an accelerated response of antidonor $CD8^+$ T cells despite the use of the same immunosuppressive protocol. Therefore, SFSG likely accelerated the antidonor response, enhanced rejection, and might result in a worse survival rate, although SFSS is multifactorial in nature. This finding is consistent with the results in an animal model (*25*) and our preliminary data using mouse model (data not shown). To our knowledge, this is the first clinical report to state that SFSG may accelerate antidonor immune responses in LDLT.

We previously reported that liver sinusoidal endothelial cells (LSECs) of grafts induce allospecific immunotolerance by suppressing reactive T cells through Fas ligand and/or PD-L1 signaling (*26–28*). Based on this concept, one possible mechanism is that the sinusoidal structure and LSECs could be damaged by portal hypertension and lose their tolerogenicity, resulting in accelerated antidonor immunoresponse and rejection. This interpretation is consistent with that of previous reports stating that portal hypertension disrupts sinusoids and LSECs in the liver (*8*). Another possibility is antirejection and/or the anti-inflammatory effect of PGE1 itself. It has been reported that PGE1 could prevent ischemia-reperfusion injury by inducing heat shock protein (*29*) or by inhibition of neutrophil adherence (*30*). It has been also reported that administration of PGE1 could prevent and suppress the rejection process in heart transplantation (*31*) and in renal transplantation (*32*). Furthermore, it has been reported that PGE1 protects human LSECs from apoptosis, which is consistent with our findings (*33*).

The main limitation of our study is its retrospective nature. Another limitation is the relatively small number of patients in each subgroup analyzed, although the background characteristics of each group and the survival rates of non-SFSG patients were similar. A randomized study is ideal; however, performing a prospective randomized study for this approach is difficult because of the high mortality of SFSG patients without PGE1 perfusion, as shown in Results.

In conclusion, continuous infusion of PGE1 is suggested to be useful in improving SFSG function and survival after LDLT. Improved understanding of underlying mechanisms may have important implications for clinical managements such as antirejection therapy or preventing ischemia-reperfusion injury in liver transplantation with SFSGs.

MATERIALS AND METHODS

Patients

From July 2003 to December 2009, LDLT was performed on 122 adult patients with end-stage liver disease. We introduced continuous portal infusion of PGE1 to five patients with SFSGs that exhibited a GRWR less than 0.72% from November 2007 to December 2009 (era 2). These patients (PG group) were the subjects of this retrospective case–control study. We compared them with a historical group of eight relevant patients who received SFSG without PGE1 infusion (non-PG group) from July 2003 to October 2007 (era 1) to determine the safety and efficacy of continuous PGE1 portal infusion for SFSGs. Because we introduced the portal infusion of PGE1 in November 2007, all patients with SFSGs in era 2 received PGE1 infusion. One SFSG case with splenectomy in era 1 was excluded from this study. The study protocol was approved by the ethics committee of Hiroshima University, and all patients provided informed consent before surgery. None of the patients receiving PGE1 portal infusion showed clinical evidence of insertion site infection or bleeding after catheter removal throughout the follow-up period.

Operation, PVP Measurement, and Continuous Portal Infusion of PGE1

The graft-harvesting technique, recipient surgery, perioperative recipient management, and immunosuppression regimens were conducted as described previously with minor modifications (*34, 35*). In brief, the right lobe without the MHV or the left lobe with the MHV was harvested from the donor as follows. Before parenchymal transection, the right or left lobe was mobilized and the short hepatic veins were transected. For the right lobe, during parenchymal transection, the major right tributaries of the MHV were clamped using a vascular clip and then transected. After hepatectomy, ex vivo perfusion of the graft was performed through the portal vein. The initial perfusate was saline solution (500 mL); then, the University of Wisconsin solution (1000 mL) was used as the perfusate.

To measure the PVP in the recipient during the operation, an 18G catheter was inserted from the mesenteric vein to the portal vein after laparotomy (*36*). The implantation was performed after total hepatectomy. The graft vein was anastomosed to the equivalent vein of the recipient in an end-to-end fashion. Thereafter, the graft was reperfused before microsurgical reconstruction of the hepatic artery (end-to-end anastomosis of the graft hepatic artery to the recipient hepatic artery). The bile duct of the graft liver was anastomosed in an end-to-end fashion to the recipient's common hepatic bile duct. In the non-PG group, the portal catheter was removed at the time of abdominal closure. In the PG group, PGE1 was administered through a portal catheter at the graft portal reflow. PGE1 was continuously administered for 1 week (0.01 g kg^{-1} min^{-1}) and then the catheter was noninvasively removed. AST, ALT, serum bilirubin, serum ammonia, and AKBR levels were measured as liver function indices. The initial immunosuppressive regimen consisted of tacrolimus and steroids. Doppler ultrasonography and computed tomographic scans were routinely performed daily and biweekly, respectively.

Immunosuppression was initiated with a protocol based on tacrolimus (Prograf; Astellas Pharma, Tokyo, Japan) and methylprednisolone. Methylprednisolone was withdrawn gradually according to the protocol. The dose of tacrolimus was controlled according to blood concentration and adjusted daily. The target trough level was set at 15 ng/mL for 2 weeks and 10 ng/mL for another 2 weeks. Continuous venous infusion of heparin for therapeutic heparinization was routinely done to prevent thrombosis, which was monitored using coagulation tests. Rejection was diagnosed and proven by biopsy histologically and MLR assay. Patients were followed for 2 years after LDLT, and survival was defined as the period between LDLT and death.

Immune Monitoring by In Vitro CFSE-MLR Assay

CFSE-MLR was routinely performed to evaluate the recipient's antidonor immune response 2 to 4 weeks after surgery.

For CFSE-MLR, peripheral blood mononuclear cells prepared from the blood of the recipients (autologous control), donors, and healthy volunteers with the same blood type as the donors (third-party control) for use as the stimulator cells were irradiated with 30 Gy. Those obtained from the recipients for use as the responder cells were labeled with 5 mM CFSE (Molecular Probes, Eugene, OR), as described previously (*24*). The stimulator and responder cells (2×10^6 each) were incubated in 24-well flat-bottomed plates in a total volume of 2 mL culture medium at 37°C under 5% CO_2 for 5 days. After culture for MLR, $CD4^+$ and $CD8^+$ T-cell proliferation, CD25 expression of proliferating T-cell subsets and SI were quantified by flow cytometry as described previously (*37, 38*) and described in the SDC Materials and Methods in detail (see http://links.lww.com/TP/A807).

Statistical Analysis

Statistical analysis and comparisons were performed using PRISM version 4.0 (GraphPad, San Diego, CA). Data are expressed as mean±SEM. An unpaired *t* test with Welch's correction was used to compare groups. *P* values <0.05 were considered statistically significant.

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INVITED COMMENTARY

Is living donor liver transplantation really equivalent to deceased donor liver transplantation?*

Hideki Ohdan

Department of Gastroenterological and Transplant Surgery, Applied Life Science, Institute of Biomedical and Health Science, Hiroshima University, Hiroshima, Japan

Correspondence
Hideki Ohdan MD, PhD,
Department of Gastroenterological and
Transplant Surgery, Applied Life Science,
Institute of Biomedical and Health Science,
Hiroshima University 1-2-3 Kasumi,
Minami-ku, Hiroshima 734-8551, Japan.
Tel.: +81-82-257-5220;
fax: +81-82-257-5224;
e-mail: hohdan@hiroshima-u.ac.jp

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*Invited commentary on "Living donor versus deceased donor liver transplantation: A surgeon-matched comparison of recipient morbidity and outcomes", by Reichman *et al.*

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Living donor liver transplantation (LDLT) has developed as an alternative to deceased donor liver transplantation (DDLT) in order to overcome the critical shortage of deceased organ donations. Particularly in regions with low deceased donation rates, like Asian, LDLT for end stage liver disease significantly reduces the risk of death or drop off the wait list without compromising post-transplant survival. A preference for LDLT to DDLT may depend on the original disease representing the indication for liver transplantation (LT). LDLT offers a timely alternative to DDLT for patients with hepatocellular carcinoma (HCC). However, the higher recurrence rate of HCC after LDLT and the indication criteria remain controversial. One of the recent quantitative meta-analyses revealed the comparable patient survival rates and no significant differences in the recurrence rates between LDLT and DDLT recipients [1]. Another meta-analysis provided evidence of lower disease-free survival (DFS) after LDLT compared with DDLT for HCC [2]. Hence, LDLT likely represents an acceptable option that does not compromise patient survival or increase HCC recurrence in comparison with DDLT at this moment.

Early data suggested that patients with Hepatitis C virus (HCV) that received a LDLT had worse outcomes, including increased rates of cholestatic HCV than did recipients of DDLT [3,4]. This is currently thought to be because of an increased rate of biliary complications or other problems seen during the learning curve of early LDLT experience. More recent data demonstrated that there is no difference in recurrent HCV between recipients of DDLT and LDLT [5,6]. The latest meta-analysis demonstrated that LDLT was equivalent to DDLT in terms of long-term patient or graft survival, HCV recurrence, and acute rejection with a potential lower short-term graft survival [7].

There are limited convincing data comparing outcomes of LDLT and DDLT for autoimmune hepatitis (AIH) and cholestatic liver diseases. It has been previously reported that the overall survival outcomes of LDLT were similar to DDLT in patients with AIH and primary biliary cirrhosis

[8]. In contrast, patients with primary sclerosing cholangitis undergoing LDLT, especially with biologically related donors, are thought to have a higher risk to develop recurrent disease compared with the DDLT setting, probably because of sharing antigens targeted by autoimmunity between recipients and the related donors [9]. Further prospective studies at transplant centers performing both LDLT and DDLT might be needed to confirm these issues.

Regardless of such original disease, LDLT offers several advantages over DDLT, which include the reduction in waiting time mortality, the reduction in cold ischemic time (CIT) and the feasibility of various preoperative interventions, such as nutritional treatment for both the donor and recipient [10]. However, it remains unclear whether those advantages offset disadvantages peculiar to LDLT, such as the smaller graft volume than DDLT and the highly technical procedure, which may be associated with higher complication rates. This seems to be caused by a fact that direct comparison of the results between LDLT and DDLT inevitably involves various biases in nature.

Reichman *et al.* [11] have performed a retrospective matched-cohort study to compare postoperative complication rate and patient survival in the two groups of patients submitted to LDLT and to DDLT. Six clinical variables for recipients: age, Meld, date of transplant, gender, primary diagnosis, and recipient surgeon were matched in each group (n = 145 in each group). They found that the overall complication rate was similar between two groups. In further detail, biliary complications were higher in LDLT although the complications that occurred in the DDLT were strongly associated with graft loss. Graft and patient survival outcomes for LDLT versus DDLT were similar. From those findings, they concluded that LDLT offers an excellent alternative to DDLT in areas of deceased donor organ shortages. This study defined surgical complications that are more frequent in LDLT, i.e., biliary complications (34% and 17% in LDLT and DDLT cohorts, respectively). Despite a higher rate of complications among LDLT recipients, complications leading to death were not significantly higher in LDLT in the experienced center. These findings, in concert with the current common consent that the incidence of complications, even biliary complications, can decline with center experience to levels comparable with DDLT [12], underscore the impact of the learning curve on this highly technical procedure. Potential recipients need to hear about both the rates of complications after LDLT and DDLT, and this study with control for recipient variables will help to define those rates. As pointed out by the authors, this study left control for donor variables out of consideration, despite a well known fact that donor age/gender and donor-recipient human leukocyte antigen matching correlate with either the incidence of certain complications or the severity of original disease recurrence. Nevertheless, this case control comparison of the outcome of LDLT and DDLT convincingly reported that these procedures had different complication profiles but the overall outcomes were similar with expert management, suggesting that the biological advantage in LDLT could compensate for a higher rate of surgical complications caused by greater technical complexity.

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ELSEVIER

Comparative Analysis of T-Cell Depletion Method for Clinical Immunotherapy—Anti-Hepatitis C Effects of Natural Killer Cells Via Interferon-γ Production

M. Ohira, S. Nishida, T. Matsuura, I. Muraoka, P. Tryphonopoulos, J. Fan, A. Tekin, G. Selvaggi, D. Levi, P. Ruiz, C. Ricordi, H. Ohdan, and A.G. Tzakis

ABSTRACT

Liver transplantation (LT) is a life-saving treatment for liver cirrhosis patients with hepatocellular carcinoma (HCC). However, 10%–20% HCC recurrence rate after LT is due to the immunosuppression inducing tumor growth. We recently reported a novel immunotherapy with donor liver natural killer (NK) cells to prevent HCC and hepatitis C virus (HCV) recurrence after LT. In this cell processing procedure, Muromonab-CD3 (Orthoclone OKT3, an anti-CD3 antibody) was added to the culture medium to deplete $CD3^+$ T cells to prevent graft-versus-host disease. However, the manufacture of OKT3 was discontinued in 2010, when other treatments with similar efficacy and fewer side effects became available. In this study, we examined alternative reagents for T-cell depletion-MACS GMP CD3 pure (GMP CD3), antithymocyte globulin, and alemtuzumab-for NK cell immunotherapy in the allogeneic setting. We observed that GMP CD3 showed exactly the same effects on liver mononuclear cells as OKT3, including activation of NK cells and depletion of T cells. Interestingly, binding of T-cell depletion antibodies to NK cells led to an anti-HCV effect via interferon-γ production. These results with the use of in vitro culture systems suggested that antibodies which produce T-cell depletion affected NK cell function.

Liver failure and hepatocellular carcinoma (HCC) caused by chronic hepatitis C virus (HCV) infection are the most common indications for liver transplantation (LT). The incidences of both conditions have been projected to increase further. On the one hand, the rate of HCC recurrence after LT is 10%–20%.[1,2] On the other hand, recurrent HCV infection in the allograft, which is universal, occurs immediately after LT and is associated with accelerated progression to liver cirrhosis, graft loss, and death.[3,4] These recurrences remains the most serious issue with LT. The use of postoperative immunosuppressants poses an additional risk for recurrences and hinders the use of chemotherapeutic or interferon (IFN) agents.[5,6] However, no definitive treatment or prevention for HCC recurrence after LT is known.

Natural killer (NK) cells are innate immune lymphocytes that are identified by their expression of the CD56 surface antigen and the absence of CD3 markers.[7,8] NK cells can directly kill targets through the release of granzymes, which are granules containing perforin and serine proteases, and/or by surface-expressed ligands that engage and activate death receptors expressed on target cells. Unlike T cells, NK cells do not require the presence of a specific antigen to kill cancer cells, modified cells, or invading infectious microbes. NK cells are abundant in the liver, in

From Department of Gastroenterologic and Transplant Surgery (H.O.), Applied Life Sciences, Institute of Biomedical & Health Sciences, Hiroshima University, Hiroshima, Japan; Division of Liver and Gastrointestinal Transplantation, (M.O., S.N., T.M., I.M., P.T., J.F., A.T., G.S., D.L.), Department of Surgery, University of Miami Miller School of Medicine, Miami, Florida; Department of Pathology and Surgery (P.R.), University of Miami Miller School of Medicine, Miami, Florida; Cell Transplant Center, (C.R.) Diabetes Research Institute, University of Miami Miller School of Medicine, Miami, Florida; Division of Liver and Gastrointestinal Transplantation (A.G.T.), Department of Surgery, University of Miami Miller School of Medicine, Miami, Florida; Department of Surgery, Cleveland Clinic, Weston, Florida.

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Address reprint requests to Seigo Nishida, MD, PhD, Division of Liver and Gastrointestinal Transplantation, Department of Surgery, University of Miami Miller School of Medicine, 1801 NW 9th Avenue, Miami, FL 33136. E-mail: snishida@med.miami.edu

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contrast to their relatively small distribution in peripheral lymph and lymphatic organs in rodents[9–11] and humans.[12,13] In addition, hepatic NK cells in humans have been shown to mediate cytotoxic activity against HCC[12] and to display anti-HCV effects[14] compared with their peripheral blood counterparts. We have successfully applied adoptive immunotherapy with liver NK cells to LT recipients with HCC in Japan and the United States.[14–16] In this regimen, LT recipients are injected intravenously with interleukin (IL) 2–activated NK cells derived from the donor liver allograft. After treatment with IL-2 and OKT3 (Orthoclone OKT3, an anti-CD3 monoclonal antibody [mAb]; Ortho Biotech, Raritan, NJ), liver NK cells expressed significantly elevated levels of the tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), a crucial molecule for killing of tumor cells. Furthermore, these cells showed great cytotoxicity against HCC without any effect on normal cells.[12]

OKT3, a potent immunosuppressant, has been shown to reverse renal allograft rejection episodes.[17,18] It has also been widely used for immunotherapy, as well as to expand cytotoxic T cells[19] and enhance the activity of lymphokine-activated killer (LAK) cells,[20–25] and prevent graft-versus-host disease (GVHD).[26–29] In the latter setting, administration of OKT3-coated T cells in vivo opsonizes for the reticuloendothelial system to subsequently trap or lyses cells.[30–32] This method has been used for clinical NK therapy in Japan, achieving protection against GVHD.[14] However, because of its numerous side effects, the availability of better-tolerated alternatives, and its declining use, OKT3 has been recently removed from the market. Therefore, alternative reagents need to be evaluated for this immunotherapy. In the present study, we evaluated the effect of alternative reagents-GMP CD3 (MACS GMP CD3 pure; Miltenyi Biotec, Bergisch Gladbach, Germany), antithymocyte globulin (Thymoglobulin; Genzyme, Cambridge, MA), and alemtuzumab (Campath; Genzyme) using culture systems with NK and T cells for subsequent application in clinical trials.

MATERIALS AND METHODS

Isolation of Liver Mononuclear Cells

Liver mononuclear cells (LMNCs) from liver perfusates were isolated by gradient centrifugation with Ficoll-Hypaque (GE Healthcare, Pittsburgh, PA) before suspension in X-Vivo 15 medium (Lonza, Walkersville, MD) supplemented with 100 μg/mL gentamicin (APP Pharmaceuticals, Schaumburg, IL), 10% human AB serum (Valley Biomedical, Winchester, VA), and 10 U/mL sodium heparin (APP Pharmaceuticals), as previously described.[16] Our Institutional Review Board (IRB) approved this study.

Cell Culture

LMNCs were cultured with 1,000 U/mL human recombinant IL-2 (Proleukin; Novartis, Emeryville, CA) in culture medium at 37°C in an atmosphere supplemented with 5% CO_2. LMNCs were exposed to a OKT3 (1 μg/mL), GMP CD3 (1 μg/mL), antithymocyte globulin (100 μg/mL), or alemtuzumab (100 μg/mL) at 1 day before cell harvest. After 4 days of culture, cells were subjected to further analyses.

Flow Cytometry

All flow cytometry (FCM) analyses were performed on an LSR II Flow Cytometer (BD Biosciences, San Jose, CA). The following mAbs were used for surface staining of the lymphocytes: fluorescein isothiocyanate–conjugated anti-CD3 (HIT3a; BD Pharmingen, San Diego, CA) or anti-CD56 (B159; BD Pharmingen); phycoerythrin (PE)-conjugated anti-TRAIL (RIK-2; BD Pharmingen), anti-NKp44 (P44-8.1; BD Pharmingen), or anti-CD158b (CH-L; BD Pharmingen); allophycocyanin (APC)–conjugated anti-CD56 (B159; BD Pharmingen), anti-CD25 (M-A251; BD Pharmingen), or anti-NKG2A (Z199; Beckman Coulter, Fullerton, CA); APC-eFluor780–conjugated anti-CD3 (UCHT1; eBioscience, San Diego, CA); PE-Cy7–conjugated anti-CD69 (FN50; Biolegend, San Diego, CA), or anti-NKG2D (1D11; Biolegend); eFluor 605NC–conjugated anti-CD16 (eBioCB16; eBioscience); Alexa Fluor 647–conjugated anti-NKp30 (P30-15; Biolegend); peridinin chlorophyll protein complex (PerCP)-Cy5.5–conjugated anti-CD158a (HP-MA4; eBioscience); and biotin-conjugated anti-CD122 (Mik-b3; BD Pharmingen), anti-NKp46 (9E2; Biolegend), or CD132 (TuGh4; BD Pharmingen). The biotinylated mAbs were visualized with the use of PerCP-Cy5.5-streptavidin (eBioscience) or PE-Cy7-streptavidin (Biolegend). Dead cells were excluded by light scatter and 4′,6-diamidino-2-phenylindole staining (DAPI; Invitrogen, Carlsbad, CA). FCM analyses were performed with Flowjo software (Tree Star, Ashland, OR).

Cytotoxic Assay

The cytotoxicity assay was performed by FCM as previously described.[16] Briefly, target cells labeled with 0.1 μmol/L carboxyfluorescein diacetate succinimidyl ester Cell Tracer Kit (Invitrogen) for 5 minutes at 37°C in 5% CO_2 were washed twice in phosphate-buffered saline solution, resuspended in complete medium, and counted with the use of trypan blue staining. The effector and target cells were coincubated at various ratios for 1 hour at 37°C in 5% CO_2. As a control, target cells or effector cells were incubated alone in complete medium to measure spontaneous cell death after DAPI was added to each tube. The data were analyzed with the use of Flowjo software. Cytotoxic activity was calculated as a percentage with the following formula: % cytotoxicity = [(% experimental $DAPI^+$ dead targets) − (% spontaneous $DAPI^+$ dead targets)]/[(100 − (% spontaneous $DAPI^+$ dead targets)] × 100.

ELISA

IFN-γ production of LMNCs during the culture was measured by enzyme-linked immunosorbent assay (ELISA) (Biolegend). Supernates collected after the incubation were stored at −80°C until further use. IFN-γ ELISA was performed according to the manufacturer's instructions.

Coculture with HCV Replicon Cells

The Huh7/Rep-Feo cell line (HCV replicon cells) was kindly provided by Dr N Sakamoto (Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo, Japan). The HCV subgenomic replicon plasmid, pRep-Feo, was derived from pRep-Neo (originally pHCVIbneo-delS).[33] pRep-Feo carries a fusion gene comprising firefly luciferase and neomycin phosphotransferase, as described elsewhere.[34,35] After culture in the pres-

ence of G418 (Invitrogen), Huh7/Rep-Feo cell lines showed stable expression of the replicons. We used transwell tissue culture plates (pore size 1 μm; Costar, Cambridge, MA) for coculture experiments. HCV replicon cells (10^5 cells) were incubated in the lower compartment with various numbers of lymphocytes in the upper compartment. The HCV replicon cells in the lower compartments were collected at 48 hours after the coculture for luciferase assays in duplicate with the use of a luminometer (TriStar LB 941; Berthold Technologies, Oak Ridge, TN) with the Bright-Glo Luciferase Assay System (Promega, Madison, WI).

Statistical Analysis

Data are presented as mean ± SEM. The statistical difference between results were analyzed by Student *t* test (2 tailed), using the Statistical Package for the Social Sciences (SPSS) software version 19 for Windows (IBM Corp, Armonk, NY). *P* values of ≤.05 were considered to be statistically significant.

RESULTS

Effect on the Surface Phenotype of LMNCs

In 5 LMNC preparations, the addition of OKT3 GMP CD3 to IL-2–stimulated LMNCs decreased $CD3^+CD56^-$ T cells to 0.2% ± 0.1% and 0.2% ± 0.1%, respectively, from the IL-2–only control value of 28.1% ± 12.3%. In contrast, $CD3^+CD56^-$ T cells were retained among LMNCs with the addition of antithymocyte globulin or alemtuzumab: 3.3% ± 2.0% and 17.2% ± 7.3%, respectively. The proportion of $CD3^-CD56^+$ NK cells increased by ~10% in all groups (Fig 1A).

Addition of OKT3 or GMP CD3 to IL-2–stimulated LMNCs maintained both activation and inhibitory markers on NK cells. Interestingly, the expressions of TRAIL, CD25 (IL-2αR), and CD132 (IL-2γR) were increased in the antithymocyte globulin group. Furthermore, both antithymocyte globulin and alemtuzumab completely blocked the expression of CD16 on NK cells (Fig 1B).

Cytotoxic Capacity

Cytotoxicity assays were performed with the use of freshly isolated cultured LMNCs as effectors and K562 cells as targets. Fig 2 shows freshly isolated LMNCs barely mediated cell death, whereas IL-2–stimulated LMNCs produced significant cytotoxicity. Although the ratios of $CD3^-CD56^+$ to $CD3^+CD56^+$ cells varied after treatment with various

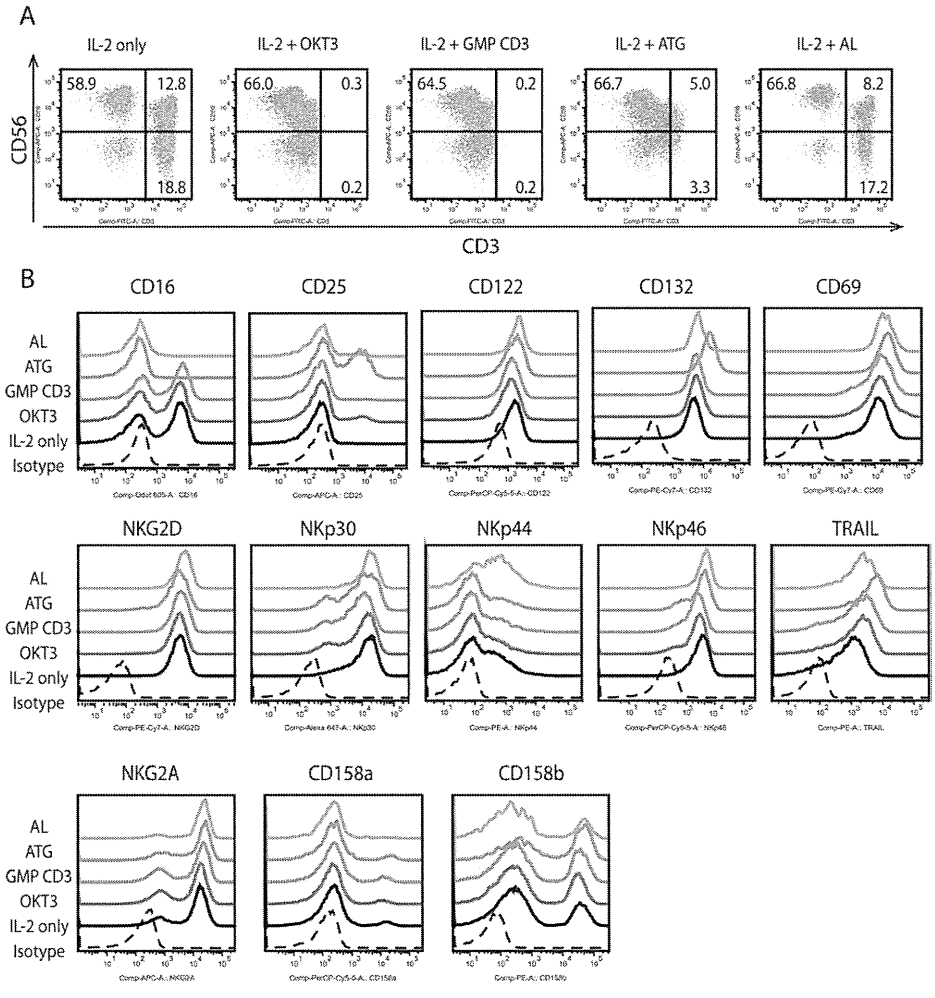

Fig 1. Effect of the T-cell depletion antibodies on the phenotypic characteristics of liver mononuclear cells (LMNCs). LMNCs obtained from cadaveric donors were stimulated with IL-2 (1000 U/mL) for 4 days. Anti-CD3 mAb (OKT3; 1 μg/mL), MACS GMP CD3 pure (GMP CD3; 1 μg/mL), antithymocyte globulin (ATG; 100 μg/mL), or alemtuzumab (AL; 100 μg/mL) was added to the culture medium 1 day before cell harvesting. (A) The LMNCs were stained with monoclonal antibodies against CD3 and CD56. The numbers indicate the mean percentages of the population. (B) Histograms show the logarithmic fluorescence intensities obtained on staining for each surface marker after gating on the $CD3^-CD56^+$ NK cells. Dotted lines indicate negative control samples with isotype-matched mAbs. The flow cytometry dot plot and histogram profiles represent 5 independent experiments. TRAIL, tumor necrosis factor–related apoptosis-inducing ligand.

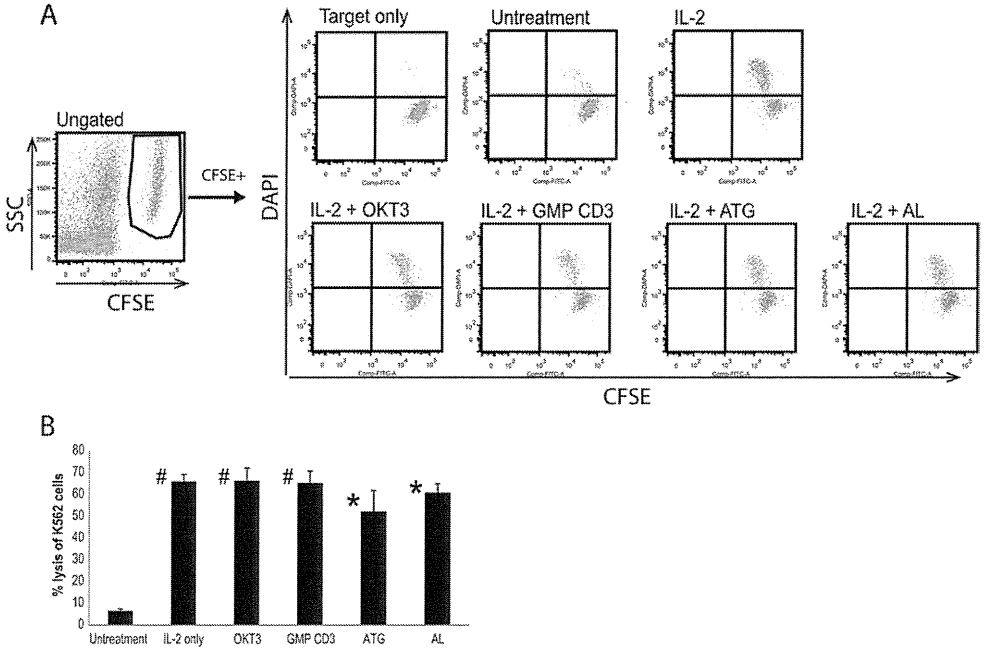

Fig 2. Antitumor effect of the T-cell depletion antibodies on IL-2–stimulated liver mononuclear cells (LMNCs). The NK cell cytotoxic activities of untreated cells and IL-2–stimulated LMNCs treated with various reagents were analyzed by a flow cytometry (FCM)–based cytotoxic assay. (A) Gate is set on cells to discriminate $CFSE^+$ targets from LMNCs. Gate is set on target to obtain the number of live and dead K562 cells. The FCM dot plot profiles represent 5 independent experiments. (B) The data represent the mean ± SEM of the percentage of target lysis at effector-to-target (E:T) ratios of 10:1 (5 LMNCs; $^{\#}P < .01$; $^{*}P < .05$ vs untreated group, *t* test).

T-cell depletion reagents for 4 days in culture, all cultured LMNCs exhibited vigorous cytotoxicity against K562. LMNCs treated with antithymocyte globulin showed slightly decreased cytotoxicity compared with the other groups, but the difference was not significant. This tendency was similar to that reported in an earlier study.[36] The cultured LMNCs did not show cytotoxicity against self-lymphoblasts (data not shown).

Anti-HCV Activity

IL-2–cultured LMNCs inhibited 40% luciferase reporter activity compared with freshly isolated LMNCs (Fig 3A). As we have reported before, the anti-HCV effect of IL-2–activated LMNCs was strongly enhanced by OKT3 treatment.[14] GMP CD3 treatment showed ~80% decreased HCV replication, which was almost the same effect as that caused by OKT3. Surprisingly, antithymocyte globulin and alemtuzumab treatment also elicited robust anti-HCV effects on LMNCs. We previously reported that IFN-γ secreted from LMNCs activated by IL-2 and OKT3 was responsible for the anti-HCV activity of these cells.[14] Cultured LMNCs also actively produced large amounts of IFN-γ (Fig 3B), which probably played a pivotal role in their anti-HCV activity.

DISCUSSION

In this study, we discovered GMP CD3 to be an alternative reagent to OKT3 for immunotherapy using liver NK cells.

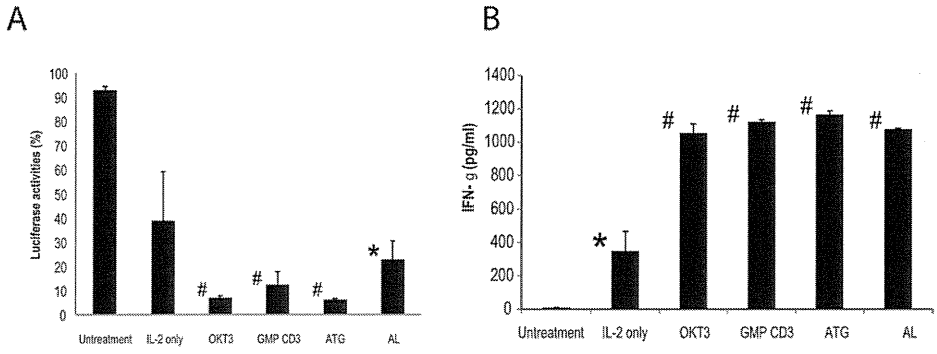

Fig 3. Anti–hepatitis C virus (HCV) effect of the T-cell depletion antibodies on IL-2–stimulated liver mononuclear cells (LMNCs). The LMNCs cultured for 4 days in the presence of IL-2 and various reagents were incubated with HCV replicon–containing cells for 48 hours in transwell tissue culture plates (effector-to-target ratio, 10:1). (A) Luciferase activity of HCV replicon–containing cells in the presence of effectors, normalized to luciferase activity in the absence of effectors. The difference in anti-HCV effect between the reagent-treated LMNCs and the freshly isolated LMNCs was statistically significant (5 LMNCs; $^{\#}P < .01$; $^{*}P < .05$ vs untreated group, *t* test). (B) IFN-γ production during the culture, as measured by ELISA [mean ± SEM (5 samples; $^{\#}P < .01$; $^{*}P < .05$ vs untreated group, *t* test)].

We compared the phenotypes and functions of LMNCs after treatment with various T-cell depletion regents, showing that GMP CD3 displayed same results as OKT3. Treatment with other T-cell depletion reagents, such as antithymocyte globulin and alemtuzumab, revealed unexpectedly strong cytotoxicity and anti-HCV effects on liver NK cells. Although antithymocyte globulin and alemtuzumab are difficult to use in immunotherapy because they completely bind the CD16 ligand on NK cells, these antibodies might affect NK cell function in in vitro culture systems.

This in vitro study showed that after treatment with GMP CD3 the degree of T-cell contamination and the NK cell phenotype and function, were similar to those after OKT3 treatment. T-Cell contamination was significantly decreased by either GMP CD3 or OKT3 treatment (Fig 1A). The 0.2% $CD3^+$ T-cell persistence in the final product represents an acceptable level for allogeneic transplantation.[16] Residual OKT3-coated T cells were dysfunctional. The NK cell percentage was the same in both groups. GMP CD3 treatment did not affect NK cell phenotype, including activation receptors, inhibitory receptors, and TRAIL. $CD3^-CD56^+$ NK cells expressed CD16, CD69, NKG2D, NKp30, NKp40, NKp46, TRAIL, and killer cell immunoglobulin–like receptors (KIRs), such as CD158a and CD158b (Fig 1B). Functional assays revealed that cytotoxicity and anti-HCV activity were maintained after GMP CD3 treatment. These results were reasonable, because both OKT3 and GMP CD3 are mouse IgG2as, whose Fc R receptor binds poorly to CD16. No animal- or human-derived components were used for the manufacture of this antibody. GMP CD3 is a reagent for research use and ex vivo cell culture processing only. It is not intended for in vivo human applications. GMP CD3 is manufactured and tested under a certificated ISO 9001 quality system in compliance with relevant GMP guidelines. It was designed following the recommendations of USP 1043 on ancillary materials.[36] GMP CD3 has been applied to expand cytokine-induced killer cells.[37]

In this study, we chose to examine the effects of other T-cell depletion antibodies. Currently, a wide variety of both polyclonal antibodies (antithymocyte globulin) and mAbs (alemtuzumab) are routinely used to deplete T cells in organ transplantation. Antithymocyte globulin contains a wide variety of antibody specificities directed toward immune response antigens, adhesion and cell trafficking molecules, and markers of heterogeneous pathways, including CD2, CD3, CD4, CD8, CD11a, CD16, CD25, CD44, CD45, HLA-DR, and HLA class I.[38] Alemtuzumab is the humanized form of a murine anti-CD52 mAb, a membrane glycoprotein with unknown function that is expressed on lymphocytes, macrophages, monocytes, and eosinophils. It is especially highly expressed on lymphocytes (up to 5% of surface antigens), explaining its powerful immunodepletion. Interestingly, antithymocyte globulin enhances the expression of IL-2 receptors (CD25 and CD132) and alemtuzumab of the activation receptor (NKp44) on NK cells (Fig 1B). Under IL-2 stimulation, either antithymocyte globulin– or alemtuzumab-treated liver NK cells showed strong cytotoxicity and anti-HCV activity (Fig 2 and 3). Our results clearly support the conclusion of other authors that binding of antithymocyte globulin to NK cells leads to cell activation and IFN-γ production.[36,39] The possible mechanism is that the binding of antithymocyte globulin or alemtuzumab to CD16 produces NK cell activation and degranulation.[40] However, antithymocyte globulin and alemtuzumab have also been reported to be potent to induce NK cell death and impair cytotoxicity.[41,42] When used for immunotherapy, antithymocyte globulin– or alemtuzumab-binding NK cells are destroyed through immunologic mechanisms such as complement-mediated and/or antibody-dependent cytotoxicity.[43]

In summary, we have shown the effects of GMP CD3 antibody to be similar to those of OKT3, namely, depletion of T cells and induction of NK cell phenotype and function. We have already applied this method to clinical immunotherapy using liver NK cells for liver transplant patients with HCC (ClinicalTrial.gov identifier: NCT01147380) after IRB and Food and Drug Administration approval in the United States. Our findings also support the hypothesis that T-cell depletion antibodies affect NK cell function with the use of in vitro culture systems.

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ORIGINAL ARTICLE

The Outcomes of Patients with Severe Hyperbilirubinemia Following Living Donor Liver Transplantation

Hajime Matsushima · Akihiko Soyama · Mitsuhisa Takatsuki · Masaaki Hidaka · Izumi Muraoka · Tamotsu Kuroki · Susumu Eguchi

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Abstract
Background Prolonged hyperbilirubinemia (HB) following living donor liver transplantation (LDLT) can be a risk factor for early graft loss and mortality. However, some recipients who present with postoperative hyperbilirubinemia do recover and maintain a good liver function.
Aim The purpose of this study was to investigate the risk factors for hyperbilirubinemia following LDLT and to identify predictors of the outcomes in patients with post-transplant hyperbilirubinemia.
Methods A total of 107 consecutive adults who underwent LDLT in Nagasaki University Hospital were investigated retrospectively. The patients were divided into two groups according to postoperative peak serum bilirubin level (HB group: ≥30 mg/dl; non-HB group: <30 mg/dl). These two groups of patients and the prognosis of patients in the HB group were analyzed using several parameters.
Results Seventeen patients (15.9 %) presented with hyperbilirubinemia, and their overall survival was significantly worse than patients in the non-HB group ($n = 90$). Donor age was significantly higher in the HB group ($P < 0.05$). Of the 17 patients in the HB group, nine survived. The postoperative serum prothrombin level at the time when the serum bilirubin level was >30 mg/dl was significantly higher in surviving patients ($P < 0.01$).
Conclusions The use of a partial liver graft from an aged donor is a significant risk factor for severe hyperbilirubinemia and a poorer outcome. However, those patients who maintain their liver synthetic function while suffering from hyperbilirubinemia may recover from hyperbilirubinemia and eventually achieve good liver function, thus resulting in a favorable survival.

Keywords Living donor liver transplantation · Hyperbilirubinemia · Partial graft · Small-for-size graft syndrome · Acute cellular rejection

Introduction

Hyperbilirubinemia following living donor liver transplantation (LDLT) can be caused by several mechanisms, such as initial poor function, acute cellular rejection, surgical complications, small-for-size syndrome, drug toxicity, among others. Hyperbilirubinemia has also been reported to be a risk factor for early graft loss and mortality [1]. However, some recipients can overcome hyperbilirubinemia, and these patients subsequently achieve and maintain a good liver function after their eventual recovery from hyperbilirubinemia. The aim of this study was to retrospectively clarify the risk factors for the development of postoperative severe hyperbilirubinemia and to identify any predictors for the outcomes in patients who present with hyperbilirubinemia following LDLT.

Patients and Methods

We retrospectively analyzed the data of 107 consecutive adult patients (67 males, 40 females, median age 55 years, age range 16–68 years) who underwent LDLT in the Department of Surgery of Nagasaki University Hospital between November 1997 and January 2010. The etiologies

H. Matsushima · A. Soyama · M. Takatsuki · M. Hidaka · I. Muraoka · T. Kuroki · S. Eguchi (✉)
Department of Surgery, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan
e-mail: sueguchi@nagasaki-u.ac.jp

of the liver disease were hepatitis C virus infection (35 patients), hepatitis B virus infection (25 patients), non-viral causes (40 patients), and fulminant liver failure (7 patients) (Table 1). During this period, we occasionally treated patients with a postoperative bilirubin level of >20 mg/dl. Marubashi et al. [1] reported that a postoperative peak serum bilirubin level of >27 mg/dl could be a predictor of short-term graft outcome. Therefore, we defined those patients who had presented with a postoperative peak serum bilirubin level of >30 mg/dl as having hyperbilirubinemia (HB group); the remaining patients formed the non-HB group.

The two groups of patients were compared for preoperative serum bilirubin level; donor age; the postoperative peak alanine aminotransferase (ALT); model for end-stage liver disease (MELD) score; graft weight (GW)/standard liver volume ratio [SLV; SLV (ml) = 706.2 × body surface area (m^2) + 2.4] [2]; type of graft; development of acute cellular rejection [as proven by biopsy within postoperative day (POD) 60]; ABO compatibility; the development of biliary complications. We defined a biliary complication as anastomotic stenosis that needed interventions by means of balloon dilatation, stent placement, or re-operation. We divided the types of grafts into those for the right lobe and left lobe, respectively. The right lobe included the right lateral sector, and the left lobe included the left lateral segment.

In the HB group, we compared surviving and non-surviving patients for all of the above-mentioned parameters as well as for serum prothrombin [PT (%)] and creatinine levels at the time when the serum bilirubin level was >30 mg/dl. In the HB group, no patients received administration of fresh frozen plasma at the time of diagnosis. We used log-rank test for survival comparison. Group data were compared with the Mann–Whitney *U* test, and differences between proportions of categorical data were compared with the χ^2 test. Furthermore, several factors detected in the univariate analysis with *P* values of <0.15 were entered into a multivariate analysis. We used multivariate logistic regression analysis for the multivariate analysis. A *P* value <0.05 was considered to be statistically significant.

Table 1 Indication for liver transplantation

Cause of liver disease	Total ($n = 107$)	HB group ($n = 17$)	Non-HB group ($n = 90$)
Liver cirrhosis (hepatitis virus C)	35	6	29
Liver cirrhosis (hepatitis virus B)	25	4	21
Alcoholism	11	2	9
Primary biliary cirrhosis	8	3	5
Fulminant hepatitis	7	0	7
Liver cirrhosis (non-B non-C)	6	0	6
Primary sclerosing cholangitis	3	0	3
Budd–Chiari syndrome	1	0	1
Caroli's disease	1	0	1
Graft failure	4	2	2
Others	6	0	6

HB Hyperbilirubinemia

Results

Of the 107 consecutive adult patients who underwent LDLT at our hospital during the study period, 17 (15.9 %) met our criteria for HB and were included in the HB group; the remaining 90 patients (84.1 %) formed to the non-HB group. The overall survival rate was significantly different between the groups ($P < 0.01$) (Fig. 1). Time-zero biopsies showed no apparent differences between patients in the HB and non-HB group. Protocol biopsy was not performed postoperatively except in cases of cellular rejection or recurrence of hepatitis was suspected. The median donor age was significantly higher in the HB versus the non-HB group [50 (range 22–63) vs. 36 (19–67) years, respectively; $P < 0.05$], and ABO incompatibility was identified as a risk factor for posttransplant hyperbilirubinemia. The median preoperative serum bilirubin level tended to be higher in the HB group than in the non-HB group [5.4 (range 1.1–39.5) vs. 3.3 (0.6–42.7) mg/dl, respectively; $P = 0.06$]. The median postoperative peak ALT level was significantly higher in the HB group than in the non-HB group [569 (range 120–1,907) vs. 339 (79–3,359) IU/l,

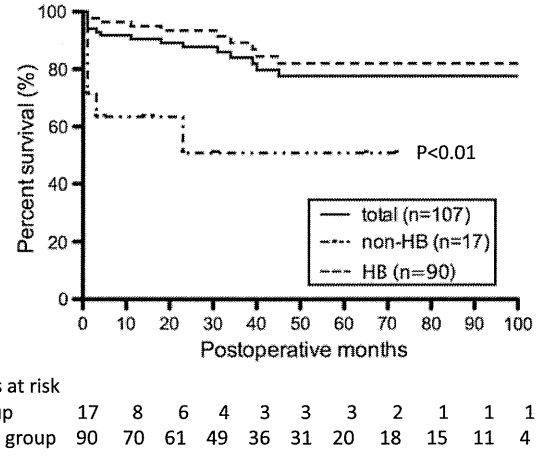

Fig. 1 Kaplan–Meier curves of the postoperative survival of patients with hyperbilirubinemia (*HB* group) and without hyperbilirubinemia (*non-HB* group)

Table 2 Analysis of predictive factors for hyperbilirubinemia (univariate analysis)

Predictive factors	HB group[a] ($n = 17$)		Non-HB group[a] ($n = 90$)		P value
GW/SLV (%)	39.9 (24.9–56.3)		44.1 (23.6–85.3)		0.139
Donor age (years)	50 (22–63)		36 (19–67)		0.035
MELD score	22 (9–32)		18 (7–40)		0.217
Preoperative serum total bilirubin (mg/dl)	5.4 (1.1–39.5)		3.3 (0.6–42.7)		0.061
postoperative peak ALT(IU/l)	569 (120–1,907)		339 (79–3,359)		0.02
	+ (n)	%	+ (n)	%	
Acute cellular rejection (<POD 60)	5/17	29	26/90	29	0.804
Biliary complication	0/17	0	18/90	20	0.07
Type of graft					
Right lobe	10/17	59	36/90	40	
Left lobe	7/17	41	54/90	60	0.241
ABO incompatibility	6/17	35	9/90	10	0.01

GW/SLV Graft weight/standard liver volume ratio, *MELD* model for end-stage liver disease, *POD* postoperative days, *ALT* alanine aminotransferase

[a] Values are presented as the median with the range in parenthesis

Table 3 Multivariate analysis of postoperative hyperbilirubinemia

Preoperative risk factors	Yes/no	P value
GW/SLV (%)	–	0.107
Donor age (years)	–	0.0125
Preoperative serum total bilirubin (mg/dl)	–	0.032
ABO incompatibility	Yes	0.163

respectively; $P = 0.02$]. There were no significant differences in the GW/SLV, MELD score, type of graft, and incidence of biliary complication and acute cellular rejection between the groups (Table 2). The multivariate logistic analysis identified donor age ($P = 0.0125$) and preoperative serum bilirubin level ($P = 0.032$) as preoperative risk factors for postoperative hyperbilirubinemia (Table 3).

Of the 17 patients in the HB group, nine were alive at the writing of this manuscript. The results of the comparison between surviving and non-surviving patients are shown in Table 4. The median postoperative PT (%) at the time when the serum bilirubin level was >30 mg/dl was significantly higher in surviving patients than in those that did not survive [52 (range 26–59) vs. 33.5 (20–60) %, respectively; $P < 0.01$]. The median postoperative serum creatinine level at the time when the serum bilirubin level exceeded 30 mg/dl tended to be lower in surviving patients than in those that had not survived [1.2 (range 0.5–2.9) vs. 1.86 (0.4–3.1) mg/dl, respectively; $P = 0.06$]. There were no significant differences between surviving patients and non-surviving patients for donor age, GW/SLV, preoperative serum bilirubin level, MELD score, postoperative duration when the serum bilirubin level was >30 mg/dl, ABO incompatibilty, and acute cellular rejection (Table 4). The multivariate logistic analysis was not performed because of the small number of patients. Table 5 summarizes the characteristics and the postoperative course of patients in the HB group. Eight patients did not survive—one patient due to severe acute cellular rejection and seven patients due to infection. The indications for liver transplantation for non-surviviors were liver cirrhosis (hepatitis C virus; 3 patients), primary biliary cirrhosis (3 patients), (hepatitis B virus; 1 patient), and graft failure (1 patients) (Table 5). None of these patients had suffered from short-term recurrence of viral hepatitis and hepatocellular carcinoma after transplantation. One patient (Table 5, case no. 10) was considered to be small-for-size syndrome with massive ascites and prolonged hyperbilirubinemia without arterial or portal occlusion and rejection. However, she had maintained PT (%) and survived. Although postoperative biopsies were performed for 11 patients in the HB group, no specific causes of hyperbilirubinemia were detected besides the findings of acute cellular rejection or recurrent hepatitis.

Discussion

In this study, we analyzed the risk factors for postoperative HB and the prognosis of patients who belonged to the HB group. Our results indicate that the donor age was most strongly correlated with the development of HB. A multivariate analysis also identified donor age and patient preoperative total bilirubin level as significant risk factors for post-transplant HB. The outcome of liver transplantation from aged donors is controversial. Some studies have shown that the outcomes of using grafts from donors older than 50 years without additional risk factors are similar to those of using grafts from donors younger than 50 years [3, 4]. However, the data from a registry of the Japanese Liver Transplantation Society show that patients who received a graft from an older donor had a significantly

Table 4 Comparison of risk factors for mortality in HB group (univariate analysis)

Risk factors	Surviving group[a] ($n = 9$)		Non-surviving group[a] ($n = 8$)		P value
GW/SLV(%)	40 (24.9–56.3)		39.2 (26.9–48.4)		0.847
Donor age	50 (22–61)		50.5 (22–63)		0.847
MELD score	22 (13–32)		22 (9–40)		1
Preoperative total bilirubin (mg/dl)	3.2 (1.9–39.5)		14.2 (1.1–28.7)		0.289
Timing of diagnosing HB	19 (5–28)		17 (6–32)		0.885
Prothrombin time (%) at HB diagnosis	52 (26–59)		33.5 (20–60)		0.004
Serum creatinine (mg/dl) at HB diagnosis	1.2 (0.5–2.9)		1.86 (0.4–3.1)		0.067
	+ (*n*)	%	+ (*n*)	%	
ABO incompatibility	3/9	33	3/8	38	1
Acute cellular rejection (<POD 60)	2/9	22	3/8	38	0.619

[a] Values are presented as the median with the range in parenthesis

Table 5 Characteristics and postoperative courses of patients in HB group

Case no.	Gender	Age	Indication for transplantation	ABO incompatibility	GW/SLV	Timing of diagnosing HB	Prothrombin (%) at HB diagnosis[a]	Outcomes	Cause of death
1	Male	63	B-LC, HCC	+	36.9	36	45	Dead	Infection
2	Female	61	PBC	+	26.9	26	25	Dead	Infection
3	Female	61	C-LC, HCC	−	43.6	12	29	Dead	Infection
4	Female	62	PBC	−	38.4	45	31	Dead	Infection
5	Male	57	C-LC, HCC	−	40	18	37	Dead	Infection
6	Male	57	C-LC, HCC	−	48.4	15	36	Dead	Infection
7	Male	41	PBC	−	44.6	16	31	Dead	ACR
8	Female	56	Graft failure	+	36.3	14	43	Dead	Infection
9	Female	54	C-LC, HCC	+	41.2	28	61	Alive	
10	Female	59	C-LC, HCC	−	24.9	26	45	Alive	
11	Male	58	B-LC, HCC	−	29.7	17	46	Alive	
12	Male	56	B-LC, HCC	−	44.2	37	76	Alive	
13	Female	53	C-LC	+	40	11	55	Alive	
14	Male	22	Graft failure	−	56.3	5	41	Alive	
15	Male	52	B-LC, HCC	+	36.1	34	52	Alive	
16	Male	62	Alcoholism	−	43.5	19	60	Alive	
17	Female	46	Alcoholism	−	37.8	17	34	Alive	

C-LC Liver cirrhosis type C, *B-LC* liver cirrhosis type B, *PBC* primry biliary cirrhosis, *ACR* acute cellular rejection

[a] At the time when the serum bilirubin level was >30 mg/dl

worse survival [5]. Notable findings of two studies which investigated non-transplanted aged livers were: 40 and 50 % decreases in vascular inflow and biliary flow, respectively, impairment of energy- and microtubule-dependent transport processes, with reduced endoplasmic reticulum mass, cumulative pigmented waste deposition, and a reduced ability to scavenge reactive oxygen intermediates [6, 7].

It has been reported that patients who receive a graft from an aged donor tend to have a greater incidence of delayed graft function [8, 9]. A multivariate analysis also revealed that the use of these grafts is associated with an increased incidence of recurrent hepatitis C [10]. A relative poorer regeneration of the liver graft from an aged donor has also been reported [11]. Taken together, these findings indicate that clinicians should be aware that the use of grafts from aged donors could lead to the development of severe hyperbilirubinemia by a multifactorial mechanism.

The HB group included significantly more patients who had undergone ABO blood type-incompatible LDLT. The outcomes of ABO blood type-incompatible LDLT have improved over the years, and many institutes have adopted ABO-incompatible LDLT owing to the various treatments that can be used to overcome antibody-mediated rejection (AMR). AMR is the result of a circulatory disturbance that is caused by injury to the endothelium due to an antibody–

antigen–complement reaction. The typical clinical manifestations of AMR are hepatic necrosis and intrahepatic biliary complications [12]. Although no patients in our study had developed hepatic necrosis or apparent intrahepatic biliary complications with the prophylaxis, including rituximab and plasma exchange, our results suggest that patients undergoing ABO-incompatible LDLT may have a greater chance of developing postoperative severe hyperbilirubinemia.

The prognosis of the HB group was significantly worse than that of the non-HB group. Marubashi et al. [1] reported devastating outcomes in patients with a postoperative peak serum bilirubin level of >27 mg/dl, with eight of their grafts resulting in early graft loss within 1 year. In contrast, we experienced a number of patients with severe hyperbilirubinemia post-LDLT who eventually recovered their liver function; in fact, nine of the 17 patients in the HB group survived. Therefore, we investigated the perioperative parameters to clarify the risk factors for decreased survival. Our analysis revealed that the postoperative PT (%) at the time when the serum bilirubin level exceeded 30 mg/dl for the first time was significantly correlated with the prognosis based on the univariate analyses. Based on these results, the patients who were able to maintain their liver synthesis function were able to recover their liver function despite a temporal deterioration in bilirubin excretion.

Cholestasis has been recognized as a clinical manifestation of small-for-size graft syndrome, and the improvement of temporal cholestasis in proportion to the liver regeneration can be expected in cases of partial liver graft transplantation. We tried to exclude small-for-size syndrome with massive ascites. Although there is no consensus on the definition of small-for-size syndrome, there was one patient in the HB group who was suspected to have small-for-size syndrome, and she recovered spontaneously [normal range PT (%)] [13, 14]. In fact, GW/SLV was not a significant risk factor for the development of hyperbilirubinemia in our present study.

In addition, the postoperative serum creatinine level at the time when the serum bilirubin level exceeded 30 mg/dl for the first time tended to be lower in surviving patients. Acute kidney injury following liver transplantation has been reported to be associated with a worse outcome [15]. It is not hard to comprehend that HB patients with multiple organ dysfunction would have be a worse prognosis.

In conclusion, the use of a partial liver graft from an aged donor is considered to be a significant risk factor for postoperative severe hyperbilirubinemia. Although the outcomes of the HB patients were worse than those for the non-HB group, we should recognize that recovery is possible even from severe hyperbilirubinemia in those patients who are able to maintain their liver synthetic function during the postoperative course.

Conflict of interest None.

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Significance of hepatitis B virus core-related antigen and covalently closed circular DNA levels as markers of hepatitis B virus re-infection after liver transplantation

Toshihisa Matsuzaki,* Ichikawa Tatsuki,* Masashi Otani,* Motohisa Akiyama,* Eisuke Ozawa,* Satoshi Miuma,* Hisamitsu Miyaaki,* Naota Taura,* Tomayoshi Hayashi,‡ Sadayuki Okudaira,† Mitsuhisa Takatsuki,† Hajime Isomoto,* Fuminao Takeshima,* Susumu Eguchi† and Kazuhiko Nakao*

Departments of *Gastroenterology and Hepatology and †Surgery, Nagasaki University Graduate School of Biomedical Sciences, and ‡Department of Pathology, Nagasaki University Hospital, Nagasaki, Japan

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cccDNA, HBcrAg, HBV, liver transplantation.

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Correspondence
Dr Toshihisa Matsuzaki, Department of Gastroenterology and Hepatology, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan. Email: tmatsuzaki6@gmail.com

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Abstract

Background and Aim: Currently, hepatitis B virus (HBV) re-infection after liver transplantation (LT) can be almost completely suppressed by the administration of HBV reverse transcriptase inhibitors and hepatitis B immunoglobulins. However, after transplantation, there is no indicator of HBV replication because tests for the serum hepatitis B surface antigen and HBV-DNA are both negative. Therefore, the criteria for reducing and discontinuing these precautions are unclear. In this study, we examined the serum HBV core-related antigen (HBcrAg) and intrahepatic covalently closed circular DNA (cccDNA) in order to determine if these could be useful markers for HBV re-infection.

Methods: Thirty-one patients underwent LT for HBV-related liver disease at Nagasaki University Hospital from 2001 to 2010. Of these, 20 cases were followed up for more than 1 year (median follow-up period, 903 days). We measured serum HBcrAg and intrahepatic cccDNA levels in liver tissue. In addition, in nine cases, we assessed the serial changes of HBcrAg and intrahepatic cccDNA levels from preoperative periods to stable periods.

Results: We examined serum HBcrAg and intrahepatic cccDNA levels in 20 patients (35 samples). HBcrAg and cccDNA levels were significantly correlated with each other ($r = 0.616$, $P < 0.001$). From a clinical aspect, the fibrosis stage was significantly lower in both HBcrAg- and cccDNA-negative patients than in HBcrAg- or cccDNA-positive patients.

Conclusions: HBcrAg and cccDNA were useful as HBV re-infection markers after LT. Keeping patients' HBcrAg and cccDNA negative after LT might contribute to long-term graft survival.

Authors' Contributions:
Toshihisa Matsuzaki: acquisition of data, study concept and design, statistical analysis, writing of manuscript.
Tatsuki Ichikawa: study concept and design, acquisition of data, critical revision of the manuscript for important intellectual content.
Masashi Otani: critical revision of the manuscript for important intellectual content.
Motohisa Akiyama: critical revision of the manuscript for important intellectual content.
Eisuke Ozawa: critical revision of the manuscript for important intellectual content.
Satoshi Miuma: critical revision of the manuscript for important intellectual content.
Sadayuki Okudaira: acquisition of data, critical revision of the manuscript for important intellectual content.
Tomayoshi Hayashi: acquisition of data, critical revision of the manuscript for important intellectual content.
Naota Taura: critical revision of the manuscript for important intellectual content.
Hisamitsu Miyaaki: critical revision of the manuscript for important intellectual content.
Susumu Eguchi: critical revision of the manuscript for important intellectual content.
Takashi Kanematsu: critical revision of the manuscript for important intellectual content.
Hajime Isomoto: critical revision of the manuscript for important intellectual content.
Fuminao Takeshima: critical revision of the manuscript for important intellectual content.
Kazuhiko Nakao: study supervision, critical revision of the manuscript for important intellectual content.

Introduction

Liver transplantation (LT) is an established procedure for the treatment of end-stage liver disease. However, the recurrence of hepatitis B virus (HBV) is implicated in life-threatening graft failure.[1] Therefore, the prevention of HBV recurrence following LT is a serious concern. The advent of hepatitis B immunoglobulins (HBIg) and the HBV reverse transcriptase inhibitor (RTI) was a major breakthrough in the management of HBV recurrence. Currently, an ideal recurrence rate for HBV has been observed in patients who received HBIg and RTI combination therapy.[2] However, several studies have reported that HBV can be detected in the transplanted liver and peripheral blood mononuclear cells of recipients even when they have a hepatitis B surface antigen (HBsAg)-negative status.[3] Therefore, prophylaxis currently must be continued for the patient's lifetime. However, there are concerns with the long-term administration of HBIg and RTI with respect to safety, medical costs, and resistant mutations of HBV.[4] In order to discontinue the prophylaxis, several groups have attempted to vaccinate LT recipients against HBV, but most of these studies involve relatively low seroconversion rates because of the immunosuppressive environment.[5]

Recently, new agents against HBV, such as adefovir and entecavir, which hardly develop resistant mutations, have become available. Some have reported that HBIg can be discontinued after LT by using the new anti-HBV agents even if the vaccination does not succeed.[6] Angus *et al.* reported that when adefovir dipivoxil was substituted for low-dose HBIg, all patients were alive at the study completion without recurrence.[7] In addition, low-risk cases, such as those with fulminant hepatitis, and hepatitis B core antibody (HBcAb)-positive donors are not necessary for the administration of high-dose HBIg.[8] However, after transplantation, RTI and HBIg may mask the appearance of HBV-DNA, regardless of the presence of intrahepatic HBV covalently closed circular DNA (cccDNA). These factors make it difficult to detect HBV dynamics following LT, and we are therefore unable to determine the feasibility of the discontinuation of prophylaxis.

Recently, a new enzyme immunoassay that detects hepatitis B core-related antigen (HBcrAg) has been reported.[9] HBcrAg changes in parallel with HBV-DNA in the serum and has a wide detection range.[10] Moreover, its levels are correlated with the intrahepatic cccDNA levels of patients with chronic hepatitis B.[11] In addition, we previously reported on the usefulness of HBcrAg in patients receiving anti-HBV prophylaxis following LT.[12]

Therefore, in this study, we simultaneously measured serum HBcrAg and intrahepatic cccDNA levels in liver tissue and studied the HBV dynamics in patients following HBV-related LTs.

Methods

Patients and samples. From 2001 to 2010, a total of 31 patients with HBV-related end-stage liver disease underwent LTs at Nagasaki University Hospital, Nagasaki, Japan. Of these, we enrolled 20 patients who could be followed up for more than approximately 1 year (median 902 days; range 323–2456 days). There were 17 men and 3 women, with a median age of 56.5 years (range 33–68 years). All 20 patients were diagnosed with liver cirrhosis, and 12 were diagnosed with hepatocellular carcinoma. In addition, two patients were coinfected with the hepatitis C virus (Table 1).

Table 1 Baseline clinical features of the enrolled patients

Case	Age	Gender	Indication disease	HBV-DNA	HBsAg	HBsAb	HBeAg	HBeAb	HBcAb	Donor HBcAb	HBcrAg
1	55	F	LC-B	< 2.6	> 2000	0.2	36.0	0.0	> 100.0	5.0	6.0
2	56	M	LC-B	< 2.6	> 2000	2.3	0.6	82.4	99.9	5.0	4.2
3	48	M	LC-B, HCC	< 2.6	562.5	0.1	1.1	57.7	> 100.0	31.3	5.0
4	60	M	LC-B	< 2.6	1789	0.1	0.2	97.6	> 100.0	70.1	5.8
5	59	M	LC-B, HCC	< 2.6	> 2000	0.1	0.1	> 100.0	> 100.0	5.0	3.2
6	57	M	LC-B, HCC	3.9	188.5	0.5	0.8	54.0	> 100.0	10.3	5.1
7	56	M	LC-B, HCC	< 2.6	> 2000	0.1	1.4	75.4	> 100.0	91.9	5.6
8	68	M	LC-B, HCC	< 2.6	> 2000	0.2	0.1	> 100.0	> 100.0	5.0	3.0
9	33	F	LC-B	3.0	> 2000	0.2	0.2	81.5	99.9	99.6	5.5
10	58	M	LC-B, HCC	3.0	> 2000	0.1	0.1	93.6	> 100.0	93.4	5.1
11	59	M	LC-B	< 2.6	378.3	0.3	0.1	61.6	> 100.0	93.0	3.8
12	57	M	LC-B + C, HCC	< 2.6	519.9	0.1	0.1	> 100.0	99.9	5.0	2.0
13	49	M	LC-B	< 2.6	> 2000	0.1	0.9	52.9	> 100.0	34.1	5.2
14	65	F	LC-B	6.9	> 2000	0.2	0.1	> 100.0	> 100.0	5.0	6.8
15	55	M	LC-B, HCC	< 2.1	> 2000	0.2	0.1	99.3	> 100.0	31.6	4.5
16	46	M	LC-B + C	4.3	1100.4	0.2	0.1	> 100.0	> 100.0	81.9	3.7
17	59	M	LC-B, HCC	< 2.1	> 2000	0.1	0.1	99.2	> 100.0	38.6	3.7
18	51	M	LC-B, HCC	2.1	> 2000	0.2	0.4	62.8	99.4	50.0	4.7
19	67	M	LC-B, HCC	3.9	> 2000	0.1	34.3	60.2	> 100.0	91.1	6.3
20	54	M	LC-B, HCC	2.1	> 2000	0.1	104.8	37.4	> 100.0	9.7	4.3

HBV, hepatitis B virus; HBcAb, hepatitis B core antibody; HBcrAg, hepatitis B core-related antigen; HBeAb, hepatitis B envelope antibody; HBeAg, hepatitis B envelope antigen; HBsAb, antibody against hepatitis B surface antigen; HBsAg, hepatitis B surface antigen; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; LC, liver cirrhosis; LC-B, LC due to HBV; LC-B + C, LC due to HBV-HCV coinfection.

All patients had been receiving RTI since preoperative periods. The HBsAg was negative in all donors, but eight donors were HBcAb-positive (cut-off, 50%), which was suggested to be due to prior exposures to HBV.

The prophylactic infusion of HBIg was administered to all patients according to a fixed-dose schedule; 10 000 units were given intravenously at the anhepatic period during the operation and the next day after the living donor LT (LDLT). Afterwards, 2000 units of HBIg were given routinely in order to keep the serum hepatitis B surface antibody (HBsAb) titers above 100 units/L. After the LDLT, serum HBsAg, hepatitis B envelope antigen (HBeAg), and HBV-DNA were not detected in any of the patients in this study.

Serum samples and biopsy specimens were obtained from 20 patients who received protocol biopsies 1 year after the LDLT at Nagasaki University Hospital after providing informed consent. Nine patients were followed up from the preoperative period to the stable period. Serum samples were obtained at the following three specified intervals: (i) in the preoperative period, samples were obtained just before the operation; (ii) in the postoperative period, samples were obtained during the operation of LT; and (iii) in the stable period, samples were obtained during admission for protocol biopsy. Liver tissue samples were obtained during the following three specified procedures: (i) biopsy from explanted liver during the operation; (ii) time-zero biopsy from the implanted liver during the operation; and (iii) protocol biopsy 1 year after the LDLT.

Serological markers for HBV. HBsAg, HBsAb, HBeAg, hepatitis B envelope antibodies (HBeAb), and HBcAb levels were assessed by the chemiluminescence enzyme immunoassay (CLEIA) method using a commercially available enzyme immunoassay kit (Lumipulse, Fuji Rebio, Inc., Tokyo, Japan). Serum concentrations of HBV-DNA were determined using a polymerase chain reaction (PCR) HBV monitoring kit (Roche Diagnostics K.K., Tokyo, Japan), which had a quantitative range from 2.6 to 7.6 log copies/mL.

HBcrAg test. Serum HBcrAg levels were measured by a CLEIA HBcrAg assay kit (Fujirebio, Inc.) with a fully automated analyzer system (Lumipulse System, FujiRebio, Inc.). HBcrAg concentrations were expressed as units/mL (U/mL). In this study, HBcrAg values were expressed as log U/mL, and the cut-off value was set at 3.0 log U/mL.[9,13]

Measurement of cccDNA. Liver tissues were stored at −80°C before DNA extraction. HBV-DNA was extracted using a high pure PCR template preparation kit (Roche Diagnostics K.K.). The concentration of purified DNA was measured at an absorbance of 260 nm.

cccDNA levels were measured with the real-time PCR method. With reference to a previous study,[11] we designed two oligonucleotide primers, cccF2 (5′-CGTCTGTGCCTTCTCATCTGA-3′, nucleotides: 1424-1444) and cccR4 (5′-GCACAGCTTGGAGGC TTGAA-3′, nucleotides: 1755-1737), and a cccP2 probe (5′-FAM-ACCAATTTATGCCTACAG-MGB-3′, nucleotides: 1672-1655). Reaction volume (20.0 μL) containing 500 ng of extracted DNA, 0.5 μmol/L of each primer, 0.2 μmol/L of the probes, and LightCycler TaqMan Master (Roche Diagnostics K.K.) was administered. The initial activation step was heated at 95°C for 10 min. The subsequent PCR conditions consisted of 60 cycles of denaturation at 95°C for 10 s, and annealing and extension at 60°C for 30 s per cycle. Real-time PCR was performed in a LightCycler (Roche Diagnostics K.K.). Serial dilutions of a plasmid containing an HBV monomer were used as quantitation standards.

Liver histology. Liver histology was evaluated by the same two pathologists. The degrees of necroinflammation and fibrosis were assessed based on the New Inuyama classification.[14] The degrees of rejection were assessed with the Rejection Activity Index according to the Banff working classification of hepatic allograft pathology.[15]

Liver function test. Blood biochemical tests were performed in all patients, and liver function was evaluated. Liver function was assessed using Pugh's modification of Child's scoring system.[16]

Statistical analyses. Student's *t*-tests and Fisher's exact tests were used for comparisons between groups of parametric quantitative data, and Mann–Whitney *U*-tests were used for comparisons between independent groups of non-parametric data. Categorical variables were compared with chi-square tests. The correlations between continuous variables were analyzed by the Pearson's correlation test. Two-tailed *P* values less than 0.05 were considered statistically significant.

Results

Correlation between HBcrAg and cccDNA. The correlation between HBcrAg and cccDNA levels in all 35 samples is summarized in Figure 1. A statistically significant positive

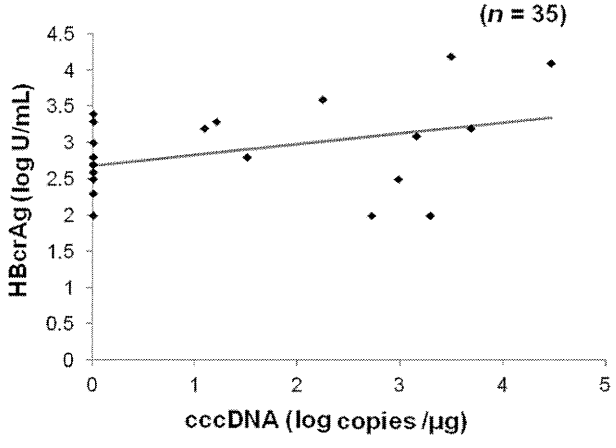

Figure 1 Correlation between serum hepatitis B core-related antigen (HBcrAg) and intrahepatic hepatitis B virus covalently closed circular DNA (cccDNA). $r = 0.616$, $P < 0.001$ ($y = 0.40x + 2.62$). Straight lines indicate the correlation between HBcrAg and cccDNA levels.

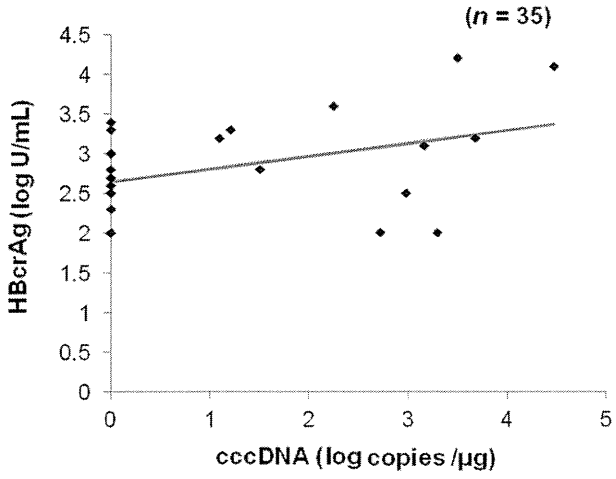

Figure 2 Correlation between hepatitis B core-related antigen (HBcrAg) and covalently closed circular DNA (cccDNA) levels after transplantation. $r = 0.402$, $P = 0.046$ ($y = 0.16 \times + 2.64$). Straight lines indicate the correlation between HBcrAg and cccDNA levels.

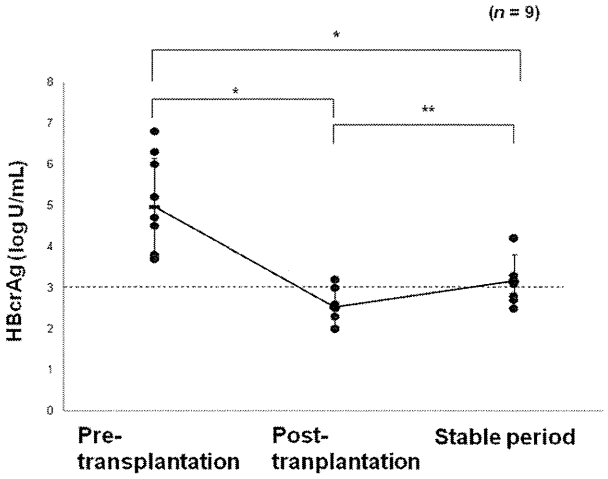

Figure 3 Serial changes of the hepatitis B core-related antigen (HBcrAg) levels. HBcrAg levels are represented as mean values; the closed circles show the values of the HBcrAg levels in all phases. The error bars indicate standard deviations. The detection range is above 3.0 log U/mL. In order to obtain the mean value, the values of 3.0 log U/mL or less, and 2.0 log U/mL or more were added to the calculation. The mean values of HBcrAg levels dropped during the postoperative period but then gradually increased again during the stable period (*$P < 0.001$ and **$P = 0.035$ indicate the significant differences between each period).

correlation was observed ($r = 0.616$, $P < 0.001$). Similarly, in the 23 samples that were obtained after LT only (that is, preoperative state samples were excluded), HBcrAg levels were significantly correlated with cccDNA levels (Fig. 2, $r = 0.402$, $P = 0.046$). These results supported the hypothesis that HBcrAg can be useful as an HBV marker instead of cccDNA after LT.

Serial changes in HBcrAg and cccDNA levels. HBcrAg and cccDNA levels showed similar dynamics during each period (Figs 3,4). All nine cases had positive levels of HBcrAg. However, seven of them were negative for HBV-DNA. During the post-transplantation period, HBcrAg levels of seven cases and cccDNA levels of eight cases became negative. Subsequently, HBcrAg and cccDNA levels of five cases became positive again during the stable period. These dynamics implicated the re-infection of HBV in the graft liver.

Comparisons of the clinical features of HBcrAg and cccDNA levels. We divided patients into two groups according to their status of HBcrAg and cccDNA, and investigated their clinical features (Table 2). Positive group includes the patients with positive cccDNA or HBcrAg, negative group includes the patients with both negative.

In comparisons between the positive group and negative group, the number of patients being treated with entecavir was significantly lower in negative group ($P = 0.022$). Additionally, the stage of the graft liver was significantly lower ($P = 0.012$) in negative group. The grafts of the HBcrAg- and cccDNA-negative patients were in good condition in the lower fibrosis stages (median 0; range 0–1).

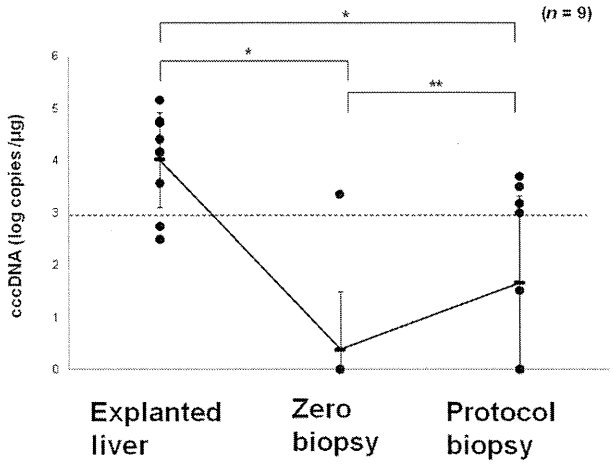

Figure 4 Serial changes of the covalently closed circular DNA (cccDNA) levels. cccDNA levels are represented as mean values; the closed circles show the values of the cccDNA levels in all phases. The error bars indicate standard deviations. The mean values of the cccDNA levels dropped during the time-zero biopsy but then gradually increased during the protocol biopsy (*$P < 0.001$ and **$P = 0.078$ indicate the significant differences between each period).

Discussion

In the present study, we demonstrated the usefulness of HBcrAg and cccDNA as markers of HBV after transplantation. As in our previous report,[12] we suggest that HBcrAg, which is a newly developed enzyme immunoassay,[9] is a possible method for detecting the dynamics of HBV after LT. However, HBcrAg consists of HBcAg, HBeAg, and p22cr, which is generated from cccDNA,

Table 2 Comparisons of the clinical features of HBcrAg and cccDNA levels

HBcrAg/cccDNA status	Positive group	Negative group	Positive versus negative
Patient M/F	10/2	7/1	NS
Day after transplantation†	854 (323–2163)	1674.5 (353–2456)	NS
Age†	55.5 (33–68)	56.5 (48–65)	NS
Serum HBV-DNA positive at LT (p/n)	7/5 (58.3%)	2/6 (33.3%)	NS
Serum HBeAg positive at LT (p/n)	1/11 (8.3%)	1/7 (14.3%)	NS
HBcAb-positive donor (p/n)	7/5 (58.3%)	1/7 (14.3%)	NS
Blood incompatibly (p/n)	1/11 (8.3%)	1/7 (14.3%)	NS
Presence of HCC at LT (p/n)	9/3 (75%)	7/1 (87.5%)	NS
RTI for prophylactic therapy after LT			
Use of LAM	3/12 (25%)	4/8 (50%)	NS
Use of ETV	9/12 (75%)	1/8 (12.5%)	$P = 0.022$
Use of ADV	0 (0%)	2/8 (25%)	NS
Use of LAM + ADV	0 (0%)	1/8 (12.5%)	NS
Immunosuppression after LT			
Use of TAC	10/12 (83.3%)	5/8 (62.5%)	NS
Use of CYA	0 (0%)	2/8 (25%)	NS
Use of MMF	2/12 (16.6%)	0 (0%)	NS
Use of TAC + MMF	0 (0%)	1/8 (12.5%)	NS
Liver function test			
Serum albumin (g/L)‡	39.2 (4.7)	40.0 (4.8)	NS
Child–Pugh score†	5.0 (5.0–9.0)	5.0 (5.0–6.0)	NS
Histology of LB			
Grade†	1.0 (0.0–3.0)	0.5 (0.0–1.0)	NS
Stage†	1.0 (0.0–3.0)	0.0 (0.0–1.0)	$P = 0.0027$
RAI score†	2.5 (0.0–5.0)	1.5 (0–4)	NS

Fisher's exact test for categorical variables.

†Mann–Whitney *U*-test for non-normally distributed variables, expressed as median (range).

‡Student's *t*-test for normally distributed variables, expressed as mean (SD).

ADV, adefovir; cccDNA, covalently closed circular DNA; CYA, cyclosporin A; ETV, entecavir; HBV, hepatitis B virus; HBcAb, hepatitis B core antibody; HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B envelope antigen; HCC, hepatocellular carcinoma; LAM, lamivudine; LB, liver biopsy; LT, liver transplantation; MMF, mycophenolate mofetil; n, negative; NS, not significant; p, positive; RAI, Rejection Activity Index; RTI, reverse transcriptase inhibitor; SD, standard deviation; TAC, taclolimus.

and thus, it is questionable if HBcrAg truly reflects the viral pattern of HBV. Therefore, we designed this study to examine the usefulness of further analysis of cccDNA, which truly functions as a reservoir of HBV replication.

In the results of this study, a positive correlation between HBcrAg and cccDNA was shown, and this was consistent with a previous report on chronic hepatitis B.[11] These findings suggest the usefulness of monitoring HBV dynamics of patients after LTs because examinations of serum HBcrAg are less invasive methods compared with examinations of cccDNA levels in liver tissue. HBcrAg enables us to frequently check the HBV dynamics of patients, and it contributes to a reduction in the risk of HBV reactivation.[13]

However, as shown in Table 2, the results of the HBcrAg and cccDNA levels were not matched in 35% (7 of 20) of the patients. This may be due to a problem with the sensitivity of these two markers. We should use these markers cautiously because HBV might exist even if these were negative. Suzuki *et al.* reported that among the 13 patients with negative results for HBsAg, HBeAg, and HBV-DNA, all had positive results with cccDNA, while HBcrAg was positive in only seven patients.[11] In addition, cccDNA was also examined in a limited way because it was extracted from tissue from only a small part of the liver. Moreover, some reports have suggested that cccDNA can be detected in extrahepatic sites,[17] and thus, it is impossible to determine whether HBV exists with only one method. Therefore, we preferred to assess HBV dynamics with these two methods in order to overcome problems with sensitivity.

Interestingly, in the group with negative results for both of the two markers, the fibrosis stage was significantly lower compared with the other. This might reflect HBV activity after the LT. In addition, it was considered that keeping the two markers negative after LT may suggest the possibility of an extension of graft survival. But we observed only a limited period, further study of long-term outcome will be required.

The goal of this study was to determine the criteria for the appropriate prophylaxis of HBV related to LT with these two markers. Lenci *et al.* reported that 80.1% of the patients with undetectable intrahepatic cccDNA levels did not exhibit signs of HBV recurrence, even after withdrawal of the prophylaxis.[18] We thought that it might be possible to select patients more efficiently and correctly by using a method that combines examinations of HBcrAg and cccDNA. We observed one patient with both HBcrAg- and cccDNA-negative discontinued antiviral therapy.