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RAPID COMMUNICATION

Prevention of *de novo* HBV infection by the presence of anti-HBs in transplanted patients receiving core antibody-positive livers

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Abstract

AIM: To analyze whether the presence of anti-HBs in liver transplant recipients is effective in preventing HBV infection.

METHODS: Twenty-three patients receiving anti-HBc positive liver were studied. Nine recipients were anti-HBc positive as a result of previous HBV infection. Of them, one also received HBV vaccine during the pre-liver transplantation period. Fourteen recipients were anti-HBs positive due to HBV vaccine administered during the pre-transplant period. Liver biopsy was obtained in 10/14 anti-HBc negative/anti-HBs positive recipients and in 4/9 anti-HBc positive recipients.

RESULTS: After a mean follow-up period of 46 months, 1 recipient with protective serum anti-HBs levels developed *de novo* HBV infection as a consequence of immune escape HBV mutants. Among the 14 vaccinated anti-HBc negative/anti-HBs positive recipients, 1/10 patients with available liver biopsy (10%) had liver HBV-DNA at 13 mo post-liver transplantation without serum viral markers and did not develop *de novo* HBV infection. The vaccinated anti-HBc positive recipient without HBV vaccine response was HBV-DNA positive in serum and liver, viral DNA was continuously negative in the following tests, so a spontaneous seroconversion was diagnosed.

CONCLUSION: The presence of anti-HBs as a result of HBV vaccine or past HBV infection seems to be effective at protecting patients receiving livers from anti-HBc positive donors. However, the emergence of immune escape HBV mutants, which can evade the anti-HBs protection, should be considered as a risk of HBV infection.

Key words: HBV vaccine; Liver transplantation; De novo HBV infection; Hepatitis B core antibody

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INTRODUCTION

It has been reported that the incidence of hepatitis B virus (HBV) infection is high in recipients after liver transplantation (LT) from hepatitis B surface antigen (HBsAg) negative but anti-core antibody (anti-HBc) positive donors[1,2]. The frequency of HBV transmission depends on the HBV serological recipient status, while the presence of anti-HBc and anti-HBs in organ recipients may confer resistance to HBV infection. Their absence results in *de novo* HBV infection[3-5]. To avoid the occurrence of *de novo* HBV infection in recipients without serum HBV markers, use of passive immunization with hepatitis B immune globulin (HBIG) in combination with lamivudine is necessary[6,7]. Another possibility to make use of these anti-HBc positive organs is to direct liver grafts to patients with anti-HBc and/or anti-HBs as a consequence of past HBV infection or HBV vaccination[8].

It is possible that HBV transmission from anti-HBc

Table 1 Features of donors and liver recipients

Pt.	Age (yr)	Sex	Liver transplant indications	Donor HBV status		Recipient pre-LT HBV status		Liver HBV-DNA	Latest	Follow-up
				anti-HBc	anti-HBs	anti-HBc	anti-HBs	(mo after LT)	anti-HBs levels	(mo)
1	58	M	HCV cirrhosis	+	-	-	+ V (>10)	13 + (RC)	ND	66
2	46	F	Cryptogenic cirrhosis	+	-	-	+ V (>10)	1 -	-	69
3	62	M	Alcoholic cirrhosis	+	+	+	+	ND	ND	70
4	66	M	HCV cirrhosis	+	+	+	+	0 -	ND	71
5	55	F	HCV cirrhosis	+	+	-	+ V (>10)	0 - 12 - 36 -	-	51
6	44	M	HCV cirrhosis	+	-	-	+ V (>100)	4 - 12 -	+	63
7	63	M	HCV cirrhosis	+	+	-	+ V (>100)	1 -	ND	74
8	54	M	HCV cirrhosis	+	-	+	+	2 - 28 -	+	76
9	41	M	HCV/alcoholic cirrhosis	+	+	+	- V (<10)	23 + (RC)	-	66
10	52	F	Alcoholic cirrhosis	+	+	-	+ V (>50)	ND	-	52
11	40	M	HCV/alcoholic cirrhosis	+	-	-	+ V (>50)	0 -	-	56
12	65	M	Alcoholic cirrhosis/ HCC	+	+	-	+ V (>10)	0 -	-	61
13	50	F	Cryptogenic cirrhosis	+	+	-	+ V (>10)	ND	-	39
14	67	F	HCV cirrhosis/HCC	+	+	-	+ V (>10)	14 -	+	36
15	67	M	HCV cirrhosis	+	+	-	+ V (>10)	ND	ND	36
16	60	M	Alcoholic cirrhosis	+	+	-	+ V (>10)	1 -	ND	32
17	62	M	HCV/HCC	+	ND	+	-	6 -	-	31
18	67	M	Alcoholic cirrhosis	+	-	-	+ V (>10)	ND	ND	28
19	46	F	HCV cirrhosis	+	+	+	-	ND	ND	24
20	58	M	Alcoholic cirrhosis	+	-	+	+	ND	ND	18
21	49	M	HCV/alcoholic cirrhosis/HCC	+	+	-	+ V (>10)	5 -	ND	18
22	56	M	Alcoholic cirrhosis	+	+	+	+	ND	ND	17
23	57	M	Alcoholic cirrhosis	+	+	+	+	ND	+	16

HCV: hepatitis C virus; HCC: hepatocellular carcinoma; HBV: hepatitis B virus; V: HBV vaccine; RC: partially double-stranded HBV DNA genome; ND: not determined.

donors to recipients via LT may be due to the persistence of HBV in tissue in a state of replication-competent that can be reactivated to form infectious particles. During initiation of HBV infection, the partially double-stranded HBV DNA genome (rcDNA) becomes a covalently closed circular DNA molecule (cccDNA) that serves as a template for viral transcription[9] and is regulated and amplified by an intracellular pathway[10]. This process establishes a pool of nuclear cccDNA, which persists in the nuclei of infected cells as long as hepatocytes survive, explaining the requirement for long-term antiviral therapies[11]. This fact could explain the reactivation of HBV under certain conditions such as administration of immunosuppressive drugs[12]. The genomic organization of the HBV direct repeat region (DR) provides a strategy to distinguish rcDNA from cccDNA using PCR primers flanking the DR region.

The purpose of this study was to analyze whether past HBV infection or positive response to HBV vaccine is effective in preventing *de novo* HBV infection in patients receiving a liver from anti-HBc positive donors.

MATERIALS AND METHODS

Patients

Anti-HBc screening is a general practice performed in organ donors at our hospital. Between February 1999 and February 2004, 31 HBsAg negative patients received anti-HBc positive liver. Thirty-one donors were anti-HBc positive and 16 of them were also anti-HBs positive (51.6%). Of the 31 recipients, 8 patients had a second LT or were under lamivudine treatment because they were anti-HBc and anti-HBs negative, so they were excluded from the study. The remaining 23 liver recipients were studied. Of them, 14 anti-HBc negative recipients developed protective anti-HBs levels after vaccination (40 mg/dose intramuscularly administered on days 0, 15 and 30; Engerix B, Smith-Kline Beechman, Belgium) and 9 were anti-HBc positive. Immunosuppression treatment included was of steroids and cyclosporin or tacrolimus. Characteristic and virological features of both donors and liver recipients are shown in Table 1.

Serum samples for detection of HBV markers anti-HBs and serum HBV-DNA were collected at the time of liver graft and during the post-transplant follow-up period. Liver tissues when possible were collected for analysis of HBV-DNA by PCR.

Hepatitis B virus markers

Serum samples were tested for HBsAg, anti-HBs and anti-HBc with commercially available radioimmunoassays kits (Abbott Laboratories, N Chicago, IL). Detection of viral DNA in serum was carried out by Abbott hybridization assay. Biopsy specimens were examined with immunohistochemical techniques for HBsAg and HBcAg

detection.

DNA extraction from paraffin-embedded liver tissues was performed using the MasterPure complete DNA purification kit (Epicentre, Madison, WI). Liver DNA was assayed for HBV DNA by 2 different sets of primers corresponding to the surface[13] and X genes. The sequences of the primers amplifying the X gene are as follows: HBVfe (TCTTGGACTCTCAGCAATGTCA nt 1438-1456), HBVre (GGTGAAAAAGTTGCATGGTGC nt 1583-1603), HBVfi (ACCGACCTTGAGGCATACTTCA nt 1463-1484), HBVri (CCAATTTATGCCTACAGCCTCC nt 1550-1571). PCR was started with the hot-start technique. The first round of PCR was performed with the outer primers for 30 cycles (at 95℃ for 15 s, at 55℃ for 10 s, and at 72℃ for 30 s) followed by an extension at 72 ℃ for 5 min. The second round was carried out with the inner primers for 30 cycles at 57℃ as the annealing temperature.

The selective detection of cccDNA was carried out by a nested PCR procedure as previously described with some modifications to distinguish between rcDNA genome present in virions and ccc HBV-DNA found in hepatocytes[14]. The rcDNA contains a single-stranded gap at the 5´end of the minus strand DNA. Since this region is sensitive to mung bean nuclease (MBN) leading to a disruption in the viral genome, no PCR product could be obtained using this rcDNA as a template. However, since cccDNA is a double-stranded covalently closed molecule and resistant to MBN, PCR could yield a fragment when this DNA was used as a template. MBN reaction was done as previously described[14]. The whole reaction was used as a template for the first round of nested PCR. The primers used to amplify cccDNA were HBVdr-s (TTACGCGGACTCCCCGT nt 1410-1424), 1900AS (GGTCAATGTCCATGCCCCAA nt 1769-1790), HBVfi (ACCGACCTTGAGGCATACTTCA nt 1463-1484), and HBVdr-as (GACATGAACAAGAGATGATTAGGCA nt 1706-1730). As a positive control, a PCR fragment containing the nick region of rcDNA was cloned into the pMosBlue vector (pMOSBlue blunt ended cloning kit, Amersham Pharmacia Biotech, UK). HBV DNA extracted from serum of HBsAg-positive individuals was used as a negative control. Both positive and negative controls were treated with MBN.

RESULTS

Only one patient developed *de novo* HBV infection after a follow-up of 45 ± 20.81 months (Table 1). This patient was vaccinated and developed a low anti-HBs response with anti-HBs title between 10 and 100 IU/mL. Seven months after LT, her routine biochemical tests showed abnormal level of liver enzymes (AST 97 U/L, ALT 151 U/L, GGT 136 U/L) but liver ultrasound was normal. Besides, virological analysis was found to be positive for HBsAg and serum HBV-DNA, even if her serum anti-HBs titer was 17 IU/mL. These virological tests did not reveal any other viral infections (CMV, CEV, HCV, HAV). Based on these results, *de novo* HBV infection was diagnosed and the patient was treated with lamivudine (100 mg/d). Serum HBV-DNA still remained positive, so a combined therapy of lamivudine and adefovir was administered 32 mo after LT. A second liver biopsy revealed marked steatosis with positive immunostaining for HBcAg (in nuclei and cytoplasm) and HBsAg (in cytoplasm and membrane). No tissue was obtained for the analysis of HBV-DNA.

It is known that the "a" determinant located within HBsAg is the target of immune response providing immunity against HBV infection[15]. The emergence of HBV with surface gene mutations is able to escape immune response against HBV vaccine, causing infection. In fact, HBV envelope mutants associated with the "a" determinant after HBV vaccination have been identified[16]. To know whether this might be the reason why *de novo* HBV infection occurred in this liver recipient, a serum sample taken 36 months after LT was used for HBV-DNA extraction, PCR amplification of the "a" determinant of the S gene and PCR fragment sequencing. Viral sequence revealed that this patient harbored a HBV variant with 2 point mutations at amino acid positions 127 and 145 of HBsAg. The first mutation resulted in a substitution of proline or lysine for threonine (Pro or Lys 127 Thr). The second mutation was a substitution of glycine for alanine (Gly 145 Ala). Several HBV mutants with amino acid changes in the "a" determinant have been reported in the post-transplant situation[16,17]. Among those mutations, the particular and almost invariably change is Gly 145 Arg, which can cause persistent infections[18]. Our patient also presented this change at position 145. However, the sequence analysis revealed the presence of an Ala instead of an Arg. This amino acid change has never been previously found in the literature. The change of Pro 127 Thr has been described in liver-transplanted patients [16].

A total of 18 biopsies taken at different post-LT time points from 14 recipients were obtained for the analysis of HBV-DNA in liver tissues. Open circular HBV-DNA (RC) was found in 2/14 patients (Table 1) and no cccDNA was detected in any of them. These results were reproducibly obtained in 3 repeated sets of PCR experiments. The anti-HBc negative patient with HBV-DNA in tissue (Table 1) had no detectable HBV-DNA and HBsAg in serum throughout a follow-up period of 66 mo. The anti-HBc positive recipient with viral DNA in liver (Table 1) was found to be serum HBV-DNA positive in one of his routine virological tests but this viral marker was continuously negative in the following tests. HBsAg was all negative during the whole follow-up period (66 months). No recurrent HBV infection could be considered in this patient.

DISCUSSION

Accumulating evidence suggests that HBV can be transmitted to the organ recipients from anti-HBc-positive donors through LT[19,20]. HBV transmission fluctuates among different studies between 50 % and 90 %. In our study only one patient receiving an anti-HBc positive liver developed *de novo* HBV infection as a consequence of an immune escape HBV mutant associated with the "a" determinant while in the absence of HBV mutants, none of the recipients developed HBV infection, suggesting that the presence of anti-HBs during LT, as a consequence of HBV vaccination or past HBV infection together with

anti-HBc, can protect against HBV infection transmitted by anti-HBc positive liver grafts.

Although less than 50% of the patients with cirrhosis due to HBV infection respond to HBV vaccine[21], a large number of patients can be considered as liver transplant recipients.

Anti-HBs levels >10 IU/mL due to HBV vaccine are considered protective in immunocompetent patients[22]. We consider this anti-HBs titer protective in those vaccinated patients[21].

We were aware of the short-term persistence of anti-HBs in these patients. Only 5 of them still maintained anti-HBs titers over the follow-up period while the rest of the patients lost this marker (Table 1). Since anti-HBs response is reduced in immunosuppressed patients[21,23], it is accepted that this rapid drop of anti-HBs levels is the consequence of their immunosuppressive therapy. Importantly, these results suggest that even with loss of anti-HBs during the post-LT period, HBV vaccination is effective as prophylaxis for the prevention of HBV infection in LT, which is in agreement with previous studies[24]. Another interesting finding that supports the efficacy of HBV vaccine against HBV infection is the continuous negativity for serum HBV DNA after LT during the follow-up period.

It is known that viral genome can persist in hepatocytes as a rcDNA molecule and as a cccDNA molecule, the later is required for viral replication[9]. Detection of intrahepatic cccDNA may indicate the possible ongoing viral replication[25]. Thus, its presence could explain the reactivation of HBV replication in patients receiving a liver from donors with anti-HBc. The only viral form detected in 2 recipients (1 anti-HBc negative, 1 anti-HBc positive) was rcDNA. The anti-HBc negative patient who had no history of previous HBV infection received HBV vaccine prior to LT with anti-HBs titers >10 IU/L. It is interesting to know that this patient, even maintaining HBV DNA in liver after one year of LT, did not show any virological evidence of *de novo* HBV infection during the follow-up period of 66 months (Table1). Moreover, he lost anti-HBs titers. The histopathological study at this time showed stage III fibrosis, confirming recurrence of HCV infection. The data may suggest that HBV graft infection may be infrequent. Likewise, in the anti-HBc positive recipient with viral DNA in liver tissue 23 months after LT (Table 1) and after 66 months of follow-up, no recurrent HBV infection occurred although he had positive serum HBV-DNA in one of his routine virological tests, suggesting that spontaneous seroconversion occurs in him.

However, even if the presence of anti-HBs in liver recipients seems to prevent recurrent or *de novo* HBV infection, the latest risk can still occur as seen in one of the recipients. Nevertheless, in this recipient HBV infection was not prevented by anti-HBs response because the cause was a circulating HBV mutant. The presence of circulating surface antigen-mutated HBV was proved when mutations in the "a" determinant region of the surface antigen were identified in this patient. In our study, the prevalence of HBV surface antibody escape mutants after liver transplantation was 6.6%, which is consistent with other studies[16, 26].

Our results are in agreement with an earlier study[27]. However, other reports have provided clear evidence that HBV genome is detectable in most anti-HBc positive donors[28]. The validity of our amplification method was confirmed by our control experiments using negative and positive controls. One possibility could be that DNA molecules isolated from preserved paraffin-embedded liver tissues are generally of poor quality because of the high degree of DNA degradation in these samples. However, HBV detection may be reduced beyond detectable levels but focal distribution of HBV infection cannot be excluded.

In conclusion, the presence of anti-HBs in liver recipients at the time of LT can prevent HBV recurrence or *de novo* HBV infection. Although we have described a new vaccine HBV mutant in a liver transplant recipient causing *de novo* HBV infection, the efficacy of HBV vaccine in organ recipients could not be considered as universal due to the development of immune escape HBV mutants associated with the "a" determinant which can evade the anti-HBs protection. Administration of HBV vaccine is mandatory in patients with chronic liver pathology potentially needing liver transplantation. Although these results are promising, the limited patient number may lead to an erroneous interpretation of the data. Most extensive studies including a large number of recipients need to be done.

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