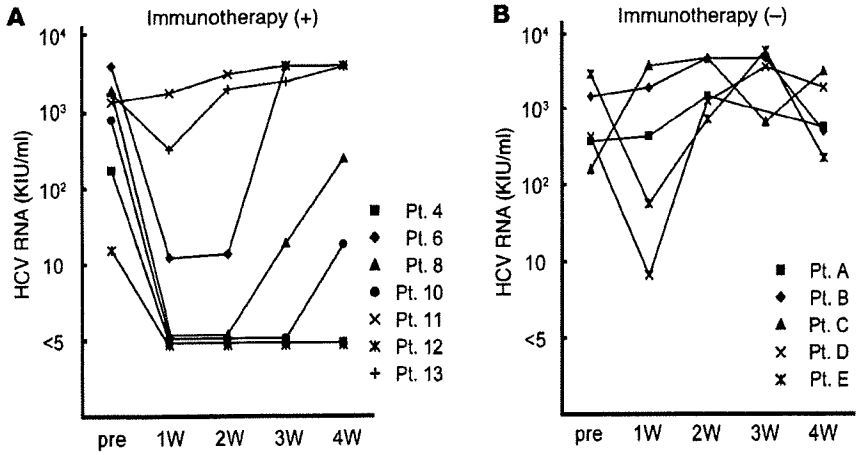

Figure 3
Serial measurement of the HCV RNA titers of LT recipients after LT. The HCV RNA titers in the sera of LT recipients who received immunotherapy were markedly lower than those in the sera of LT recipients who did not receive the therapy during the first month after LT. Each line with a different symbol represents serial HCV RNA titers from an LT recipient who received (+) (**A**; $n = 7$) and 1 who did not receive (–) (**B**; $n = 5$) the immunotherapy after LT. KIU, kilo international unit; pre, pre LT; W, week.

tion that NK cell–conditioned media have an enhanced expression of signal transducer and activator of transcription 1, a nuclear factor that is essential in IFN-γ–mediated antiviral pathways. It has also been reported that hepatocytes cultured in NK cell–conditioned media express higher levels of IFN-α/β, IFN regulatory factor 3, and IFN regulatory factor 7, confirming that NK cells play a key role in suppressing HCV infection of and replication in human hepatocytes in an IFN-dependent manner (23). Similar to recent reports, in the present study, we demonstrated that the NK cells among the IL-2/OKT3–treated liver lymphocytes released soluble factors, predominantly IFN-γ, thus suppressing HCV replication (Figures 5–7).

In addition to NK cells, NKT cells are thought to be involved in eliciting innate responses against infection; however, the role of NKT cells in controlling HCV infection/replication remains unclear. One report has indicated that the number of NKT cells in patients with chronic HCV infection does not differ from that in healthy donors; however, activated NKT cells in HCV-infected patients produce higher levels of IL-13 — but comparable levels of IFN-γ — than those in healthy subjects, showing that NKT cells are biased toward T-helper 2-type responses in chronic HCV infection (24). Another recent report has shown that the sustained response of patients with chronic hepatitis C to treatment with IFN-α and ribavirin is closely associated with increased dynamism of NK and NKT cells in the liver, implicating an NKT cell–mediated mechanism in anti-HCV activity (25). Here, we have described that NKT as well as NK cells in the IL-2/

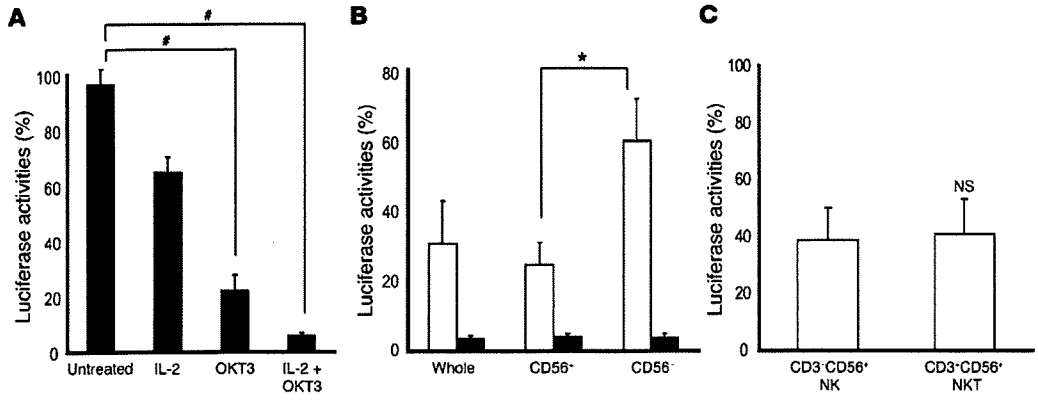

Figure 4
The cultivation of liver lymphocytes with IL-2/OKT3 markedly promoted anti-HCV activity. (**A**) Activation by IL-2 and OKT3 significantly promoted the anti-HCV effect of the liver allograft–derived lymphocytes that were cultured in complete medium with and without IL-2 (100 JRU/ml) for 3 days. OKT3 (1 μg/ml) was then added 1 day before coculturing with HCV replicon cells, at the indicated time. The bar graphs indicate the luciferase activities of the cells in each group. Data are presented as mean ± SEM ($n = 5$). Statistical analyses were performed using the Mann-Whitney *U* test with Bonferroni correction after the Kruskal-Wallis *H* test. $^{\#}P < 0.01$ for OKT3 and IL-2/OKT3 treatment versus no treatment. (**B**) $CD56^+$ fraction, including NK and NKT cells, strongly inhibited HCV replication. The culture conditions are described in **A**. By magnetic cell sorting, $CD56^+$ and $CD56^-$ fractions were isolated from the activated lymphocytes and analyzed for anti-HCV activity. The bar graphs indicate the luciferase activities of the cells in each group (IL-2–treated group, white bars; IL-2 plus OKT3–treated group, black bars). Whole, whole lymphocytes. Data are presented as mean ± SEM ($n = 5$). Statistical analyses were performed using the Mann-Whitney *U* test. $^{*}P < 0.05$ for $CD56^+$ fraction versus $CD56^-$ fraction. (**C**) Anti-HCV effect of NK cells was almost identical to that of NKT cells after IL-2 activation. The liver allograft–derived lymphocytes were cultured in complete medium with IL-2 (100 JRU/ml) for 3 days. By magnetic sorting, $CD3^-CD56^+$ (NK) and $CD3^+CD56^+$ (NKT) fractions were isolated from the activated lymphocytes and analyzed for anti-HCV activity. Data are presented as mean ± SEM ($n = 6$).

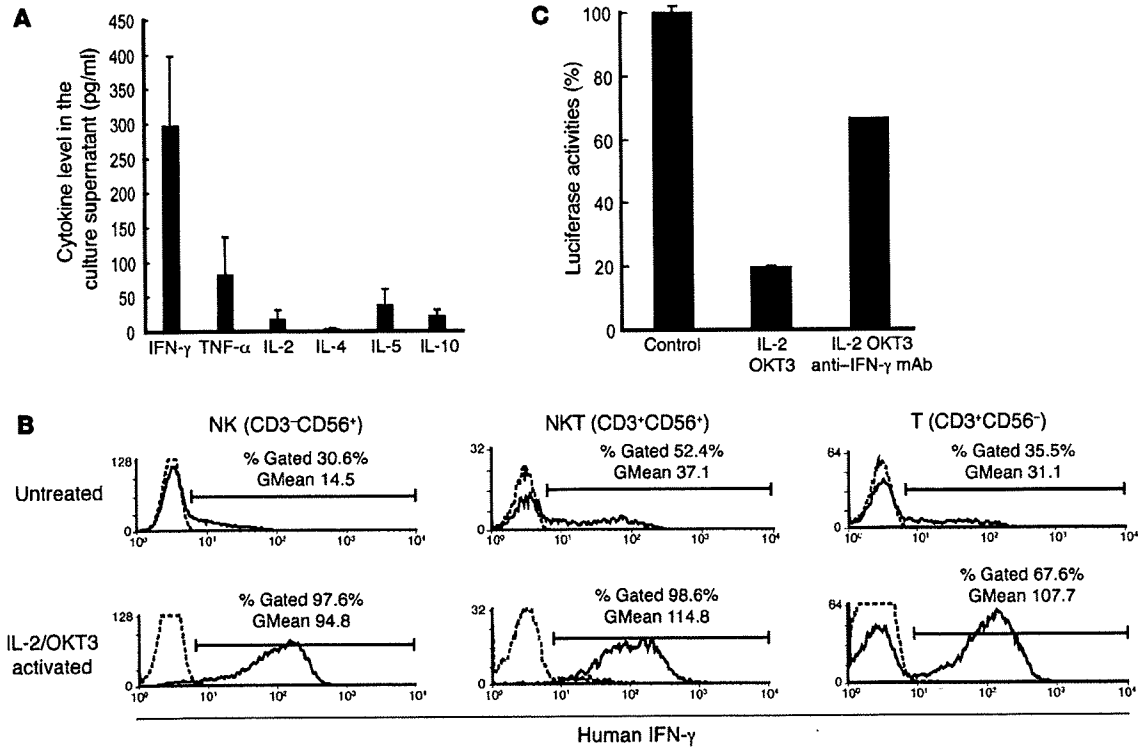

Figure 5

Anti-HCV activity of IL-2/OKT3–treated liver lymphocytes was dependent on their IFN-γ secretion ability. (**A**) IFN-γ was the major cytokine released from the cultured cells. The bar graphs indicate the concentrations of various cytokines (IFN-γ, TNF-α, IL-2, IL-4, IL-5, and IL-10) detected in the coculture supernatant by CBA. Data are presented as mean ± SEM (n = 3). (**B**) The effects of IL-2 and OKT3 (100 JRU/ml and 1 μg/ml, respectively) on IFN-γ production by stimulated $CD3^-CD56^+$ NK, $CD3^+CD56^+$ NKT, and $CD3^+CD56^-$ T cells were evaluated by a combination of cell surface and cytoplasmic mAb staining and subsequent flow cytometric analysis. Histograms represent the log fluorescence intensities obtained upon staining for IFN-γ after gating of each fraction. Dotted lines represent negative control staining with isotype-matched mAbs. Horizontal lines indicate the gated portion of lymphocytes. GMean, geometric mean fluorescent intensity. (**C**) Blocking of IFN-γ with mAb (100 μg/ml) elucidated the marked role played by IFN-γ in producing the anti-HCV effect. The bar graphs indicate the luciferase activities of the cells in each group. Data are presented as mean ± SEM of a representative triplicate sample.

OKT3-treated liver lymphocytes could play a vital role in controlling HCV replication in hepatic cells via an IFN-γ-associated mechanism (Figures 5 and 6).

Therefore, in the early phase of HCV reinfection after LT, the effects of IFN-γ secretion from adoptively injected liver lymphocytes may include inhibition of HCV virion production, which is probably caused by suppression of viral RNA and protein synthesis without immune lysis of intact hepatic cells. This IFN-γ secretion from both $CD3^+CD56^+$ NKT cells and $CD3^+$ T cells was markedly upregulated after treatment with OKT3, which was originally used to prevent GVHD (Figure 5B). This is possibly because of the potent mitogenic activity of OKT3 that induces the activation of $CD3^+CD56^+$ NKT cells and $CD3^+$ T cells. However, the administration of OKT3-coated cells in vivo results in the opsonization and subsequent trapping and/or lympholysis of cells by the reticuloendothelial system (26–28). Thus, GVHD is prevented in LT recipients treated with adoptive immunotherapy.

Our finding that the IL-2/OKT3-treated liver lymphocytes controlled HCV replication via an IFN-γ-associated mechanism can lead to the clinical application of recombinant IFN-γ for anti-HCV treatment. However, a clinically applicable dose of recombinant IFN-γ could not induce significant inhibitory effects on HCV viremia in the previous study (29). Based on the accumulation of adoptively injected IL-2/OKT3-treated liver lymphocytes in the liver of human hepatocyte-chimeric mice (data not shown), the immunotherapy with the liver lymphocytes would provide sufficient IFN-γ to the HCV-infected site.

It has been recently reported that HCV-specific $CD8^+$ T cells exert strong antiviral effects by both cytopathic and IFN-γ-mediated noncytopathic effector functions (30). However, in patients with chronic HCV infection, dysfunction and functional restoration of HCV-specific $CD8^+$ T cell responses have been reported (31). Since HCV-specific $CD8^+$ T cell defects may be important in persistent HCV infections, correcting these defects is considered to our knowledge to be a novel approach to treat HCV infection. Further studies are required to investigate whether activation of NK or NKT cells functionally restores HCV-specific $CD8^+$ T cells.

In conclusion, adoptive immunotherapy using IL-2/OKT3–treated liver lymphocytes containing abundant NK and NKT cells could mount remarkable anti-HCV responses in HCV-infected LT recipients, although its effects were incomplete or transient. Treatment-related improvements, such as defining the best schedule and frequency of cell inoculation and developing more potent effectors, could improve clinical benefits.

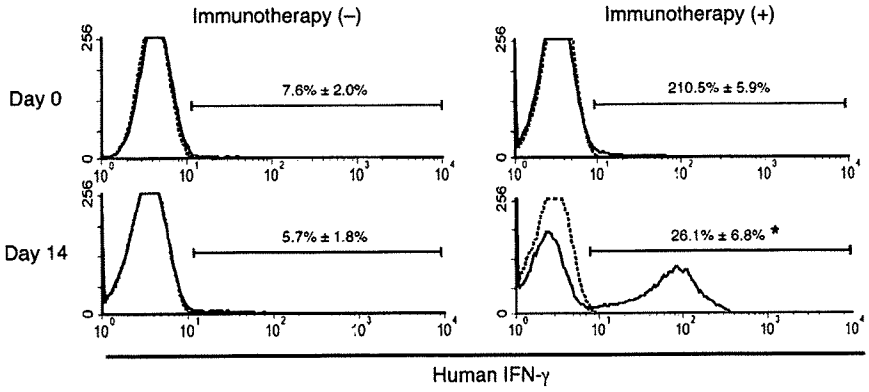

Figure 6
Adoptive immunotherapy with IL-2/OKT3–treated liver lymphocytes induced the production of IFN-γ in the LT recipients. At 14 days after LT, the number of IFN-γ–secreting cells in the peripheral blood of LT recipients treated with the adoptive immunotherapy (+) with IL-2/OKT3–treated liver lymphocytes, including NK and NKT cells, was significantly higher than that in the peripheral blood of untreated LT recipients (–). Histograms represent the proportion (percentage) of IFN-γ–positive cells among the mononuclear cells obtained from the peripheral blood of the immunotherapy (n = 4) and control group (n = 4) LT recipients. Dotted lines represent negative control staining with isotype-matched mAbs. Horizontal lines indicate the gated portion of lymphocytes. Data are presented as mean ± SEM. Histogram profiles shown are representative of 4 independent experiments. Statistical analyses were performed using the Mann-Whitney U test. *P < 0.05 for immunotherapy group versus control group.

Methods

Subjects. All the human liver samples were collected at Hiroshima University Hospital. Tissue specimens were collected after approval from the Institutional Review Board of Hiroshima University and after written informed consent was obtained from the patients. The use of immunotherapy with IL-2/OKT3–treated liver lymphocytes was approved by the Clinical Institutional Ethical Review Board of Hiroshima University. Written informed consent was obtained from all of the patients. This approach was successfully used in 14 cirrhotic patients with HCC undergoing clinical LT (Tables 1 and 2). Of these 14 patients, 7 had chronic HCV infection. Five other LT recipients with chronic HCV infection did not agree to receive this immunotherapy during the trial period. HCV RNA was qualitatively detected in the sera of these patients by a standardized qualitative RT-PCR assay (Amplicor HCV monitor, version 2.0; Roche Diagnostics) every week during the first month after LT.

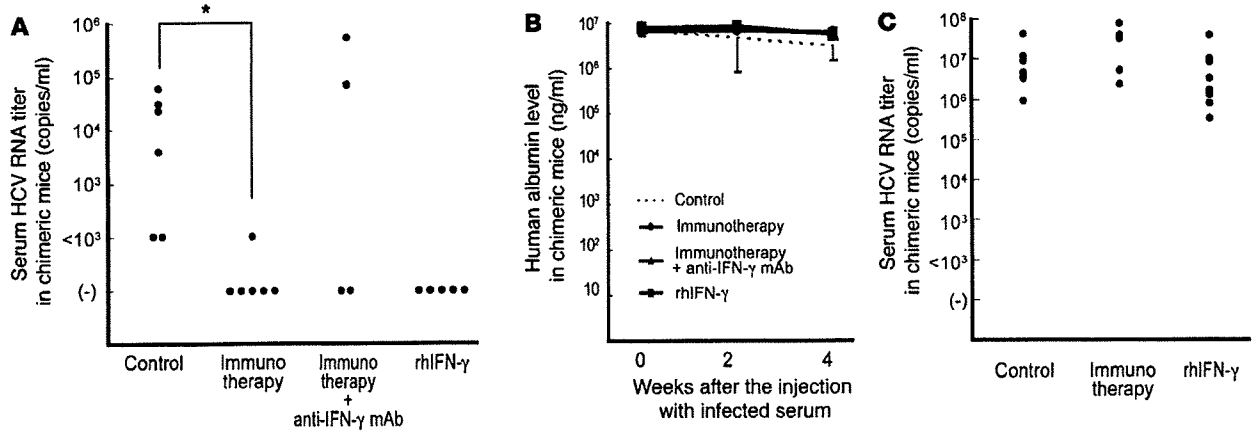

Figure 7
Adoptive immunotherapy with IL-2/OKT3–treated liver lymphocytes prevented HCV infection in human hepatocyte–chimeric mice. (**A**) Human hepatocyte–chimeric mice were intravenously injected with human serum samples positive for HCV genotype 1b. Two weeks after injecting the infected serum, the mice were intraperitoneally inoculated with IL-2/OKT3–treated liver lymphocytes (20 × 10^6 cells/mouse; n = 6) for adoptive immunotherapy. When indicated, anti-human IFN-γ mAb was injected intraperitoneally 1 day before the immunotherapy (n = 4). Intraperitoneal injection of recombinant human IFN-γ (rhIFN-γ) was commenced at 2 weeks after injecting the infected serum (n = 5). The untreated mice served as controls (n = 6). The dot plots represent serum HCV RNA titers in each chimeric mouse 4 weeks after the injecting the infected serum. Statistical analyses were performed using the Mann-Whitney U test. *P < 0.01 for immunotherapy group versus control group. (**B**) The lines represent serial changes in human serum albumin levels in the sera of the mice indicated above. Data are presented as mean ± SEM. (**C**) IL-2/OKT3–treated liver lymphocytes (20 × 10^6 cells/mouse) were intraperitoneally inoculated 4 weeks after the injection with the infected serum (n = 5) for adoptive immunotherapy. Intraperitoneal injection of recombinant human IFN-γ was commenced 4 weeks after the injecting the infected serum (n = 9). The untreated mice served as controls (n = 9). The dot plots represent serum HCV RNA titers in each chimeric mouse 6 weeks after injection with the infected serum.

Isolation of lymphocytes from liver allograft perfusate. Donor hepatectomy and the transplantation procedure were performed as described previously (32). After hepatectomy, ex vivo perfusion of the liver allograft was performed through the portal vein. Liver allograft–derived lymphocytes were isolated by gradient centrifugation with Ficoll-Paque (GE Healthcare Bio-Sciences AB).

Adoptive transfer of IL-2/OKT3–treated liver lymphocytes. Liver lymphocytes were cultured with human recombinant IL-2 (100 Japanese reference units/ml [JRU/ml]; Takeda) in complete medium at 37°C in a 5% CO_2 incubator for 3 days. One day before the infusion, 1 μg/ml of OKT3 (Janssen-Kyowa) was added in order to opsonize the $CD3^+$ fraction. On the day of infusion, the cells were washed twice with 0.9% sodium chloride and resuspended with 5% human serum albumin in 0.9% sodium chloride for injection (Figure 1). The viability of the cells was assessed by the dye-exclusion test, and the cells were checked twice for possible contamination by bacteria, fungi, and endotoxins.

Cytotoxicity assay. A ^{51}Cr-release assay was done as previously described (5), using HepG2 tumor cells (Japanese Cancer Research Resources Bank) as targets. Briefly, ^{51}Cr-labeled target tumor cells were added for 4 hours at 37°C to effector cells in round-bottomed 96-well microtiter plates (BD Biosciences — Discovery Labware). The percentage of specific ^{51}Cr release was calculated as follows: % cytotoxicity = [(cpm of experimental release − cpm of spontaneous release)/(cpm of maximum release − cpm of spontaneous release)] × 100. All the assays were performed in triplicate.

Flow cytometry. Flow cytometric analyses were performed using a FACSCalibur dual-laser cytometer (BD Biosciences). The following mAbs were used for the surface staining of the lymphocytes: FITC-conjugated anti-CD3 mAb (clone HIT3a; BD Biosciences — Pharmingen); PE-conjugated anti-CD56 mAb (clone B159; BD Biosciences — Pharmingen); and biotinylated anti-TRAIL (biotin-conjugated anti-TRAIL) mAb (clone RIK-2; eBioscience). The biotinylated mAb was visualized using APC-streptavidin (BD Biosciences — Pharmingen). Dead cells identified by light scatter and propidium iodide staining were excluded from the analysis. IFN-γ production in the lymphocytes was measured by a combination of cell surface and cytoplasmic mAb staining and subsequent flow cytometric analysis, as described previously (33).

Isolation of $CD56^+$ and $CD56^-$ fractions and that of NK and NKT cells. Liver allograft–derived lymphocytes were separated into a $CD56^+$ fraction — including NK and NKT cells — and a $CD56^-$ fraction by using auto MACS (Miltenyi Biotec) with anti-human CD56 microbeads (Miltenyi Biotec) according to the manufacturer's instructions. The NK and NKT cells were also isolated by magnetic cell sorting, using the human NK cell isolation kit or human $CD3^+CD56^+$ NKT cell isolation kit (Miltenyi Biotec). The purity of the isolated fractions was assessed by flow cytometric analysis, and only the fractions with purities greater than 90% were used for functional studies.

Coculture with HCV replicon–containing hepatic cells. An HCV subgenomic replicon plasmid, pRep-Feo, was derived from pRep-Neo (originally, pHCVIbneo-delS; ref. 34). The pRep-Feo carries a fusion gene comprising firefly luciferase (*Fluc*) and neomycin phosphotransferase, as described elsewhere (35, 36). After culture in the presence of G418 (Invitrogen), pRep-Feo cell lines stably expressing the replicons were established. For coculture experiments, transwell tissue culture plates (pore size, 1 μm; Costar) were used. HCV replicon–containing hepatic cells (10^5 cells) were incubated in the lower compartment with different numbers of lymphocytes in the upper compartment. The hepatic cells in the lower compartments were collected 48 hours after coculture for the luciferase assay. Luciferase activities were measured with a luminometer (Lumat LB9501; Promega), using the Bright-Glo Luciferase Assay System (Promega).

Cytometric bead array. Cytokine (IFN-γ, TNF-α, IL-2, IL-4, IL-5, IL-10) levels in the coculture assay supernatants were measured with the FACSCalibur dual-laser cytometer (BD Biosciences), using a BD Human Th1/Th2 Cytokine Cytometric Bead Array (CBA) Kit according to the manufacturer's instructions.

Generation of human hepatocyte–chimeric mice. Generation of the *uPA*$^{+/+}$ *SCID*$^{+/+}$ mice and transplantation of human hepatocytes were performed as described recently by our group (20, 37). Mouse serum concentrations of human serum albumin correlated with the repopulation index (20), and these were measured as described previously (37).

In vivo studies using human hepatocyte–chimeric mice. Human hepatocyte–chimeric mice were intravenously injected with 50 μl of the human serum samples positive for HCV genotype 1b. The serum HCV RNA titer in human hepatocyte–chimeric mice was detected by nested PCR, as previously described (38, 39). All animal protocols described in this study were performed in accordance with the guidelines and with approval of the Ethics Review Committee of Animal Experimentation of the Graduate School of Biomedical Sciences, Hiroshima University. Either 2 or 4 weeks after injecting the infected serum, the mice were intraperitoneally inoculated with IL-2/OKT3–treated liver lymphocytes (20×10^6 cells/mouse) for adoptive immunotherapy. When indicated, anti-human IFN-γ mAb (R&D Systems) (1.5 mg/mouse) was injected intraperitoneally 1 day before the immunotherapy. In a separate experiment, intraperitoneal injection of recombinant human IFN-γ (Imunomax-γ; Shionogi & Co. Ltd.) was commenced at either 2 or 4 weeks after injecting the infected serum. IFN-γ was administered as follows: 1×10^5 IU on the first day and thereafter 2×10^4 IU/day for 13 days.

Statistics. Data are presented as mean ± SEM. The statistical differences of the results were analyzed by 2-tailed, paired Student's *t* test, Mann-Whitney *U* test, and Mann-Whitney *U* test with Bonferroni correction after the Kruskal-Wallis *H* test, using the Stat View program. *P* values of 0.05 or less were considered statistically significant.

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Address correspondence to: Hideki Ohdan, Department of Surgery, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan. Phone: 81-82-257-5220; Fax: 81-82-257-5224; E-mail: hohdan@hiroshima-u.ac.jp. Or to: Kazuaki Chayama, Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan. Phone: 81-82-257-5190; Fax: 81-82-257-5194; E-mail: chayama@hiroshima-u.ac.jp.

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ORIGINAL ARTICLE

Successful hepatitis B vaccination in liver transplant recipients with donor-specific hyporesponsiveness

Hiroyuki Tahara,[1,2] Yuka Tanaka,[1,2] Kohei Ishiyama,[1,2] Kentaro Ide,[1,2] Masayuki Shishida,[1,2] Toshimitsu Irei,[1,2] Yuichiro Ushitora,[1,2] Masahiro Ohira,[1,2] Masataka Banshodani,[1,2] Hirotaka Tashiro,[1,2] Toshiyuki Itamoto,[1,2] Toshimasa Asahara,[1,2] Michio Imamura,[2,3] Shoichi Takahashi,[2,3] Kazuaki Chayama[2,3] and Hideki Ohdan[1,2]

1 Department of Surgery, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Science, Hiroshima University, Hiroshima, Japan
2 Liver Research Project Center, Hiroshima University, Hiroshima, Japan
3 Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Science, Hiroshima University, Hiroshima, Japan

Keywords
antiviral prophylaxis, CFSE-MLR assay, HB vaccination, liver transplantation.

Correspondence
Hideki Ohdan MD, PhD, Department of Surgery, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical 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
Kazuaki Chayama MD, PhD, Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University 1-2-3 Kasumi, Minami-Ku, Hiroshima 734-8551, Japan. Tel.: +81 82 257 5190; fax: +81 82 255 6220; e-mail: chayama@hiroshima-u.ac.jp

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Summary

Currently, patients are prescribed lifelong treatment with hepatitis B immunoglobulin (HBIg) after liver transplantation (LT) for hepatitis B virus (HBV)-related diseases in order to prevent reinfection with HBV. Active immunization with an HBV vaccine would be a preferable alternative; however, the immunosuppressive environment in LT recipients is believed to elicit a poor response to vaccination. Minimizing the exposure of the HBV-infected LT recipients to immunosuppressants would be beneficial in inducing adaptive immunity against HBV by vaccination. In this study, in addition to efforts to minimize immunosuppression, prophylaxis with HBV vaccination combined with continuous HBIg administration was performed in 17 LT recipients who had undergone transplantation attributable to HBV-related diseases. During the observation period, the overall response rate to HBV vaccination was 64.7%. The immune status of the recipients was evaluated by a mixed lymphocyte reaction assay in response to allostimulation. Patients showing a donor-specific hyporesponse with a well-maintained response to the third-party stimulus always achieved a sustained immune response to the vaccine, whereas patients showing a hyporesponse to both the donor and the third-party stimulus were unable to do so. Thus, inducing an anti-donor-specific immunosuppressive status by minimizing immunosuppression should enable post-transplant HBV vaccination to be a promising prophylactic strategy.

Introduction

Patients face a high risk of endogenous hepatitis B virus (HBV) reinfection in the absence of postoperative prophylaxis after liver transplantation (LT) caused by HBV-related disease. Combined treatment with either a nucleoside or nucleotide analog and hepatitis B immunoglobulins (HBIg) has been the gold standard for prophylaxis of HBV reinfection after LT [1–3]. According to current recommendations, HBIg should be administered indefinitely after LT [4–6]. However, indefinite prophylaxis with HBIg has substantial drawbacks, such as increasing costs [7] and the risk of emergence of HBV envelope protein mutations [8,9]. Therefore, induction of an active immune response against the hepatitis B surface antigen (HBsAg), leading to the continuous production of specific antibodies would be

an enormous advantage, and it would eliminate the need for lifelong replacement with HBIg [10,11].

Several groups have attempted vaccination of LT recipients against HBV [11–20]. In most of these studies, relatively low seroconversion rates as well as serum anti-HBs concentrations were observed among chronic HBV-infected LT recipients; only a minority of vaccinees developed stable antibody levels >100 IU/l, the maintenance of which is required for prevention of HBV reinfection [21]. The poor response to vaccination was probably because of the immunosuppressive environment in LT recipients. Minimizing the exposure of HBV-infected LT recipients to immunosuppressants appears to be beneficial in inducing adaptive immunity against HBV by vaccination; however, the relevance of the immune status of LT recipients to the outcome of HBV vaccination remains to be elucidated.

In this study, prophylactic HBV vaccination combined with continuous HBIg administration was performed in 17 LT recipients who had undergone transplantation because of an HBV-related disease and had not experienced signs of recurrence for at least 12 months after treatment with HBIg. The immune status of these patients was evaluated by a mixed lymphocyte reaction (MLR) assay in response to anti-donor and third-party allostimulation using an intracellular carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeling technique.

Patients and methods

Patients

In this study, we included 17 living donor LT recipients at the Hiroshima University Hospital. All patients had normal liver function without any virologic and biochemical evidence of HBV recurrence. The following were the inclusion criteria: (i) at least 3 months of HBIg plus lamivudine (100 mg/day) with/without adefovir (10 mg/day) administration and (ii) no findings of recurrent infection and negativity for HBsAg and hepatitis B viral deoxyribonucleic acid (HBV DNA) (by PCR) at the time of vaccination. For prophylaxis against reinfection, all transplanted patients were on a stable schedule of 1000–2000 IU of intravenous HBIg every 4 weeks in order to maintain an anti-HBs titer of >100 IU/l. We attempted to minimize immunosuppression in all patients with good liver function by adopting the policy of tapering off the immunosuppressants. The study protocol was approved by the Ethics Committee of Hiroshima University, and all patients provided informed consent before entering into the trial. None of the vaccinees showed clinical evidence of recurrence of HBV graft infection and the episode of rejection throughout the follow-up period, and all of them were persistently negative for both HBsAg and HBV DNA, except for one vaccinee (Patient #3) who showed temporarily positive for HBV DNA.

Vaccination protocol

All participants received a yeast-derived recombinant, adsorbed HBV vaccine (Bimmugen®; Chemotherapy and Serotherapy Laboratories Inc., Kumamoto, Japan) subcutaneously every 4 weeks at a dose of 10–20 μg (0.5–1.0 ml) in combination with HBIg and lamivudine/adefovir. HBIg immunoprophylaxis was continued during primary immunization (dose, 1000–2000 IU every 4 weeks). The response to vaccination was defined as (i) a confirmed increase in the anti-HBs titer to >100 IU/l that could not be explained by HBIg administration and (ii) sustained anti-HBs titer to >100 IU/l after discontinuation of combined administration of the vaccine and HBIg. If the anti-HBs titer exceeded the responsive increasing level, HBIg substitution and vaccine administration were discontinued. Lamivudine/adefovir prophylaxis was additionally discontinued, if the anti-HBs titer was maintained effectively without HBIg administration. The vaccine was continuously and indefinitely administered till acquired immunity was elicited.

Serologic markers and virologic assays

Serum HBsAg, hepatitis Be antigen (HBeAg), hepatitis B core antibody (HBcAb), and anti-HBsAb were measured monthly using an enzyme-linked immunoassay (Abbott Diagnostics, Chicago, IL, USA). HBV DNA was detected by the Amplicor HBV monitor test (Roche Diagnostics, Tokyo, Japan). The measurement range of the assay is $10^{2.6}$–$10^{7.6}$ copies/ml (2.6–7.6 log copies/ml). These quantitative assays of HBV DNA were performed at the Special Reference Laboratory, Tokyo, Japan. Positive levels of HBV DNA were defined as levels >2.6 log copies/ml. HBV recurrence was diagnosed on the basis of appearance of HBsAg or HBV DNA.

Immune monitoring by *in vitro* CFSE-MLR assay

For patients who showed completely normal liver function, CFSE-MLR was performed to determine whether immunosuppression could be further minimized. In patients with hyporesponse of anti-donor T cells, immunosuppression was successfully reduced.

For CFSE-MLR, the peripheral blood mononuclear cells prepared from the blood of the LT recipients (autologous control), donors, and healthy volunteers with same blood type as the donors (third-party control) for use as the stimulator cells were irradiated with 30 Gy and those obtained from the recipients for use as the responder cells

were labeled with 5 μM CFSE (Molecular Probes Inc., Eugene, OR, USA), as described previously [22]. The stimulator and responder cells (2×10^6 each) were incubated in 24-well flat-bottomed plates (BD Labware, Franklin Lakes, NJ, USA) in a total volume of 2 ml of culture medium at 37 °C under 5% CO_2 for 5 days. After culture for MLR, the harvested cells were stained with either phycoerythrin (PE)-conjugated anti-human CD4 or PE-conjugated anti-human CD8 monoclonal antibodies (mAbs; BD Pharmingen, San Diego, CA, USA) and subjected to analysis by flow cytometry (FCM). All analyses were performed on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA). Dead cells were excluded from the analysis by forward scatter or propidium iodide gating. T-cell proliferation was visualized by serial-halving of the fluorescence intensity of CFSE. $CD4^+$ and $CD8^+$ T-cell proliferation and stimulation index (SI) were quantified using a previously described method [23,24]. Briefly, the number of division precursors was extrapolated from the number of daughter cells of each division, and the number of mitotic events in each $CD4^+$ and $CD8^+$ T-cell subset was calculated. Using these values, the mitotic index was calculated by dividing the total number of mitotic events by the total number of precursors. The SIs of allogeneic combinations were calculated by dividing the mitotic index of a particular allogeneic combination by that of self-control.

Statistical analysis

The values are presented as the median and the range. The Mann–Whitney *U*-test was performed to analyze whether the age of the vaccinees at the time of vaccination, the time elapsed since LT, the anti-HBsAb titers at the start of the vaccination, the median tacrolimus trough levels, and the SI in anti-donor and anti-third-party MLR differed significantly between the good and poor responders and also between the moderate and poor responders. A Fisher's exact test was performed to determine whether there were differences between both the above groups with regard to gender, indication for LT, ratio of HBV DNA and HBeAg negative before LT, ratio of donor HBc and HBsAb positive before LT, and immunosuppressive monotherapy at the time of vaccine administration. *P*-values below 0.05 were considered statistically significant.

Results

Demographics

A total of 17 HBV vaccinees (four female- and 13 male subjects; age range, 20–65 years; median age, 49 years) participated in this study. The demographic and clinical data of the participants are shown in Table 1. Of them, 14 patients underwent LT for HBV-related cirrhosis and three underwent transplantation for HBV-related fulminant hepatic failure. Among the 17 vaccinees, five (29.4%) had been HBV DNA positive before LT with levels >2.6/ml, and five (29.4%) had been HBeAg positive before LT. Immunosuppressive treatment comprised either cyclosporine or tacrolimus monotherapy in 11 patients (64.7%) and additional steroid therapy (methylprednisolone, 2–4 mg/day) in six patients. Steroids were withdrawn at after a median duration of 13 months (range, 1–50 months) after LT. At the time of vaccination, a median duration of 21 months (range, 3–41 months) had elapsed since LT. The median follow-up time after commencement of vaccination was 26 months (range, 8–72 months). At the start of vaccination, a median anti-HBsAb titer was 161.4 (range, 37.7–328.4) IU/l.

Response to vaccination

During the observation period, 11 of the 17 HBV vaccinees (64.7%) achieved a sustained immune response to the HBV vaccine, which was defined as a confirmed increase in the anti-HBs titer to >100 IU/l that could not be explained by HBIg administration and no decrease in the anti-HBs titer to <100 IU/l even after discontinuation of combined administration of the vaccine and HBIg (Table 1). Within 1 year, 5/11 responders responded to the vaccine, and other six responded after 1 year from the commencement of vaccination (Fig. 1a and b). The other six HBV vaccinees did not respond to the vaccine during the study period (Fig. 1c). When the subjects were divided into three distinct groups, i.e., patients who responded to the vaccine within 1 year after commencement of vaccination (good responders), patients who responded to the vaccine after 1 year since commencement of vaccination (moderate responders), and patients who did not respond to the vaccine within 1 year and still remain receiving the vaccine (poor responders), the following factors did not exhibit statistically significant differences between the good and poor responders and also between the moderate and poor responders: age, gender, indication for LT, HBV viremia, donor HBcAb and HBsAb before LT, immunosuppressive regimen and tacrolimus trough levels and anti-HBsAb titers at the time of vaccination, duration between vaccination and transplantation and also duration between steroid withdrawal and transplantation. (Table 2) (Fig. 2).

Estimation of immunosuppressive status during vaccination by CFSE-MLR assay

Eleven patients (#1, 2, 4, 5, 7, 9, 11, 12, 13, 14 and 17) and their donors consented to be subjected to an

Table 1. The demographic and clinical characteristics of patients.

Patient	Age*	Gender	Underlying disease	HBV DNA before LT	Recipient HBeAg before LT	Donor HBcAb before LT	Donor HBsAb before LT	Time of vaccination†	Time of steroid withdrawal†	Duration of follow-up‡	Immuno-suppressive drugs*	Tac/CsA trough level (ng/ml)*	Anti-HBsAb titer (IU/l)*
Patients who responded to the vaccine within 1 year after commencement of vaccination (good responders)													
1	62	M	Cirrhosis/HCC	<2.6	Negative	NA	Negative	41	3	34	CsA 50 mg	39.3 (CsA)	152.6
2	54	M	Cirrhosis/HCC	<2.6	Negative	NA	NA	26	2	35	CsA 50 mg	15.0 (CsA)	189.1
3	58	M	Cirrhosis	6.4	Positive	Negative	Negative	9	2	43	Tac 2 mg	4.6	161.0
4	43	M	Cirrhosis/HCC	3.4	Negative	Negative	NA	35	45	20	Tac 3 mg + mPSL 2 mg	1.5	220.6
5	57	M	Fulminant	<2.6	Negative	Negative	Negative	9	1	15	Tac 2 mg	3.4	37.7
Patients who responded to the vaccine after 1 year since commencement of vaccination (moderate responders)													
6	34	M	Fulminant	<2.6	Negative	NA	NA	3	7	72	Tac 6 mg + mPSL 4 mg	4.2	152.1
7	38	M	Cirrhosis/HCC	<2.6	Negative	NA	NA	35	7	49	Tac 1 mg	4.4	146.6
8	57	M	Cirrhosis/HCC	<2.6	Negative	NA	Negative	40	50	31	Tac 2 mg + mPSL 4 mg	4.4	68.3
9	46	F	Cirrhosis/HCC	4.6	Positive	Negative	Positive	17	2	33	Tac 3 mg	4.7	93.4
10	46	F	Cirrhosis	<2.6	Negative	Positive	Positive	20	1	22	Tac 1 mg	4.2	214.9
11	53	M	Cirrhosis/HCC	<2.6	Negative	Positive	Positive	13	4	15	Tac 2 mg	4.2	160.5
Patients who did not respond to the vaccine during the study period (poor responders)													
12	20	M	Fulminant	>7.6	Positive	Negative	Positive	18	29	20	Tac 3 mg + mPSL 2 mg	5.0	222.7
13	46	M	Cirrhosis	<2.6	Negative	Negative	Negative	16	1	20	Tac 1 mg	6.6	188.9
14	58	F	Cirrhosis/HCC	<2.6	Negative	NA	NA	18	20	11	Tac 2 mg + mPSL 2 mg	1.5	92.3
15	65	M	Cirrhosis/HCC	4.5	Positive	NA	NA	12	21	13	Tac 4 mg + mPSL 4 mg	8.0	328.4
16	45	F	Cirrhosis/HCC	<2.6	Negative	NA	Negative	25	23	8	Tac 0.5 mg	3.7	193.3
17	54	M	Cirrhosis/HCC	<2.6	Positive	Positive	Positive	13	1	9	Tac 2 mg	2.9	122.2

LT, liver transplantation; Tac, tacrolimus; CsA, cyclosporine; mPSL, methylprednisolone; NA, not available.

*At the time of vaccination.

†Months after liver transplantation.

‡Months after commencement of vaccination.

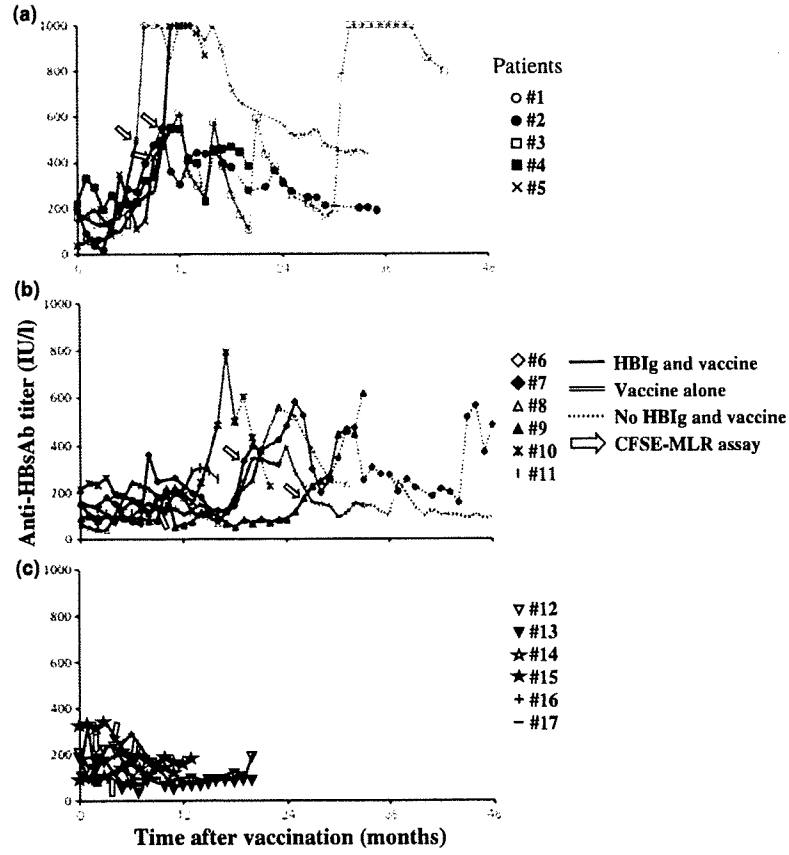

Figure 1 Anti-HBs titer kinetics in patients who responded to the vaccine within 1 year after commencement of vaccination (good responders) (a), in patients who responded to the vaccine after 1 year since the commencement of vaccination (moderate responders) (b), and in patients who did not respond to the vaccine (poor responders) (c).

Table 2. Age, gender, indication for LT, HBV viremia, immunosuppresive regimen, duration between vaccination and transplantation, and duration between steroid withdrawal and transplantation.

	Good responders (n = 5)	Moderate responders (n = 6)	Poor responders (n = 6)	P-value
Age at vaccination (years)*	55 (43–62)	46 (34–57)	48 (20–65)	NS
Gender (male/female)	5/0	4/2	4/2	NS
Indication for LT (fulminant hepatitis/cirrhosis)	1/4	1/5	1/5	NS
HBV DNA before LT (positive/negative)	2/3	2/4	2/4	NS
Recipient HBeAg before LT (positive/negative)	1/4	1/5	3/3	NS
Donor HBcAb before LT (positive/negative)	0/3	2/1	1/2	NS
Donor HBsAb before LT (positive/negative)	0/3	3/1	2/2	NS
CsA or Tac monotherapy/combination with steroid†	4/1	4/2	3/3	NS
Duration between vaccination and transplantation (months)*	24 (9–41)	21 (3–40)	17 (12–25)	NS
Duration between steroid withdrawal and transplantation (months)*	11 (1–45)	12 (1–50)	16 (1–29)	NS
Anti-HBsAb titer (IU/l)*†	152 (38–221)	139 (93–215)	191 (92–328)	NS

NS, not significant; LT, liver transplantation; CsA, cyclosporine A; Tac, tacrolimus.
*Median (range).
†At the time of vaccination.

MLR assay using a CFSE-labeling technique. In all the seven patients who responded to the HBV vaccine, limited $CD4^+$ T-cell proliferation was observed in the anti-donor MLR assay as compared with the anti-third-party MLR assay, i.e., a hyporesponse in the anti-donor MLR assay and a normal response in the anti-third-party MLR assay (Fig. 3). In these patients, the average of SIs for $CD4^+$ T cells in response to anti-third-party stimulation was >2 (average value in healthy volunteers without any immunosuppressive treatment). In contrast,

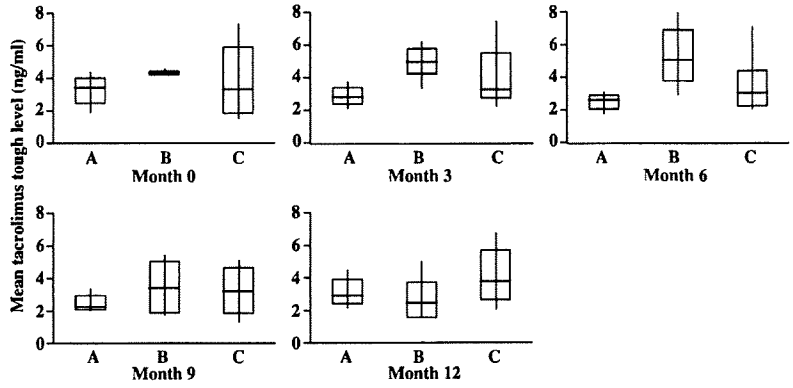

Figure 2 Tacrolimus trough levels in patients who responded to the vaccine within 1 year after commencement of vaccination (good responders) (A), in patients who responded to the vaccine after 1 year since the commencement of vaccination (moderate responders) (B), and in patients who did not respond to the vaccine (poor responders) (C). The Mann–Whitney *U*-test was used to compare the tacrolimus trough levels between the good and moderate responders with those of poor responders. The box plot represents the 25th to 75th percentile, the dark line is the median, and the extended bars represent the 10th to the 90th percentile. Statistical analyses at none of the time-points at 0, 3, 6, 9 and 12 months were significant.

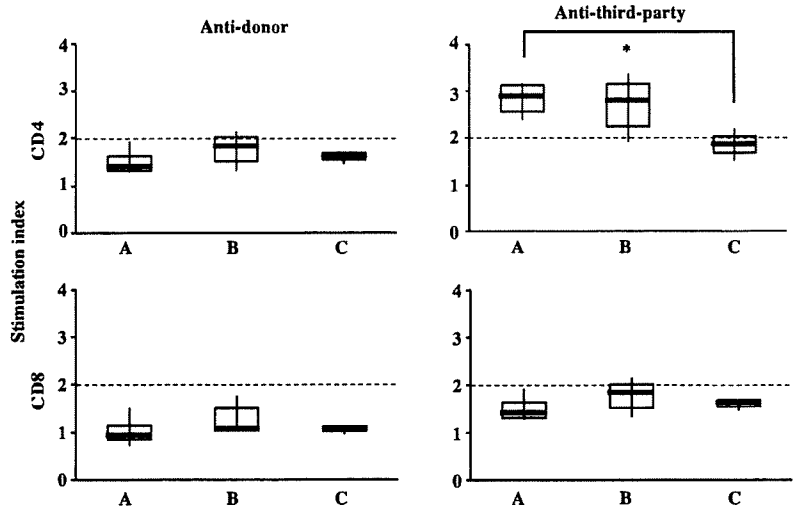

Figure 3 Stimulation indices (SIs) of each of the $CD4^+$ and $CD8^+$ T-cell subsets in the anti-donor and anti-third-party MLR in patients who responded to the vaccine within 1 year after commencement of vaccination (good responders) (A), in patients who responded to the vaccine after 1 year since the commencement of vaccination (moderate responders) (B), and in patients who did not respond to the vaccine (poor responders) (C). $CD4^+$ and $CD8^+$ T-cell proliferation and their SIs were quantified as follows. The number of division precursors was extrapolated from the number of daughter cells of each division, and the number of mitotic events in each of the $CD4^+$ and $CD8^+$ T-cell subsets was calculated. Using these values, the mitotic index was calculated by dividing the total number of mitotic events by the total number of precursors. The SIs of allogeneic combinations were calculated by dividing the mitotic index of a particular allogeneic combination by that of the self control. The Mann–Whitney *U*-test was used to compare the tacrolimus trough levels between the good and moderate responders with those of poor responders. The box plot represents the 25th to 75th percentile, the dark line is the median, and the extended bars represent the 10th to the 90th percentile. $*P = 0.04$.

in the four patients who did not respond to the HBV vaccine, limited $CD4^+$ and $CD8^+$ T-cell proliferation was observed in both the anti-donor and the anti-third-party MLR assay, i.e., a hyporesponse in both cases. In these patients, the average of SIs for $CD4^+$ T cells in response to both anti-donor and anti-third-party stimulation was <2. Thus, the SIs for $CD4^+$ T cells in response to anti-third-party stimulation in good responders was higher than that of poor responders ($P = 0.04$) (Fig. 3).

Discussion

The strategy of HB vaccination after LT to achieve protective immunity and to allow discontinuation of long-term HBIg administration has been investigated in a number of studies [7,11,12,15–20]. However, those attempts to immunize these patients with HB vaccine have been equivocal and generally less than successful. It is common practice to immunize these patients against hepatitis B; however, the response of LT recipients could be below adequate standard. Although the currently available HBV vaccines are extremely safe and have an efficacy of more than 90% in the general population, it has been reported that the response rate is slightly lower in obese individuals, smokers, and men and is significantly lower in patients with cirrhosis or chronic renal failure, patients undergoing long-term hemodialysis, organ transplant recipients, and immunocompromised patients [21]. In particular, because of the impairment in T-cell-dependent functions in cirrhotic patients, the results of vaccination in transplant candidates have been very disappointing [25–29]. Moreover, even in responder patients, immunosuppressive treatment frequently leads to a decrease in the serum antibody titers after transplantation [21]. Among the previous HBV vaccination trials in multiple institutions, most of the results did not show significant promise with regard to HBV vaccine response rates. Each vaccination protocol differed with respect to the dose of vaccine, the time of commencement and frequency of vaccination, the route of vaccination, combination with HBIg, and the immunosuppressive regimen at the time of vaccination. It has been reported that successful vaccination is attributed to the long time-interval that had elapsed after transplant, which allowed them to markedly reduce the immunosuppressive therapy [11]. It has also been proposed that the administration of the vaccine through the intradermal route in preference to the intramuscular route might prove to be more responsive to HB vaccination, because the epidermis is known to be rich with antigen-presenting cells, making it an appropriate target for vaccine delivery [18]. Based on these hypotheses in this study, vaccination through the intradermal route was administered to the LT recipients against HBV with an effort to minimize immunosuppression. In addition to the different vaccination protocols, the difference in the immune status of the subjects likely influences their HBV vaccine response.

In order to evaluate the immune status of the LT recipient vaccinees, we employed a MLR assay using a CFSE-labeling technique [22]. CFSE stably stains intracellular proteins without toxicity, and the fluorescence of each stained cell segregates equally to the daughter cells upon cell division, resulting in sequential halving of cellular fluorescence intensity with each successive generation [30]. When analyzed by FCM, this sequential halving of fluorescence is visualized as distinct peaks or populations of cells and can be used to track cell division in populations of proliferating cells. This, then, allows phenotypic analysis of the proliferating cells and determination of the number of cells produced in each generation by multicolor FCM analysis, i.e., the number of viable $CD4^+$ and $CD8^+$ responder T cells that proliferate in response to allostimulation can be quantified separately. The lack of proliferation of $CD4^+$ T cells in anti-donor MLR reflects the suppression of the anti-donor response [22]. In this study, all of the good responders showed a normal response of the anti-third-party $CD4^+$ T cells (Fig. 3). In contrast, the poor responders showed a hyporesponse of both anti-donor and anti-third-party $CD4^+$ T cells, suggesting an excessively immunosuppressive state. The development of an effective immune response to HB vaccination requires coordinated immune activity comprising the interaction of T cells, cytokines, antigen-presenting cells, and B cells [31]. It is important to note that these immunocompetent cells can be sufficiently activated to acquire immune activity at the time of vaccination even in a state of immunosuppression. T-cell interaction should lead to (i) activation of anti-HBsAg-specific T cells in order to achieve a successful response to vaccination and (ii) suppression of anti-donor-specific T cells to avoid transplant rejection. Patients showing a donor-specific hyporesponse with a well-maintained response to the third-party stimulus always achieved a sustained immune response to the vaccine in this study; based on this observation, we propose a concept that inducing anti-donor-specific immunosuppressive status by minimizing immunosuppression enables post-transplant HBV vaccination to become a promising prophylactic strategy, although further studies are needed to establish the optimal HBV vaccination protocol. A larger and prospective trial might be required to evaluate whether or not the MLR response can actually predict successful HBV vaccination. The higher rate of response to vaccination than that of this study has been shown in a previous report [17]. An adjuvant preparation of vaccine that used in the previous study is thought to attribute to the successful induction of a strong response. It remains to elucidate whether patients with hyporesponse to both anti-donor and anti-third-party $CD4^+$ T cells can respond to such an adjuvant preparation of vaccine.

Authorship

HT, KC, and HO: designed research. HT and YT: performed research. HT, KI, KI, MS, TI, YU, MO, MB,

HT, TI, and TA: collected data. HT, YT, and HO: analyzed data. HT and HO: wrote the paper.

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ORIGINAL ARTICLE

Significant Correlation Between Spleen Volume and Thrombocytopenia in Liver Transplant Patients: A Concept for Predicting Persistent Thrombocytopenia

Masahiro Ohira,[1] Minoru Ishifuro,[2] Kentaro Ide,[1] Toshimitsu Irei,[1] Hirotaka Tashiro,[1] Toshiyuki Itamoto,[1] Katsuhide Ito,[2] Kazuaki Chayama,[3] Toshimasa Asahara,[1] and Hideki Ohdan[1]

[1]Department of Surgery, Division of Frontier Medical Science, Programs for Biomedical Research, [2]Department of Radiology, Division of Medical Intelligence and Informatics, Programs for Applied Biomedicine, and [3]Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan

Interferon (IFN) therapy with or without ribavirin treatment is well established as a standard antiviral treatment for hepatitis C virus (HCV)–infected patients. However, susceptibility to thrombocytopenia is a major obstacle for initiating or continuing this therapy, particularly in liver transplant (LTx) recipients with HCV. Studies have reported that splenectomy performed concurrently with LTx is a feasible strategy for conditioning patients for anti-HCV IFN therapy. However, the relationship between the severity of splenomegaly and alterations in the blood cytopenia in LTx recipients remains to be clarified. Here, we analyzed the relationship between spleen volume (SV) and thrombocytopenia in 45 patients who underwent LTx at Hiroshima University Hospital. The extent of pre-LTx splenomegaly [the SV to body surface area (BSA) ratio in an individual] was inversely correlated with both the post-LTx white blood cell count and platelet (PLT) count ($P < 0.001$). Furthermore, the PLT count of patients with thrombocytopenia (PLT count $\leq 5 \times 10^4/mm^3$) increased significantly in the group without splenomegaly (SV/BSA value < 400) versus that in the group with splenomegaly ($P = 0.005$). Thus, if both splenomegaly and thrombocytopenia coexist (PLT count $\leq 5 \times 10^4/mm^3$ and SV/BSA value ≥ 400), persistent thrombocytopenia is predictable after LTx. ***Liver Transpl 15:208-215, 2009.*** © **2009 AASLD.**

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Postoperative thrombocytopenia is a common feature in liver transplant (LTx) patients.[1,2] The mechanism underlying this thrombocytopenia is considered to involve the peripheral destruction and/or consumption of platelets (PLTs)[2,3] because megakaryotic hyperplasia has been observed in the bone marrow aspirates of LTx recipients.[2] Severe thrombocytopenia resulting from bleeding complications during the postoperative period may lead to increased morbidity and mortality.[4,5] Furthermore, the PLT count is one of the crucial determinants for the discontinuation of interferon (IFN) administration, which is used as a preemptive measure or as a treatment strategy for recurrent hepatitis C virus (HCV) infections.[6] Thrombocytopenia in patients with cirrhosis has been reported to be caused by an increased PLT pool in the enlarged spleen.[7-9] Splenectomy may alleviate the postoperative thrombocytopenia in LTx patients; however, the septic complications following this procedure have generally been reported to have an adverse effect on LTx outcome.[10-13] Therefore,

Abbreviations: ALT, alanine aminotransferase; BSA, body surface area; HCV, hepatitis virus C; Hgb, hemoglobin; IFN, interferon; LTx, liver transplant; MELD, Model for End-Stage Liver Disease; PLT, platelet; PSE, partial splenic embolization; SD, standard deviation; SV, spleen volume; T-Bil, total bilirubin; WBC, white blood cell.

Address reprint requests to Hideki Ohdan, M.D., Ph.D., Department of Surgery, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan. Telephone: +81-82-257-5222; FAX: +81-82-257-5224; E-mail: hohdan@hiroshima-u.ac.jp

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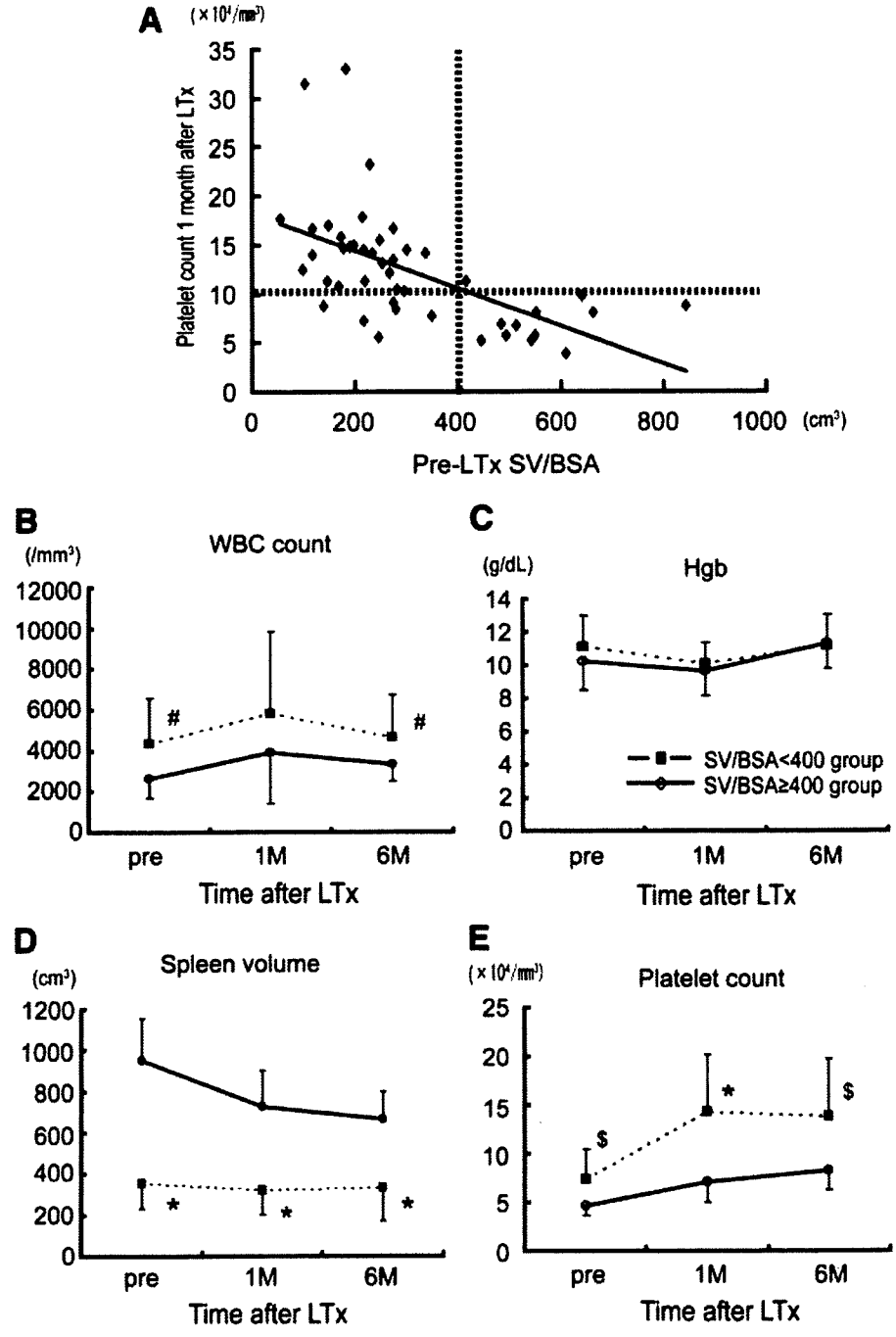

Figure 1. (A) Correlation between the pre-LTx SV/BSA value and PLT count at 1 month after LTx ($r = 0.67$, $P < 0.0001$). A regression line is superimposed on the plot: $y = -0.02x + 18.23$ (x axis: SV/BSA value; y axis: post-LTx PLT count at 1 month). Changes in (B) the WBC count, (C) hemoglobin concentration, (D) spleen volume, and (E) PLT count. The post-LTx values of these variables in the SV < 400 group (broken line with closed squares) and SV ≥ 400 group (thick line with open circles) are shown. There was a significant difference between the groups with respect to the WBC count, PLT count, and spleen volume ($^{\#}P < 0.05$, $^{\$}P < 0.01$, and $^{*}P < 0.001$ for the SV < 400 group versus the SV ≥ 400 group). Abbreviations: BSA, body surface area; Hgb, hemoglobin; LTx, liver transplant; PLT, platelet; SV, spleen volume; WBC, white blood cell.

topenia and eventually underwent splenectomy so that IFN therapy could be commenced only 9 months after LTx.

DISCUSSION

Thrombocytopenia is an extremely common complication in LTx patients. Several causes have been postulated for this reduced concentration of PLTs, including hypersplenism,[16,17] decreased thrombopoietin levels,[18,19] and destruction by anti-PLT antibodies.[20,21] It has also been reported that serum thrombopoietin levels or anti-PLT antibodies levels correlate with the spleen size.[22-24] This fact is consistent with the finding that the spleen size correlates with portal hypertension and the PLT count in patients with cirrhosis.[16] Our data also demonstrate that pre-LTx splenomegaly is associated with the pre-LTx PLT count. Uneventful LTx is expected to improve splenomegaly.[25,26] However, our data show that splenomegaly remained unchanged in

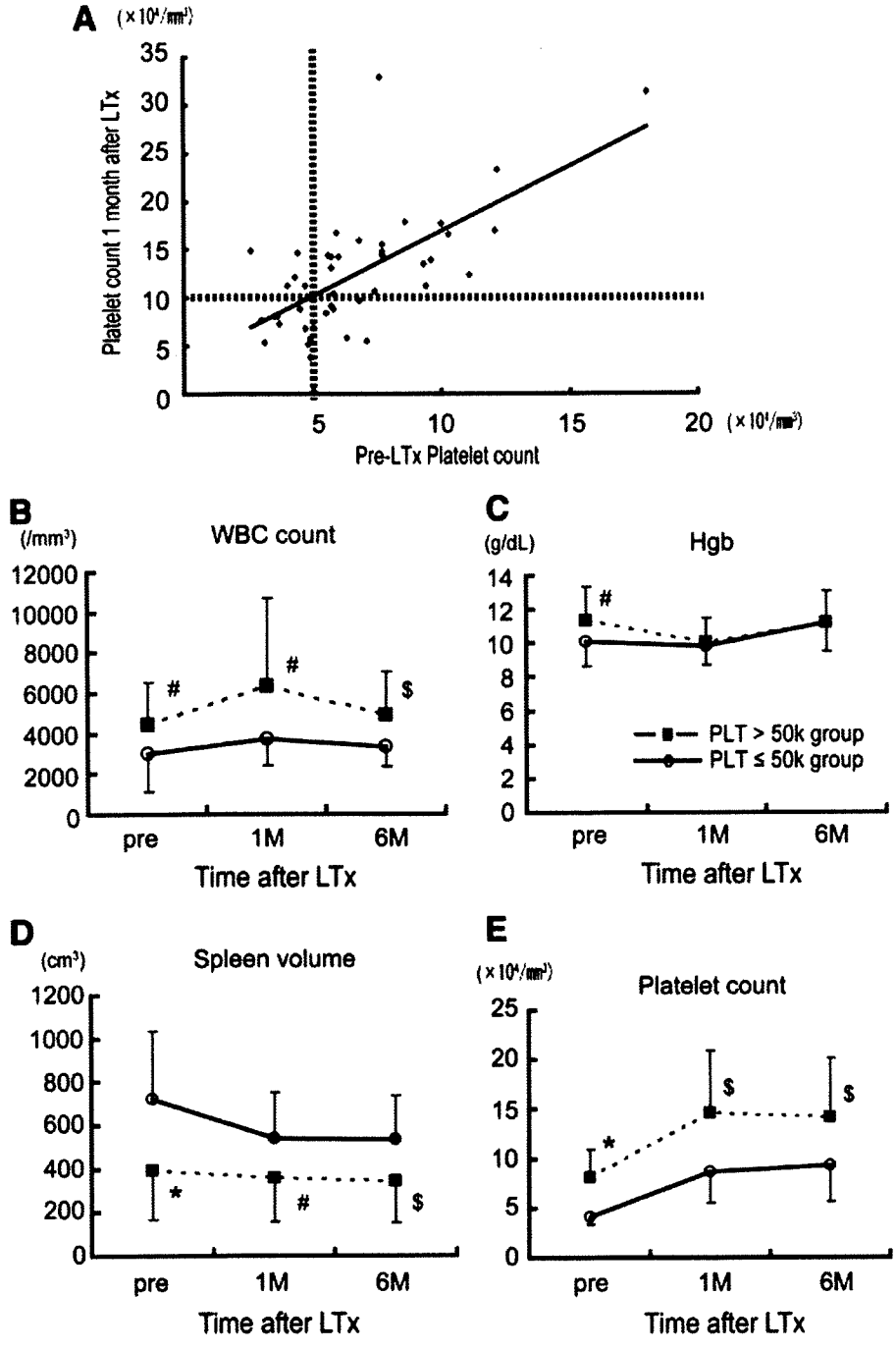

Figure 2. (A) Correlation between the pre-LTx PLT count and PLT count at 1 month after LTx ($r = 0.61$, $P < 0.0001$). A regression line is superimposed on the plot: $y = 1.35x + 3.48$ (x axis: pre-LTx PLT count; y axis: post-LTx PLT count at 1 month). Changes in (B) the WBC count, (C) hemoglobin concentrations, (D) spleen volume, and (E) PLT count. The values of these variables after LTx in the PLT > 50K group (broken line with closed squares) and PLT ≤ 50K group (thick line with open circles) are shown. There was a significant difference between the groups with respect to the WBC count, hemoglobin concentration, PLT count, and spleen volume ($^{\#}P < 0.05$, $^{\$}P < 0.01$, and $^{*}P < 0.001$ for the PLT > 50K group versus the PLT ≤ 50K group). Abbreviations: Hgb, hemoglobin; LTx, liver transplant; PLT, platelet; WBC, white blood cell.

LTx recipients whose pre-LTx SV/BSA level exceeded 400. Among the various perioperative clinical factors, the SV/BSA level was the most significant determinant of the PLT count after LTx. In the present analysis, the PLT count of patients with pre-LTx thrombocytopenia (PLT count ≤ $5 \times 10^4/mm^3$) increased significantly after LTx in the group with no pre-LTx splenomegaly (SV/BSA value < 400) versus the group with pre-LTx splenomegaly ($P < 0.01$).

We recently reported that splenectomy should be performed simultaneously with LTx in HCV patients with a PLT count < $6 \times 10^4/mm^3$ in order to complete preemptive IFN therapy at an earlier time point in the postoperative period.[27] Several authors have reported that the only indication for simultaneous splenectomy in LTx is the preoperative PLT count[12,28,29] because thrombocytopenia in the immediate posttransplant period is correlated with a low preoperative PLT count.[30]

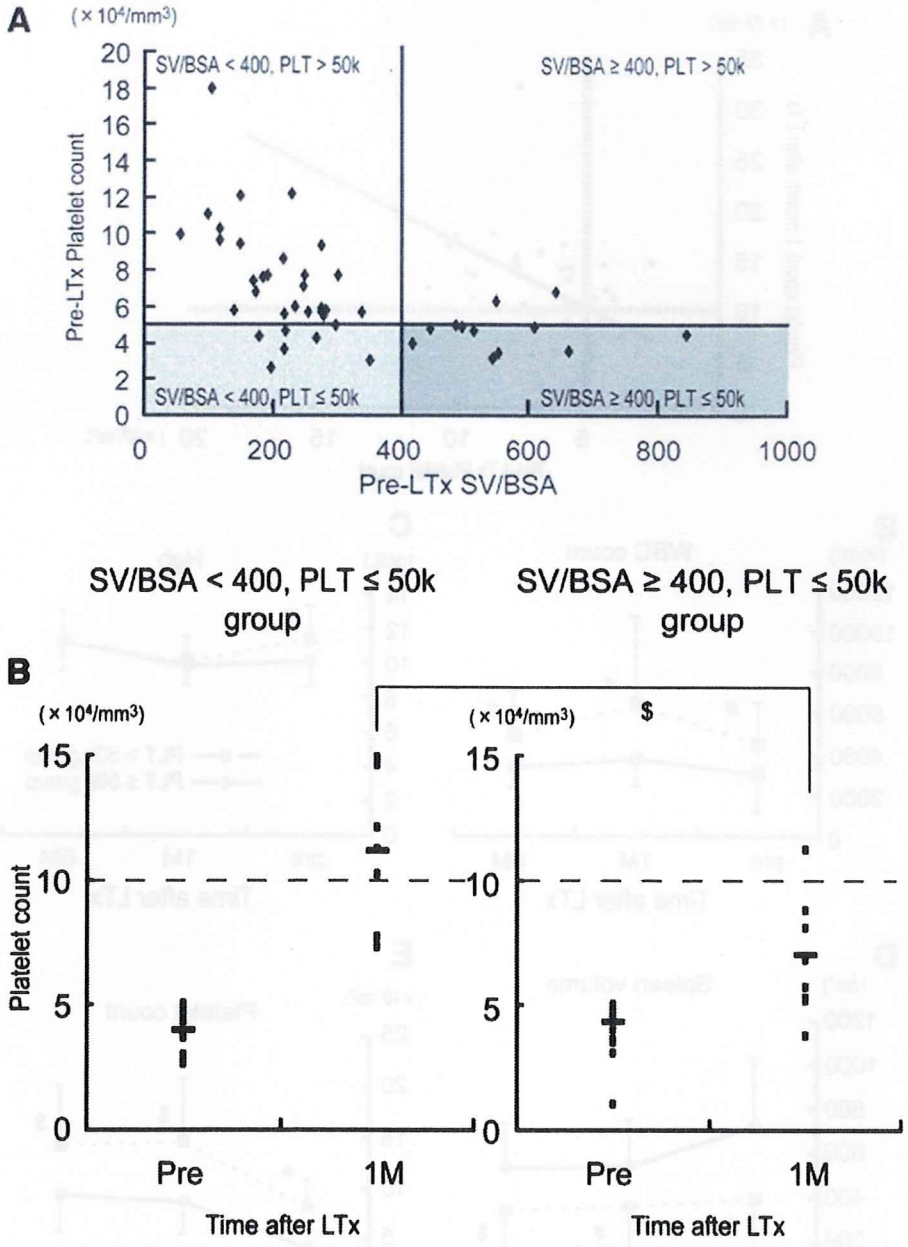

Figure 3. (A) Correlation between the pre-LTx SV/BSA value and pre-LTx PLT count. The patients were categorized as follows: the PLT > 50K, SV < 400 group, which consisted of patients without severe thrombocytopenia (pre-LTx PLT count > 5 × 10^4/mm^3) and without severe splenomegaly (pre-LTx SV/BSA level < 400); the PLT > 50K, SV ≥ 400 group, which consisted of patients without severe thrombocytopenia and with severe splenomegaly (pre-LTx SV/BSA value ≥ 400); the PLT ≤ 50K, SV < 400 group, which consisted of patients with severe thrombocytopenia (pre-LTx PLT count ≤ 5 × 10^4/mm^3) and without severe splenomegaly; and the PLT ≤ 50K, SV ≥ 400 group, which consisted of patients with severe thrombocytopenia and with severe splenomegaly. (B) Changes in the PLT count in the PLT ≤ 50K, SV < 400 group and PLT ≤ 50K, SV ≥ 400 group. The PLT count in the PLT ≤ 50K, SV < 400 group was significantly elevated versus that in the PLT ≤ 50K, SV ≥ 400 group ($^{\$}P$ < 0.01). Abbreviations: BSA, body surface area; LTx, liver transplant; PLT, platelet; SV, spleen volume.

Studies have also reported that the routine administration of simultaneous splenectomy and LTx in all HCV patients conditions them for anti-HCV IFN therapy.[31] Although splenectomy strongly affects thrombocytopenia, it might predispose patients to develop portal vein thrombosis or increase the risk of sepsis, which is particularly lethal for immunosuppressed subjects.[32] Thus, caution is advised when recommending splenectomy for patients undergoing LTx. Compared with splenectomy, splenic artery ligation is a technically simpler procedure that can easily be included in a complicated transplant operation.[33] However, the benefit of splenic artery ligation in reducing posttransplant thrombocytopenia is controversial.[34,35] Recently, partial splenic embolization (PSE) has been described as a useful procedure for severe post-LTx thrombocytopenia,[36,37] and PSE could also be an option for pre-LTx.[38] However, several groups have reported complications generally observed after PSE, including splenic infarction, abscess formation, reduced immunity-related septic complications, and portal thrombosis.[39,40] Thus, the most appropriate methods among the strategies or alternative methods for avoiding persistent thrombocytopenia remain to be elucidated.

In conclusion, the pre-LTx SV/BSA value and PLT count have been correlated with post-LTx thrombocytopenia. If both splenomegaly and thrombocytopenia coexist (PLT count ≤ 5 × 10^4/mm^3 and SV/BSA

value ≥ 400), persistent thrombocytopenia is predictable after LTx.

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