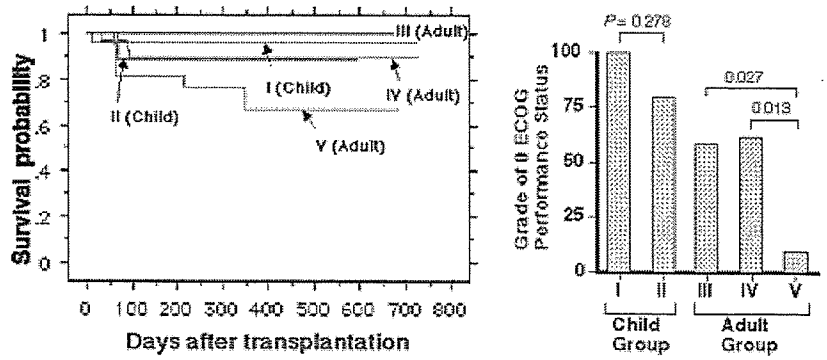

Figure 3. Kaplan-Meier curves showing recipient survival probability in the 5 (Groups I and II in child; Groups III, IV, and V in adult) groups (left). Percentage of grade 0 Eastern Cooperative Oncology Group performance status at 1 yr after LDLT (right).

TABLE 4. Comparison of Frequencies of Rejection and Infection in 5 Groups

Group	*n*	Rejection (%)	*P**	Infection (%)	*P**
Child					
I	24	58.3	—	54.2	—
II	9	66.7	1.000 (vs. group I)	44.4	0.708 (vs. group I)
Adult					
III	26	30.8	—	30.8	—
IV	30	23.3	0.561 (vs. group III)	46.7	0.279 (vs. group III)
V	23	30.4	1.000 (vs. group III)	69.6	0.010 (vs. group III)

*P-values are based on Fisher's exact test.

cytotoxic activity. In Groups IV and V, in contrast, $CD8^+$ T cells with very low proportions of naive T cells already had high cytotoxic activity prior to LDLT. The greater the $CD8^+$ CTL activity prior to LDLT, the smaller the capacity to generate CTLs for new invasion of bacteria and virus after LDLT (data not shown). Accordingly, the capacity to generate CTLs for infection after LDLT decreases progressively from Group I to Group V recipients, indicating progressive reduction in the latent ability to generate CTLs for clearance of new antigen. As a result, frequencies of postoperative complications are highest in Group V.

Current immunosuppressive induction protocols involve calcineurin inhibitors (cyclosporin, tacrolimus), corticosteroids, mono- and polyclonal antibodies, azathioprine, and mycophenolate mofetil. Use of steroids significantly increased the level of viremia in HCV-positive patients. Gane et al.[35] showed clearly that steroid pulse therapy is associated with a 4- to 100-fold increase in HCV-ribonucleic acid levels and subsequent development of acute hepatitis. In the present study, a Group IV recipient developed graft failure following repeated injection with steroid. On the other hand, in Groups IV and V, various immunosuppressors such as tacrolimus, cyclosporin, and others did not reduce preexisting CTL levels prior to transplantation. These levels had been reached during a lifetime of antigen exposure; there was also reduced thymopoiesis, characteristic of advancing age. In this regard, various conventional immunosuppressive agents remain limited in their ability to reduce preexisting CTLs. Drastic lymphocyte-depleting agents, such as rabbit anti-thymocyte globulin and anti-interleukin-2 antibodies, are able to induce cell regeneration by homeostasis-driven proliferation of T cells, and consequently provide the conditions involved in lymphocyte repopulation, favoring phenotypes with a low CTL activity with high naive T cells. Use of a preoperative shot pulse of antibody followed by low-dose immunosuppressive maintenance monotherapy may be reasonable compromise for a given transplant patient, based on preoperative high CTL activity.[36,37] However, we routinely perform anti-infective prophylaxis with less immunosuppression in Group V. It is very difficult to adjust the dose of immunosuppressive drugs specific for each recipient during infection.

Although this study had a short posttransplantation follow-up, the effect of the immunological status of T cells on the outcome has been definitively settled. Longer follow-up of larger cohorts is needed to decide whether the impairment of innate and adaptive immunoresponses by various dangerous factors after LDLTs has a significant adverse effect on long-term graft and patient survival.

ACKNOWLEDGMENTS

We thank Yasumichi Tamura, Seiji Kanazawa, and Shinsaku Yamashita of Shionogi & Co. for kindly transporting blood, and the members of the Department of Liver Transplantation and Immunology of Kyoto University Medical School for their assistance in this work.

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American Journal of Transplantation 2006; 6: 2680–2685
Blackwell Munksgaard

doi: 10.1111/j.1600-6143.2006.01542.x

Beneficial Effects of Short-Term Lamivudine Treatment for *de novo* Hepatitis B Virus Reactivation After Liver Transplantation

M. Umeda[a], H. Marusawa[a], M. Ueda[b], Y. Takada[b], H. Egawa[b], S. Uemoto[b] and T. Chiba[a,*]

[a] *Department of Gastroenterology and Hepatology and* [b] *Department of Transplantation Immunology, Graduate School of Medicine, Kyoto University, Kyoto, Japan*
** Corresponding author: Tsutomu Chiba, chiba@kuhp.kyoto-u.ac.jp*

Clearance of hepatitis B surface antigen (HBsAg) by lamivudine is achieved in only a small proportion of patients with chronic hepatitis B virus (HBV) infection. We investigated the effect of lamivudine on *de novo* HBV reactivation after living-donor liver transplantation when the number of HBV was expected to be very small. Thirty-eight HBV-naive recipients who received liver grafts from antibodies to core antigen-positive donors receiving hepatitis B immunoglobulin (HBIG) were studied. HBsAg appeared in nine cases (23.7 %) despite receiving HBIG for 12–71 months (mean: 35.1 months) after transplantation. Lamivudine treatment was started in six recipients during the acute phase of HBV reactivation. Five of the six recipients achieved complete clearance of HBsAg in sera at a median of 4.6 months (ranging from 21 to 330 days) after lamivudine administration. Although lamivudine was stopped in four cases, all remained negative for HBsAg. Our findings suggested that short-term lamivudine treatment during acute phase of HBV reactivation could achieve complete clearance of HBsAg in a significant number of liver transplant recipients.

Key words: Anti-HBc, hepatitis B, hepatitis B immunoglobulin, lamivudine, liver transplantation

Received 11 May 2006, revised 3 July 2006 and accepted for publication 1 August 2006

Introduction

There is growing recognition that the majority of healthy individuals who are negative for hepatitis B surface antigen (HBsAg) but positive for antibodies to core antigen (anti-HBc), who had once been assumed to denote previous exposure to hepatitis B virus (HBV), have persistent viral infection of the liver tissues (1,2). Recently, we demonstrated that latent HBV infection is accompanied by ongoing viral replication in the livers, but not in the sera, of healthy anti-HBc-positive liver transplant donors (3). In support of the concept of occult HBV infection, HBV was transmitted from anti-HBc-positive donors to HBV-naive recipients at a high frequency via liver grafts in living-donor liver transplantation (LDLT) and orthotopic liver transplantation (4–8). Because of the persistent shortage of organs and the increasing number of patients awaiting transplantation, the use of liver grafts from anti-HBc-positive donors cannot be avoided, especially in areas where the prevalence of HBV is high (4). Thus, adequate prophylaxis against HBV is required to prevent viral reactivation in HBV-naive recipients after liver transplantation.

To date, strategies to prevent viral breakthroughs in the recipients of anti-HBc-positive livers have been empirical, and hepatitis B immunoglobulin (HBIG) has been widely used as the standard prophylaxis after liver transplantation (9). Several reports, including ours, suggested that HBIG prophylaxis was effective for preventing HBV exacerbation in recipients who received hepatic allografts from anti-HBc-positive donors (4,10). However, long-term passive immunization with HBIG is associated with problems, such as high cost, limited availability, and selection of viral strains containing mutations in the surface gene of HBV-DNA (9). Moreover, the difficulty of maintaining serum antibodies to HBsAg (anti-HBs) titer and the poor compliance of HBIG could result in viral reactivation after liver transplantation (7).

The aim of this study was to evaluate the efficacy of short-term administration of lamivudine for the treatment of *de novo* HBV exacerbation in transplant recipients with anti-HBc-positive donors.

Patients and Methods

Patients

From July 1995 to July 2004, 902 patients underwent LDLT at Kyoto University Hospital. Before operations, serological evaluation for HBV markers, including HBsAg, anti-HBs, anti-HBc, hepatitis B e antigen (HBeAg), and antibodies to HBeAg (anti-HBe), was carried out using commercial enzyme immunoassay kits (Dainabot, Tokyo, Japan). HBV-DNA was analyzed using a commercial polymerase chain reaction (PCR) assay (Amplicor HBV Monitor, Roche, Branchburg, NJ, USA). Among 902 donors, 121 (13.4%) were positive for anti-HBc in the absence of HBsAg. Of these, all recipients fulfilling both of the following criteria were included: (a) none of the HBV-related

serological markers positive before transplantation; (b) post-operative survival and well-tolerated prophylaxis with HBIG for longer than 6 months after LDLT. Accordingly, 55 recipients were excluded because they were positive for HBV-serological markers before LDLT. Twenty-six patients were also excluded from the study because of their short duration of survival, and all of these patients died from causes not related to the HBV reactivation. Two patients refused to receive HBIG for financial reasons, and were lost to follow-up. A total of 38 recipients were considered eligible for this study. These HBV-naive recipients with anti-HBc-positive donors underwent LDLT for the following liver diseases: biliary atresia (n = 21), hepatitis C virus (HCV)-related chronic liver disease (n = 3), primary biliary cirrhosis (n = 3), primary sclerosing cholangitis (n = 2), chronic rejection (n = 2), post-LDLT graft failure (n = 2) and others (n = 5). The male/female ratio was 1/1 and the age range was 0–58 years (mean age: 15.9 years, age ≤18 years: n = 28, age >18 years: n = 10). HBV reactivation after LDLT was diagnosed by confirming the appearance of HBsAg in sera of the recipients. Liver tissue and serum samples of all anti-HBc-positive donors were obtained at the time of operation and subjected to analysis for HBV-DNA. All subjects provided written informed consent, and the study was conducted in accordance with the principles of the Declaration of Helsinki.

Prophylaxis with HBIG and immunosuppressive protocol

HBIG monotherapy was given to all the recipients with grafts from anti-HBc donors, as reported previously (4). The first dose of HBIG at 200 IU/kg body mass was administered during the anhepatic phase of LDLT, and the same dose was given every day during the first 6 post-operative days. Subsequently, HBV-serological markers were examined at monthly intervals after the transplant operation and 1000 IU of HBIG was periodically administered to maintain serum anti-HBs titers at more than 200 IU/L throughout the follow-up period.

Immunosuppressive therapy for all recipients consisted of tacrolimus and low-dose steroids. Target trough levels of tacrolimus in whole blood were 10 to 15 ng/mL in the first week, and then 5 to 10 ng/mL during the first month after transplantation. Methylprednisolone (10 mg/kg) was administered intravenously (IV) during the anhepatic phase of surgery, followed by 2 mg/kg administered IV for the first 3 days, then tapered to 1 mg/kg for 3 days and converted to 0.3 mg/kg/day of prednisone, which was decreased gradually and discontinued between 3 and 6 months.

PCR amplification of HBV-DNA and sequencing of the surface gene

Preparation of DNA samples and detection of HBV genomes by nested PCR have been described previously (3). The nucleotide sequence spanning the S region was amplified by PCR using specific primer sets, followed by subcloning of PCR products using a pGEM-T Easy Vector System I (Promega, Madison, WI, USA). A total of 15 clones derived from each serum specimen were subjected to sequencing analyses (3).

Results

HBV reactivation despite HBIG prophylaxis

Post-operative HBIG prophylaxis was given to 38 HBV-naive recipients with grafts from anti-HBc-positive donors. Among them, 29 showed no evidence of HBV recurrence during the follow-up period (mean: 41.1 months, range: 10 months to 9.5 years). Unfortunately, in 9 of 38 cases (23.7 %), anti-HBs titer decreased concurrently with the appearance of HBsAg in the serum despite HBIG prophylaxis after LDLT. Table 1 shows the serological characteristics of the donors and pre-transplant status of the recipients who suffered from HBV reactivation despite HBIG administration. Baseline characteristics including age, gender and HBV-related serology were similar between these nine recipients and the remaining recipients without HBV recurrence (data not shown). Consistent with our previous analyses, 31 of the 38 donors with anti-HBc (81.6%) were positive for HBV-DNA in the liver specimens, indicating a high frequency of latent HBV infection in the livers of anti-HBc-positive individuals (3). In contrast, HBV-DNA was negative in the sera of all anti-HBc-positive donors. All allografts of nine recipients with HBsAg appearance were positive for HBV-DNA by PCR analyses, suggesting that the *de novo* HBV reactivation originated from the liver graft with latent HBV infection after LDLT.

To define the factors associated with HBV reactivation in these nine recipients, variables related to the donors and recipients, transplant procedures, and HBIG prophylaxis were analyzed. The indications for LDLT in these recipients with HBV reactivation were biliary atresia (n = 7), Wilson's disease (n = 1) and primary sclerosing cholangitis (n = 1). The mean period between LDLT and *de novo* HBsAg appearance was 35.1 months (range: 12–71 months; Table 2).

Table 1: HBV-serological status of recipients with HBV reactivation post-LDLT and of their corresponding donors with anti-HBc

Recipient				Donor			
Case #	Age/Sex	Indication for LDLT	HBsAg/ Anti-HBs	Anti-HBc	HBsAg/ Anti-HBs	HBeAg/ Anti-HBe	HBV-DNA in liver graft
1	13/F	BA	−/−	+	−/+	−/+	+
2	3/M	BA	−/−	+	−/+	−/−	+
3	9/M	BA	−/−	+	−/+	−/+	+
4	22/F	BA	−/−	+	−/+	−/−	+
5	0/M	BA	−/−	+	−/+	−/+	+
6	16/F	BA	−/−	+	−/+	−/+	+
7	16/M	Wilson	−/−	+	−/−	−/+	+
8	23/F	BA	−/−	+	−/−	−/−	+
9	25/M	PSC	−/−	+	−/−	−/+	+

LDLT = living-donor liver transplantation; HBsAg = hepatitis B surface antigen; anti-HBs = antibody to HBsAg; anti-HBc = antibody to hepatitis B core antigen; HBeAg = hepatitis B e antigen; anti-HBe = antibody to HBeAg; BA = biliary atresia; PSC = primary sclerosing cholangitis.

Table 2: Clinical features of recipients with HBV reactivation

Case #	Anti-HBs titer[1] (mIU/mL)	Duration until HBV reactivation[2] (months)	Clinical features at the time of HBV reactivation		Possible reasons for HBV reactivation
			ALT (IU/L)	Histology	
1	N.D.	21	251	N.D.	Noncompliance
2	N.D.	32	190	CAH	Noncompliance
3	N.D.	16	13	N.D.	Noncompliance
4	23.8	71	699	N.D.	Immunosuppression
5	140.6	15	24	N.D.	Escape mutant
6	117	30	153	CAH	Escape mutant
7	11.7	12	1409	CAH	Unknown
8	N.D.	61	25	CAH	Unknown
9	34.7	58	65	CAH	Unknown

anti-HBs = antibody to HBsAg; ALT = alanine aminotransferase; N.D. = not determined; CAH = chronic active hepatitis; noncompliance = noncompliance of HBIG; escape mutant = emergence of surface escape mutant.
[1]Anti-HBs titer before HBsAg appearance.
[2]Period between liver transplantation and HBsAg appearance.

A liver biopsy was performed on five of the nine patients at the time of the *de novo* HBV recurrence, and all exhibited evidence of chronic active hepatitis accompanied by mild inflammatory activity and mild fibrosis. HBV appearance was attributed to the decrease in serum anti-HBs titer despite HBIG prophylaxis in four of nine recipients. Among them, three recipients (cases #1, #2 and #3) were considered to have suffered from HBV recurrence because of non-compliance of HBIG. Although post-operative HBIG prophylaxis was given to these three patients, they had a transient cessation of HBIG treatment for personal reasons 12, 7 and 11 months after LDLT. They experienced a decrease in anti-HBs titer and, consequently, HBsAg became detectable in the sera after cessation of HBIG treatment. The mean period between the cessation of HBIG treatment and the emergence of HBsAg in their sera was 12.3 months (range, 3–26 months). An immunosuppressive condition was presumed to be associated with viral activation, with consequent decreases of anti-HBs in one case (case #4). Recipient #4 showed an anti-HBs titer of less than 23.8 IU/L 2 weeks after the 2000 IU-HBIG infusion, followed by the appearance of HBsAg. Continuous medication with prednisolone for the treatment of chronic rejection suggested the underlying possible immunocompromised condition in this case.

In the remaining five cases (cases #5, #6, #7, #8 and #9), HBsAg eventually became positive despite the continuous treatment with periodical HBIG prophylaxis. HBV clones comprising mutations in the S gene have been reported in OLT recipients who developed recurrent hepatitis B despite HBIG prophylaxis (11). To ascertain whether the HBIG failure in these cases was associated with changes in antigenicity of the S protein, the S gene sequence of HBV-DNA was determined in the HBV strain of two patients whose sera at the acute phase of HBV exacerbation were available for further analyses (cases #5 and #6). The sequence analyses of the two cases revealed that the detected HBV clone contained several mutations, including G- to -A substitutions at nucleotide 586 (subtype adr) and 587 (subtype adw) within the 'a' determinant region on the HBsAg-encoding gene, suggesting that the cloned HBV variants might be responsible for HBV recurrence despite HBIG administration in these two cases. Unfortunately, we could not determine the factors that were related to HBV reactivation in cases #7, #8 and #9.

Short-term lamivudine treatment

Among recipients with *de novo* HBV recurrence, six (cases #1, #2, #4, #6, #7 and #9) of nine cases had elevated levels of serum alanine aminotransferase, suggesting that recurrent active hepatitis was present. Moreover, of these six cases, all four cases that were examined exhibited histological evidence of inflammation with lymphocytes infiltration around the portal area at the time of *de novo* HBV reactivation (Table 2). Lamivudine (100 mg) was given in six cases to suppress the viral activity (Figure 1). Of them, five patients started the treatment immediately after HBsAg appearance (average: 27 days; range: 1 day to 2 months). One patient (case #1) did not take lamivudine at the time of HBsAg appearance because of personal reasons, but finally received lamivudine therapy 23 months after HBV reactivation.

After the administration of lamivudine, HBsAg decreased in the sera of five out of the six recipients, and in all these five cases (cases #1, #2, #4, #7 and #9), HBsAg disappeared from the sera at a median of 4.6 months (range: 21–330 days) after the beginning of lamivudine treatment. Suppression of HBsAg by lamivudine treatment was invariably associated with a decline in serum transaminase levels in these five cases. After confirming the stable seroconversion to anti-HBs-positive status, lamivudine treatment was stopped in four of the five recipients after 60 months, 10 months, 1.5 months and 4 months (cases #1, #2, #7 and #9). The remaining individual (case #4) is currently receiving lamivudine treatment because she started treatment only 4 months ago; her most recent blood test

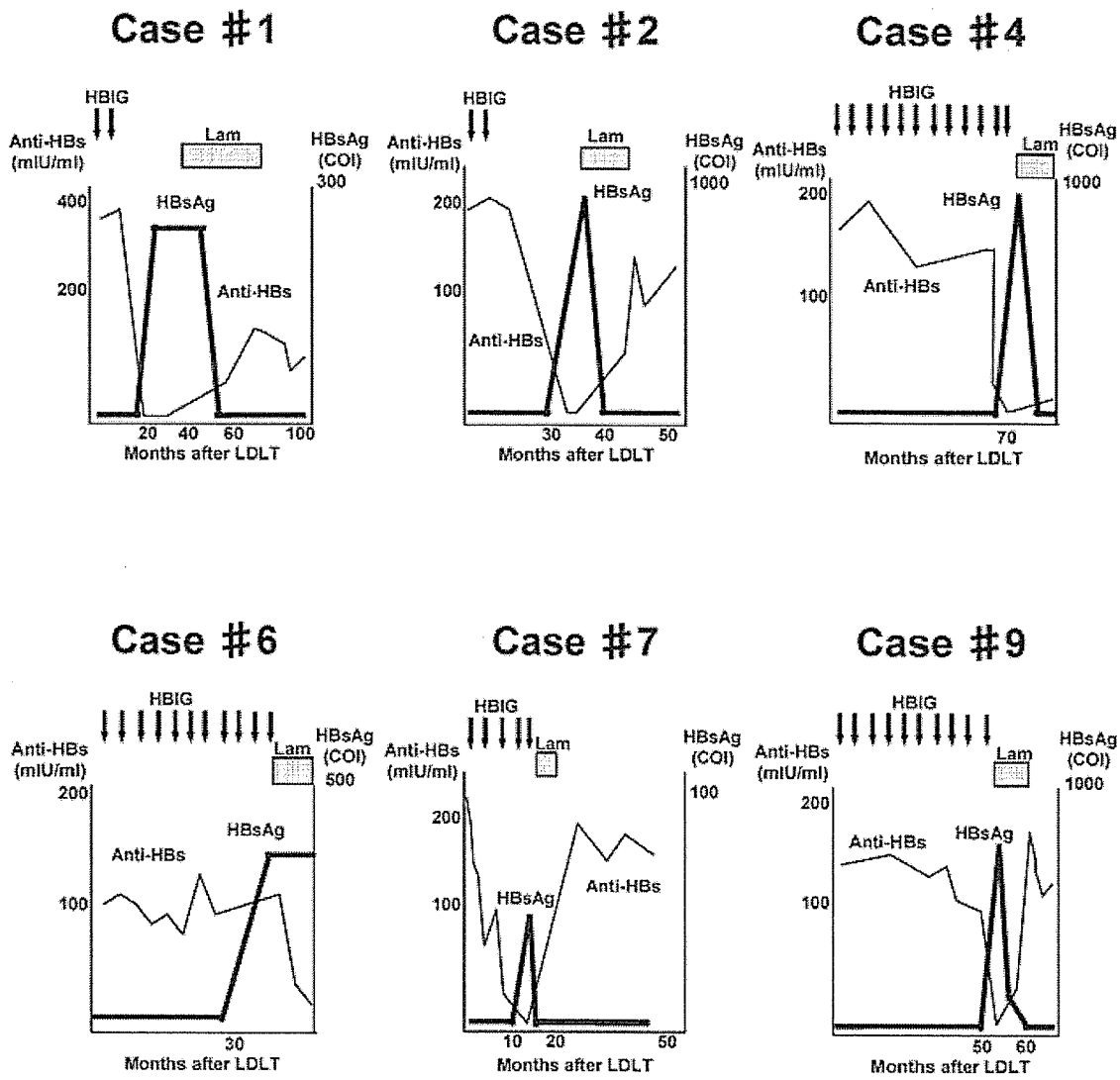

Figure 1: Clinical course of the six liver transplant recipients with HBV reactivation who received lamivudine treatment. The bold line represents HBsAg and the fine line represents anti-HBs. The treatment with HBIG is shown as arrows and treatment with lamivudine as shaded boxes. HBIG = hepatitis B immunoglobulin; Lam = lamivudine.

was negative for HBsAg. The presence of circulating exogenous anti-HBs derived from HBIG complicates detection of the endogenous anti-HBs, which reflect the development of anti-HBV immunity in recipients. Thus, we also gradually withdrew the HBIG prophylaxis after the administration of lamivudine, and confirmed the sustained positivity of anti-HBs without HBIG treatment in two recipients (cases #2 and #7). Since then, they consistently showed evidence of immunity against HBV with endogenous anti-HBs titers greater than 100 IU/L without any prophylaxis. Consequently, HBIG was not given to these two recipients, even after termination of lamivudine treatment. Serum HBsAg of these two recipients remained negative during the follow-up periods of 14 and 35 months after the withdrawal of HBIG and lamivudine. Recipient #7 died of progressive deterioration of liver function 35 months after the complete loss of HBsAg in sera, and the histological autopsy findings were consistent with chronic rejection in the liver graft. The remaining recipient (case #6) is currently being treated with lamivudine monotherapy. None of the recipients developed tyrosine-methionine-aspartate-aspartate (YMDD) mutants during the course of lamivudine therapy (data not shown).

Three patients (recipients #3, #5 and #8) did not receive lamivudine treatment after HBsAg appearance because the alanine aminotransferase levels of these patients showed no evidence of active hepatitis.

Discussion

In this study, we demonstrated that short-term lamivudine therapy for LDLT recipients with *de novo* HBV reactivation

could achieve a significant HBsAg seroconversion rate without inducing drug-resistant YMDD mutants. The important point to note is that after the transient use of lamivudine, some of the recipients who had been naive against HBV infection before transplantation maintained anti-HBs positivity despite withdrawal of HBIG even during immunosuppressive therapy, indicating that they were likely to acquire endogenous immunity to HBV infection.

Passive immunoprophylaxis with HBIG has been used to prevent *de novo* HBV recurrence after liver transplantation in HBV-naive recipients who received liver grafts from anti-HBc-positive donors (8). However, not only the efficacy, but also the safety of long-term HBIG treatment after liver transplantation has not been determined, including the risk of development of hepatitis B 'surface' escape mutants during passive immunoprophylaxis. In this study, we demonstrated that a considerable number of patients developed *de novo* HBV reactivation during the long-term course of HBIG prophylaxis. Our findings suggested that factors affecting the recurrence of *de novo* HBV in those recipients included non-compliance of HBIG, an immunosuppressive condition, and possible emergence of 'surface' escape mutants.

Lamivudine is a potent inhibitor of HBV replication, and several investigators have reported that lamivudine therapy is effective for hepatitis B treatment following liver transplantation (12,13). The present data show that early initiation of lamivudine treatment for post-transplant HBV *de novo* reactivation efficiently induced a sustained loss of HBsAg, suggesting that the effect of lamivudine on *de novo* HBV reactivation is stronger than its effect on chronic HBV infection. In general, HBV replication decreases immediately after administration of lamivudine; however, it has been shown that lamivudine treatment achieves clearance of HBsAg in only a very small proportion of cases. Indeed, loss of HBsAg occurred in only 0–2% of patients with chronic HBV infection that were treated with lamivudine (14). In transplant recipients with HBV-associated liver disease, lamivudine treatment induced the disappearance of HBsAg in only 3 of 52 cases (6%) by week 52, 2 of whom acquired anti-HBs (12). The better results observed in this study can be attributed to the fact that lamivudine treatment was commenced during the acute phase of *de novo* HBV reactivation when the number of HBV was expected to be very small. The beneficial effect of early lamivudine therapy for acute hepatitis has been reported previously (15,16). We suggest that the timing of the lamivudine administration in patients with HBV activation, specifically in the acute phase of HBV reactivation, is important to achieve complete viral suppression and successful seroconversion from HBsAg to anti-HBs.

It is well recognized that prolonged administration of lamivudine can lead to viral breakthrough because of the emergence of viral variants with reduced sensitivity to the drug resulting from one or more mutations in the YMDD locus of the HBV polymerase gene (17). Many investigators have reported a high rate of virological breakthrough with prolonged lamivudine therapy in liver transplant recipients with active HBV infection (17,18). In contrast, we confirmed the beneficial effect of lamivudine on *de novo* HBV reactivation and showed that it does not cause the emergence of YMDD mutants. Reduced risk for virological breakthrough during lamivudine therapy may be attributed to the short-term use of the drug and the low levels of HBV-DNA at the acute phase of *de novo* HBV reactivation, as prolonged use of lamivudine and high HBV-DNA levels before treatment were shown to be associated with the emergence of the drug-resistant mutants (19). Prevention of drug-resistant viral clones by transient use of lamivudine has also been demonstrated in patients with chronic HBV infection (20). Although short-term lamivudine therapy could reduce the incidence of YMDD mutants, the relapse rate was high after the withdrawal of lamivudine in HBV-related chronic liver disease patients (20). Thus, short-term lamivudine treatment might be applicable specifically to the acute phase of *de novo* HBV reactivation.

In conclusion, we have shown in this study that short-term use of lamivudine resulted in complete clearance of HBsAg in the majority of patients with *de novo* HBV reactivation, and that the effects of lamivudine were stronger in patients with *de novo* HBV reactivation than in patients with HBV-related chronic liver disease. More importantly, sustained clearance of HBV was obtained in two cases who stopped both HBIG and lamivudine administration, suggesting acquired immunity against HBV; however, a study of a large number of patients with *de novo* HBV reactivation is needed to reach any definitive conclusion. Thus, further studies with greater patient numbers are required to determine whether short-term lamivudine administration induces clearance of HBsAg in the sera, as well as acquired immunity against HBV in HBV-naive recipients receiving anti-HBc-positive allografts.

Acknowledgments

This study was supported by a grant-in-aid for scientific research (15209024 and 16790378) from the Japan Society for the Promotion of Science (JSPS).

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American Journal of Transplantation 2006; 6: 2812–2816
Blackwell Munksgaard

doi: 10.1111/j.1600-6143.2006.01531.x

Case Report

Liver Transplantation from an Identical Twin without Immunosuppression, with Early Recurrence of Hepatitis C

A. Yoshizawa[a], Y. Takada[a,*], Y. Fujimoto[a], T. Koshiba[b], H. Haga[c], S. Nabeshima[d] and S. Uemoto[a]

[a] *Department of Surgery, Graduate School of Medicine,*
[b] *Horizontal Medical Research Organization, Faculty of Medicine*
[c] *Department of Pathology and*
[d] *Department of Gastroenterology and Hepatology, Graduate School of Medicine, Kyoto University, Kyoto, Japan*
** Corresponding author: Yasutsugu Takada, takaday@kuhp.kyoto-u.ac.jp*

Hepatitis C virus reinfection after liver transplantation is universal and more severe than in nontransplant patients. Rejection episodes and immunosuppressive agents are considered risk factors for deterioration of recurrent hepatitis C. We report 2 cases of living donor liver transplantation for patients with hepatitis C-related cirrhosis who received right-lobe grafts from an identical twin. Thanks to genetic identity, no immunosuppressive drugs were administered during or after transplantation without rejection. Hepatitis C virus RNA kinetics showed a rapid increase following transplantation and liver biopsies 1 month after transplantation showed acute lobular hepatitis in both cases. Antiviral therapy using interferon α and ribavirin was started immediately, and both cases showed virological and histological response. In conclusion, avoidance of immunosuppression did not delay hepatitis C recurrence following transplantation, while early antiviral therapy without risk of rejection or immunosuppression led to successful viral eradication.

Key words: Hepatitis C, immunosuppression, liver transplantation, living-related liver donors, reinfection

Received 1 May 2006, revised 14 July 2006 and accepted for publication 17 July 2006

Introduction

The first successful kidney transplantation between identical twins was performed by Murray in 1954 (1). Solid-organ transplantation between identical twins has been successfully performed with the small intestine (2), kidney, pancreas (3), combined pancreas and kidney (4), parathyroid gland (5) and liver (6,7). This historical success has proved that for solid-organ transplantation from a syngeneic donor, allografts can be transplanted without immunosuppressive therapy. We performed living donor liver transplantation (LDLT) for two patients with hepatitis C virus (HCV)-related cirrhosis, using a right-lobe graft from an identical twin in each case without any immunosuppression.

HCV-related disease is one of the leading indications for liver transplantation worldwide. However, reinfection with HCV is immediate and universal following surgery, and jeopardizes both graft and patient survival (8). The spectrum of allograft injuries related to HCV recurrence ranges from no evidence of injury to graft failure requiring retransplantation in a subset of patients. Several factors have been proposed to explain these variable outcomes, including HCV viral load, genotype, rejection episodes and immunosuppression (9). Immunosuppression accounts for a major part of the accelerated progression. Steroid pulse therapy for rejection reportedly aggravates recurrent HCV (10). Furthermore, intraoperative bolus steroid injection has been shown to increase serum HCV RNA immediately after transplantation (11).

Liver transplantation for HCV is analogous to acute HCV infection, in that a never-infected liver is placed into a viremic host and inevitably becomes infected. Human liver transplantation thus offers a unique opportunity to study viral kinetics and the immunopathogenic mechanisms of acute HCV hepatitis. In the present cases of LDLT between identical twins, the influences of rejection and immunosuppression can also be excluded. We report herein the kinetics of serum HCV RNA levels, clinical course with antiviral therapy and pathological features for acute hepatitis C after LDLT free from both alloimmune response and immunosuppressive therapy.

Case 1

The recipient was a 51-year-old man diagnosed with HCV cirrhosis and multiple hepatocellular carcinoma. Genotype was 1b and HCV RNA level was 160 kIU/mL, while Child-Pugh classification was A. LDLT was performed using a right-lobe graft (graft-to-recipient weight ratio, 1.25%) from his identical twin. No immunosuppressive agent was administered, including intraoperative bolus steroid injection.

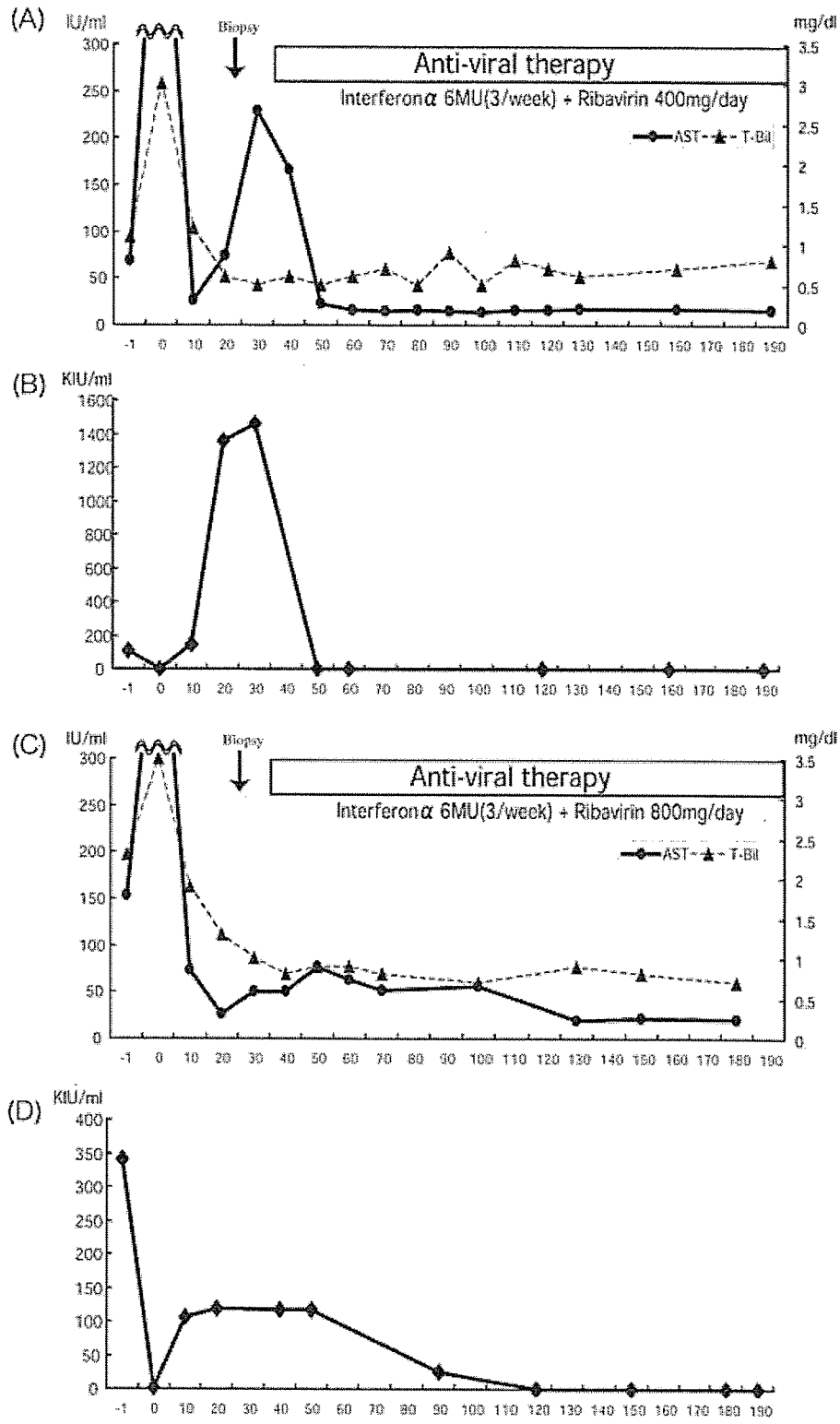

Figure 1: Results of liver function tests, serum HCV RNA level and clinical courses of Cases 1 and 2. (A) AST and total bilirubin (T-Bil) levels in Case 1. AST and T-Bil levels were elevated on POD 1 and decreased to within normal ranges by POD 10. On POD 17, AST elevated again and liver biopsy was performed on POD 24. Combined antiviral therapy was started from POD 31. (B) Kinetics of serum HCV RNA levels in Case 1. After reperfusion, HCV RNA could not be detected from serum. On POD 1, serum HCV RNA became positive and elevated continuously until peaking on POD 21 at 1600 kIU/mL. After antiviral therapy, serum RNA level decreased rapidly to be undetectable within 2 weeks. (C) AST and T-Bil levels in Case 2. AST and T-Bil elevated on POD 1 and gradually decreased. T-Bil gradually decreased to normal range after POD 20, but AST level remained abnormal until 3 months after antiviral therapy. T-Bil and AST have since remained within normal range. (D) Kinetics of serum HCV RNA level in Case 2. After reperfusion, serum HCV RNA became negative and continued until POD 7. On POD 10, serum HCV RNA level became positive and elevated to a peak of 100 kIU/mL on POD 27. After antiviral therapy started, serum HCV RNA gradually decreased and became negative on POD 120.

AST level was elevated to 1000 IU/mL on postoperative day (POD) 1, but subsequently improved before again elevating after POD 17 (Figure 1A). HCV RNA became negative after reperfusion, then turned positive on POD 1 and elevated continuously to a peak of 1600 kIU/mL on POD 21 (Figure 1B). Liver biopsy on POD 24 demonstrated the following findings: mild portal inflammation with infiltration of lymphocytes and eosinophils, but without bile duct

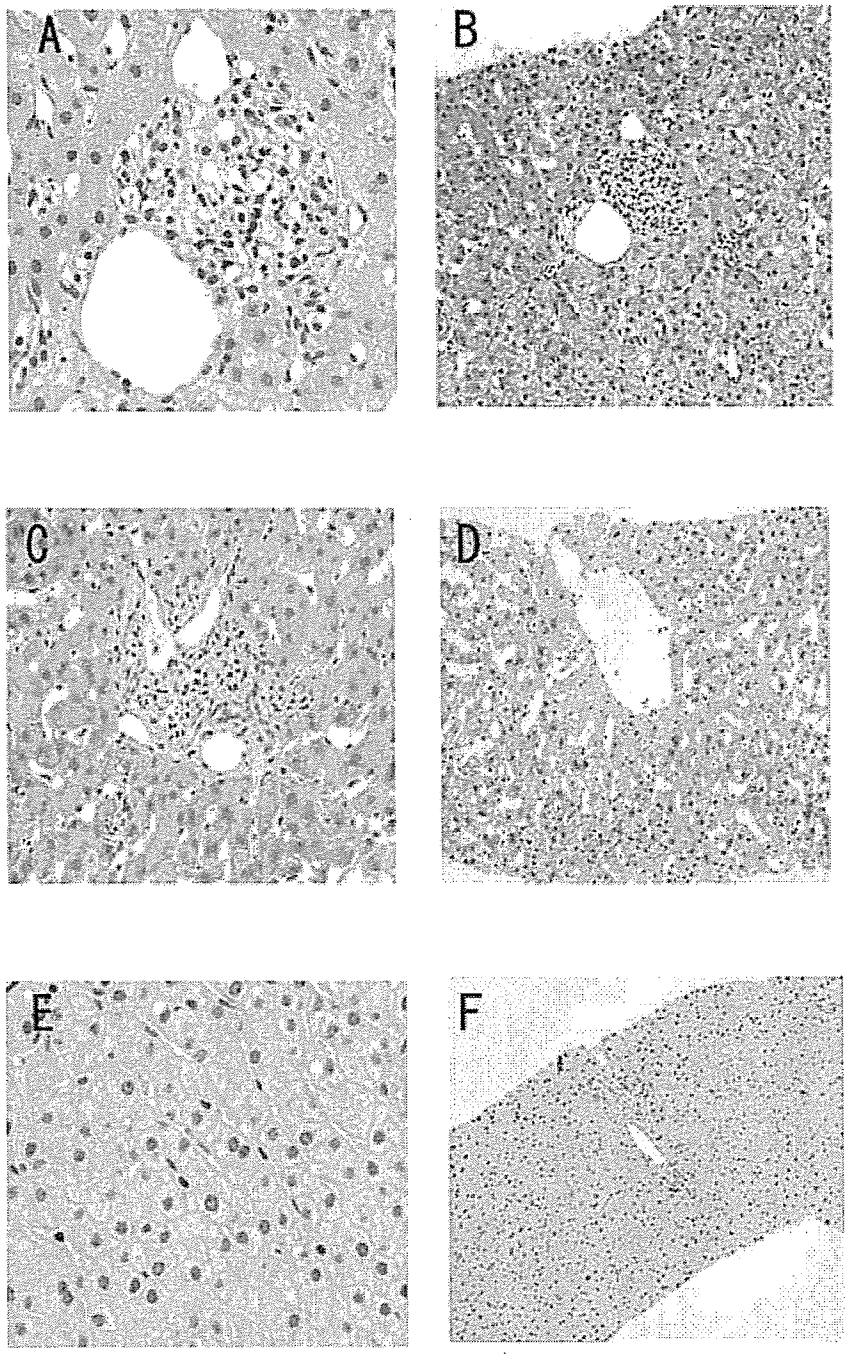

Figure 2: Histopathological findings from liver posttransplant biopsy. (A, B) Liver biopsy was obtained on POD 24 in Case 1 after LFTs elevated. (A) Mild portal inflammation with lymphocytes and eosinophils and no bile duct damage or endothelialitis. (B) Hepatocyte degeneration, steatosis and sinusoidal dilatation. (C, D) Liver biopsy was obtained on POD 27 in Case 2 after LFTs elevated. These biopsies show similar findings to Case 1. (E, F) Liver biopsy in Case 1 was obtained 7 months after transplantation, when HCV RNA was not detected in peripheral blood. These biopsies showed no signs of hepatitis.

damage or endothelialitis; lobular inflammation with acidophilic bodies; hepatocyte degeneration and steatosis and sinusoidal dilatation (Figure 2A, B). With a histological diagnosis of lobular hepatitis (A1, F0, according to METAVIR score (12)), antiviral therapy was started using interferon α-2b (6 MU 3 times/week) and ribavirin (400 mg/day) from POD 31. Ribavirin dose was reduced because the patient was anemic (Hb, 9.4 g/dL). HCV RNA turned negative within 2 weeks after starting treatment. Treatment was continued for 48 weeks, and sustained virological response has been maintained for >1 year after the cessation of therapy with good liver function. Liver biopsy results as of 8 months after LDLT showed no sign of hepatitis (Figure 2E, F).

Case 2

The recipient was a 38-year-old man diagnosed with HCV cirrhosis (Child-Pugh grade B, genotype 1b, HCV-RNA 340 kIU/mL). He had been treated using interferon

monotherapy for HCV-related chronic hepatitis at 20-years old, but this was ineffective and liver disease progressed to cirrhosis. The patient underwent LDLT using a right-lobe graft (graft-to-recipient weight ratio, 1.28%) from his identical twin without any immunosuppression. Serum HCV RNA rapidly decreased to undetectable levels after reperfusion and remained negative until POD 7. On POD 10, HCV RNA became positive and elevated to a peak of 100 kIU/mL on POD 25 (Figure 1D). Liver biopsy on POD 27 showed lobular hepatitis (A1, F0) with similar findings to those in Case 1 (Figure 2C, D). Combined antiviral therapy comprising interferon α-2b (6 MU 3 times/week), and ribavirin (800 mg/day) was administered from POD 34. Biochemical and virological responses were attained. After 12 weeks of therapy, HCV RNA was 0.57 kIU/mL. By 7 months after LDLT, the patient showed normal results on liver function test (LFTs) and HCV RNA was not detectable in serum. Liver biopsy 7 months after LDLT showed no signs of hepatitis. The patient remains on this treatment.

Discussion

These cases revealed three key findings. First, increased serum HCV RNA levels and histological findings of acute lobular hepatitis accompanied by mild elevation of LFT results were observed soon after LDLT despite the absence of immunosuppression. This absence of immunosuppression was expected to delay or alleviate HCV reinfection, if immunosuppression represents one of major factors in the progression of HCV recurrence. However, recurrence of hepatitis C proved inevitable after liver transplantation for HCV-infected patients, even without immunosuppression. A possible explanation for the early reinfection and progression of acute hepatitis was the genetic identity between donor and recipient. As HCV demonstrates cell tropism (13), a grafted liver from an identical twin would be more easily infected than an allograft. However, whether sharing the same HLA loci represents a risk factor for recurrence of HCV remains controversial. Another explanation is the graft type used in these cases. Right-lobe grafts inevitably undergo regeneration immediately after transplantation, and hepatocyte proliferation promotes viral replication (14). Whether hepatitis C recurrence occurs earlier and with greater severity for LDLT than for deceased donor liver transplantation has recently become a subject of debate (15,16).

The second key finding was that in the present 2 cases, liver biopsy results 1 month after LDLT showed similar features of acute lobular hepatitis, even though viral kinetics differed. Generally, early histopathological features of recurrent hepatitis C may be modified by immunosuppressive therapy and can be difficult to differentiate from acute rejection. The presence of eosinophils in portal inflammatory infiltrate is reportedly a significant variable associated with acute rejection (17,18). In the present cases where the possibility of acute rejection could be excluded, eosinophils were seen in areas of portal inflammation in both patients. Interestingly, this suggests that eosinophil infiltration is not necessarily specific to acute rejection, but also appears in early acute hepatitis. Sinusoidal dilatation, which is reported as the only specific feature of recurrent hepatitis C (17), was identified in both cases.

The third key finding was that, though sustained virological response could not be assessed in Case 2, antiviral therapy was effective in both cases. Antiviral therapy can reportedly stimulate immune responses and may in turn increase the risk of allograft rejection (19). However, in the present cases, treatment could be started without risk of rejection as soon as recurrent hepatitis was confirmed. Early treatment of acute hepatitis C with interferon is reportedly more effective compared to that for chronic infection (20). In terms of early introduction of antiviral therapy and remaining free from immunosuppression, the rapid response of these 2 patients with genotype 1b viruses may display some analogy to acute hepatitis C in nontransplant recipients.

In conclusion, we have reported 2 cases of LDLT between identical twins for HCV cirrhosis without any immunosuppressive drugs. Despite avoidance of immunosuppression, rapid increases in serum HCV RNA levels and histological recurrence of HCV by 1 month after LDLT were observed. However, antiviral therapy for acute hepatitis yielded good responses in both cases.

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Journal of Hepatology 46 (2007) 26–36

Journal of Hepatology

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Serum-derived hepatitis C virus infectivity in interferon regulatory factor-7-suppressed human primary hepatocytes

Hussein H. Aly[1,2,3], Koichi Watashi[2], Makoto Hijikata[2], Hiroyasu Kaneko[2], Yasutugu Takada[1], Hiroto Egawa[1], Shinji Uemoto[1], Kunitada Shimotohno[2,*]

[1] *Graduate School of Medicine, Department of Transplant Surgery, Kyoto University Hospital, Kyoto, Japan*
[2] *Laboratory of Human Tumor Viruses, Institute of Virus Research, Kyoto University, Japan*
[3] *Hepatology Department, National Hepatology and Tropical Medicine Research Institute, Cairo, Egypt*

See Editorial, pages 1–5

***Background/Aims*: The development of an efficient in vitro infection system for HCV is important in order to develop new anti-HCV strategy. Only Huh7 hepatocyte cell lines were shown to be infected with JFH-1 fulminant HCV-2a strain and its chimeras. Here we aimed to establish a primary hepatocyte cell line that could be infected by HCV particles from patients' sera.**

***Methods*: We transduced primary human hepatocytes with human telomerase reverse transcriptase together with human papilloma virus 18/E6E7 (HPV18/E6E7) genes or simian virus large T gene (SV40 T) to immortalize cells. We also established the HPV18/E6E7-immortalized hepatocytes in which interferon regulatory factor-7 was inactivated. Finally we analyzed HCV infectivity in these cells.**

***Results*: Even after prolonged culture HPV18/E6E7-immortalized hepatocytes exhibited hepatocyte functions and marker expression and were more prone to HCV infection than SV40 T-immortalized hepatocytes. The susceptibility of HPV18/E6E7-immortalized hepatocytes to HCV infection was further improved, in particular, by impairing signaling through interferon regulatory factor-7.**

***Conclusions*: HPV18/E6E7-immortalized hepatocytes are useful for the analysis of HCV infection, anti-HCV innate immune response, and screening of antiviral agents with a variety of HCV strains.**

***Keywords*: Immortalization; Primary hepatocytes; HCV infection; IRF-7; IRF-3; HPV18/E6E7; Innate immune response**

1. Introduction

Infection with Hepatitis C virus (HCV) is a serious problem worldwide since 3% of the world's population is chronically infected [1]. Chronic HCV may lead to liver cirrhosis and hepatocellular carcinoma. Current standard therapy utilizes the combination of pegylated interferon-α and ribavirin, which results in a sustained response in only 30–60% of patients [2–5]. Many patients, however, do not qualify for or tolerate standard therapy [6]. Thus, it is important to develop an efficient in vitro infection system for HCV to facilitate the discovery of new anti-HCV strategies. Only Huh7 cell line is permissive for replication, infection and release of the fulminant hepatitis-derived HCV-2a (JFH-1) *strain* and its chimeric derivatives [7–9]. No other hepatocyte cell lines are able to support HCV replication efficiently.

Received 5 June 2006; received in revised form 24 July 2006; accepted 1 August 2006; available online 30 October 2006

* Corresponding author. Tel.: +81 75 751 4000; fax: +81 75 751 3998.
E-mail address: kshimoto@virus.kyoto-u.ac.jp (K. Shimotohno).

0168-8278/$32.00
doi:10.1016/j.jhep.2006.08.018

Normal human hepatocytes are the ideal system in which to study HCV infectivity. When cultured in vitro, however, they proliferate poorly and divide only a few times [10]. Continuous proliferation could be achieved however by introducing oncogenes, such as Simian virus large tumor antigen (SV40 T) [11]. This often resulted in tumor development [12] together with numerical (aneuploidy) and structural (aberrations) chromosome abnormalities [13]. The human papilloma virus E6E7 genes (HPV/E6E7) immortalized multiple cell types that were phenotypically and functionally similar to the parental cells [14–20]. As yet, no human hepatocytes have been immortalized with HPV18/E6E7.

We established a human primary non-neoplastic hepatocyte cell line transduced with the HPV18/E6E7 that retained primary hepatocyte characteristics even after prolonged culture, and were more prone to HCV infection than those cells immortalized with SV40 T antigen. We further improved the susceptibility of HPV18/E6E7-immortalized hepatocytes to HCV infectivity by impairing interferon regulatory factor-7 (IRF-7) expression. These cells are useful to assay infectivity of HCV strains other than JFH-1, HCV replication, innate immune system engagement of HCV, and screening of anti-HCV agents. This infection system using non-neoplastic cells also suggested that IRF-7 plays an important role in eliminating HCV infection.

2. Materials and methods

2.1. Cell cultures

We obtained the approval of the Ethical Committee of Kyoto University for the use of human hepatocytes and sera obtained from HCV-positive patients. Informed consent was obtained from both the hepatocyte donor and HCV-positive patients. Primary hepatocytes (P.H.) were cultured as described [21]. HeLa, 293, Huh-7.5, and PH5CH8 cells were cultured as previously described [22]. For three-dimensional (3D) cultures, Mebiol Gel (Mebiol Inc.) was prepared according to the manufacturer's instructions.

2.2. Plasmids construction

The SV40 T, hTERT and HPV/E6E7 fragments from pAct-SVT, PCX4neo/hTERT, and pLXSN-E6E7 plasmids were inserted into pCSII-EF-RFA plasmid creating the pCSII-EF-SVT, pCSII-EF-hTERT, and pCSII-EF-E6E7 plasmids, respectively. The full-length IRF-3 and IRF-7 genes were cloned by RT-PCR using total RNA isolated from 293 cells as a template and were inserted into pcDNA3 vector. Dominant-negative forms of IRF-3 (DNIRF-3) and IRF-7 (DNIRF-7) were constructed by PCR amplification of the coding region for amino acid residues 108–427 of IRF-3 and 237-514 of IRF-7, respectively. The amplified IRF-3 fragment was cloned into pcDNA3 in frame with a FLAG epitope tag generating pcFLAG-DNIRF-3. The amplified IRF-7 fragment was cloned into pLXSH in frame with HA epitope tag generating pLXSH-HA-DNIRF-7. The pIFNβ promoter-luc and pIFNα promoter-luc plasmids were gifts from Dr. Taniguchi of the Tokyo University. The psiRNA-hIRF-3 and psiRNA-hIRF-7 plasmids were purchased from Invivogen (USA).

2.3. Immunoblot analysis

Immunoblot analysis was performed as described previously [22]. We used anti-SV40 T (Santa Cruz), anti-HPV18/E7 (Santa Cruz), anti-tubulin (Sigma), anti-FLAG (Sigma), and anti-HA (Sigma) antibodies.

2.4. Transfection, small interfering RNA silencing and luciferase assays

Transfection of plasmid DNA was performed using Effectene transfection reagent (Qiagen) as recommended by the manufacturer. The pLXSH-HA-DNIRF-7 plasmid was transfected into the HuS-E/2 clone; transfectants were selected in 100 μg/ml hygromycin B (Gibco). The psiRNA-hIRF-3 and psiRNA-hIRF-7 plasmids were separately transfected into HuS-E/2 cells followed by Zeocin (250 μg/ml) selection. After two weeks of continuous selection, cells were infected with HCV. Luciferase assays were conducted as previously described [22]. The results are presented as relative light units (RLU) normalized to the total content of protein in the cell lysates.

2.5. Reverse transcriptase polymerase chain reaction (RT-PCR) and real-time RT-PCR

Using 250 ng of total RNA as a template, we performed RT-PCR with a one-step RNA PCR kit (Takara) according to the manufacturer's instructions. The primer sets and reaction conditions used are detailed in Table 1. To measure HCV-RNA titers by real-time RT-PCR, we collected RNA from infected wells. Five hundred nanograms of total cellular RNA was analyzed for the quantity of HCV-RNA as previously described [23].

2.6. HCV infection experiment

HCV infection experiment from serum was done as mentioned before [22]. HCV-infected-serums were titrated and 1×10^5 HCV-RNA copies/ml were used for each infection experiment. Concentrated culture medium for HCV/JFH1-producing cells was prepared as previously described [7]. HCV titer in the concentrated medium was measured, adjusted and added to the cells as mentioned above.

2.7. Blocking of HCV infectivity by anti-CD81

Inhibition of HCV infectivity was performed by blocking CD81 as previously described [7].

3. Results

3.1. Establishment of immortalized primary human hepatocytes

Primary hepatocytes were isolated from liver tissue obtained from a 9-year-old male patient with Primary Hyperoxaluria who had undergone liver transplantation. Hepatocytes were left unmanipulated or transduced with CSII-EF-hTERT alone or in combination with CSII-EF-SVT or CSII-EF-E6E7 to enhance the efficiency of immortalization. After six weeks only cells transduced by the combination of hTERT and either LT or HPV18/E6E7 continued to proliferate. Initially appearing colonies with a growth advantage were picked up and expanded. SV40 T-immortalized cell clones were named HuS-T cells and given numbers from 1 to 7,

Table 1
Primer sequences and RT-PCR parameters

Genes	Primer sequence 5′–3′	PCR parameters[a]
HGF	F: AGGAGCCAGCCTGAATGATGA R: CCCTCTGATGTCCCAAGATTAGC	95, 56, 72 1 min, 45 s, 1 min
TGFα	F: ATGGTCCCCTCGGCTGGA R: GGCCTGCTTCTTCTGGCTGGCA	95, 59, 72 45 s, 30 s, 1 min
TGFβ1	F: GCCCTGGACACCAACTATTGCT R: AGGCTCCAAATGTAGGGGCAGG	95, 58, 72 45 s, 30 s, 1 min
TGFβ2	F: GATTTCCATCTACAAGACCACGAGGGACTTGC R: CAGCATCAGTTACATCGAAGGAGAGCCATTCG	95, 58, 72 45 s, 30 s, 1 min
HGFR	F: TGGTCCTTGGCGTCGTCCTC R: CTCATCATCAGCGTTATCTTC	95, 54, 72 30 s, 45 s, 1 min
EGFR	F: CTACCACCACTCTTTGAACTGGACCAAGG R: TCTATGCTCTCACCCCGTTCCAAGTATCG	95, 58, 72 45 s, 30 s, 1 min
TGFβ1R	F: CGTGCTGACATCTATGCAAT R: AGCTGCTCCATTGGCATAC	95 s, 54, 72 30 s, 45 s, 1 min
TGFβ2R	F: TGCACATCGTCCTGTGGAC R: GTCTCAAACTGCTCTGAAGTGTTC	95, 58, 72 45 s, 30 s, 1 min
FGFR	F: ATGTGGAGCTGGAAGTGCCTC R: GGTGTTATCTGTTTCTTTCTCC	95, 54, 72 30 s, 45 s, 1 min
IGF-1R	F: ACCCGGAGTACTTCAGCGCT R: CACAGAAGCTTCGTTGAGAA	95, 54, 72 30 s, 45 s, 1 min
HNF1α	F: GTGTCTACAACTGGTTTGCC R: TGTAGACACTGTCACTAAGG	95, 52, 72 45 s, 30 s, 1 min
HNF1β	F: GAAACAATGAGATCACTTCCTCC R: CTTTGTGCAATTGCCATGACTCC	95, 52, 72 1 m, 45 s, 1 min
HNF3β	F: CACCCTACGCCTTAACCAC R: GGTAGTAGGAGGTATCTGCGG	95, 56, 72 1 m, 45 s, 1 min
HNF4	F: CTGCTCGGAGCCACAAAGAGATCCATG R: ATCATCTGCCACGTGATGCTCTGCA	95, 58, 72 45 s, 30 s, 1 min
Albumin	F: AGTTTGCAGAAGTTTCCAAGTTAGTG R: AGGTCCGCCCTGTCATCAG	95, 55, 72 45 s, 30 s, 1 min
Apolipoprotein-a	F:AGGCTCGGCATTTCTGGCAG R: TATCCCAGAACTCCTGGGTC	95, 55, 72 45 s, 30 s, 1 min
HTF	F: TCGCTACAGCCTTTGCAATG R: TTGAGGGTACGGAGGAGTTCC	95, 55, 72 45 s, 30 s, 1 min
E-cadherin	F: TCCATTTCTTGGTCTACGCC R: TTTGTCCTACCGACTTCCAC	95, 55, 72 45 s, 30 s, 1 min
CYP 1B1	F: CACCAAGGCTGAGACAGTGA R: GCCAGGTAAACTCCAAGCAC	94, 57, 72 30 s, 30 s, 1 min
CYP 2C9	F: GGACAGAGACGACAAGCACA R: TGGTGGGGAGAAGGTCAAT	94, 57, 72 30 s, 30 s, 1 min
CYP 2B	F: GGCACACAGCCAAGTTTACA R: CCAGCAAAGAAGAGCGAGAG	94, 57, 72 30 s, 30 s, 1 min
CYP 3A4	F: TGTGCCTGAGAACACCAGAG R: GCAGAGGAGCCAAATCTACC	94, 57, 72 30 s, 30 s, 1 min
CYP 2E1	F: CCGCAAGCATTTTGACTACA R: GCTCCTTCACCCTTTCAGAC	94, 57, 72 30 s, 30 s, 1 min
CYP 1A1	F: AGGCTTTTACATCCCCAAGG R: GCAATGGTCTCACCGATACA	94, 57, 72 30 s, 30 s, 1 min
GAPDH	F: CCATGGAGAAGGCTGGGG R: CAAAGTTGTCATGGATGACC	95, 8, 72 45 s, 30 s, 1 min

Table 1 (*continued*)

Genes	Primer sequence 5′–3′	PCR parameters[a]
CD81	F: CTCAACTGTTGTGGCTCCAAC R: CCAATGAGGTACAGCTTCCC	95, 55, 72 45 s, 30 s, 1 min
TLR3	F: GATCTGTCTCATAATGGCTTG R: GACAGATTCCGAATGCTTGTG	95, 55, 72 45 s, 30 s, 1 min
TLR7	F: CCAGACATCTCCCCAGCGTC R: GGCAAAACAGTAGGGACGGC	95, 55, 72 45 s, 30 s, 1 min
TLR8	F: CTGTGAGTTATGCGCCGAAG R: CGGGATTTCCGTTCTGGTGC	95, 55, 72 45 s, 30 s, 1 min
Myd88	F: GGTCTCCTCCACATCCTCCC R: CCAGCTTGGTAAGCAGCTCG	95, 55, 72 45 s, 30 s, 1 min
IRF3	F: GAACCCCAAAGCCACGGATC R: CCTCCCGGGAACATATGCAC	95, 55, 72 45 s, 30 s, 1 min
IRF7	F: GTGCTGTTCGGAGAGTGGCTC R: CAGCCCAGGCCTTGAAGATG	95, 55, 72 45 s, 30 s, 1 min

CYP, cytochrome P450; EGFR, epidermal growth factor receptor; F, forward primer; FGFR, fibroblast growth factor receptor; GAPDH, glyceraldehyde phosphate dehydrogenase; HGF, hepatocyte growth factor; HGFR, hepatocyte growth factor receptor; HNF, hepatocyte nuclear factor; HTF, human transferrin; IGF-1R, insulin-like growth factor-type I receptor; IRF, interferon regulatory factor; R, reverse primer; TGF, transforming growth factor; TGFR, transforming growth factor receptor; TLR, toll like receptor.

[a] Temperatures are tabulated in the first lane in degrees celsius and the corresponding times in the second lane. Performing one-step RT-PCR, reverse transcription was carried out at 42 °C for 20 min with a pre-PCR denaturation at 95 °C for 10 min.

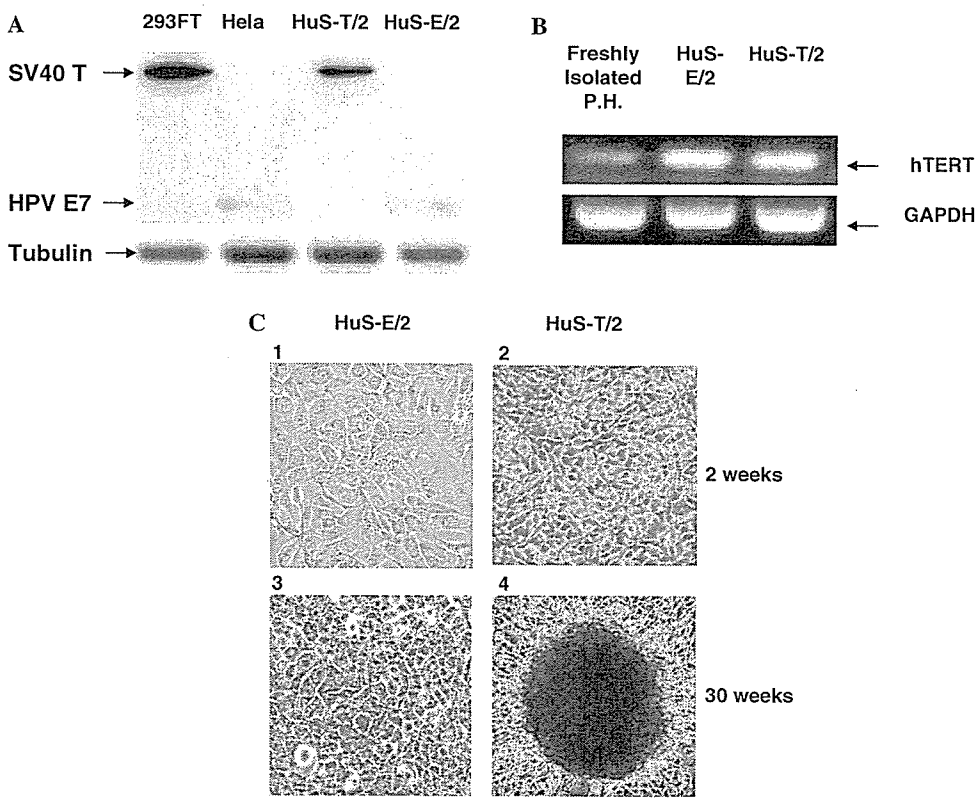

Fig. 1. (A) Immunoblot detection of SV40 T and HPV E7 expression in HuS-T/2 and HuS-E/2 cells, respectively. 293-FT and HeLa cells were used as positive controls for SV40 T and HPV E7 expression, respectively. The specific bands representing the targets are indicated. Detection of tubulin expression in all cells served as an internal control. (B) Human Telomerase Reverse Transcriptase (hTERT) expression was examined by RT-PCR in freshly isolated hepatocytes and the HuS-E/2 and HuS-T/2 cell lines. GAPDH expression was used as an internal control. The hTERT-specific bands are shown. (C) Morphological characteristics of HuS-E/2 and HuS-T/2 cells after two (panels 1 and 2) and 30 (panels 3 and 4) weeks in culture. [This figure appears in colour on the web.]

while the HPV18/E6E7-immortalized clones were named HuS-E cells and given numbers from 1 to 4. Expression of SV40 T and HPV E7 proteins was detected in the appropriate cells by immunoblot analysis (Fig. 1A). In both immortalized cell lines, expression of hTERT-mRNA was enhanced in comparison to non-transduced, freshly isolated hepatocytes as determined by RT-PCR (Fig. 1B). HuS-E cells were larger in size and exhibited slower growth than HuS-T cells (Fig. 1C).

3.2. Characterization of HuS-E and HuS-T immortalized hepatocytes

The HuS-E/2 and HuS-T/2 clones demonstrated the highest expression of hepatocyte-specific markers and transcription factors by RT-PCR (data not shown); these cells were used as representative for each group in this study. To address if HuS-E/2 and HuS-T/2 maintained similar characteristics as primary hepatocytes, they were both cultured continuously for 30 weeks and the expression profiles of a variety of growth factors (Fig. 2A), growth factor receptors (Fig. 2B), hepatocyte-specific nuclear factors (Fig. 2C), albumin, apolipoprotein-A1, transferrin (Fig. 2D), cytochrome p450 (CYP) genes (Fig. 2E), and GAPDH were compared with freshly isolated primary hepatocytes after isolation or two weeks of culture, Huh-7.5 cells, and 293 cells. After two weeks in culture, the expression of nearly all examined genes was similar between freshly isolated hepatocytes and the HuS-E/2 cell line. HuS-E/2 cells, however, exhibited higher expression of TGFβ2 (Fig. 2A), TGFβ2R, and HGFR (Fig. 2B) and lower expression of CYP 3A4 and 2C9 (Fig. 2E) in comparison to freshly isolated hepatocytes. Primary hepatocytes displayed reduced expression of TGFβ1 and TGFβ2 (Fig. 2A) and a loss of CYP1A1 expression (Fig. 2E) after two weeks of culture. HuS-E/2 cells exhibited higher expression of HGF (Fig. 2A), HGF receptor (Fig. 2B), HNF-4, (Fig. 2C), albumin, apolipoprotein-A1, HTF, and E-cadherin (Fig. 2D) in comparison to HuS-T/2 cells. Expression of CYP 3A4 (Fig. 2E) was lost from both HuS-T/2 and HuS-E/2 cells, while HuS-T/2 cells also lost the expression of HNF-1α (Fig. 2D), and CYPs 2B, 2E1 (Fig. 2E).

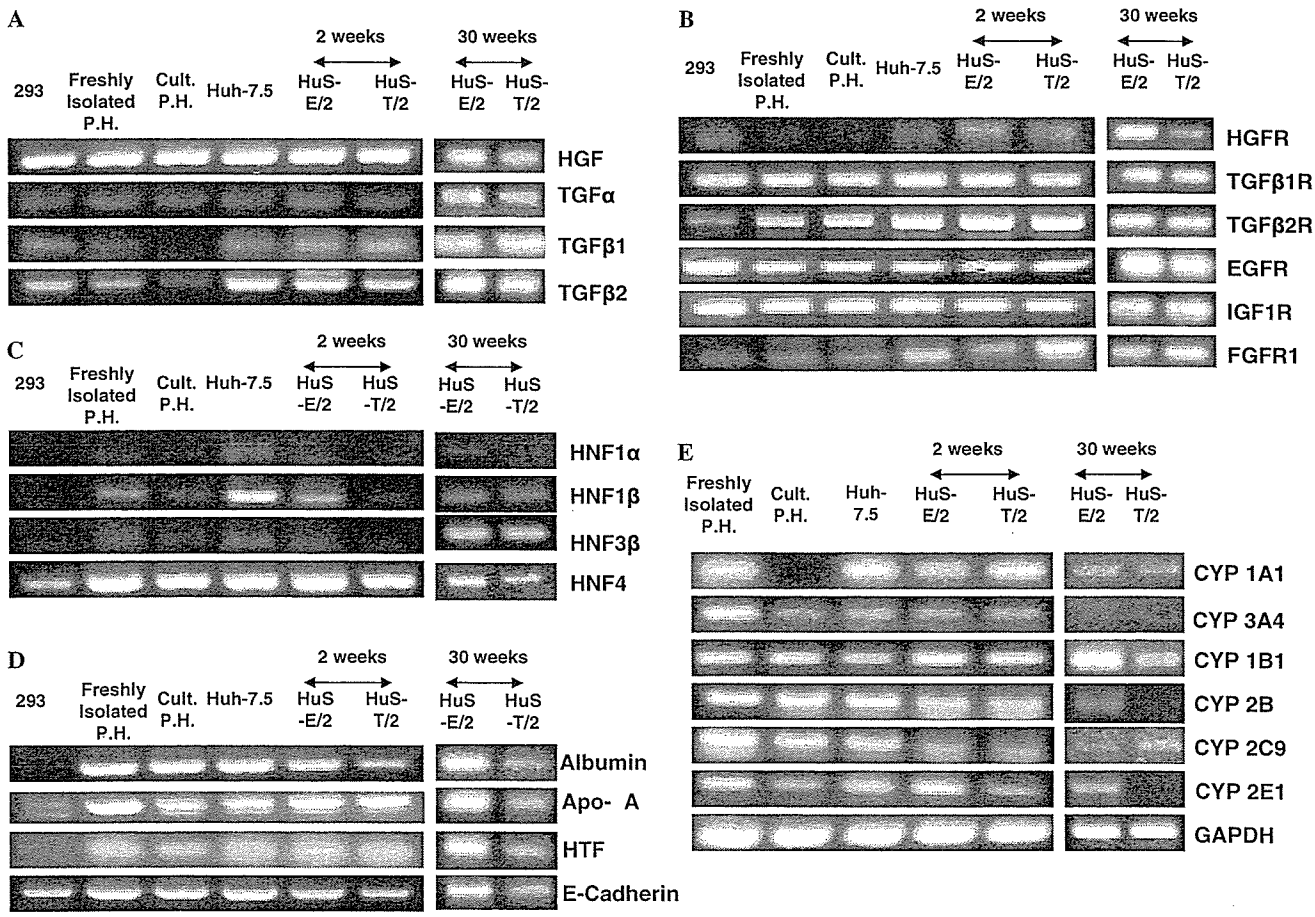

Fig. 2. Expression of the genes encoding growth factors (A), growth factor receptors (B), hepatocyte-specific nuclear factors (C), hepatocyte differentiation and functional markers (D), and CYP enzymes (E) in 293 cells, freshly isolated primary hepatocytes (P.H.), primary hepatocytes cultured for two weeks (Cult. P.H.), Huh-7.5 cells, and HuS-E/2 and HuS-T/2 cells cultured for two and 30 weeks were investigated by RT-PCR. The bands representing specific targets are indicated in the representative reactions.

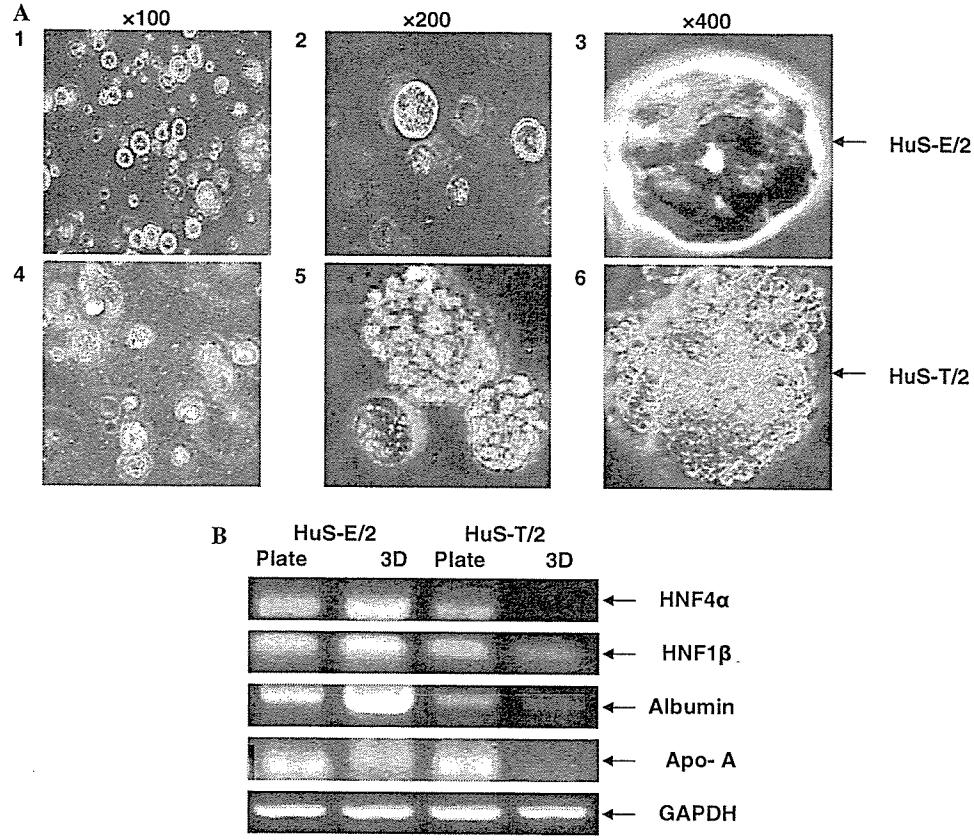

Fig. 3. (A) The morphology of HuS-E/2 and HuS-T/2 cells in 3D culture. HuSE/2 and HuS-T/2 cells were cultured in Mebiol Gel in 12-well plates at a concentration of 5×10^5 cells/well. The microscopic characteristics of these cells after one week of 3D culture are shown. (B) The expressions of HNF4α, HNF1β, albumin, and apo-A by HuS-E/2 and HuS-T/2 cells in both flat and 3D cultures are detailed. After one week of culture of HuS-E/2 and HuS-T/2 cells in flat and 3D cultures, the expressions of HNF4α, HNF1β, albumin, and apo-A were measured by RT-PCR in 250 ng total RNA.

HuS-T/2 but not in HuS-E/2 cells showed a transformed-like character starting from the 13th week of culture. This was demonstrated by continuing proliferation after confluence, pile-up formations (Fig. 1C), and proliferating in serum-deprevied condition. However, HuS-E/2 cells did not show any transformed-like characters even after 30 weeks of culture.

3.3. The characteristics of HuS-E and HuS-T immortalized hepatocytes in 3D culture

After one week in 3D culture, HuS-E/2 (Fig. 3A, panels 1, 2, and 3) cells adopted a donut-shaped structure with a central pore, while HuS-T/2 cells (Fig. 3A, panels 4, 5, and 6) displayed irregular mass formations (similar to the growth pattern of Huh-7.5 cells in 3D culture (data not shown)). In 3D culture, while the expression of HNF4, HNF1β, and albumin was enhanced in HuS-E/2, it was decreased in HuS-T/2 cells (Fig. 3B).

3.4. HCV infection to HuS-E/2

We further assessed the HCV infectivity of HuS-E- and HuS-T-derived clones by infection with HCV-1b-infected serum. Of the three HuS-E clones examined, HuS-E/2 clone demonstrated the highest infectability with HCV genotype 1b in comparison to Huh-7.5, PH5CH8 (Fig. 4A), and HuS-T cells (data not shown), which were excluded from further experiments.

3.5. Anti-CD81 blocked HCV infectivity

CD81 is involved in the entry of HCV pseudoparticles [24] and in vitro-synthesized JFH-1 [7]. To determine if authentic viral particles follow the same route of entry when infecting HuS-E/2 cells, we first examined the CD81 expression by RT-PCR. Both HuS-E/2 and HuS-T/2 cells expressed similar amounts of CD81 as freshly isolated hepatocytes and Huh-7.5 cells (Fig. 4B). Antibodies against CD81 reduced HCV infectivity of HuS-E/2 cells from the levels seen using a non-specific control antibody, confirming the importance of CD81 in HCV infectivity (Fig. 4C).

3.6. IFNα blocked HCV infectivity

We treated HuS-E/2 cells with HCV-containing serum. Cells were then cultured in fresh medium supplemented

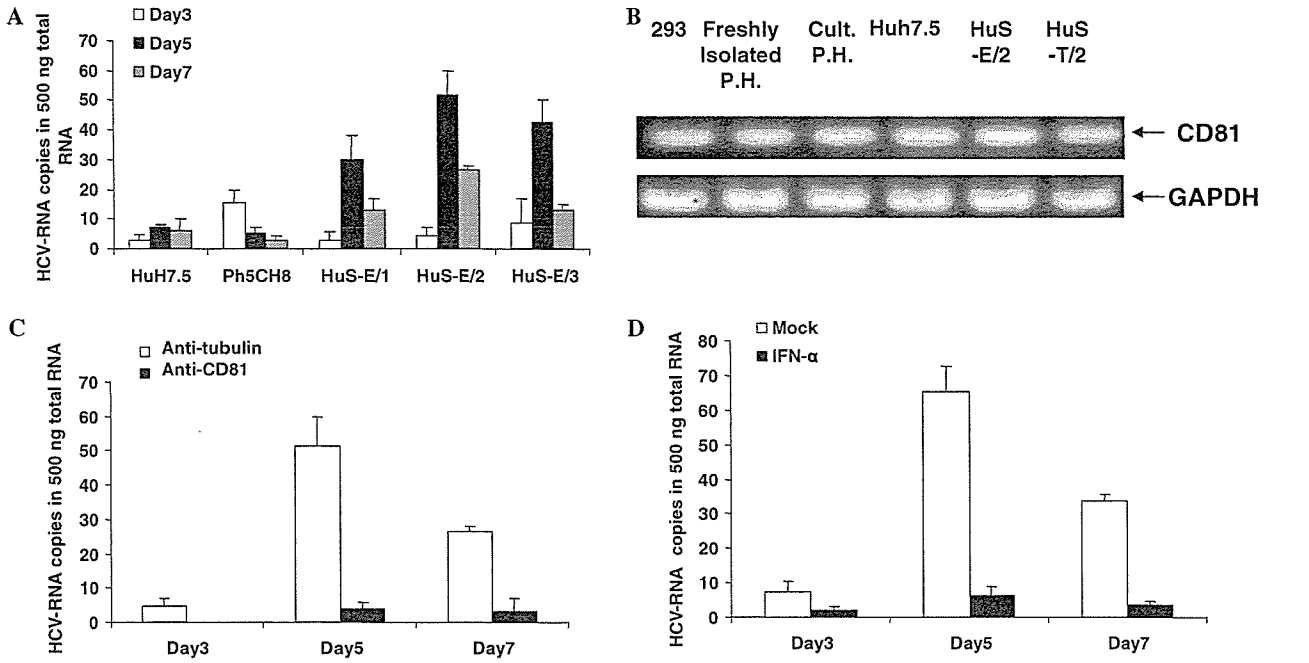

Fig. 4. (A) Serum from an HCV patient was used to infect Huh-7.5 cells, PH5CH8 cells, and three HPV E6E7-immortalized clones (HuS-E/1-3) for 24 h. After washing three times in phosphate-buffered saline (PBS), cells were cultured in fresh medium. Cells were then harvested and lysed at the indicated time points. The quantity of HCV genome RNA per 500 ng total RNA was determined by real-time RT-PCR analysis. (B) HuS-E/2 and HuS-T/2 cells both expressed CD81. Expression of CD81 (upper panel) and GAPDH as an internal control (lower panel) in 293 cells, freshly isolated P.H., cultured P.H., and Huh-7.5, HuS-E/2, and HuS-T/2 cells was investigated by RT-PCR. (C) Anti-CD81 antibodies blocked HCV infectivity. HCV infection was performed as described in (A) with the addition of CD81-specific (black bar) or anti-tubulin antibodies (control, white bar). (D) IFNα inhibits HCV multiplication in HuS-E/2 cells infected with HCV-containing serum. HuS-E/2 cells were infected with HCV as described in (A). After washing three times with PBS, cells were cultured in fresh medium supplemented with (black bar) or without (white bar) 100 U/ml IFNα.

without or with 100 U/ml IFNα. The enhancement of the HCV-RNA genome titers on the fifth day (about 10-fold) was not observed in cells treated continuously with IFNα (Fig. 4D). This result suggests that IFNα inhibited HCV replication in infected HuS-E/2 cells.

3.7. The effect of blocking IRF-3 and IRF-7 signaling on HCV infectivity

Production of interferon-alpha (IFNα) and interferon-beta (IFNβ) limits viral replication and spread, providing one of the most effective innate antiviral responses [25]. Signaling through IRF-3 and IRF-7 plays important roles in the stimulation of IFN-α/β production [25]. To determined which molecules (IRF-3 or IRF-7) play an important role in modulation of the innate immune response against HCV infection in these cells, we first detected intrinsic expression of double-stranded RNA-stimulated Toll-like receptor (TLR) 3, the downstream effector IRF-3, single-stranded RNA-stimulated TLR7, and 8, and the downstream effectors MyD88 and IRF-7 by RT-PCR. TLR3 exhibited very low expression in freshly isolated hepatocytes, Huh-7.5, HuS-E/2, and HuS-T/2 cells, while TLR7, TLR8, MyD88, and IRF-7 were easily detectable in both freshly isolated and immortalized cell lines (Fig. 5A).

The abilities of DNIRF-3 and DNIRF-7 to inhibit IFNβ and IFNα production by HuS-E/2 cells infected with Sendai virus were confirmed using assays of IFNβ or IFNα promoter-driven luciferase reporters. DNIRF-3 exhibited strong inhibition of IFNβ production (Fig. 5B) and weaker inhibition of IFNα transcription (Fig. 5C), while DNIRF-7 strongly inhibited IFNα production (Fig. 5C) and only weakly inhibited IFNβ production (Fig. 5B).

We then assessed the inhibition of HCV infectivity by DNIRF-3 and DNIRF-7. Transient transfection with DNIRF-3, DNIRF-7, or an empty vector was performed prior to HCV infection. Using Effectene reagent, the efficiency of plasmid transfection into HuS-E/2 cells was approximately 70% (data not shown). While there was no significant effect of DNIRF-3 on HCV infectivity, DNIRF-7 demonstrated a marked increase in HCV titers on days 3 and 5 after infection in comparison to control cells (Fig. 5D). To confirm that the enhancement of HCV replication by DNIRF-7 is not mediated by the impairment of IRF-3 signaling by heterodimeric interactions between IRF-3 and DNIRF-7, we performed siRNA inhibition of IRF-3 and IRF-7. The reduction of IRF-3 and IRF-7 expression by siRNA was obvious by RT-PCR (Fig. 5E). siRNA-mediated suppression of either IRF-3 or IRF-7 inhibited IFNβ and IFNα production