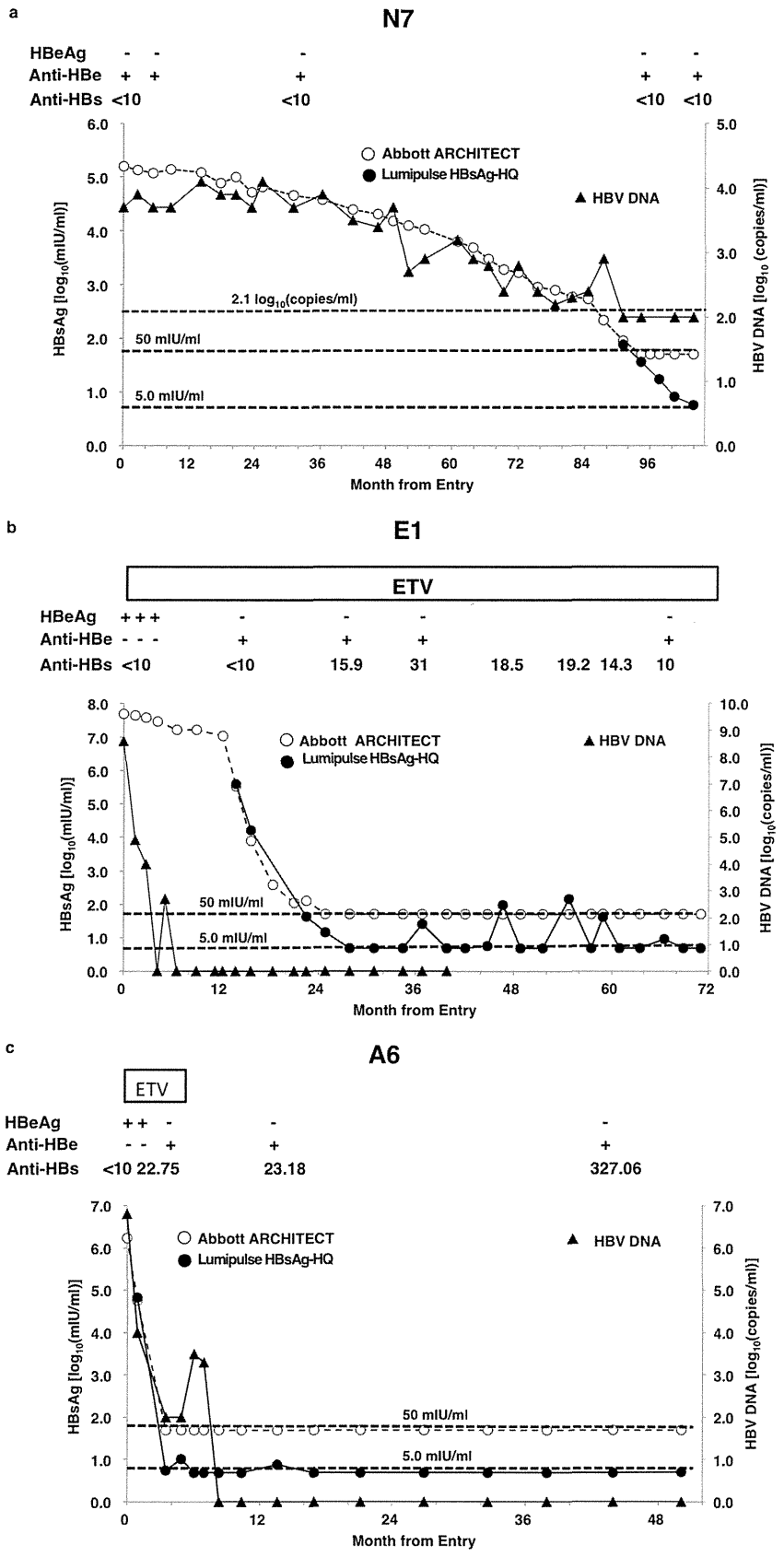


FIG 3 (a) HBsAg and HBV DNA dynamics of case no. N7. The Lumipulse HBsAg-HQ was still positive even 10 months after Abbott Architect results became negative. (b) HBsAg and HBV DNA dynamics of case no. E1. The HBsAg level as measured by the Lumipulse HBsAg-HQ assay was detectable for 3 months after HBsAg became negative by the Abbott Architect assay. After 1 year, HBsAg became detectable by the Lumipulse HBsAg-HQ assay, although HBV DNA was undetectable by the Cobas TaqMan and HBsAg was undetectable by the Abbott Architect assay. At 5 points, HBsAg was detectable by the Lumipulse HBsAg-HQ assay, and the anti-HBs concentration was >10 mIU/ml. (c) HBsAg and HBV DNA dynamics of case no. A6. HBsAg was detectable by the Lumipulse HBsAg-HQ assay for 3 months after HBsAg became negative by the Abbott Architect assay.

In conclusion, the automatic, highly sensitive HBsAg CLEIA Lumipulse HBsAg-HQ assay is a very convenient and precise assay for HBV monitoring in clinical practice.

ACKNOWLEDGMENTS

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The authors declare no conflicts of interest.

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A novel TK-NOG based humanized mouse model for the study of HBV and HCV infections

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ABSTRACT

The immunodeficient mice transplanted with human hepatocytes are available for the study of the human hepatitis viruses. Recently, human hepatocytes were also successfully transplanted in herpes simplex virus type-1 thymidine kinase (TK)-NOG mice. In this study, we attempted to infect hepatitis virus in humanized TK-NOG mice and urokinase-type plasminogen activator-severe combined immunodeficiency (uPA–SCID) mice. TK-NOG mice were injected intraperitoneally with 6 mg/kg of ganciclovir (GCV), and transplanted with human hepatocytes. Humanized TK-NOG mice and uPA/SCID mice were injected with hepatitis B virus (HBV)- or hepatitis C virus (HCV)-positive human serum samples. Human hepatocyte repopulation index (RI) estimated from human serum albumin levels in TK-NOG mice correlated well with pre-transplantation serum ALT levels induced by ganciclovir treatment. All humanized TK-NOG and uPA–SCID mice injected with HBV infected serum developed viremia irrespective of lower replacement index. In contrast, establishment of HCV viremia was significantly more frequent in TK-NOG mice with low human hepatocyte RI (<70%) than uPA–SCID mice with similar RI. Frequency of mice spontaneously in early stage of viral infection experiment (8 weeks after injection) was similar in both TK-NOG mice and uPA–SCID mice. Effects of drug treatment with entecavir or interferon were similar in both mouse models. TK-NOG mice thus useful for study of hepatitis virus virology and evaluation of anti-viral drugs.

1. Introduction

Hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are serious health problems worldwide. More than 350 and 170 million people are infected with HBV and HCV, respectively [1,2]. Both types of hepatitis viruses result in the development of chronic liver infection and potentially death due to liver failure and hepatocellular carcinoma [3]. Although the chimpanzee is a useful animal model for the study of HBV and HCV infection, there are ethical restrictions and hampered by the high financial cost on the use of this animal. The immunodeficient mice with a urokinase-type plasminogen activator (uPA) transgene [4,5] or a targeted disruption of the murine fumaryl acetoacetate hydrolase (FAH) [6–10] were shown to be excellent recipients for human hepatocyte. These small animal models are available for hepatitis viruses infection [4,11], and are useful for the study of HBV and HCV biology [12–14]. However, there are disadvantages that limit the utility of this model for many applications, including excessive mortality [9].

Recently, human hepatocytes were successfully transplanted into severely immunodeficient NOG mice with the herpes simplex virus type-1 thymidine kinase (HSVtk) expressing in mouse hepatocytes (TK-NOG) [15]. Mouse liver cells expressing HSVtk

Abbreviations: ALT, alanine aminotransferase; GCV, ganciclovir; HBV, hepatitis B virus; HCV, hepatitis C virus; HSA, human serum albumin; HSVtk, herpes simplex virus type-1 thymidine kinase; IFN, interferon; PegIFN-alpha, pegylated interferon-alpha; RI, repopulation index; RT-PCR, reverse transcript-polymerase chain reaction; SCID, severe combined immunodeficiency; uPA, urokinase-type plasminogen activator.

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were ablated after a brief exposure to ganciclovir (GCV), and transplanted human hepatocytes were stably maintained within the mouse liver without exogenous drug administration [15]. The analyses of drug interactions and pharmacokinetics have previously been reported using TK-NOG mice transplanted with human hepatocytes [15–18]. In the present study, we succeeded in infecting human hepatocyte-transplanted TK-NOG mice with HBV and HCV and showed that this mouse model is as useful as the uPA/SCID model for the study of hepatitis viruses.

2. Materials and methods

2.1. Animal treatment

TK-NOG mice were purchased from Central Institute for Experimental Animals (CIEA, Kawasaki, Japan). Eight-weeks-old mice were injected intraperitoneally with 6 mg/kg of GCV twice a day. After two days, mice were re-injected with the same amount of GCV. Seven days after 1st GCV injection, mice were transplanted with 1 or 2 $\times$ 10^6 of human hepatocytes obtained from human hepatocyte transplanted uPA–SCID chimeric mice by collagenase perfusion method by intra-splenic injection. Transplanted human hepatocytes used in this study were obtained from a same donor. One week after the first GCV treatment, serum alanine aminotransferase (ALT) levels were measured (Fuji DRI-CHEM, Fuji Film, Tokyo, Japan). Infection, extraction of serum samples, and sacrifice were performed under ether anesthesia. Mouse serum concentration of human serum albumin (HSA), which correlated with the human hepatocyte repopulation index (RI) [15], was measured as previously described [5]. Generation of the uPA/SCID mice and transplantation of human hepatocytes were performed as described previously [5,12,19]. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of the Graduate School of Biomedical Sciences, Hiroshima University.

2.2. Human serum samples

Human serum samples containing high titers of either genotype C HBV (5.3 $\times$ 10^6 copies/mL) or genotype 1b HCV (2.2 $\times$ 10^6 copies/mL) were obtained from patients with chronic hepatitis who provided written informed consent. The individual serum samples were divided into small aliquots and stored separately in liquid nitrogen until use. Mice were injected intravenously with 50 μL of either HBV- or HCV-positive human serum. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved a priori by the institutional review committee.

2.3. Quantitation of HBV and HCV

DNA and RNA extraction and quantitation of HBV and HCV by real-time polymerase chain reaction (RT-PCR) were performed as described previously [12,13,19]. Briefly, DNA was extracted using SMITEST (Genome Science Laboratories, Tokyo, Japan) and dissolved in 20 μL H_2O, and RNA was extracted from serum samples using SepaGene RVR (Sankojunyaku, Tokyo, Japan) and reverse transcribed with a random hexamer and a reverse transcriptase (ReverTraAce; TOYOBO, Osaka, Japan) according to the instructions provided by the manufacturer. Quantitation of HBV DNA and HCV RNA was performed using Light Cycler (Roche Diagnostic, Japan, Tokyo). The lower detection limits of real-time PCR for HBV DNA and HCV RNA are 4.4 and 3.5 log copies/mL, respectively.

2.4. Histochemical analysis of mouse liver

Liver specimens of HBV-infected TK-NOG mice were fixed with 10% buffered-paraformaldehyde and embedded in paraffin blocks for histological examination. Hematoxylin-eosin and immunohistochemical staining using antibodies against HSA (Bethyl Laboratories Inc., Montgomery, TX) and hepatitis B core antigen (HBc-Ag) (DAKO Diagnostika, Hamburg, Germany) were performed as described previously [12].

2.5. Treatment with antiviral agents

Mice were treated with antiviral agents eight weeks after HBV or HCV infection, by which time stable viremia had developed. HBV-infected mice were administered either food containing 0.3 mg of entecavir/kg of body weight/day or daily intramuscular injections with 7000 IU/kg of IFN-alpha (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan). HCV-infected mice were administered intramuscular injection with either 1000 IU/kg of IFN-alpha daily or 10 μg/kg of PegIFN-alpha-2a (Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) twice a week for three weeks.

2.6. Statistical analysis

Differences in HSA levels between TK-NOG mice and uPA–SCID mice, and incidence of infection between highly and poorly repopulated mice were examined for statistical significance using the Mann–Whitney *U*-test.

3. Results

3.1. Correlation between serum ALT level after GCV administration and the human hepatocyte index in TK-NOG mice

We analyzed the correlation between serum ALT levels after GCV injection and the human hepatocyte RI using 194 TK-NOG mice. Seven days after GCV injection when serum ALT levels had reached maximum levels [15], mice were transplanted with human hepatocytes. After transplantation of human hepatocytes, serum concentrations of HSA increased and reached plateau at 6–8 weeks. Serum ALT levels one week after GCV administration and HSA levels 8 weeks after hepatocyte transplantation showed a positive correlation, indicating that the higher serum ALT level, the higher the RI (Fig. 1A). HSA levels 8 weeks after human hepatocyte transplantation in TK-NOG mice were lower than in uPA–SCID mice (Fig 1B), which indicates that mice livers were more efficiently replaced with human hepatocytes in uPA–SCID mice than in TK-NOG mice.

3.2. Infection with hepatitis viruses in humanized TK-NOG mice and uPA–SCID mice

Eight weeks after human hepatocyte transplantation, TK-NOG mice and uPA–SCID mice with HSA levels over 1.0 mg/mL were inoculated with either HBV- or HCV-positive human serum samples. Eight weeks after injection, the frequency of the development of viremia was compared between the mice with lower (<70%) and higher ($\geqslant$70%) human hepatocyte RI. 70% of RI corresponds to 5.4 and 6.3 mg/dl of serum HAS in TK-NOG mice and uPA–SCID mice, respectively [5,15]. All humanized TK-NOG and uPA–SCID mice inoculated with HBV developed viremia 8 weeks after injection, irrespective of the RI (Fig. 2A). Incidence of HCV viremia was also high in TK-NOG mice regardless of the RI. In contrast, the frequency of HCV viremia was much lower in uPA–SCID mice with the RI. Only 20% (1 of 5) of uPA–SCID mice with low RI became

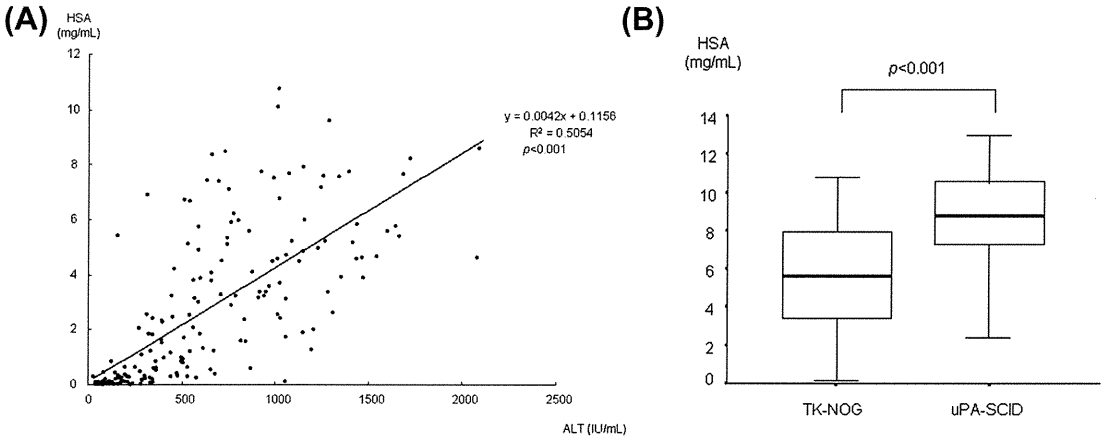

Fig. 1. Human hepatocyte repopulation index in humanized mice. Serum alaninaminotransferase (ALT) levels in TK-NOG mice were measured one week after ganciclovir treatment. Human serum albumin (HSA) levels were measured eight weeks after transplantation of human hepatocytes. (A) Correlation between serum ALT level after ganciclovir administration and human hepatocyte repopulation index in TK-NOG mice. Points represent single mouse measurements. *r* (Spearman rank) and *P* value are shown. (B) HSA levels in TK-NOG mice and uPA–SCID mice. 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; and the upper and lower bars outside the boxes represent the 90th and 10th percentiles, respectively.

positive for HCV, whereas 94.3% (50 of 53) of mice with high RI became positive ($p = 1.07 \times 10^{-6}$). Serum viral titers gradually increased in mice that developed viremia. Eight weeks after infection, HBV DNA and HCV RNA titers increased to approximately 8 and 6 log copies/mL, respectively in both TK-NOG and uPA–SCID mice (Fig. 2B). Viremia levels were slightly higher in uPA–SCID mice than TK-NOG mice, probably due to higher human hepatocyte RI (HSA levels) in uPA–SCID mice. In HBV-infected TK-NOG mice, histological analysis showed that hepatocytes positive for HSA were also positive for HB core antigen (Fig. 2C), which is in line with our previous findings using uPA–SCID mice [12].

3.3. The effect of antiviral agents on hepatitis virus-infected humanized mice

We analyzed the effect of antiviral agents on HBV- and HCV-infected humanized mice. Eight weeks after HBV-infection, 2 humanized TK-NOG mice were orally administrated 0.3 mg/kg day of entecavir, and 2 other mice received intramuscular injections with 7000 IU/g of IFN-alpha daily for 3 weeks. Both treatments resulted in a rapid reduction of mouse serum HBV DNA titers (Fig. 3A). Two HCV-infected humanized TK-NOG mice were administrated IFN-alpha daily, and 2 other mice received PegIFN-alpha-2a injections twice a week for 3 weeks. Both treatments resulted in a reduction of HCV RNA titers in mouse serum. The effects of these antiviral agents on HBV and HCV in TK-NOG mice were similar to those in uPA–SCID mice (Fig. 3B).

3.4. Incidence of unexpected death

The incidence of unexpected death is high in human hepatocyte chimeric uPA–SCID mice [20]. Incidence of unexpected death in the early stages of viral infection (within 8 weeks of viral infection) was similar between TK-NOG mice and uPA–SCID mice (6.3% vs 10.6%, $p = 0.465$) (Fig. 4).

4. Discussion

Human hepatocyte chimeric mice are valuable tool for hepatitis virology and drug assessment [12–14]. To establish human hepatocyte chimerism, two conditions are necessary: immunodeficiency and mouse-specific liver cell damage. For immune deficiency, SCID mice [4,5,12–14,20], NOG mice [8,21] and RAG-2 deficient mice [6,9,10] have been reported. We previously reported that the level of immunodeficiency in SCID mice, which are the most weakly immunodeficient of the three types, is sufficient to prevent rejection of transplanted human hepatocytes [5]. However, preventive treatments for human liver cell rejection via mice NK cells, such as an anti-asialo GM1 antibody, are necessary in SCID mice [5].

To evoke mouse liver cell injury, uPA and FAH transgene techniques were used [4–10]. Recently, successful human liver cell transplantation to TK-NOG mice in the absence of ongoing drug treatment after a brief exposure to a non-toxic dose of GCV has been reported [15]. We thus attempted to use TK-NOG mice to establish high levels of replacement with human hepatocytes and tried to infect hepatitis viruses.

In this study, we transplanted human hepatocytes to 194 TK-NOG mice and analyzed whether elevated serum ALT levels, which results from liver damage caused by GCV exposure, reflects HSA levels, as it is known that HSA levels are correlated with the human hepatocyte RI and can serve as a surrogate measure [15]. We found a positive correlation between ALT and HSA levels (Fig. 1A), indicating that higher levels of liver damage are associated with establishment of higher levels of repopulation of the liver with human hepatocytes. As the human hepatocyte RI obtained in this study using TK-NOG mice is lower than in uPA–SCID mice (Fig 1B), dose escalation of GCV or alternative treatment timing might result in more highly repopulated mice.

We infected humanized TK-NOG mice with hepatitis viruses and compared infection rates and serum viral titers with humanized uPA–SCID mice. HBV inoculation resulted in development of viremia without regard for the human hepatocyte replacement index in both TK-NOG mice and uPA–SCID mice (Fig. 2A). Incidence of HCV viremia was also high in TK-NOG mice regardless of HSA levels, whereas HCV viremia was infrequent in uPA–SCID mice with low HSA levels. These results are consistent with those of Vanwolleghem et al. [20] who showed, using a large number of human hepatocyte chimeric uPA–SCID mice, that an HSA level well above 1 mg/mL is important for successful HCV infection. The reason for the higher infection rate in TK-NOG mice with low human hepatocyte RI in this study is unknown. Although the level of immunodeficiency is higher in TK-NOG mice, it is difficult to conclude that this difference in immunodeficiency alone is responsible for the enhanced HCV infection rate. Although some studies have

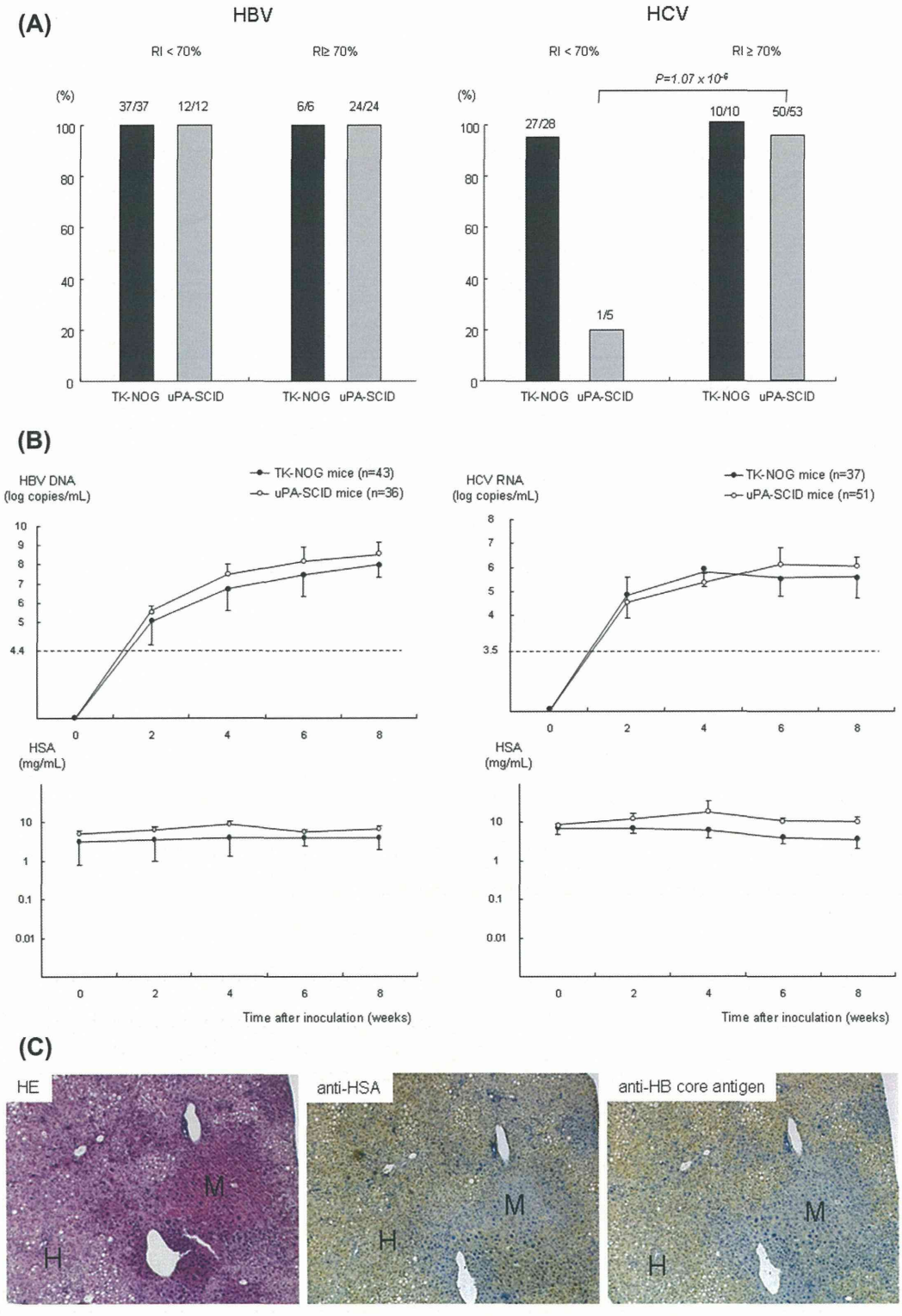

Fig. 2. Hepatitis viruses infection in chimeric mice. (A) Eight weeks after human hepatocyte transplantation, mice with serum HSA level over 1 mg/mL were inoculated with HBV- or HCV-positive human serum samples. Percentages of mice that became positive for HBV DNA (left panel) or HCV RNA (right panel) 8 weeks after inoculation according to human hepatocyte repopulation index (RI) in TK-NOG mice and uPA–SCID mice are shown. 70% of RI corresponds to 5.4 and 6.3 mg/dl of serum HAS in TK-NOG mice and uPA–SCID mice, respectively. (B) Changes in serum titers of HBV DNA (left panel) and HCV RNA (right panel) (upper panels) and HSA levels (lower panels) of TK-NOG mice and uPA–SCID mice. The horizontal dashed lines represent the lower detection limit of HBV DNA and HCV RNA (4.4 and 3.5 log copies/mL, respectively). (C) Histochemical analysis of liver samples obtained from HBV-infected TK-NOG mice. Hematoxylin-eosin staining (HE) and immunohistochemical staining using monoclonal antibodies against HSA and HB core antigen are shown. Regions are shown as human (H) and mouse (M) hepatocytes, respectively (Original magnification 100×).

reported structural differences between wild type and chimeric mice [22,23], the influence of such structural differences on HCV infectivity remains to be determined.

Human hepatocyte transplanted uPA–SCID mice are useful for evaluating antiviral agents [12–14]. In this study, we analyzed the efficacy of antiviral agents such as entecavir, IFN-alpha and

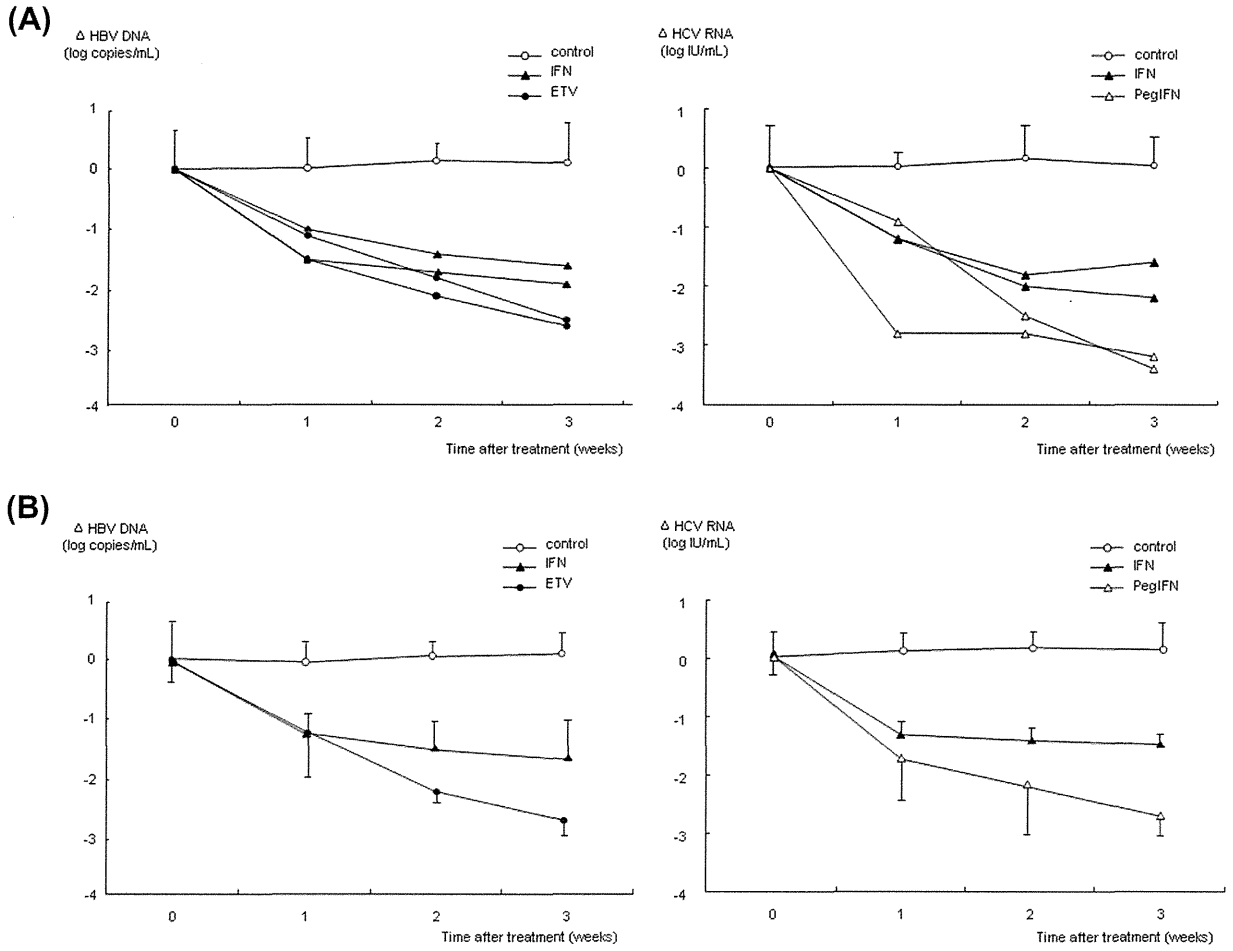

Fig. 3. Reduction of serum viral titers in mice treated with anti-viral agents. (A) HBV- (left panel) or HCV-infected (right panel) TK-NOG mice were treated with entecavir, interferon (IFN)-alpha or PegIFN-alpha-2a. Control: HBV- and HCV-infected mice without antiviral treatment. (B) HBV- (left panel) or HCV-infected (right panel) uPA–SCID mice were treated with entecavir, IFN-alpha or PegIFN-alpha-2a. Data are shown using the mean ± SD ($n = 4$).

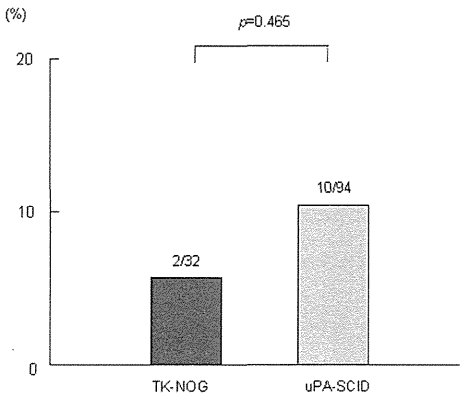

Fig. 4. Frequency of unexpected death within 8 weeks in mice. The numbers of sudden deaths occurring within 8 weeks of viral infection in TK-NOG mice and uPA–SCID mice are shown as bars.

PegIFN-alpha using HBV- and HCV-infected TK-NOG mice and compared them with uPA–SCID mice (Fig. 3). The results showed that both mouse models are equally useful for evaluation of antiviral drugs.

Human hepatocyte chimeric uPA–SCID mice are weak and prone to unexpected death [20], and this limitation appears to apply to TK-NOG mice as well. Incidence of unexpected death in the early stages of viral infection was not significantly different between TK-NOG mice and uPA–SCID mice (Fig. 4). The cause of these unexpected deaths is unknown. Further study is necessary to develop a more robust and easy to manipulate animal model.

In summary, we established a hepatitis virus infection mouse model using the human hepatocyte transplanted TK-NOG mouse. This model is useful for the study of hepatitis virology and evaluation of antiviral agents.

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Serum interleukin-6 associated with hepatocellular carcinoma risk: A nested case–control study

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Inflammatory markers have been associated with increased risk of several cancers, including colon, lung, breast and liver, but the evidence is inconsistent. We conducted a nested case–control study in the longitudinal cohort of atomic-bomb survivors. The study included 224 hepatocellular carcinoma (HCC) cases and 644 controls individually matched to cases on gender, age, city and time and method of serum storage, and countermatched on radiation dose. We measured C-reactive protein (CRP) and interleukin (IL)-6 using stored sera obtained within 6 years before HCC diagnosis from 188 HCC cases and 605 controls with adequate volumes of donated blood. Analyses with adjustment for hepatitis virus infection, alcohol consumption, smoking habit, body mass index (BMI) and radiation dose showed that relative risk (RR) of HCC [95% confidence interval (CI)] in the highest tertile of CRP levels was 1.94 (0.72–5.51) compared to the lowest tertile ($p = 0.20$). RR of HCC (95% CI) in the highest tertile of IL-6 levels was 5.12 (1.54–20.1) compared to the lowest tertile ($p = 0.007$). Among subjects with BMI > 25.0 kg/m^2, a stronger association was found between a 1-standard deviation (SD) increase in log IL-6 and HCC risk compared to subjects in the middle quintile of BMI (21.3–22.9 kg/m^2), resulting in adjusted RR (95% CI) of 3.09 (1.78–5.81; $p = 0.015$). The results indicate that higher serum levels of IL-6 are associated with increased HCC risk, independently of hepatitis virus infection, lifestyle-related factors and radiation exposure. The association is especially pronounced among subjects with obesity.

Epidemiology

Key words: C-reactive protein, interleukin-6, obesity, hepatocellular carcinoma, nested case–control study

Abbreviations: BMI: body mass index; CI: confidence interval; CRP: C-reactive protein; HBV: hepatitis B virus; HCC: hepatocellular carcinoma; HCV: hepatitis C virus; IL-6: interleukin-6; RERF: Radiation Effects Research Foundation; RR: relative risk; SD: standard deviation

Conflict of interest: Nothing to report

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Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide. Chronic infections with hepatitis B virus (HBV) or hepatitis C virus (HCV) are recognized as crucially important risk factors for HCC, whereas an increase of HCC without HBV and HCV infection (non-B, non-C HCC) has been noted recently in Japan.[1,2] Although periodic follow-up with imaging, tumor markers such as alpha-fetoprotein (AFP) and fibrosis markers are recommended, these strategies have not been sufficient for early detection of HCC in chronic liver disease, especially in non-B, non-C liver disease. Therefore, it is necessary to identify biomarkers that may be useful to narrow down a high-risk subgroup for HCC.

A large number of epidemiologic studies have shown that obesity and diabetes mellitus are associated with increased risks of such malignant tumors as colon, prostate and breast, as well as HCC.[3–11] Our earlier study also demonstrated that obesity [body mass index (BMI) > 25.0 kg/m^2] 10 years before HCC diagnosis was significantly associated with increased risk of HCC, independently of HBV and HCV infection, alcohol consumption, smoking habit and radiation exposure.[12] It has been suggested that cell proliferation activity of insulin due to hyperinsulinemia or chronic inflammation may promote

What's new?
According to previous research, alcohol consumption, obesity, and radiation exposure as well as hepatitis virus infection are all independent risk factors for hepatocellular carcinoma (HCC). Inflammatory markers have also been associated with increased risk of liver cancer, but the evidence is inconsistent. In this nested case-control study in the longitudinal cohort of atomic-bomb survivors, which took into account hepatitis virus infection, lifestyle-related factors, and radiation exposure, elevated IL-6 levels were found to be associated with increased risk of HCC. The findings also indicated that association of IL-6 levels with increased risk of HCC is especially pronounced among subjects with obesity.

carcinogenesis by DNA damage, enhancement of cellular proliferation and inhibition of apoptosis.[4,13] In recent years, some studies have suggested that blood levels of inflammatory markers or cytokines also related to insulin resistance—such as C-reactive protein (CRP), interleukin (IL)-6 and tumor necrosis factor (TNF)—may reveal a biological mechanism by which risks of colon, lung and breast cancers increase,[14–17] but other studies have not supported such associations.[18,19]

Several studies have demonstrated that elevated serum levels of IL-6 are associated with increased risk of HCC in female chronic hepatitis C patients,[20] and that the combination of serum levels of IL-6 and alpha-fetoprotein improves sensitivity in diagnosing HCC or predicting future HCC development in chronic hepatitis B patients.[21] A few experimental studies using a mouse model have demonstrated that estrogen-mediated inhibition of IL-6 production by Kupffer cells reduces liver cancer risk in females,[22] and that obesity-promoted HCC development was bound up with elevated production of the tumor-promoting cytokines, such as IL-6 and TNF, which cause hepatic inflammation and activation of the oncogenic transcription factor STAT3.[23] In several other cancers,[24,25] it has been suggested that IL-6 and STAT3 may also contribute toward a general enhancement of cancer risk by high BMI.

With the aim of investigating whether serum levels of CRP and IL-6 are associated with risk of HCC and, if so, whether that risk is independent of HBV and HCV infection, alcohol consumption, smoking habit, BMI and radiation exposure, we conducted a nested case–control study using sera collected from a prospective cohort study of atomic-bomb survivors. We subsequently evaluated whether the association between serum IL-6 levels and HCC risk is modified by alcohol consumption, smoking habit, BMI or radiation dose to the liver using analyses based on subgroups of those factors.

Material and Methods

Cohorts

The Atomic Bomb Casualty Commission (ABCC) and its successor, the Radiation Effects Research Foundation (RERF), established the prospective Adult Health Study cohort in 1958, in which more than 20,000 gender-, age- and city-matched proximal and distal atomic-bomb survivors and persons not present in the cities at the time of bombings have been examined biennially in outpatient clinics in Hiroshima and Nagasaki.

Cases and controls

Incident cancer cases were identified through the Hiroshima Tumor and Tissue Registry and Nagasaki Cancer Registry, confirmed and supplemented by additional cases detected *via* pathological review of related diseases.[26] As described in our previous studies,[3,27] 359 primary HCC cases were diagnosed among 18,660 Adult Health Study participants between 1970 and 2002, who visited our outpatient clinics before their diagnosis. Of these, 229 cases had serum samples obtained within 6 years before HCC diagnosis (average: stored sera obtained 1.2 years before diagnosis). After excluding five cases with inadequate stored serum, 224 cases remained for our previous studies. There were no important differences in characteristics such as alcohol consumption, smoking habit, BMI or radiation dose to the liver (among exposed persons) between HCC cases excluded because of nonavailability of stored serum and those included in our study.

As described in our previous studies,[3,27] 644 controls were selected from the at-risk cohort members matched to the case on gender, age, city and time and method of serum storage, and countermatched on radiation dose in nested case–control fashion.[28] Counter matching (to increase statistical efficiency for studying joint effects of radiation and other factors) was performed using four strata based on whole-body (skin) dose: zero dose (<0.0005 Gy), <0.05 Gy, <0.75 Gy and ≥ 0.75 Gy (nonzero categories correspond roughly to tertiles of skin dose among all eligible exposed cases). At the time of each case diagnosis, one control serum was selected at random from each of the three dose strata not occupied by the case in the cohort risk set.

Laboratory tests

Virological assays of HBV and HCV were performed on 211 cases and 640 controls with sufficient stored sera for these assays as previously described.[29,30] HBV infection (HBV+) status was defined as positive for HBsAg or having a high titer of anti-HBc Ab (positive for anti-HBc Ab of samples diluted 200-fold). HCV infection (HCV+) status was defined as positive for HCV RNA. Non-B, non-C status was defined as negative for HBsAg and not having a high titer of anti-HBc Ab (HBV-) as well as negative for HCV RNA (HCV-).

Serum levels of CRP were measured using an autoanalyzer (Hitachi 7180, Hitachi, Tokyo, Japan) and a high-sensitivity assay kit (Nissui Pharmaceutical, Tokyo, Japan) containing anti-CRP monoclonal antibodies. The detection limit of CRP was 0.08 mg/L. The intra-assay variability was determined by assaying two pooled serum samples (mean CRP level: 0.62 and 1.68 mg/L, respectively) 20 times in a single day, and the respective coefficients of variation (CVs) were 1.12 and 0.95%. The interassay variability was determined by assaying two quality control samples (mean CRP level: 2.14 and 4.71 mg/L, respectively) once a day 12 for days; the respective CVs were 4.1 and 1.2%. Serum levels of IL-6 were measured using the multiplex bead array assay on the Luminex Complete System 200 (Luminex Corp., Austin, TX),[31] with MILLIPLEX™ MAP kits (Millipore, Billerica, MA) according to the manufacturer's instructions. Human serum adipokine panel B (HADK2-61K-B) was used for IL-6. The intra-assay variability was determined by assaying two pooled serum samples with and without including a quality control sample (mean IL-6 level: 4.29 and 144.82 pg/mL, respectively) 15 times in a single day, and the respective CVs were 8.6 and 7.5%. The interassay variability was determined by assaying two quality control samples (mean IL-6 level: 31.02 and 171.62 pg/mL, respectively) once a day for 7 days; the respective CVs were 7.9 and 13.7%.

Radiation dose

Radiation dose to the liver was estimated for each subject according to Dosimetry System DS02.[32] A weighted sum of the gamma dose in gray plus ten times the neutron dose in gray was used.

Alcohol consumption, smoking habit and BMI

Self-administrated questionnaires on lifestyle-related factors were given to Adult Health Study participants in 1965 during attendance at the outpatient clinic and in 1978 by mail survey. Information on alcohol consumption was obtained from the 1965 questionnaire when available, with missing data complemented using the 1978 mail survey. Mean ethanol amounts were calculated as grams per day, as previously described.[33] Information on smoking habit was obtained from the 1965 questionnaire. Subjects were categorized as never, current or former smoker. BMI (kg/m^2) was calculated from height and weight measured in the outpatient clinic of the Adult Health Study. Subjects were classified based on BMI quintiles with cut points of 19.5, 21.2, 22.9 and 25.0. Following the recommendations for Asian people by the WHO, the International Association for the Study of Obesity and the International Obesity Task Force,[34] 21.3–22.9 kg/m^2 was considered as normal, 23.0–25.0 kg/m^2 as overweight and >25.0 kg/m^2 as obese.

Ethical consideration

This study (RERF Research Protocol 1-09) was reviewed and approved by the Research Protocol Review Committee and the Human Investigation Committee of RERF.

Statistical analyses

The nested case–control design is analyzed using a partial likelihood method analogous to that used for cohort follow-up studies,[35] which is in practice the same as the conditional binary data likelihood for matched case–control studies[36] except that the subjects (cases and controls) in the study are not completely independent owing to the possibility of repeated selection. Radiation risk was estimated using an excess relative risk (ERR) model (ERR = RR − 1) to conform to other analyses of the atomic-bomb survivor cohort.[3,27,37] Bias in control doses due to selecting controls using countermatching was corrected using weights as described elsewhere.[28] Risks for all other factors were assessed using a log-linear model. In analyses based on continuous values, CRP and IL-6 were transformed using the natural logarithm. Analyses using CRP or IL-6 groups used tertiles computed among controls. A two-degree-of-freedom heterogeneity test was performed by comparing the deviance of the model with tertiles to that without, using the lowest tertile as the comparison group. We fit log-linear regression models for the effect of a 1-standard deviation (SD) increase in IL-6 and tested for interaction with each of the other risk factors individually using the same heterogeneity test, with degrees of freedom depending on the number of categories of the other risk factor; we report the p value for the pairwise test comparing the interaction parameter in the highest to lowest level of each other risk factor. We also assessed various models for log relative risk of HCC with continuous level of IL-6—linear, linear-quadratic and linear spline—using the Akaike information criterion (AIC).[38] Analyses were conducted using Epicure (HIROSOFT International Corp., Seattle, WA).

Results

Characteristics of cases and controls

Table 1 shows characteristics of HCC cases and matched controls. Because of matching, cases and controls were comparable with respect to gender, age, city and time and method of serum storage. Prevalence of HBV and/or HCV infection status in HCC cases is higher than in controls. Compared to the controls, higher proportions of HCC cases had a history of alcohol consumption exceeding 40 g of ethanol per day, were obese (BMI > 25.0 kg/m^2) and were current smokers. Median serum levels of CRP were 0.72 mg/L among HCC cases and 0.59 mg/L among controls. Median serum levels of IL-6 were 4.88 pg/mL among HCC cases and 2.90 pg/mL among controls. HCC cases also received on average higher radiation doses to the liver compared to controls.

Correlations among CRP, IL-6, alcohol, BMI and radiation dose

Table 2 shows Spearman rank-correlation coefficients (r) between serum levels of CRP and IL-6, alcohol consumption,

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				
Age at HCC diagnosis (years)[1]	224	67.6 (10.1)	–	–
Age at serum storage (years)[1]	224	66.4 (10.2)	644	63.7 (9.8)
Gender	224		644	
Male		136 (60.7)		387 (60.1)
Female		88 (39.3)		257 (39.9)
City	224		644	
Hiroshima		155 (69.2)		444 (68.9)
Nagasaki		69 (30.8)		200 (31.1)
Unmatched variables				
Viral etiology	211		640	
HBV−/HCV−		45 (21.3)		579 (90.5)
HBV+ and/or HCV+		166 (78.7)		61 (9.5)
Alcohol consumption (g ethanol/day)	199		577	
None		97 (48.7)		315 (54.6)
0< <40		57 (28.6)		194 (33.6)
≥40		45 (22.6)		68 (11.8)
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)
BMI (kg/m^2) 10 years before diagnosis	210		633	
≤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)
Inflammatory markers				
CRP (mg/L), median (IQR)	188	0.72 (0.18, 1.89)	605	0.59 (0.25, 1.52)
IL-6 (pg/mL), median (IQR)	182	4.88 (2.88, 8.77)	589	2.90 (1.53, 5.42)
Radiation dose to the liver (Gy)[1,2]	204	0.46 (0.69)	606	0.34 (0.56)

[1]Mean (SD).
[2]Control values were adjusted for countermatched selection.

BMI 10 years before HCC diagnosis and radiation dose to the liver. Serum levels of CRP were positively correlated with serum levels of IL-6 among both cases (r = 0.46) and controls (r = 0.29). Serum levels of CRP were modestly correlated with BMI among both cases (r = 0.15) and controls (r = 0.28), whereas correlations between serum levels of IL-6 and BMI were not significant among either cases (r = 0.11) or controls (r = 0.06). Neither alcohol consumption nor radiation dose showed any evidence of correlation with either marker.

Risk of HCC according to serum levels of CRP and IL-6

Table 3 shows the association between CRP and HCC risk based on tertiles of serum CRP levels. Analyses with adjustment for HBV and HCV infection, alcohol consumption, smoking habit, BMI 10 years before HCC diagnosis and radiation dose showed that relative risks (RRs) of HCC [95% confidence interval (CI)] in the middle tertile (0.37–0.96 mg/L) and highest tertile (>0.96 mg/L) of CRP levels were 2.11 (0.73–6.54; p = 0.17) and 1.94 (0.72–5.51; p = 0.20), respectively, compared to

Table 2. Spearman rank-correlation coefficients between CRP, IL-6, alcohol, BMI and radiation dose among HCC cases and controls

	CRP		IL-6	
Variables	Correlation	*p* Value	Correlation	*p* Value
HCC cases				
CRP	–	–	–	–
IL-6	0.46	<0.001	–	–
Alcohol consumption (g ethanol/day)	0.01	0.9	−0.02	0.83
BMI 10 years before diagnosis	0.15	0.049	0.11	0.14
Radiation dose to the liver	−0.09	0.26	−0.08	0.30
Controls				
CRP	–	–	–	–
IL-6	0.29	<0.001	–	–
Alcohol consumption (g ethanol/day)	−0.003	0.94	0.05	0.28
BMI 10 years before diagnosis	0.28	<0.001	0.06	0.13
Radiation dose to the liver	−0.02	0.64	−0.06	0.13

Table 3. Relative risks of HCC by tertile of serum levels of CRP

	Tertile of CRP			*p* Value for heterogeneity
	Low < 0.37 mg/L	Middle 0.37–0.96 mg/L	High > 0.96 mg/L	
No. of cases/controls[1]	**49/120**	**29/98**	**59/109**	
Crude RR (95% CI)	1.00	0.64 (0.36–1.15)	1.16 (0.71–1.88)	0.10
p Value	–	0.14	>0.50	
Adjusted RR (95% CI)[2]	1.00	1.54 (0.62–3.92)	1.90 (0.87–4.36)	0.28
p Value	–	0.36	0.11	
Adjusted RR (95% CI)[3]	1.00	2.11 (0.73–6.54)	1.94 (0.72–5.51)	0.32
p Value	–	0.17	0.20	

[1]Number of subjects for whom information available for all factors included in a log-linear model: 137 HCC cases and 327 controls.
[2]Adjusted for HBV/HCV infection, excluding three HBV+/HCV+ individuals.
[3]Adjusted for HBV/HCV infection, alcohol consumption, smoking habit, BMI 10 years before diagnosis and radiation dose to the liver.

Table 4. Relative risks of HCC by tertile of serum levels of IL-6

	Tertile of IL-6			*p* Value for heterogeneity
	Low < 2.01 pg/mL	Middle 2.01–4.46 pg/mL	High > 4.46 pg/mL	
No. of cases/controls[1]	**13/103**	**48/107**	**71/103**	
Crude RR (95% CI)	1.00	3.78 (1.87–8.26)	6.44 (3.24–14.0)	<0.001
p Value	–	<0.001	<0.001	
Adjusted RR (95% CI)[2]	1.00	2.87 (1.02–8.91)	4.09 (1.46–12.9)	0.025
p Value	–	0.045	0.007	
Adjusted RR (95% CI)[3]	1.00	3.85 (1.16–14.7)	5.12 (1.54–20.1)	0.023
p Value	–	0.027	0.007	

[1]Number of subjects for whom information available for all factors included in a log-linear model: 132 HCC cases and 313 controls.
[2]Adjusted for HBV/HCV infection, excluding three HBV+/HCV+ individuals.
[3]Adjusted for HBV/HCV infection, alcohol consumption, smoking habit, BMI 10 years before diagnosis and radiation dose to the liver.

those in the lowest tertile (<0.37 mg/L; heterogeneity $p = 0.32$).

Table 4 shows the association between IL-6 and HCC risk based on tertiles of IL-6. Analyses with adjustment for HBV and HCV infection, alcohol consumption, smoking habit, BMI 10 years before HCC diagnosis and radiation dose showed that RRs of HCC (95% CI) in the middle tertile (2.01–4.46 pg/mL) and highest tertile (>4.46 mg/L) of IL-6

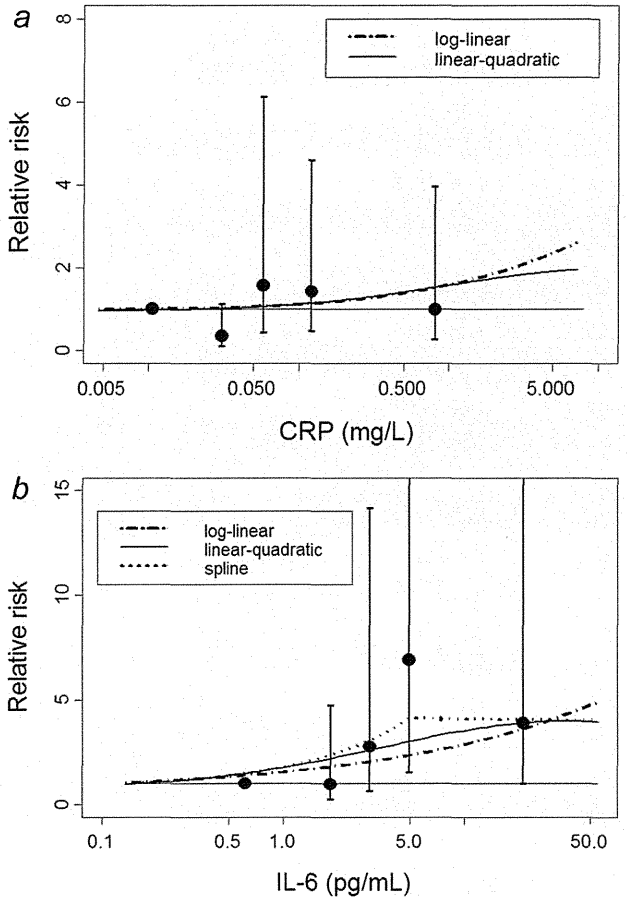

Figure 1. (*a*) Continuous risk of HCC by CRP. RR (95% CI) of HCC with adjustment for alcohol, smoking habit, BMI and radiation dose is plotted according to serum levels of CRP. A test for overall significance of the log-linear curve was not significant (p = 0.23, dashed line). Fit of a linear-quadratic model (solid line) was not as good as the log-linear model according to the AIC model-comparison criterion. (*b*) Continuous risk of HCC by IL-6. RR (95% CI) of HCC with adjustment for alcohol, smoking habit, BMI and radiation dose is plotted according to serum levels of IL-6. A test for overall significance of the log-linear curve was significant (p = 0.015, dashed line). Fits of linear-quadratic (solid line) and linear spline (dotted line) were not as good as the log-linear model according to the AIC model-comparison criterion.

levels were 3.85 (1.16–14.7; p = 0.027) and 5.12 (1.54–20.1; 0.007), respectively, compared to those in the lowest tertile (<2.01 pg/mL; heterogeneity p = 0.023).

Additional analyses were conducted to examine the association between CRP or IL-6 and non-B, non-C HCC risk, although there were relatively few cases with non-B, non-C status (31 cases). Analyses with adjustment for alcohol consumption, smoking habit, BMI 10 years before HCC diagnosis and radiation dose showed that RRs of non-B, non-C HCC (95% CI) in the middle and highest tertiles of CRP were 7.77 (1.13–78.5) and 7.40 (1.26–64.6), respectively, compared to those in the lowest tertile (heterogeneity p = 0.065). RRs of non-B, non-C HCC (95% CI) in the middle and highest tertiles of IL-6 were 56.3 (4.27–2,000) and 98.0 (6.74–4,500), respectively, compared to those in the lowest tertile (heterogeneity $p < 0.001$) after the same adjustment. The wide confidence bounds are presumably due to the small numbers of non-B, non-C HCC cases.

We also examined the possibility of a nonlinear relation between serum levels of CRP or IL-6 and HCC risk. There was no evidence of any systematic relationship between CRP and HCC risk (Fig. 1*a*). The log RR of HCC increased linearly with logarithm of serum IL-6 level after adjustment for alcohol consumption, smoking habit, BMI and radiation dose (p = 0.015, AIC = 132.63; Fig.1*b*). Although HCC risk appears to level off or decline at high values of IL-6 (Fig.1*b*), neither a negative quadratic term (p = 0.17, AIC = 132.73) nor a linear spline (p = 0.10, AIC = 133.95, with best fit obtained using a join point at log IL-6 = 1.6 or IL-6 = 4.95) revealed any statistically significant departure from the log-linear model. Although the appearance of a downturn at high values of IL-6 may be spurious, lack of statistical significance could also be due to the large uncertainty in estimated risk for IL-6 (high upper bound on confidence intervals for IL-6 groups).

Interaction between IL-6 level and gender, lifestyle-related factors or radiation for risks of HCC

Table 5 shows the association between IL-6 and HCC risk by selected subgroups. Stronger association was found between a 1-SD increase in log IL-6 and HCC risk among subjects with BMI of >25.0 kg/m^2 (obese) 10 years before diagnosis than among subjects with BMI of 21.3–22.9 kg/m^2 (normal), resulting in adjusted RR (95% CI) of 3.09 (1.78–5.81; p for interaction = 0.015). However, there was no significant difference in association between IL-6 and HCC risk among females compared to males, among subjects with alcohol consumption of 40 g of ethanol per day compared to never drinkers, among current smokers compared to never smokers or among subjects exposed to ≥1.0 Gy radiation compared to subjects exposed to <0.001 Gy radiation.

Additional analyses were conducted to examine the association between IL-6 and non-B, non-C HCC risk by selected subgroups. Similarly, a stronger association was found between a 1-SD increase in log IL-6 and non-B, non-C HCC risk among subjects with BMI of >25.0 kg/m^2 than among subjects with BMI of 21.3–22.9 kg/m^2, resulting in adjusted RR (95% CI) of 5.01 (1.51–34.0; p for interaction = 0.025). The results suggest that elevated serum levels of IL-6 among obese subjects are more strongly associated with increased risks of non-B, non-C HCC as well as overall HCC compared to subjects with normal weight.

Discussion

Our study demonstrated that elevated serum levels of IL-6 are associated with increased risk of HCC, independently of hepatitis virus infection, lifestyle-related factors—such as alcohol consumption, smoking habit and BMI—and radiation

Table 5. Relative risks of HCC associated with a 1-SD increase in log IL-6 level

	RR	95% CI	*p* Value for interaction[1]
All HCC	1.84	1.50, 2.28	
Gender			
Males	1.78	1.36, 2.38	
Females	1.91	1.41, 2.68	>0.5
Alcohol consumption (g ethanol per day)			
None	1.91	1.40, 2.69	
≥40	1.88	1.69, 3.53	>0.5
Smoking habit			
Never	2.09	1.48, 3.07	
Current smoker	1.61	1.19, 2.23	0.28
BMI (kg/m^2) 10 years before diagnosis			
21.3–22.9	1.26	0.80, 1.99	
>25.0	3.09	1.78, 5.81	0.015
Radiation dose to the liver (Gy)			
0≤ <0.001	2.01	1.43, 2.89	
≥1.0	2.50	1.38, 5.10	>0.5
Non-B, non-C HCC	1.62	1.14, 2.39	
Gender			
Males	1.09	0.60, 1.96	
Females	2.13	1.32, 3.84	0.09
Alcohol consumption(g ethanol per day)			
None	1.86	1.09, 3.73	
≥40	2.09	0.57, 11.0	>0.5
Smoking habit			
Never	2.04	1.13, 4.16	
Current smoker	1.35	0.78, 2.39	0.33
BMI (kg/m^2) 10 years before diagnosis			
21.3–22.9	0.84	0.31, 2.02	
>25.0	5.01	1.51, 34.0	0.025
Radiation dose to the liver (Gy)			
0≤ <0.001	1.71	0.89, 3.44	
≥1.0	2.66	1.06, 10.1	0.47

[1]*p* Value for interaction is from the likelihood ratio test for a difference in IL-6 risk between high-risk and reference categories of the other factor, while adjustment was made for main effects and interactions of all categories of the other factor.

exposure. Significant association was observed between elevated serum levels of IL-6 and increased risk of non-B, non-C HCC, whereas the association with elevated serum levels of CRP was only marginally significant. Among subjects with obesity, an even stronger association was observed between elevated serum levels of IL-6 and increased risk of HCC (non-B, non-C HCC as well as all HCC).

Several studies have demonstrated that elevated serum level of CRP is associated with poor prognosis in HCC patients, whereas few cohort studies have shown a significant association between CRP level and HCC risk.[39] In our study, the association between serum level of CRP and HCC risk was not significant, after adjusting for HBV and HCV infection, lifestyle-related factors and radiation dose. However, it has been reported that positive association between CRP level and degree of hepatic steatosis occurs among obese patients with nonalcoholic fatty liver disease,[40] and CRP level is useful not only for distinguishing nonalcoholic steatohepatitis (NASH) from simple nonprogressive fatty liver but also for predicting the severity of liver fibrosis in steatohepatitis