planted BMC referred to the numbers of bone marrow and hepatocyte transplantation, as described previously.[4,9,18–20] The abdomen was closed in two layers.

Experimental design

BMC transplantation to FHF model rats

Fulminant liver failure-induced rats underwent intrasplenic injection of BMC (group I; $n = 12$) or normal saline (group II; $n = 12$). Eight rats in each group were monitored until death to determine the survival time, while the other four rats in each group were killed at 12 h after the BMC transplantation for blood analysis. The experiment for the survival rate and the experiments for biochemical blood analysis were separately done.

BMC transplantation to HIR model rats

Two days after induction of HIR, BMC (group A; $n = 15$) or normal saline (group B; $n = 20$) were injected into the spleen. Long-living rats were killed at 1, 3 or 5 months after the operation.

Postoperative evaluation

Blood chemistry

Blood samples were analyzed for total bilirubin (T-Bil) levels, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in the clinical laboratory.

Morphological evaluation (DPPIV staining)

After death of the rats, tissues were rapidly cooled and stored at −80 °C until analysis. To detect DPPIV enzyme expression, cryostat sections (5 μm thick) were made. DPPIV stain was performed as previously described.[21] Briefly, sections were fixed for 5 min in 95% ethanol 5% glacial acetic acid (99:1 v/v) at 0 °C to −10 °C, followed by a 5-min wash in 95% ethanol at 4 °C. Air-dried slides were incubated for 10–20 min at 37 °C in the substrate solution: 2.5 mg Gly-Pro-4-methoxy-b-naphthylamide (Sigma-Aldrich Japan) dissolved in 150 mL of dimethylformamide and mixed with 5 mL of a solution of Fast Blue BB salt (Sigma-Aldrich Japan) in 0.1 M Tris maleate, 0.1 M NaCl, pH 6.5. The slides were rinsed two times in 0.14 M NaCl, incubated for 2 min in 0.1 M $CuSO_4$, and rinsed again in 0.14 M NaCl. The slides were fixed for 10 min in cold 4% paraformaldehyde/PBS/5 μM $MgCl_2$ and washed in 0.14 M NaCl. The slides were washed in water and counterstained with Harris hematoxylin. Finally, the percentage of $DPPIV^-$ cells were counted using Scion image software (Scion Image Beta 4.03 for Windows XP version; Scion, Baltimore, MD, USA) from three independent rats.

Statistical analyses

Data were analyzed statistically using Mann–Whitney *U*-test and Welch test where appropriate. $P < 0.05$ was considered significant. Data are presented as the mean ± standard deviation.

RESULTS

BMC transplantation to the FHF model rats

Survival time

IN FHF RATS, more than 90% of them died within 48 h (survival time, 33 ± 9 h), but mature hepatocyte transplantation was able to prolong the survival time (73 ± 22 h).[15] However, BMC transplantation did not prolong the survival time. The survival times of group I (30 ± 11 h) versus group II (28 ± 14 h) were not significantly different, and the 48-h survival rate for each group was 12.5% (Fig. 2).

Blood chemistry

The average serum T-Bil levels (group I, 2.93 ± 0.72 mg/dL; group II, 2.82 ± 0.95 mg/dL), AST (group I, 5484 ± 1627 IU/L; group II, 6803 ± 3700 IU/L) and ALT activities (group I, 3008 ± 1531 IU/L; group II, 4123 ± 3868 IU/L) were not significantly different (Fig. 3). As

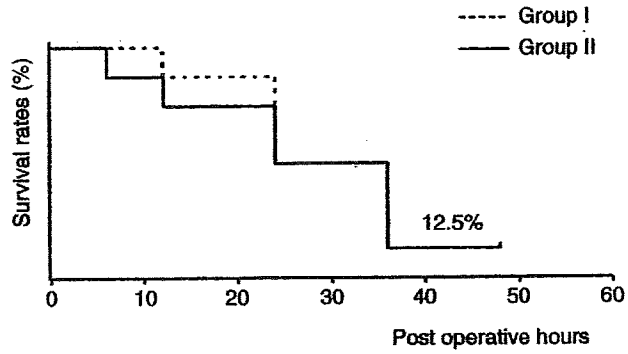

Figure 2 Effect of bone marrow cells (BMC) in the treatment of the fulminant hepatic failure model. Comparison of the survival rate for group I vs group II was not significant (12.5%). The mean survival time was 30 ± 11 and 28 ± 14 h for group I and group II, respectively. Group I, acute liver failure with BMC; group II, acute liver failure without BMC.

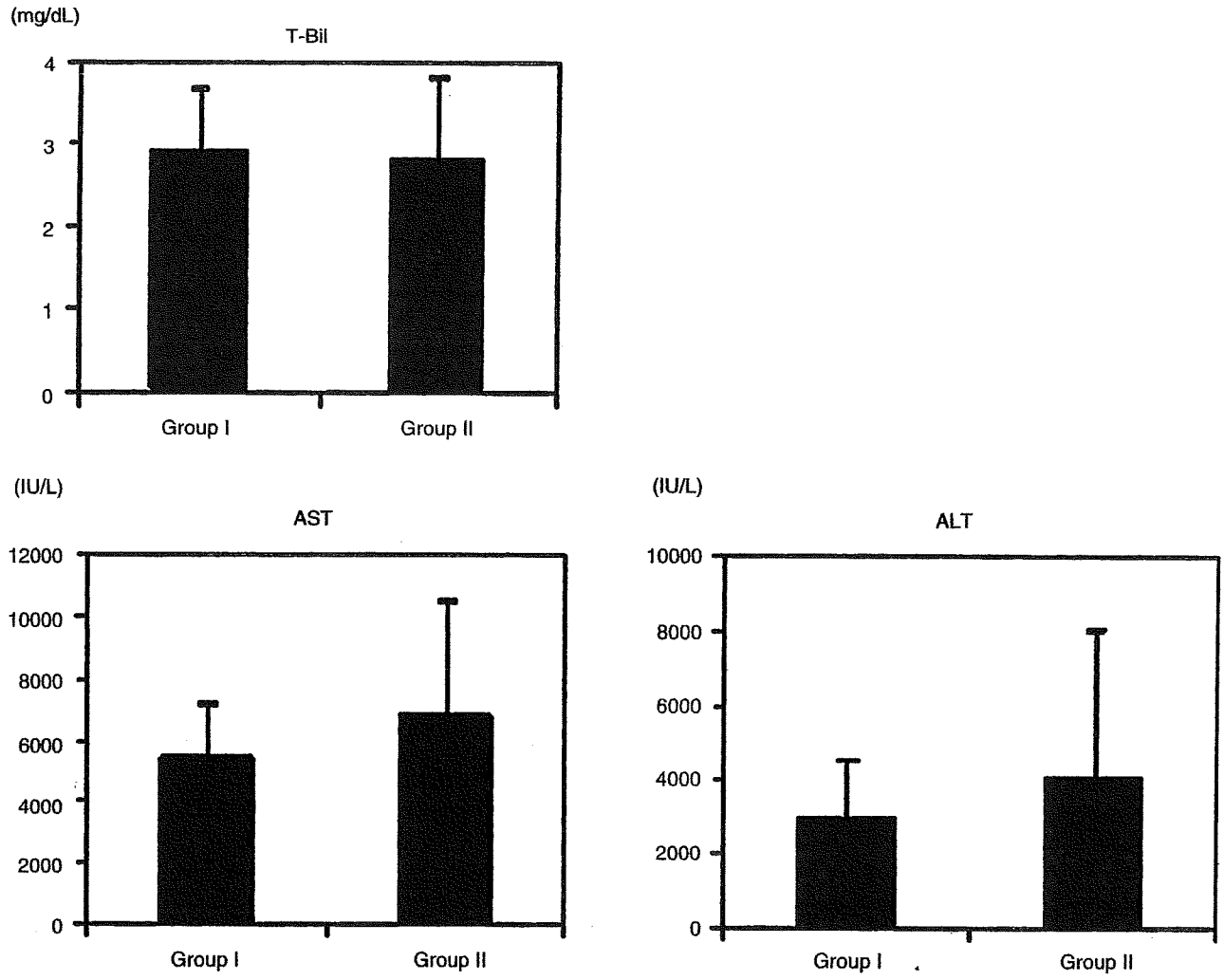

Figure 3 Biochemical changes after bone marrow cell (BMC) treatment in the fulminant hepatic failure model. Between groups I and II, the average serum total bilirubin (T-Bil) levels, and aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities 12 h after treatment were not significant.

for the HIR model, data from group A was only available 24 h after treatment: T-Bil 0.93 ± 0.49, AST 1007 ± 223 and ALT 457 ± 140.

BMC transplantation to the HIR model rats

Survival rate

Thirteen of 15 rats in group A survived up to 1 month, but 13 of 20 rats in group B did not survive up to 1 month. The survival times of group A (127 ± 109 days) versus group B (35 ± 50 days) were significantly different. The 150-day survival rate of group A (74.2%) was significantly greater than group B (17.1%) (Fig. 4).

DPPIV staining

Morphologically, clusters of DPPIV$^+$ hepatocytes were found in the liver of rats in group A (Fig. 5a) but there were no DPPIV$^+$ cells in the liver in group B. The DPPIV$^+$ hepatocytes in the rats in group A were considered to be bone marrow-derived hepatocytes that replaced a maximum of 13% of the recipient liver (Fig. 5b).

DISCUSSION

WE TESTED WHETHER BMC have the potential to support the diseased liver in two distinct, well-established animal models. One is the surgically

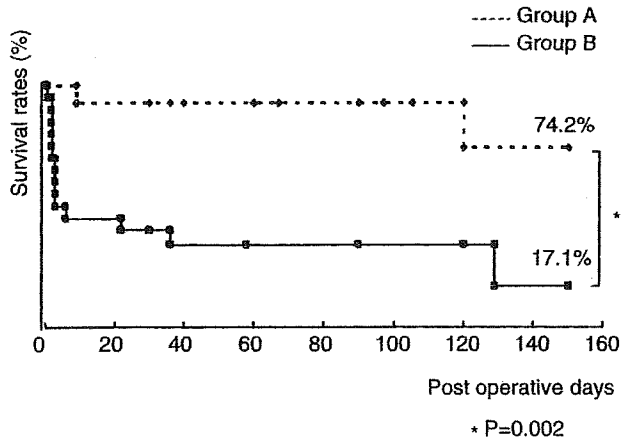

Figure 4 Effect of bone marrow cells in the treatment hepatic irradiation model. The survival rate of group A (74.2%) vs group B (17.1%) was significantly improved ($P = 0.002$).

induced FHF model, in which liver failure is caused by 68% hepatectomy followed by occlusion of the remnant liver lobes. The other is the HIR model, in which 68% hepatectomy is performed followed by irradiation of the remnant liver resulting in fatal liver failure associated with inhibition of liver regeneration. Of note, the HIR model does not emulate human liver disease, but an artificial model to show proliferative advantage to the transplanted cells. Radiation induces critical deterioration in the liver microenvironment that is conducive to the selective proliferation of normal, transplanted hepatocytes as demonstrated previously.[17,20] The precise mechanisms underlying this phenomenon have yet to be elucidated. Radiation is known to inhibit liver growth for extended periods of time; thus, its effect would appear to be similar, at least in principle, to that exerted by retrorsine; blocking that would block of the endogenous hepatocyte cell cycle.

Using these models, we demonstrated that BMC may have the potential to morphologically *trans*-differentiate into hepatocytes in the HIR model. However, our study also demonstrated potential limitations of BMC transplantation in the treatment of fulminant liver failure. In the FHF model, previous studies have demonstrated that mature hepatocyte transplantation can provide survival benefit in liver failure.[15,22] However, a large number of hepatocytes are necessary to adequately treat liver disease. On the other hand, some investigators have suggested that BMC transplantation can be used to treat chronic liver diseases through its regenerative potential.[9] Therefore, we investigated the potential of BMC transplantation to treat FHF in an animal model of acute liver failure induced by the loss of a large amount of hepatocytes followed by lethal systemic inflammation.

As a result, BMC transplantation did not exhibit any survival benefit in rats in this FHF model. This may because BMC require certain conditions and/or adequate time to differentiate into functional hepatocytes. Regarding the numbers of transplanted BMC, we injected 2×10^6 cells under the fibrous capsule of the spleen. In previous reports, the numbers of transplanted cells ranged from 10^6 to 10^7 cells. However, the injection of too many cells can sometimes cause venous thrombosis. Furthermore, our strategy required fewer cells to treat liver damage; namely, 2×10^6 BMC were consid-

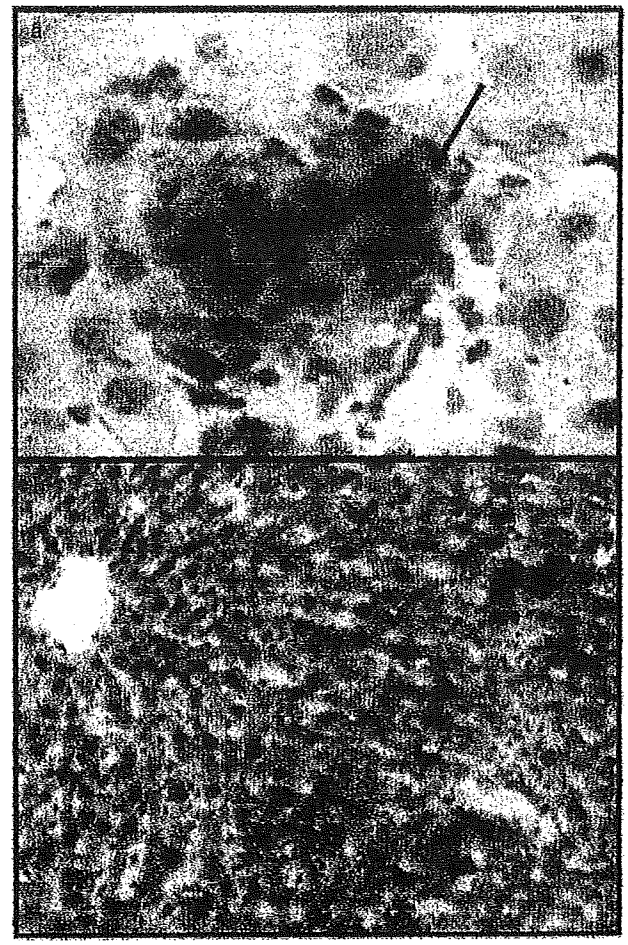

Figure 5 (a) Histological presence of bone marrow-derived hepatocytes. Clusters of dipeptidyl peptidase IV$^+$ hepatocytes were found in the livers of rats from group A (arrow), but no DPPIV$^+$ cells were found in the livers of group B rats. (b) DPPIV$^+$ hepatocytes replaced the recipient liver to a maximum of 13%.

ered sufficient. Accordingly, while BMC transplantation may be promising, acute liver failure may not be a good target for this treatment strategy.

Similar to previous reports that have indicated the potential of BMC to differentiate into various types of cells, DPPIV staining revealed that several clusters of hepatocytes exhibited the features of BMC derived from transplanted donor cells in the HIR model. It remains unclear whether transplanted BMC were *trans*-differentiated into hepatocytes or were fused with the host hepatocytes. However, the presence of $DPPIV^+$ cells in the HIR model indicates the possibility that these cells are committed stem cells of the liver. This is because the $DPPIV^+$ hepatocytes are morphologically hepatocytes, but their potential may include self-replication and vigorous proliferation. Therefore, we anticipate the therapeutic potential of BMC against acute liver injury. In the present study, total replacement of the liver by BMC was not observed, however, several rats in the BMC transplantation group survived longer than those of the control group. Although the etiology of the survival benefit remains unknown, we speculate that BMC transplantation can contribute not only to the repopulation of hepatocytes but also to the improvement of the internal environment of the liver. For instance, it is hypothesized that BMC can contribute to the suppression or resolution of fibrosis in liver cirrhosis or can activate hepatic mesenchymal cells. The "fibrolysis" effect of BMC transplantation has been demonstrated.[9] Further investigations of the contribution of BMC transplantation to improve the intrahepatic microcondition are required.

Furthermore, these results suggest that the HIR model itself can be a useful model for cell transplantation in which high-dose irradiation of the liver can injure the host hepatocytes and impart a selective proliferative advantage to the unirradiated donor cells.[21,23] Histologically, extensive loss of hepatocytes occurs in the irradiated liver, and various degrees of micro- and macrovesicular steatosis in the centrizonal areas. Furthermore, preparation of partial hepatectomy induces several chemical growth factors to regenerate the remnant liver. We speculate that this particular condition may lead to selective growth of transplanted cells. Thus, the HIR model could be applied to clinical cases, such as congenital or metabolic liver disease, by transplantation of normal cell sources. Cell transplantation therapy is expected to become a novel and effective therapy against liver diseases, as an alternative to liver transplantation. However, cell transplantation therapy requires a large number of donor cells that are able to survive in the host liver. In general, a sufficient number of mature hepatocytes cannot be provided, but stem cells may solve this problem. Among the currently available stem cell sources, BMC have several advantages, as previously described. To further elucidate this, further studies concerned with the evaluation of pure bone marrow stem cells and the etiology of *trans*-differentiation to hepatocytes are needed. Furthermore, it is still unknown whether "so called" bone marrow-derived hepatocytes are actually *trans*-differentiated from BMC or fused with BMC. We are currently investing the cell fusion phenomenon to determine the contribution of BMC to support liver failure.

In conclusion, BMC transplantation may have limitations in the treatment of these models of fulminant or acute liver failure because they do not have sufficient time to develop into functional hepatocytes. Preparative HIR may be beneficial and help convert the transplanted BMC into host hepatocytes to provide survival benefit, although the precise mechanism warrants further investigation.

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Case Report

Living-donor liver transplantation from second generation children for atomic bomb survivors

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No report has been available regarding organ transplantation for atomic bomb survivors, even with renal graft. We experienced a living-donor liver transplantation for two atomic bomb survivors using grafts from second-generation children. Post transplant course was uneventful without any systemic disorders under regular immunosuppression schema during 3-year follow-up. The detailed results are herein reported for the first time in the literature.

Key words: atomic bomb, survivor, living donor liver transplantation, second generation children, T cell function

INTRODUCTION

SIXTY YEARS SINCE atomic bomb exploded in Nagasaki, the survivors have become old enough to suffer from end-stage liver disease, which requires liver transplantation.[1,2] However, no report has been available regarding organ transplantation for atomic bomb survivors, even with renal graft. It has been reported that atomic bomb survivors might have significant immunological alteration, especially T cell function such as interleukin-2 production.[3] We recently performed living-donor liver transplantation (LDLT) for two atomic bomb survivors. The results are herein reported for the first time.

CASE REPORT

OF 93 PATIENTS who had undergone LDLT in Nagasaki University Hospital (Nagasaki, Japan) between 1997 and October 2008, two patients (2.2%) were atomic bomb survivor. A survivor was defined in the present study as a person who received the "Atomic Bomb Survivor's Health Handbook" produced by Nagasaki city authorities since the establishment of the Atomic Bomb Survivors' Medical Treatment Law in April 1957. All information, including exposure distance, had been recorded before the present study.

The initial indication for LT was hepatocellular carcinoma in decompensated cirrhotic liver due to hepatitis B with model for end-stage liver disease (MELD) score of 19 in one patient, while it was due to hepatitis C in other patient with MELD score of 12 with intractable pleural effusion. The detailed information was described in Table 1. Since they were A-bombed in their childhood, both patients did not remember the wartime. Therefore, their psychiatric status before LDLT was stable. Usual thorough systemic survey was performed for both recipients, which included no specific additional screening. LDLT was performed with a right lobe graft without the middle hepatic vein for first patient in June, 2005 and with extended left lobe graft with middle hepatic vein for second patient in September, 2005. The living donors were a second generation son and a daughter of the recipients. Human leukocyte antigens mismatches were 3 and 2, respectively. The first patient had been treated with tacrolimus based immunosuppression with trough level of 8–12 mg/dL during first 3 months and 5–8 ng/mL thereafter. The second patient had been on cicrosporine with a trough level around 150 ng/mL within first 3 months and subsequent interferon treatment for hepatitis C recurrence.[4] Both patients have experienced neither acute cellular rejection nor fatal infection throughout the

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Table 1 Patient demographics and peripheral blood lymphocyte activation of the patients

	Case 1	Case 2
Age (years)	59	64
Sex	M	F
Age at A-bomb detonation (years)	0	4
Distance from A-bomb at detonation (km)	3.3	2.5
Indication for LDLT	HCC/LC-B	LC-C
Living donor relationship and age (years)	Son (32)	Daughter (34)
CD4(%)/CD8(%)	35.0/43.4 (ratio 0.8)	28.0/32.9 (ratio 0.9)
T cell function tests		
Con-A stimulation	57 430	64 535
SI (Con-A)	354.6	136.7
Control	162	472
PHA stimulation	140 234	100 830
SI (PHA)	865.6	162
Control	162	472

Con-A, concanavalin A ; HCC, hepatocellular carcinoma; LC-B, liver cirrhosis due to hepatitis B infection; LC-C, liver cirrhosis due to hepatitis C infection; LDLT, living-donor liver transplantation; PHA, phytohemagglutinin; SI, stimulation index.

post-transplant course. Both patients have been well with normal liver function tests 3 years after the LDLT. Both recipients have undergone close follow-up using computed tomography, magnetic resonance imaging and bone scintigram for systemic survey.

DISCUSSION

WE FEEL THAT medical doctors in Nagasaki have a responsibility to report the results of interventions undertaken for atomic bomb survivors. At the time of writing, both of the patients reported herein are well following their liver transplantation with the allografts from living donors, who are second generation children of atomic bomb survivors. Given that atomic bomb survivors have a higher risk of multiple primary cancer development,[5] longer-term follow-up might be needed to ensure subsequent development of new cancer, on the top of susceptibility to cancer development in patients under immunosuppression. No immunological derangement or fetal infectious complications occurred after LDLT under regular regimen of immunosuppression probably because our patients did not have T cell dysfunction as shown in Table 1. Since LDLT were performed in our case using allograft from second-generation children, we also need to make careful observation in the graft liver, which might also have a risk with genetic instability.[6] Therefore, careful surveillance for simultaneous cancer and follow-up after LDLT are needed for those patients.

The exposure distance was used as a substitute for the estimated irradiated dose. Several unique epidemiological studies on Nagasaki survivors have already been documented with exposure distances.[5] In general, survivors who were less than 1.5 km from the hypocenter were exposed to a significant dose of radiation. The estimated doses in Nagasaki survivors who were not shielded at the time of explosion are: 924.7 cGy at 1.0 km, 120.7 cGy at 1.5 km, 17.9 cGy at 2.0 km, and 2.9 cGy at 2.5 km from the hypocenter.[5] In our patients, the distance from the hypocenter was 2.5 and 3.3 km. Since the distance was not too close, the influence between those patients and eradiation might not be strong. Latent period to develop hepatocellular carcinoma in case 1 patient was not unusual as compared to regular population of its development.

Usually, LDLT might not be indicated for a patient with impaired T cell function. For example, patients with T cell dysfunction with human immunodeficiency virus sometimes cannot be indicated for LDLT to avoid opportunistic infection.[7] Therefore, if T cell function is impaired in atomic bomb survivors, LDLT should not be undertaken due to the risk of infectious complications. Function of T cell could also decrease with aging, various drugs, stress and etc. Further evidence for optimal immunosuppressive therapy for atomic bomb survivors is needed.

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Original Paper

Low-dose Recombinant Human Hepatocyte Growth Factor Enhances Effect of Hepatocyte Transplantation in Rats Treated with Retrorsine

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KEY WORDS: Hepatocyte transplantation; Hepatocyte growth factor; Regeneration; Retrorsine

ABBREVIATIONS: Recombinant Human Hepatocyte Growth Factor (rhHGF); Nagase Analbuminemic Rats (NARs); Hepatocyte Transplantation (HcTx); Orthotopic Liver Transplantation (OLT); Hepatocyte Growth Factor (HGF); Sprague-Dawley (SD); Continuous Systemic Administration (c.s.a.)

ABSTRACT

Background/Aims: The aim of this study was to regenerate transplanted hepatocytes selectively in a recipient using retrorsine and recombinant human hepatocyte growth factor (rhHGF).

Methodology: Nagase analbuminemic rats (NARs) received pretreatment with retrosine and were divided into three experimental groups. Group1: Hepatocyte transplantation (HcTx) + 50μg/kg/day rhHGF. Group2: HcTx + 250μg/kg/day rhHGF. Group3: HcTx + normal saline. The serum levels of albumin and the albumin-positive hepatocytes in the liver were investigated. The rat endogenous HGF of the rats given only retrorsine was measured.

Results: The serum albumin levels of Group1 were higher than those of Group2, while there was no significant difference between Group2 and Group3. Histological examination of Group1 and 3 showed the presence of a large number of albumin-positive hepatocytes, which frequently consisted of large clusters and occupied 53.90±2.31% and 31.25±5.36% of host liver, respectively. The liver sections of Group2 showed numerous albumin-positive hepatocyte, which were not seen as clusters. The rat endogenous HGF concentration was extremely high.

Conclusion: Low-dose rhHGF enhances the effect of HcTx under the suppressive state of proliferation of host hepatocytes. Because of the high endogenous HGF, the administration of a high concentration of rhHGF suppressed the regenerative activity of the transplanted hepatocytes.

INTRODUCTION

Although the therapy for hepatic metabolic deficient disease still remains orthotopic liver transplantation (OLT), the donor organ shortage has been problematic. HcTx has been proposed as an alternative therapy to OLT, but it is difficult to regenerate transplanted hepatocytes and to achieve the long term correction of a liver-related metabolic defect (1-3). Therefore, partial hepatectomy and portal branch ligation have been added as a growth stimulus for transplanted hepatocytes (4-8). However, these invasive methods are not suitable in a clinical setting for patients with congenital hepatic metabolic deficiency disease.

Human hepatocyte growth factor (HGF) was isolated and purified from the plasma of patients with fuluminant hepatic failure and was found to stimulate DNA synthesis even in adult rat hepatocytes (9, 10). A recombinant form of human rhHGF has also been developed and is as effective as the native HGF in terms of proliferative activities in rat and human hepatocytes (11). Furthermore, the cytoprotective effect of HGF through the immunoregulation action and antiapoptosis action has also been reported (12-15).

Recently, nearly total liver replacement by transplanted normal hepatocytes was reported in rats treated with retrorsine (16-18). Retrorsine is a naturally occurring pyrrolizidine alkaloid that is taken up selectively by the liver and metabolized to bioactive compounds that alkylate DNA, which causes a long-lasting block of hepatocyte cell division (19-23). To selectively enhance the proliferation of transplanted hepatocytes, combined therapy with HGF and retrorsine seems to be an attractive strategy, although it has never been tested in vivo.

In the present study, was investigated the effect of rhHGF for HcTx in rats treated with retrorsine.

METHODOLOGY

Animals

All rats used were purchased from Japan SLC Inc.(Shizuoka, Japan) and were maintained at the

Hepato-Gastroenterology 2009; 56:1466-1470

Animal Center at Nagasaki University School of Medicine. NARs were used as the recipients, while male Sprague-Dawley (SD) rats were used as the hepatocyte donors. All animals were maintained in a climate-controlled (24°C) room with a 12-hour light-dark cycle and were provided tap water and standard laboratory chow *ad libitum*. All procedures were done in accordance with the guidelines of the University of Nagasaki Research Animal Resources.

Chemicals

rhHGF was manufactured and provided by Mitsubishi Pharma Corporation (Yokohama, Japan). Retrorsine, Collagenase type IV, bovine serum albumin, and purified rat albumin were purchased from Sigma Chemical Co. (St. Louis, MO). Purified rabbit anti-rat albumin IgG antibodies and peroxidase conjugated rabbit anti-rat albumin were obtained from ICN Pharmaceuticals Inc (Aurora, OH).

Hepatocyte Isolation and transplantation

Hepatocytes were isolated from SD rats according to a standard two-step collagenase perfusion technique (24). After enrichment through a Percoll gradient, hepatocytes viability was determined by the trypan blue exclusion test. The final viability of the purified hepatocytes suspension was always 90-95%. A suspension of 2×107 viable hepatocytes in one milliliter of saline was directly infused via the portal vein. The NARs were given two injections of retrorsine, 30mg/kg each, intraperitoneally, 2 weeks apart. Four weeks after the second injection, each animal received 2×107 viable hepatocytes via the portal vein. After the HcTx, the continuous systemic administration (c.s.a.) of rhHGF or normal saline was done using an osmotic pump as described below. All animals were given Cyclosporin A, at a dose of 15mg/kg, intramuscularly one day prior to HcTx and every other day until sacrifice to limit rejection of the transplanted cells.

Continuous systemic administration of rhHGF

Alzet osmotic pumps were purchased from ALZA Co.(Palo Alto, CA). The pump was connected to the catheter canulated into the jugular vein and placed in the subcutaneous layer of the anterior wall. Continuous infusion of rhHGF or normal saline was done using an osmotic pump.

Experimental designs

The rats were divided into three experimental groups (**Figure 1**). The Group 1 (n=5) animals received retrorsine+HcTx followed by c.s.a. of 50μg/kg/day of rhHGF for 7 days. The Group 2 (n=5) animals received retrorsine+HcTx followed by c.s.a. of 250μg/kg/day of rhHGF for 7 days. The Group 3 (n=5) animals received retrorsine+HcTx followed by c.s.a. of normal saline for 7 days. HGF concentration was set according to our previous study (25). Blood samples were obtained from the tail vein on days 0 (before HcTx), 3, 7, 14, 21, 28, 35, 42 and 56, respectively, for the determination of serum albumin. All animals were euthanized at the 56th day. At sacrifice, the liver specimens were fixed in 10% buffered formalin.

Serum Albumin determination

Quantitative analysis of the serum levels of albumin was carried out by sandwich ELISA using rabbit anti-rat albumin IgG described previously (26).

Investigation of albumin positive hepatocytes

Serial sections of the liver were immunostained utilizing rabbit anti-rat albumin IgG antibody and an immunoperoxidase avidin-biotin peroxidase complex method. Albumin-positive area in the liver was quantitated with the help of a computer-assisted image analyzer.

Measurement of serum human HGF concentration

The human HGF concentration was measured by the Quantikine Human HGF EIA kit (R&D Systems, Inc., Minneapolis, MN) on day 3 to confirm the rise of the human HGF level for each administration of rhHGF.

Measurement of serum rat endogenous HGF concentration

The rat endogenous HGF concentration of rats given only retrorsine was measured by a rat HGF EIA kit (Institute of Immunology Co., Tokyo, Japan) on days 0 and 56 to check the host response

FIGURE 1 NARs were given two injections of retrorsine, 30mg/kg each, intraperitoneally (i.p.), 2 weeks apart. Four weeks after the second injection, each animal received HcTx. After theHcTx, c.s.a. ofrhHGF or normal saline was done.

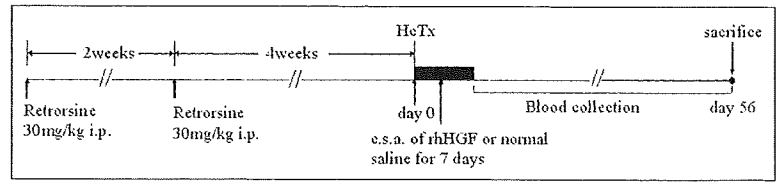
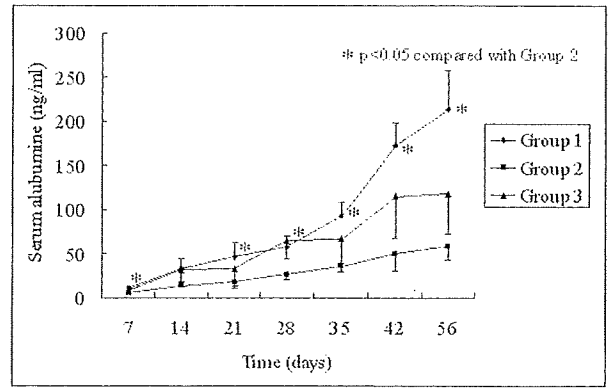
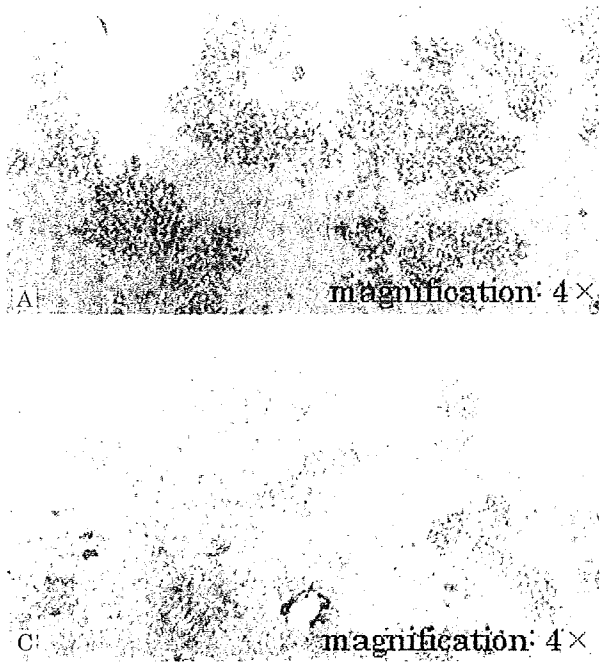


FIGURE 2 A significant increases in the serum albumin level was observed in each group following hepatocyte transplantation. The serum albumin level of Group1 was statistically higher than that of Group 2 at each point except for day 14 ($p<0.05$). There was no statistically significant difference between Groups 2 and 3.





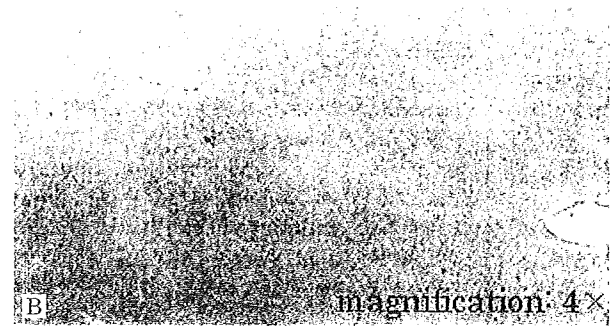


FIGURE 3
(A) A large number of albumin–positive hepatocytes can be seen in Group 1, frequently as large clusters, which occupied 53.90±2.31% of host liver.
(B) There are numerous albumin–positive hepatocytes in Group 2, which are distributed throrough the liver parenchyma, but not seen as clusters.
(C) A large number of albumin–positive hepatocytes can be seen in Group 3. They are frequently seen as large clusters, which occupied 31.25±5.36% of host liver.

against retrorsine (n=5).

Statistical Analysis

All data were expressed as a median and their range. Mann-Whitney's test was used for the data analysis. The differences were considered statistically significant when the p values were less than 0.05.

RESULTS

Serum albumin level

A significant increase in serum albumin levels were observed in each group following hepatocyte transplantation (v.s. base line levels; 1.26±0.04ng/ml) (**Figure 2**). The serum albumin level of Group 1 was statistically higher than that of Group 2 at each point except for day 14 ($p<0.05$). There was no statistically significant difference in the serum albumin level between Groups 2 and 3.

Albumin-positive hepatocytes in the liver

The liver sections obtained from Group 1 showed a markedly large number of cells that stained positive for albumin. They were frequently observed as large clusters and occupied 53.90±2.31% of host liver (**Figure 3A**). The liver sections obtained from Group 2 showed sparse albumin-positive hepatocytes. They were uniformly distributed throughout the liver parenchyma, and were not observed as clusters (**Figure 3B**). The liver sections obtained from Group 3 showed a large number of cells that stained positive for albumin. They were frequently observed as large clusters and occupied 31.25±5.36% of host liver, although there were fewer clusters than in Group 1 (**Figure 3C**). There was statistically difference in the albumin-positive hepatocyte area between group 1 and 3 ($p<0.05$).

Serum human HGF concentration

The serum human HGF concentration through a c.s.a. of 50 and 250μg/kg/day of rhHGF were 5.59±1.20 and 8.44±0.51 ng/ml, respectively ($p<0.05$).

Serum endogenous rat HGF concentration

The endogenous rat HGF concentration on days 0 and 56 were 25.01±0.19 and 20.14±1.29 ng/ml, respectively, after retrorsine treatment. There was no statistically significant difference between the concentration on day 0 and that on day 56.

DISCUSSION

The present study demonstrated the enhanced effect of hepatocyte transplantation by the administration of low- dose rhHGF in rats treated with retrorsine as compared to that with high-dose rhHGF.

The hepatocytes were transplanted under the host's hepatocyte proliferation potency control by retrorsine processing, and attempted selective proliferation of the transplanted hepatocytes. Pyrrolizidine alkaloids including retrorsine were studied originally because of their toxicity in animals, particularly sheep and cattle, in which they cause both acute and chronic injury (19, 20). The administration of a high dose of pyrrolizidine alkaloid is lethal, but a low dose induces only chronic hepatic megalocytosis (27). In our study, there was no death in the rats that only received retrorsine. Pyrrolizidine alkaloids are established hepatocarcinogens (28-32). However, Laconi E *et al.* (16) and Laconi S *et al.* (18, 33) maintained that animals treated with retrorsine for a long time without neoplastic change in the host's hepatocytes and transplanted hepatocytes. To date, in order to inhibit the proliferation of endogenous hepatocytes, irradiation (34, 35) and anti-cancer drugs (36) have been used. Nevertheless, it is thought that those are not suitable in a clinical setting for co-effects to cells except hepatocytes. For the above reasons, we thought that retrorsine could likely be a drug which would the inhibit host's hepatocyte proliferation potency in clinical application.

In the present study, as a proliferation stimulus, instead of partial hepatectomy or portal branch ligation, rhHGF was administered, which was thought to be less invasive. In addition, carbon tetrachloride

has been used as a proliferation stimulus for transplanted hepatocytes (37, 38), but it is not thought to be suitable for use in a clinical setting. Strain *et al.* (11) reported that 0.63ng/ml of rhHGF was the minimum concentration needed to stimulate hepatocyte proliferation in a culture system. According to our previous study (25), when the rats were given a continuous systemic administration of rhHGF in a dose of 50μg/kg/day, the mean concentration of rhHGF in the portal blood was almost similar to the minimum concentration needed to stimulate hepatocyte proliferation in vitro. Based on this investigation, we considered that 50μg/kg/day should be given. We set the concentration of 250μg/kg/day as the high dose HGF, but could have set various concentrations of rhHGF between 50μg/kg/day and 250μg/kg/day, or lower concentration than 50μg/kg/day. Furthermore, we planned to administer rhHGF by continuous systemic infusion because the half-life of HGF in plasma was very short in vivo and vitro (39-42).

In the present study, the cell proliferation and function of the transplant hepatocytes of low-dose rhHGF were better than that with high-dose rhHGF. For that reason, since the rat endogenous HGF was already elevated by retrorsine treatment itself, high dose exogenous rhHGF administration was thought to counteract the proliferation of the transplanted hepatocytes. In fact, it was reported that there is a synergistic effect between rat HGF and rhHGF (40, 43). Furthermore, on the HGF concentration of 5-10ng/ml as growth stimuli, the DNA synthesis of the hepatocyte reaches a plateau, while the DNA synthesis is suppressed with an HGF concentration of more than 10ng/ml (44). This is presumably the reason why the elevation of serum albumin was observed in Group 2 in the present study. In addition, cytoprotective effect rhHGF may be affecting the data. Whether infused rhHGF affect only transplanted hepatocytes, or only native hepatocytes injured by retrorsine, or both awaits further investigation.

In conclusion, low-dose rather than high-dose rhHGF enhances the therapeutic effect of hepatocyte transplantation under retrorsine treatment. Since we only investigated 50μg/kg/day and 250μg/kg/day, further investigation is needed using a more optimal concentration of rhHGF.

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INTERNAL MEDICINE

□ ORIGINAL ARTICLE □

The Significance of Enzyme Immunoassay for the Assessment of Hepatitis B Virus Core-Related Antigen following Liver Transplantation

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Abstract

Purpose Recently, a new enzyme immunoassay for the detection of hepatitis B virus (HBV) core-related antigen (HBcrAg) has been reported. In this study, we proposed to account for feasibility of HBcrAg assay, and discuss the dynamics of HBV seen in patients following HBV-related living donor liver transplantation (LDLT).

Methods and results This study involved 12 patients; 11 patients had positive serum HBcrAg, and 6 patients had negative HBV-DNA. In the post-operation period, all cases were negative for HBV-DNA and HBsAg in sera under prophylaxis therapy. At post-operation, 5 of the 12 had positive serum HBcrAg, and at stable state, 6 had positive serum HBcrAg postoperatively. The mean levels of HBcrAg following LDLT were significantly lower than those seen in the preoperative-operation stage.

Conclusion This enzyme immunoassay is a readily utilizable marker of HBV replication in the post transplantation stage. Furthermore, the evaluation of HBV activity by HBcrAg assay must be studied to determine the appropriate prophylaxis for controlling replication of HBV following LDLT.

Key words: hepatitis B virus, liver transplantation, hepatitis B virus core-related antigen

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Introduction

Liver transplantation (LT) is a long established procedure for the treatment of end-stage liver disease. Patients with chronic or fulminant hepatitis B virus (HBV) infection are major candidates for LT. However, the recurrence of HBV following LT is implicated in severe and life-threatening graft failure (1). Therefore, the prevention of HBV recurrence following LT has been a serious concern. The advent of anti-HBsAg immune globulin (HBIG, Hebsbulin-IH, Mitsubishi Pharma Corporation, Tokyo, Japan), and HBV reverse transcriptase inhibitor, namely lamivudine (Lam, Zeffix, GlaxoSmithKline K.K., Tokyo, Japan) and adefovir dipivoxil (Adv, Hepsera, GlaxoSmithKline K.K., Tokyo, Japan), was a major breakthrough in controlling HBV recurrence in patients who received transplants for HBV-related liver disease. The ideal recurrence rate for HBV (<10%), has been observed in patients receiving HBIG and Lam combination prophylaxis versus just HBIG monotherapy (2, 3) or Lam monotherapy (4, 5). Lam monotherapy has been shown to be ineffective in controlling recurring HBV, and the long term administration of HBIG was necessary (6, 7). Therefore, presently, continuous combination therapy is the standard prophylaxis in the control of HBV recurrence following HBV-related LT.

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Table 1. Clinical Characteristics

Case	Age at LDLT	Gender	Indication disease	HCC	HBV-DNA at LDLT (logcopy/mL)	Adefovir before LDLT	HBsAg (COI)	HBsAb (IU/mL)	HBeAg (IU/mL)	HbeAb (COI)	HBcrAg (logU/mL)
1	56	male	LC	+	<2.6	-	>2000	2.3	0.6	82.4	4.2
2	60	male	LC	+	<2.6	-	1789	0.1	0.2	97.6	5.8
3	59	male	LC	+	<2.6	-	>2000	0.3	0.1	>100	3.2
4	55	female	LC	-	<2.6	-	>2000	0.2	36.0	0	6.0
5	56	male	LC	+	<2.6	-	>2000	0.1	1.4	75.4	5.6
6	68	male	LC	+	<2.6	-	47.7	0.1	0.1	>100	<3.0
7	37	female	LC	+	2.6	+	>2000	0.1	0.2	81.5	5.5
8	57	male	LC	+	<2.6	+	188.5	0.5	0.8	54.0	5.1
9	48	male	LC	-	<2.6	+	562.5	0.1	1.1	57.7	5.0
10	53	male	LC	-	4.4	+	>2000	0.1	49.2	96.1	7.5
11	34	female	FHF	-	4.9	-	374	7.9	0.8	93.9	5.7
12	28	female	FHF	-	4.6	-	19.5	133.8	4.6	54.4	7.4

Previous reports showed only trace amounts of HBV replication in extra-hepatic sites following LT (8). If HBV was present in hepatocytes, Lam would have masked the appearance of HBV-DNA regardless of the presence of intrahepatic HBV covalently closed circular (ccc)DNA (9, 10). These factors make it difficult to understand HBV dynamics following LT. Recently, new enzyme immunoassays for detecting HBV core antigen (HBcAg) (11) and HBV core-related antigen (HBcrAg) (12, 13) have been reported. These antigens move parallel with HBV-DNA in the serum and have a wide detection range (14). In particular, the assay for HBcrAg is able to detect both HBcAg and HBeAg even in anti-HBc antibody and anti-HBe antibody-positive specimens. Additionally, it has shown a higher sensitivity than HBV-DNA transcription mediated amplification (TMA), and equivalent sensitivity to in-house real time detection PCR (15). Different from the assay for HBV genome, the HBcrAg assay detects translational products of HBV and is presumed to be a reflection of cccDNA (16, 17). The HBcrAg assay has never been used to assess transplant patients undergoing HBV prophylaxis, and the status of HBV replication markers has also never been discussed in the case of post-transplanted patients, negative for HBsAg and HBV-DNA, who were undergoing anti-HBV prophylaxis. Therefore, in this study, we proposed to account for availability of HBcrAg assay, and discuss HBV dynamics in patients following HBV-related LT.

Abbreviation: HBsAg: hepatitis B virus s antigen, HBeAg: hepatitis B virus e antigen, HBcAg: hepatitis B virus core antigen, HBcrAg: Hepatitis B virus core-related antigen, cccDNA: covalently closed circular DNA, Lam: Lamivudin, HBIG: anti-HBs antigen immune globulin

Materials and Methods

Patients and clinical samples

From 2001 to 2006, a total of 12 patients with HBV-related severe liver disease, were admitted to Nagasaki University Hospital, Nagasaki, Japan, and enrolled in this study (Table 1). There were 8 men and 4 women with a median age of 52.0 years (range 28-68 years). All 12 patients had received LDLT at this hospital. The graft survival rate was 100%, and not one showed evidence of graft hepatitis. Of the 12 patients, 10 had been diagnosed with liver cirrhosis (LC) (with 7 of those having hepatocellular carcinoma), and 2 patients had been diagnosed with fulminant hepatic failure (FHF). All patients had been receiving a daily dose of 100 mg Lam since the pre-operation period in order to prevent the recurrence of HBV infection [range 0.1-22 months, mean (standard deviation: SD); 7.81 (8.17) months] and following LT, 4 patients began receiving Adv therapy [range; 19-250 days, mean (SD) 102.3 (128.2) days] in addition to Lam due to Lam resistant HBV mutations present before and after LT. Donor status of HBV serological makers such as HBsAg, HBsAb and HBcAb were negative. Prophylactic infusion of HBIG was administered to all patients using a fixed dosing schedule: 10,000 units intravenously at the anhepatic period and on the day following LDLT. Afterwards, a dose of 2,000 units of HBIG was given routinely over the long term with the aim of keeping the serum titer greater than 100 units/L. After LDLT, serum HBsAg, HBeAg or HBV-DNA were not detected in any patient. Serum samples for HBV were collected from each patient at the following three specified intervals: 1) at the pre-operation period during Lam or Lam/Adv treatment (ranges; 7.9±8.7 days prior to LT). 2) at the immediate post-operation period during which patients received combined prophylaxis, and immunosuppression with steroid and calcineurin inhibitor (within 37.7±20.3 days after LT), and 3) at the stable state period when patients received combined prophylaxis, and immunosuppression without steroid (18.1±16.7 months after LT). Serum concentration of aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (T-Bil), prothrombin time (PT), and albumin (Alb) were obtained from patient's medical records. All patients underwent needle liver biopsy every year after transplantation.

Abbreviation: HBsAb: hepatitis B virus s antibody, HBcAb: hepatitis B virus core antibody

Serological markers for HBV

HBsAg, HBsAb, HBeAg, HBeAb, and HBcAb were assessed by the chemiluminescence enzyme immunoassay (CLEIA) method, using a commercially available enzyme immunoassay kit (Lumipulse, Fuji Rebio, Tokyo, Japan). Serum concentrations of HBV DNA were determined using a

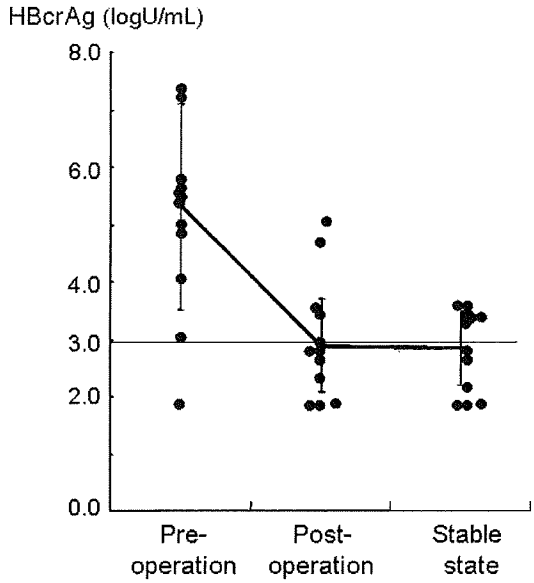

Figure 1. Serial changes of the HBcrAg levels. The HBcrAg levels are represented as mean values; the closed circles show the value of HBcrAg levels in all phases. Error bar is standard deviation. The mean value of HBcrAg levels in the post-operation period and in the stable state period were significantly lower than that in the pre-operation period (t-test, $p<0.05$). The detection range is above 3.0 logU/mL. To obtain the mean value, the value of 3.0 logU/mL or less and 2.0 logU/mL or more was added to the calculation.

polymerase chain reaction HBV monitoring kit (Roche, Tokyo, Japan), which had a quantitative range from 2.6 to 7.6 log copy/mL.

Serum concentrations of HBcrAg were measured using the CLEIA method reported previously (12, 18). In brief, 100 mL serum was mixed with 50mL of a pretreatment solution containing 15% sodium dodecylsulfate, and 2% Tween 60. After incubation at 70℃ for 30 minutes, 50mL of pretreated serum was added to test wells coated with monoclonal antibodies specific for denatured HBcAg and HBeAg (HB44, HB61, and HB114), and then filled with 100 mL assay buffer. The plate was incubated for 2 hours at room temperature and the wells were then washed with buffer. Alkaline phosphatase-labeled monoclonal antibodies specific for denatured HBcAg and HBeAg (HB91 and HB 110), were added to the wells, and the plate was again incubated at room temperature, this time for 1 hour. After washing, CDP-Star with Emerald II (Applied Biosystems, Bedford, MA) was added and the plate was incubated at room temperature one more time for 20 minutes. The relative chemiluminescent intensity was measured, and the HBcrAg concentration was determined by comparison with a standard curve generated using recombinant pro-HBeAg (amino acids, 10-183 of the precore/core gene product). The HBcrAg concentration was expressed as units/mL (U/mL) and the immunoreactivity of recombinant pro-HBeAg at 10 fg/ml corresponded to 1 U/mL. In this study, the cutoff value was tentatively set at 3.0 logU/mL (12).

Table 2. Comparison of the HBcrAg Levels between Lam Group and Combination Lam/Adv Group at Each Period

Group	Number	pre-operation	post-operation	stable
L: Lam	6	4.47 (1.62)	2.92 (1.19)	5.14 (0.72)
A: Lam+Adv	4	5.78 (1.17)	3.58 (0.78)	3.45 (0.17)

Abbreviation: HBeAb: hepatitis B virus e antibody

Statistical analyses

Statistical analyses were performed using the SPSS 11.0.1 J statistical software package (SPSS, Inc., Chicago, IL). The p-values of less than 0.05 were considered statistically significant.

Results

Serial changes in HBcrAg levels at indicated periods

Results of the HBcrAg assay showed differences in titers during the specific periods (Table 1 and Fig. 1); 11 cases had positive levels of HBcrAg, however 8 of them were negative for HBV-DNA. In the post-operation period, all cases were negative for HBV-DNA and HBsAg, however 5 of them (cases 4, 5, 7, 9 and 10) had positive levels of HBcrAg. In the stable state period, 6 of the cases (cases 2, 5, 7, 8, 9 and 10) had positive levels of HBcrAg. The 2 cases with FHF (cases11 and 12) had negative levels of HBcrAg in both post operation and the stable state periods. Of the 4 patients who received combined Lam/Adv treatment, 3 patients (cases 7, 9, and 10) also had positive HBcrAg levels in both post operation and the stable state periods. Two cases (cases 2 and 8) had negative levels of HBcrAg in the post operation period, but positive levels in the stable state period. The overall mean level of HBcrAg following LT [post-operation 3.05 (1.026) logU/mL and stable state periods 2.875 [(0.66) logU/mL] was significantly lower than that at pre-operation period [mean (SD); 5.25 (2.445) logU/mL] (Fig. 1). After LT, the levels of serum HBcrAg were decreased and steroid administration on early post-operation period did not seem to influence HBcrAg levels.

Comparison of the HBcrAg levels between combination Lam/Adv group and Lam group

A comparison of the mean values of HBcrAg levels between the group receiving only Lam treatment (6 patients with LC: Group L) and the group receiving combination Lam/Adv treatment (4 patients with LC: Group A) was made (Table 2). The mean value of HBcrAg in Group A was higher than that in Group L through all periods of the study [mean (SD) value (logU/mL) is as follows: pre-operation, Group A; 5.78 (1.17), Group L; 4.47 (1.62), post-operation, Group A; 3.58 (0.78), Group L; 2.92 (1.19), stable state, Group A 3.45 (0.17), Group L: 5.14 (0.72)]. No

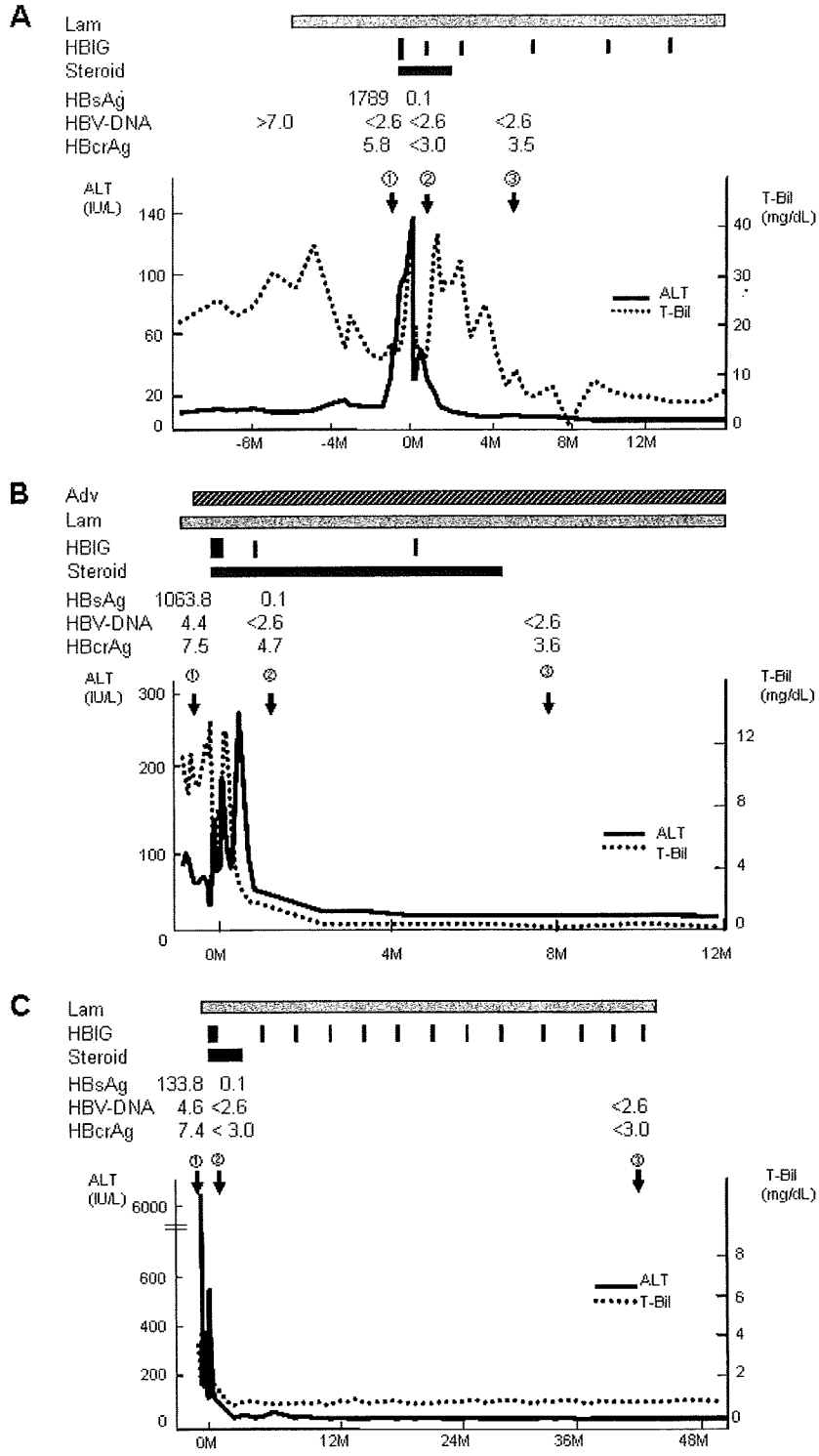

Figure 2. Clinical course of representative cases. A: Case 2 had suffered from LC. The value of crAg was below sensitivity in the immediate post-LT phase, then returned to a positive value in the post-LT late phase. B: Case 7 suffered from LC with Lam resistance. Adv was added to the Lam treatment at the pre-LT period, and continued after LT. C: Case 9 suffered from fulminant hepatic failure.

significant difference was noted between these groups in repeated statistical analysis of the data.

Patterns of serum HBcrAg levels compared with the clinical courses of selected cases

No correlation was made between the serum HBcrAg levels and AST, ALT, total bilirubin, prothrombin time, or albumin during any phase of this study (data not shown). Patterns of variation in serum HBcrAg levels were compared to the clinical courses of selected cases. A representative case (Fig. 3A, case 2) is a 60-year-old man with LC and hepatoma. He had been receiving Lam therapy for 5 months

prior to LT. His serum HBV-DNA became negative 1 month prior to LT. Liver function became worse and LT was performed for hepatic failure. His level of serum HBcrAg prior to LT was 5.8 logU/mL, and HBsAg was positive. Following LT, HBsAg became negative. His HBsAb titer was high due to HBIG. At post operation, his serum HBcrAg level fell below the cut-off level (2.8 logU/mL). However, levels of HBcrAg then rose to 3.5 logU/mL despite normal levels of ALT and total bilirubin. Negative levels of both HBsAg and HBV-DNA have continued to present. In case 8, whose HBcrAg level was negative in the post operation period but became positive in the stable state period, the levels of ALT and total bilirubin remained at normal levels in the observation period.

Case number 10 (Fig. 3B), a 53-year-old man with LC, began receiving Lam therapy 19 months prior to LT, however his ALT and T-Bil were in relapse due to Lam resistant HBV mutation. Therefore, the addition of Adv therapy was started 3 weeks prior to LT. Hepatic failure could not be prevented despite the addition of Adv, and LT was performed. At the time of LT, serum HBV-DNA and HBcrAg were 4.4 logU/mL and 7.5 logU/mL, respectively. Following LT, Lam and Adv therapy was continued and his levels of HBV-DNA have remained below the cut-off level (<2.6 logU/mL), but levels of HBcrAg have been positive, throughout both the post-operation period; 4.7 logU/mL, and the stable state period; 3.6 logU/mL. His liver function became stable following after LT.

Case number 12 (Fig. 3C), a 28-year-old woman with FHF, suffered from acute HBV infection. Since several courses of plasma aphaeresis, along with Lam therapy did not improve her condition, she underwent LT despite positive levels both of HBV-DNA (4.6 logU/mL) and HBcrAg (7.4 logU/mL). Following LT, serum HBV-DNA became negative, and serum HBcrAg levels have remained below the cut-off level (post-operating period; 2.0 logU/mL, stable state period; 2.3logU/mL). Her liver function became stable following LT.

Every case entered in this study underwent an annual liver biopsy in our hospital. All of the biopsy specimens in the stable state did not show any pathological features of chronic viral hepatitis despite the titer of HBcrAg in serum.

Discussion

This newly developed enzyme immunoassay for HBcrAg could be a useful measure of HBV activity in patients receiving anti-HBV prophylaxis following LT. Serum HBcrAg was detected prior to LT in all patients, and the levels varied in the early and late post operation period. Our use of HBcrAg assay shows that HBV replication is occurring in patients receiving combination prophylaxis following LT, and that LT itself decreased levels of HBcrAg. Since LT decreased the levels of serum HBcrAg, then the use of steroid did not have any influence on HBcrAg levels. The value of HBcrAg varies over time, but it has no relationship to hepatic function. However, further observation is necessary to evaluate the relationship between the detection of HBcrAg and the long-term prognosis of these patients.

It has been reported that serum HBcrAg levels can be thought of as a non-relapse marker at the time of Lam cessation (15), and a risk marker for HBV resistance at the 6 month point in Lam treatment (16). Lam blocked the reversed transcription of HBV-RNA to HBV-DNA, but did not inhibit translation or transcription. Cessation of Lam at the absence of serum HBV-DNA causing a flare up of HBV replication, due to the existence of HBV cccDNA, which is a template for the HBV pregenome RNA, may be a source of Lam resistant HBV strains in hepatocytes (8, 19). The levels of cccDNA in hepatocytes, as well as HBcrAg in serum, but not HBV-DNA in serum, are also a prediction marker of sustained anti-viral response in Lam treatment (20, 21). Production of HBcrAg in hepatocytes as a reflection of HBV replication activity, indicates the existence of cccDNA in hepatocytes. Therefore, the concentration of HBcrAg in the serum of a patient receiving Lam treatment may indicate an altered HBV replication status within the hepatocytes (22). We feel the HBcrAg assay is a reliable means for identifying HBV replication following HBV-related LT, and think that HBV replication continues following LT despite combination HBIG and Lam prophylaxis. The sensitivity of HBcrAg is not very high in HBsAg seroclearance patients (17). Since HBsAg and HBV-DNA had not been detected in post LT patients receiving combination prophylaxis, HBcrAg assay can be a predictive maker of HBV replication at this stage. Recently, it was reported that HBV cccDNA in hepatocytes (23), HBV-RNA (22, 24) and serum HBsAg quantitative (25) are HBV replication markers. In addition to the HBcrAg assay, we should evaluate these markers to fully understand HBV dynamics after LT.

Previous reports have suggested that Lam resistant, HBV-infection related-LT was as safe as wild type HBV-infection-related LT (26). These reports concluded that a combination of Adv and Lam therapy provides effective prophylaxis in patients with pre-LT Lam resistant HBV mutants (26, 27). However, positive HBV-DNA was observed in all of the patients in the present study, and Adv and Lam resistant HBV has recently been observed (28). In our study, in the stable period, the titer of HBcrAg in Lam group was relatively higher than Adv add-on group. Further study is needed to evaluate Adv add-on Lam combined prophylaxis.

The production site of HBcrAg was unclear in the post-LT period. In cases of HCV-related LT, non-hepatic virus sources, at the most, account for 4% of the total viral production, and post-LT viral clearance, after rapid initial decline, slows, possibly due to the filling of absorption sites in newly grafted liver (29). HBV re-infection may be caused by the over-production of HBV in extrahepatic sites or HBV circulating particles following LT (30). Escaped mutants from HBIG and Lam may also cause re-infection (31). According to a recent report (32), highly sensitive real-time PCR of cccDNA found that cccDNA in PBMCs is detected

only to a small degree. As such, PBMCs are unlikely to function as a reserve of HBV. In HCV-related LT, it has been reported that the virus immediately re-infects liver grafts (33, 34), but re-infection of the graft is not apparent in HBV. We can not disregard production of HBcrAg in hepatocytes following LT, but further studies are necessary to fully understand HBV replication sites following LT.

In addition to HBsAg and HBV-DNA, HBcrAg assessment could be a practical tool as a marker of HBV replication after LT. Because the levels of HBcrAg are a reflection of cccDNA, we think that the HBcrAg positive cases need continual prophylaxis following LT. In addition, the evaluation of HBV dynamics by HBcrAg assay must be studied to determine the appropriate prophylaxis against replication of HBV following LT.

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