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

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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

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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

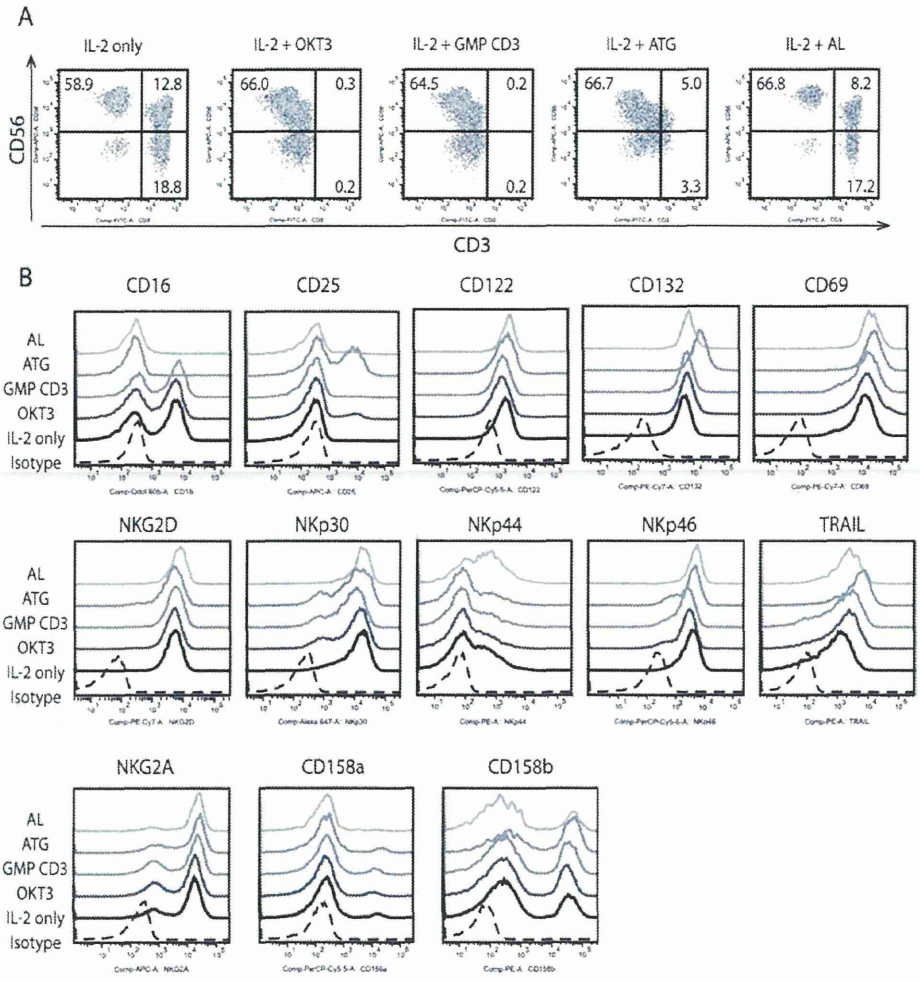

Fig 1. Effect of the T-cell depletion antibodies on the phenotypic characteristics of liver mononuclear cells (LMNCs). LMNCs obtained from cadaveric donors were stimulated with IL-2 (1000 U/mL) for 4 days. Anti-CD3 mAb (OKT3; 1 μg/mL), MACS GMP CD3 pure (GMP CD3; 1 μg/mL), antithymocyte globulin (ATG; 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.

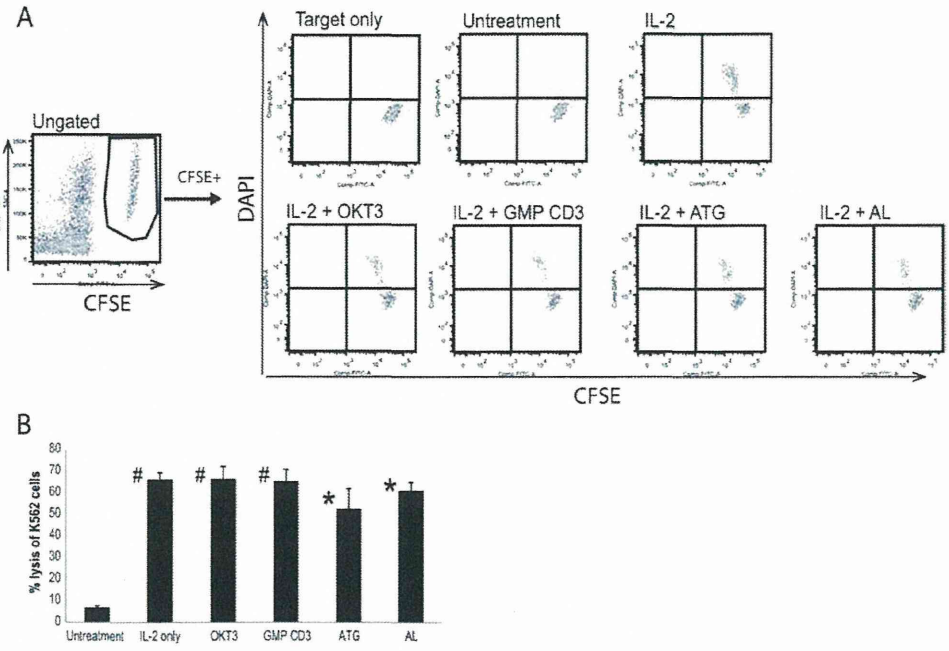

Fig 2. Antitumor effect of the T-cell depletion antibodies on IL-2–stimulated liver mononuclear cells (LMNCs). The NK cell cytotoxic activities of untreated cells and IL-2–stimulated LMNCs treated with various reagents were analyzed by a flow cytometry (FCM)–based cytotoxic assay. (A) Gate is set on 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.

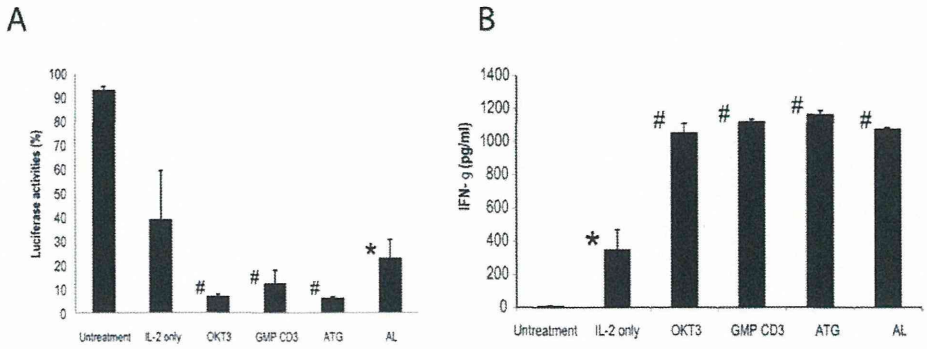

Fig 3. Anti–hepatitis C virus (HCV) effect of the T-cell depletion antibodies on IL-2–stimulated liver mononuclear cells (LMNCs). The LMNCs cultured for 4 days in the presence of IL-2 and various reagents were incubated with HCV replicon–containing cells for 48 hours in transwell tissue culture plates (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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Attenuation of Portal Hypertension by Continuous Portal Infusion of PGE1 and Immunologic Impact in Adult-to-Adult Living-Donor Liver Transplantation

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Background. Small-for-size syndrome remains the greatest limiting factor of expanding segmental liver transplantation from living donors. Portal hyperperfusion is considered to substantially contribute to small-for-size syndrome. We investigated the impact of continuous portal infusion of prostaglandin E1 (PGE1) on small-for-size grafts (SFSGs) in adult-to-adult living-donor liver transplantation (LDLT).

Methods. From July 2003 to December 2009, LDLT was performed in 122 patients. We introduced continuous portal infusion of PGE1 to five SFSG patients (PG group) from November 2007 to December 2009 and retrospectively compared them with a historical control group of eight relevant SFSG patients without PGE1 infusion (non-PG group) from July 2003 to October 2007 to determine the safety and efficacy of continuous PGE1 portal infusion for SFSGs. Splenectomy cases were excluded from analysis.

Results. The PG group demonstrated significantly lower postoperative portal pressure than the non-PG group. Moreover, the PG group demonstrated significantly improved liver function in the early posttransplantation period and significantly better recovery from hyperammonemia at 1 week after transplantation and from hyperbilirubinemia in the late posttransplantation period. Overall survival was significantly better in the PG group than in the non-PG group. Three patients in the non-PG group died of rejection-related reasons. Interestingly, immunomonitoring assay revealed that antidonor immune responses were significantly accelerated in the non-PG group compared with the PG group after LDLT. In contrast, the PG group showed well-suppressed antidonor immune responses.

Conclusion. Continuous portal infusion of PGE1 for SFSG attenuated portal hypertension, improved graft function, and suppressed antidonor immune responses, resulting in better survival.

Keywords: Living-donor liver transplantation, Small-for-size graft, Portal hypertension, Alloimmune response, Prostaglandin E1.

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Segmental liver transplantation based on cadaveric splitting or living-donor liver transplantation (LDLT) has been developed for treating patients with end-stage liver disease. It is also a means of overcoming organ shortage and wait-list mortality. However, small-for-size syndrome (SFSS) remains the greatest limiting factor for the expansion of segmental liver transplantation from either cadaveric or living donors (*1, 2*). If the volume of the engrafted liver is considerably less than the standard liver weight in patients with end-stage liver disease who are undergoing partial liver transplantation, excessive portal venous inflow might cause early portal hypertension (*3, 4*) and increased morbidity and mortality due to SFSS (*5*). Previous data have suggested that, in recipients of adult-to-adult LDLT, one of the most challenging tasks is to match a good size graft. Emphasis has more recently been placed not only on the evaluation of the ratio between donor and recipient liver volume but also on the degree of portal hypertension and the stage of liver disease in the recipient, consistent with the result in a pig model (*6–8*). Therefore, the importance of portal pressure during LDLT is now recognized.

We have demonstrated that continuous portal infusion of prostaglandin E1 (PGE1) considerably improved the congestion

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of the residual liver after extended hepatectomy in a rat model (9). Based on this result, we applied a continuous portal infusion of PGE1 for small-for-size grafts (SFSGs) in LDLT in the clinical setting.

We here investigated the clinical significance of controlling portal pressure by continuous portal infusion of PGE1 after surgery in LDLT with SFSGs, focusing on portal decompression, postoperative liver function, survival, and the antidonor immune status of the recipient retrospectively.

RESULTS

Patients' Demographic and Clinical Characteristics

Thirteen patients receiving SFSGs were retrospectively analyzed in this study. The patients' demographic and clinical characteristics are shown in Table 1. Of these patients, five received a continuous portal injection of PGE1 after transplantation (PG group) from November 2007 to December 2009 (era 2), whereas eight were historical controls from July 2003 to October 2007 (era 1) without PGE1 infusion (non-PG group). There was no significant difference in age or underlying disease between the two groups. Preoperative examination of the hepatic reserve showed similar Child-Pugh scores (PG group, 10.0±0.71; non-PG group, 9.00±0.83). Patients' model for end-stage liver disease scores, which were used as recipient severity indices, was similar between groups (mean [range], 16.8 [8–30] and 15.1 [9–28], respectively). Portal vein pressure (PVP) at laparotomy was also similar between the two groups (25.2 [17–34] and 20.3 [17–24] mm Hg, respectively). Concerning the graft, one patient in each group showed minimal fatty metamorphosis (<0.1%) on histology and there was no significant difference in graft-to-recipient body weight ratio (GRWR) between the two groups (0.680 [0.63–0.71] and 0.655 [0.51–0.72], respectively).

Furthermore, factors related to surgical invasiveness in those two groups, such as hemorrhage level, operation time, and graft ischemia duration, were similar. No donor had donor-specific antigens, and there was no difference in the number of human leukocyte antigen (HLA) mismatch (Table 1). Three donor candidates in each group underwent liver biopsy. Among them, one in each group showed minimal fatty metamorphosis (<0.1%) on histology. Of note, three of five patients in the PGE1 group and three of eight patients in the non-PGE1 group received right-lobe grafts. All patients receiving right lobes in both groups had grafts with middle hepatic vein (MHV) tributaries more than 5 mm in diameter, and all draining tributaries were reconstructed with the

TABLE 1. Patients' demographic and clinical characteristics

Variables	PG group (n=5)	Non-PG group (n=8)	*P*
Recipient factors			
Age, years	56.4±3.4	57.9±4.4	0.510[a]
Gender, male/female	5/0	3/5	0.075[b]
Child-Pugh score	10.0±1.6	9.0±1.9	0.325[a]
MELD score	16.8±8.2	15.1±5.8	0.702[a]
PVP, mm Hg, at laparotomy	25.2±6.1	20.9±3.0	0.199[a]
Disease background			
Viral hepatitis (B/C)	1/2	1/5	>0.999[b]
Alcoholic	1	1	>0.999[b]
Acute hepatic failure	1	0	0.385[b]
Cholestatic disease	0	1	>0.999[b]
Donor factors			
Age, years	26.2±3.3	33.3±10.5	0.113[a]
Gender, male/female	0/5	5/3	0.075[b]
Graft factors			
Graft type, right/left	3/2	3/5	0.592[b]
GRWR, %	0.68±0.03	0.66±0.09	0.510[a]
Reconstruction of hepatic vein	3	3	0.592[b]
HLA class I mismatch	1.20±0.49	1.63±0.23	0.453[a]
HLA class II mismatch	0.60±0.24	1.00±0.00	—
DSA	0	0	—
Surgical factors			
Operation time, min	781.0±153.6	755.9±106.0	0.758[a]
Bleeding, mL	5322.0±2295.3	5751.4±6371.2	0.866[a]
Total ischemia time, min	117.0±35.5	118.9±31.4	0.925[a]

[a] Unpaired *t* test with Welch's correction.
[b] Fisher's exact test.
DSA, donor-specific antibody; HLA, human leukocyte antigen; GRWR, graft-to-recipient body weight ratio; MELD, model for end-stage liver disease; PVP, portal vein pressure.

recipients' native MHV trunk as reported previously (*10*). There was no thrombosis in those reconstructed tributaries after surgery. One patient of each group had grafts with inferior right hepatic vein, which were reconstructed using direct anastomosis to inferior vena cava in each case.

Continuous PGE1 Infusion Attenuated Portal Hypertension After Reperfusion in SFSGs

After laparotomy, we inserted a catheter from the mesenteric vein to the distal side of the portal vein and measured the PVP during the operation. All patients exhibited portal hypertension during laparotomy. In the PG group, after reflow of the portal and hepatic veins was confirmed, we started PGE1 infusion into the portal vein through a catheter. Continuous infusion of PGE1 resulted in a significant reduction of PVP at the time of abdominal closure in the PG group compared with the non-PG group ($P<0.005$; Fig. 1A). The mean PVP at the time of abdominal closure was 15.4±1.17 mm Hg in the PG group and 20.5±1.47 mm Hg in the non-PG group (Fig. 1A). Furthermore, the PVP ratio at the end of the operation, compared with that at laparotomy, showed effective portal decompression in the PG group and non-PG group, respectively (0.62±0.04 vs. 0.99±0.06; $P<0.001$; Fig. 1B). Importantly, none of the patients in the PG group developed hypoperfusion after PGE1 portal infusion.

Clinical Course of Graft Liver Function

Graft liver function markers, including serum transaminases, arterial ketone body ratio (AKBR), ammonia, and total bilirubin, after surgery were compared between the PG group and the non-PG group.

Elevated serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were significantly attenuated in the PG group compared with the non-PG group on days 1 and 2 (Fig. 2). Similarly, the AKBR, which reflects the hepatic mitochondrial redox state and is considered an accurate index of the functional reserve of the graft liver after transplantation, was significantly higher in the PG group. However, these values became comparable between the two groups after day 3. Strikingly, significantly better recovery from hyperammonemia was seen in the PG group for 1 week after surgery. The serum total bilirubin level was comparable between the two groups by day 28 after LDLT. Nonetheless, hyperbilirubinemia was significantly improved in the PG group after day 28 but remained prolonged in the non-PG group. These results indicate that continuous infusion of PGE1 significantly improved the liver function after LDLT with SFSGs.

Complications and Prognosis

In the PG group, no complications associated with the portal vein catheter were observed after surgery (e.g., post-removal bleeding, catheter infection, or portal thrombosis). One patient in the non-PG group and none in the PG group developed SFSS. Postoperative death occurred in 5 patients of the non-PG group and in none in the PG group. In the non-PG group, the 1- and 2-year survival rates were 62.5% and 37.5%, respectively. In contrast, in the PG group, the 1-and 2-year survival rates were both 100%, a difference that was statistically significant ($P<0.05$; Fig. 3). The main causes of death in the non-PG group were graft dysfunction, rejection, and subsequent infection as well as bacterial sepsis after biliary stenosis. No patients in the PG group had a

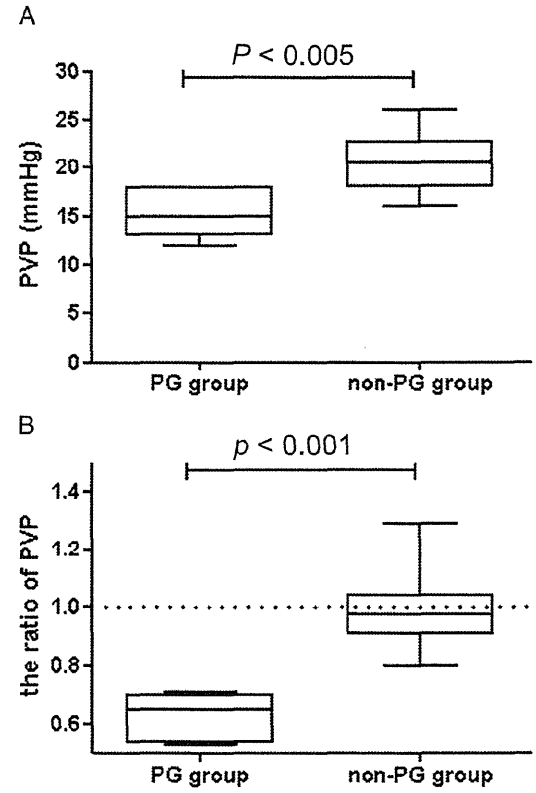

FIGURE 1. PVP value at the end of the operation (A) and ratio of PVP at the end of the operation to that at laparotomy (B) in the PG group and the non-PG group. An unpaired *t* test with Welch's correction was used to compare PVP and the ratio of PVP between the PG group and the non-PG group. The box plot represents the 25th to 75th percentiles, the dark line is the median, and the extended bars represent the 10th to the 90th percentiles. *$P<0.05$; ***$P<0.001$. PVP, portal vein pressure.

rejection episode. Rejection was diagnosed by liver biopsy and histologic findings showed features of SFSG and/or portal hypertension with rejection (see **Figure S1, SDC**, http://links.lww.com/TP/A807). The 2-year survival of SFSG patients (non-PG group) in era 1 (July 2003 to October 2007) was significantly worse than that of the non-SFSG patients in the same period (37.5% vs. 77.8%; $P<0.05$), whereas the 2-year survival of SFSG patients (PG group) in era 2 (November 2007 to December 2009) was not statistically different from that of the non-SFSG patients in the same period (100% vs. 77.1%). Of note, the 2-year survival of non-SFSG patients was similar between eras 1 and 2 (Fig. 4).

Estimation of Immunosuppressive Status After Surgery by Using the Carboxyfluorescein Diacetate Succinimidyl Ester-Mixed Lymphocyte Reaction Assay

Because the main cause of death in 3 patients in the non-PG group was related to rejection, we retrospectively analyzed the immunosuppressive postoperative status of both groups. All patients and their donors consented to be

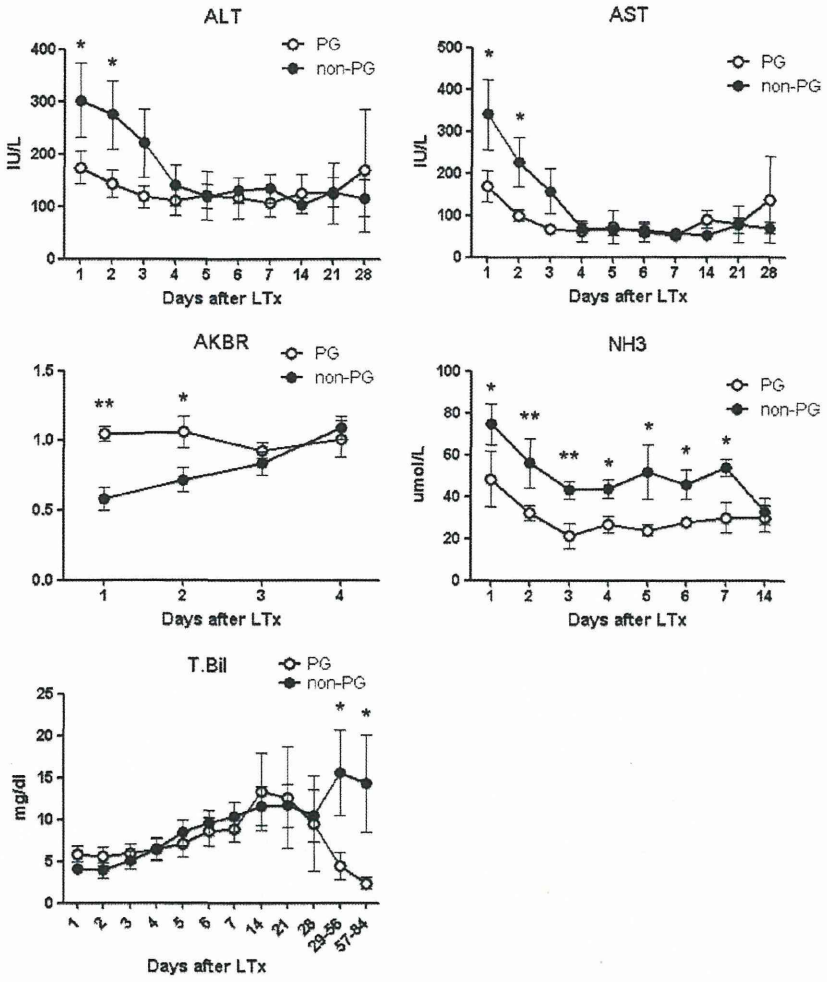

FIGURE 2. Liver function tests (ALT, AST, AKBR, NH_3, and T.Bil) of patients with (PG group; open circle) or without PGE1 portal infusion (non-PG group; closed circle) after LDLT. Data are mean±SEM for individual groups. An unpaired *t* test with Welch's correction was used to compare each of the indicated parameters between the PG group and the non-PG group. **P*<0.05; ****P*<0.01. AKBR, arterial ketone body ratio; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDLT, living-donor liver transplantation; LTx, liver transplantation; NH_3, ammonia; T.Bil, total bilirubin.

subjected to a mixed lymphocyte reaction (MLR) assay with the carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling technique. In all five patients of the PG group, suppressed $CD8^+$ T-cell proliferation, which is defined as a stimulation index (SI)<2, was observed in the antidonor MLR assay (i.e., a hyporesponse to donor; mean SI, 1.10±0.13; Fig. 4A). The mean percentage of $CD25^+$ cells among the proliferating $CD8^+$ T cells, which are activated cytotoxic T cells, was 9.24±5.93 (Fig. 4B). In contrast, in five of the eight patients in the non-PG group, accelerated $CD8^+$ T-cell proliferation was observed in the antidonor MLR assay (i.e., a hyperresponse to donor; mean SI, 2.85±0.50; Fig. 4A). Furthermore, the mean percentage of $CD25^+$ cells among the proliferating $CD8^+$ T cells was 63.82±8.63 (Fig. 4B). These differences between the two groups were significant. Of note, three patients in the non-PG group who showed high antidonor response (i.e., SI of $CD8^+$ T cells>3) required steroid pulse treatment and died of graft dysfunction or infection after rejection. Two patients who showed a relatively high antidonor response (i.e., SI of $CD8^+$ T cells>2) required an increase in immunosuppressant doses. These results indicated that patients with SFSGs show accelerated antidonor immune responses and that continuous portal infusion of PGE1 suppressed this type of antidonor immune response.

DISCUSSION

Various approaches to controlling excessive portal flow and pressure have been proposed, such as dual grafting to increase graft volume (*11, 12*). Although this concept is simple, it requires two healthy living donors and involves increased risk to donors. Another approach is portal decompression with a portosystemic shunt (*13, 14*) or splenic artery manipulation, including splenectomy, embolization, and ligation (*15–17*). This method is more favored in terms of availability and donor risk. Nonetheless, there is