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Supporting Information

Additional Supporting Information may be found the online version of this article.

Figure S1: Changes in peripheral T_{EM}/T_{reg} ratio of a long-survived animal (#8).

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ORIGINAL ARTICLE

Successful transplantation of rat hearts subjected to extended cold preservation with a novel preservation solution

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Keywords
cold preservation, deuterium oxide, heart transplantation, preservation solution, rat.

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Summary

Since prolonged cold preservation of the heart deteriorates the outcome of heart transplantation, a more protective preservation solution is required. We therefore developed a new solution, named Dsol, and examined whether Dsol, in comparison to UW, could better inhibit myocardial injury resulting from prolonged cold preservation. Syngeneic heterotopic heart transplantation in Lewis rats was performed after cold preservation with UW or Dsol for 24 or 36 h. In addition to graft survival, myocardial injury, ATP content, and Ca^{2+} -dependent proteases activity were assessed in the 24-h preservation group. The cytosolic Ca^{2+} concentration of H9c2 cardiomyocytes after 24-h cold preservation was assessed. Dsol significantly improved 7-day graft survival after 36-h preservation. After 24-h preservation, Dsol was associated with significantly faster recovery of ATP content and less activation of calpain and caspase-3 after reperfusion. Dsol diminished graft injury significantly, as revealed by the lower levels of infarction, apoptosis, serum LDH and AST release, and graft fibrosis at 7-day. Dsol significantly inhibited Ca^{2+} overload during cold preservation. Dsol inhibited myocardial injury and improved graft survival by suppressing Ca^{2+} overload during the preservation and the activation of Ca^{2+} -dependent proteases. Dsol is therefore considered a better alternative to UW to ameliorate the outcome of heart transplantation.

Introduction

Graft dysfunction in the early phase of reperfusion, due to ischemia and reperfusion injury (IRI), is still the critical problem to be conquered in clinical heart transplantation [1]. Since the heart is susceptible to cold IRI [2], the time limit for a safe heart graft is 4–6 h in clinical settings using University of Wisconsin solution (UW) [3]. Improvement of the graft quality after cold preservation is thus a very important issue, but the method of cardiac cold preservation has not been dramatically changed since the UW was introduced in 1988 [4]. For this reason, a better, alternative organ preservation solution is needed.

During cold preservation, harmful processes such as ATP depletion [5], Ca^{2+} overload [6], production of reactive oxygen species (ROS) [7], cellular acidosis [8], swelling [9], and cytoskeletal disruption [10] are initiated and progress. During subsequent re-warming ischemia and reperfusion, some of these harmful cascades, including ROS production, Ca^{2+} overload and downstream

activation of proteases [11], and delayed recovery of ATP production [12], are further enhanced. Prolonged cold preservation exacerbates these processes, and eventually causes cardiac graft injury.

We therefore developed Dsol, a novel organ preservation solution based on UW solution with a high sodium and low potassium component, modified impermeants, and deuterium oxide (D_2O) as solvent (Table 1). We expect the extracellular-type composition of this solution without hydroxyethyl starch (HES) to inhibit coronary endothelial injury and subsequent graft infarction after reperfusion [13,14]. In addition, the impermeants sucrose and mannitol, which cost less than raffinose, are expected to give the solution potent cellular protection and antioxidant effects [15,16]. Deuterium oxide (D_2O) has unique biological effects, including inhibition of cytosolic Ca^{2+} overload [17], and the stabilization of microtubules [18], actin cytoskeleton [19], plasma membrane [20], and membrane-bound proteins [21]. D_2O also accelerates ATP production by stimulation of glucose uptake, glycolysis [22], and mitochondrial respiration [23]. These properties could suppress Ca^{2+}-induced cellular damage, and maintain structural and functional homeostasis of cardiomyocytes. In previous studies, the efficacy of D_2O for liver and heart preservation [24], and D_2O-containing solutions for kidney [25], pancreas [26], and vascular tissue preservation [27] has been reported. However, the effects of D_2O-containing solution have not yet been explored in a heart preservation and transplantation model.

The aims of the present study were to test whether Dsol, in comparison to the widely accepted UW, could better inhibit myocardial injury in extended cold preservation and subsequent syngeneic transplantation of rat hearts.

Table 1. Composition of the preservation solutions.

	Dsol	UW
Additives (mM)		
NaOH	125	25
KOH	–	100
$MgSO_4$	5	5
KH_2PO_4	25	25
Lactobionate	100	100
Raffinose	–	30
Sucrose	20	–
Mannitol	10	–
Adenosine	5	5
Allopurinol	1	1
Glutathione	3	3
HES (g/l)	–	50
Solvent (%)		
H_2O	70	100
D_2O	30	–
Freezing point (°C)	0.3	−0.9

HES, Hydroxyethyl starch.

Materials and methods

Chemicals and reagents

All the chemicals and reagents were of the highest grade commercially available, and purchased from Wako Pure Chemical Co. (Osaka, Japan) unless otherwise noted.

Preparation of preservation solutions

UW solution (Viaspan®) was purchased from Bristol–Myers Squibb Co. (New York, NY, USA). Dsol was developed in our laboratory (Table 1). Deuterium oxide was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). The freezing point of Dsol was 0.3 °C and we confirmed that Dsol would not freeze at 4 °C under the conditions employed herein. All solutions were filtered (0.45 μm) before use.

Animals

The experiments were approved by the institutional Animal Care Committee, and were conducted under the guidelines for animal care and use of Hokkaido University. Inbred male Lewis (LEW) rats weighing 250–350 g were purchased from Kyudo Co., Ltd. (Saga, Japan), and were used as both donors and recipients. They were maintained in a specific pathogen-free facility, and were used for the experiments without fasting.

Cell culture and reagents

H9c2 cells (passage 18–25; CRL-1446™; American Type Culture Collection, Rockville, MD, USA) were cultured in DMEM (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 10% (v/v) heat inactivated bovine serum (Gibco–Invitrogen, Carlsbad, CA, USA), and penicillin–streptomycin (Gibco), under 95% air/5% CO_2 at 37 °C. To assess the cytosolic Ca^{2+} concentration, a FRET-based Ca^{2+} indicator, the Premo Cameleon Calcium Sensor™ (Molecular Probes Inc. Eugene, OR, USA), was transduced into the H9c2 cells according to the manufacturer's instructions. Briefly, the cells were incubated in a growth medium containing an appropriate amount of vector at room temperature for 4 h, then incubated for another 16 h in a fresh growth medium containing expression-enhancer solution. Cells (4×10^4 cells/well) were plated on a 96-well culture plate for fluorescent measurement overnight under the normal growth conditions.

Heterotopic cardiac transplantation

Heterotopic heart transplantation was performed as previously described [28]. Briefly, after anesthetization with isoflurane inhalation, sodium heparin (1000 U/kg) was intravenously administered to the donor. Then, the heart was perfused in situ with 4 °C UW or Dsol from the aorta. The heart was rapidly excised and preserved in each solution at 4 °C. Recipients underwent a mid-line abdominal incision after anesthesia. The ascending aorta and pulmonary artery of the donors were anastomosed to the recipient's infra-renal abdominal aorta and inferior vena cava, respectively. The warm ischemic time was strictly adjusted to 25 min.

Experimental protocol *in vivo*

The grafts were transplanted after 24-h cold preservation in UW or Dsol solution (UW24 or Dsol24 group, respectively), 36-h preservation in UW or Dsol solution (UW36 or Dsol36 group, respectively), or no preservation (non-preserved control: NPC group). Graft survival was followed for 7 days. In the 24-h preservation groups, rats were sacrificed at 1 and 24 h after reperfusion (R1h and R24h, respectively). Grafts at the end of 24-h cold preservation (CP24h) in UW and Dsol solution, and normal heart controls (NHC) were also sampled. Graft infarction, apoptosis, serum biochemistry, inflammatory cells infiltration, high energy phosphates content, calpain and caspase 3 activities were assessed. At 7 days after reperfusion, rats were sacrificed to examine the level of graft fibrosis.

Graft survival

Graft survival was examined by palpation through the abdominal wall by two independent examiners in a blinded manner. Graft loss was defined as total stasis or the absence of any wall movement by direct inspection.

Infarction

Cardiac infarct size was assessed at R1h and R24h by triphenyltetrazolium chloride (TTC) staining as previously described [29]. Briefly, the excised hearts were incubated for 12 min in 1.5% TTC (w/v) in PBS at 37 °C, and fixed in 10% formalin-PBS thereafter. After taking microscopic images, the infarct area was calculated using computerized planimetry.

Apoptosis

Graft apoptosis was assessed at R24h by terminal dUTP nick end-labeling (TUNEL) staining as previously described [30]. Nuclei were counterstained with hematoxylin. TUNEL-positive cells were counted in five randomly selected HPFs (magnification ×400) adjacent to the necrotic area, the so-called area at risk, in a blinded manner. Mononuclear cells, cells without myofiber, or cells located at the interstitium were excluded as inflammatory cells. Results were expressed as the average number of TUNEL-positive cells per single HPF.

Infiltration of polymorphonuclear neutrophils (PMNs) and monocytes

The numbers of infiltrating inflammatory cells were assessed by counting the number of PMNs and monocytes at R24h. The grafts were fixed in 10% formalin-PBS, embedded in paraffin, and stained with hematoxylin-eosin (HE) for the PMNs count. Graft samples were also embedded and frozen in an OCT compound. Immunohistochemical (IHC) staining for monocytes/macrophages was performed with a mouse anti-rat CD68 antibody (AbD Serotec, Oxford, UK). Then the samples were incubated with a biotinylated goat anti-mouse IgG secondary antibody (DAKO, Cambridge, UK) and streptavidin-biotin-peroxidase (DAKO) in sequence. Detection of antibody binding was performed with 3,3'-diaminobenzidine (DAKO). Cells were counterstained with hematoxylin.

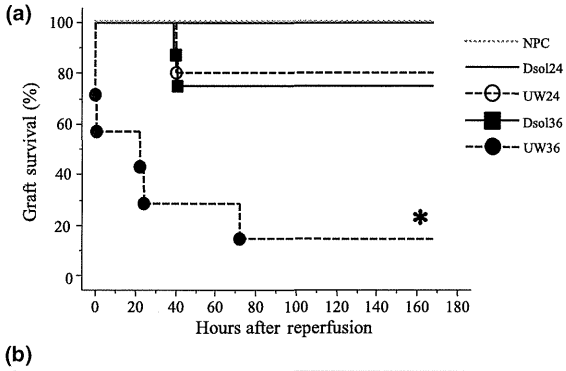
The numbers of PMNs and monocytes/macrophages were counted in 10 randomly selected HPFs for each section.

High energy phosphates

The levels of tissue adenine nucleotides (ATP, ADP, AMP) before preservation, at the end of 24-h cold preservation, and at R1h were measured as previously described [5]. Grafts were snap-frozen and homogenized in 20 μl/mg of ice cold 0.3 M perchloric acid with 0.01% (w/v) EDTA using a Polytron homogenizer (Kinematica Inc., Bohemia, NY, USA). After centrifugation (2200 *g*, 10 min, 4 °C), the supernatant was neutralized by 5 N KOH. An aliquot (20 μl) was analyzed by HPLC (Eicom, Kyoto, Japan). The dry-to-wet weight ratio of the tissue was separately measured by lyophilization. Myocardial adenine nucleotides were expressed as micromole per gram dry weight (μmol/g dw). Total adenine nucleotide (TAN) was calculated as the sum of ATP, ADP, and AMP.

Fibrosis

Grafts excised at R7d were fixed in 10% formalin-PBS, embedded in paraffin, and stained with Masson's trichrome. After microscopic images were taken with a BIOREVO BZ9100 fluorescence microscope (KEYENCE, Osaka, Japan), they were processed using computerized



(b)

	Graft survival							
NPC	>7d	>7d	>7d	>7d	>7d	>7d		
Dsol24	>7d	>7d	>7d	>7d	>7d			
UW24	40 h	>7d	>7d	>7d	>7d			
Dsol36	39 h	41 h	>7d	>7d	>7d	>7d	>7d	>7d
UW36	0 h	0 h	1 h	22 h	24 h	3d	>7d	

Figure 1 Seven-day cardiac isograft survival. Cardiac grafts were preserved for 24 h (UW24: $n = 5$; Dsol24: $n = 5$) or 36 h (UW36: $n = 7$; Dsol36: $n = 8$) following syngeneic heterotopic transplantation. Grafts without preservation were used as a non-preservation control (NPC; $n = 6$). (a) Survival curve after reperfusion. (b) Survival time of individual hearts in each group after reperfusion. Dsol significantly improved 7-day graft survival after 36-h cold preservation. $^*P < 0.05$ by log-rank test, UW36 vs. Dsol36.

planimetry software (KEYENCE). The fibrotic area was expressed as the percentage of the total LV area.

Calpain and caspase 3 activation

To determine the levels of activation of calpain and caspase 3, calpain-specific cleavage of cytoskeleton-bound proteins (α-fodrin and talin) and cleavage of caspase 3 were assessed by a standard Western blot analysis [31,32]. The graft was homogenized with a glass-Teflon homogenizer in 4 ml/g of lysis buffer containing 25 mmol/l Tris–HCl, 150 mmol/l NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/l EDTA, and 1% protease inhibitor cocktail (Sigma–Aldrich, St. Louis, MO, USA) at pH 7.6. The homogenate was centrifuged for 15 min at 14000× *g* and 4 °C. The protein concentration of the resulting supernatant was determined with a bicinchoninic acid assay (Thermo Scientific, Rockford, IL, USA). Then, the proteins were separated with standard SDS–PAGE techniques. After transfer to a PVDF membrane, the proteins were probed with mouse anti-α-fodrin mAb (1:1000; Biomol, Plymouth Meeting, PA, USA), mouse anti-talin mAb (1:200; Sigma), and rabbit anti-caspase 3 Ab (1:1000; Cell Signaling, Danvers, MA, USA). Then, IgG-horseradish peroxide-conjugated anti-mouse or anti-rabbit secondary antibody (1:2500–1:10000; Amersham Bioscience, Buckinghamshire, UK) was applied for chemiluminescence detection (Amersham Bioscience). α-tubulin was detected with rabbit mAb to α-tubulin (1:1000; Cell Signaling) as an internal control. The cleaved bands of α-fodrin and talin were then normalized by the respective intact bands. Cleaved bands of caspase 3 were normalized by α-tubulin. The values were finally expressed as a percentage of the value in the normal heart controls.

Cytosolic Ca^{2+} concentration *in vitro*

Cells expressing a FRET based Ca^{2+} indicator, Premo Cameleon Calcium Sensor™, were subjected to 24-h cold preservation in UW or Dsol. Cameleon was excited at 370 nm to produce fluorescence from CFP detected at 480 nm in the Ca^{2+} -unbound form. In the Ca^{2+} -bound form, FRET occurred from CFP to YFP, resulting in the production of additional fluorescence at 535 nm. The mean fluorescent intensity at 535 nm (MFI_{535}) was expressed as a percentage of the MFI_{535} before preservation.

Statistical analysis

Data were expressed as the mean ± standard deviation or mean ± standard error of the mean as annotated. Graft survival was plotted by the Kaplan–Meier method, and was applied to a log-rank test for comparisons. One-factor ANOVA followed by *post hoc* test was applied as appropriate. A value of $P < 0.05$ was considered statistically significant.

Results

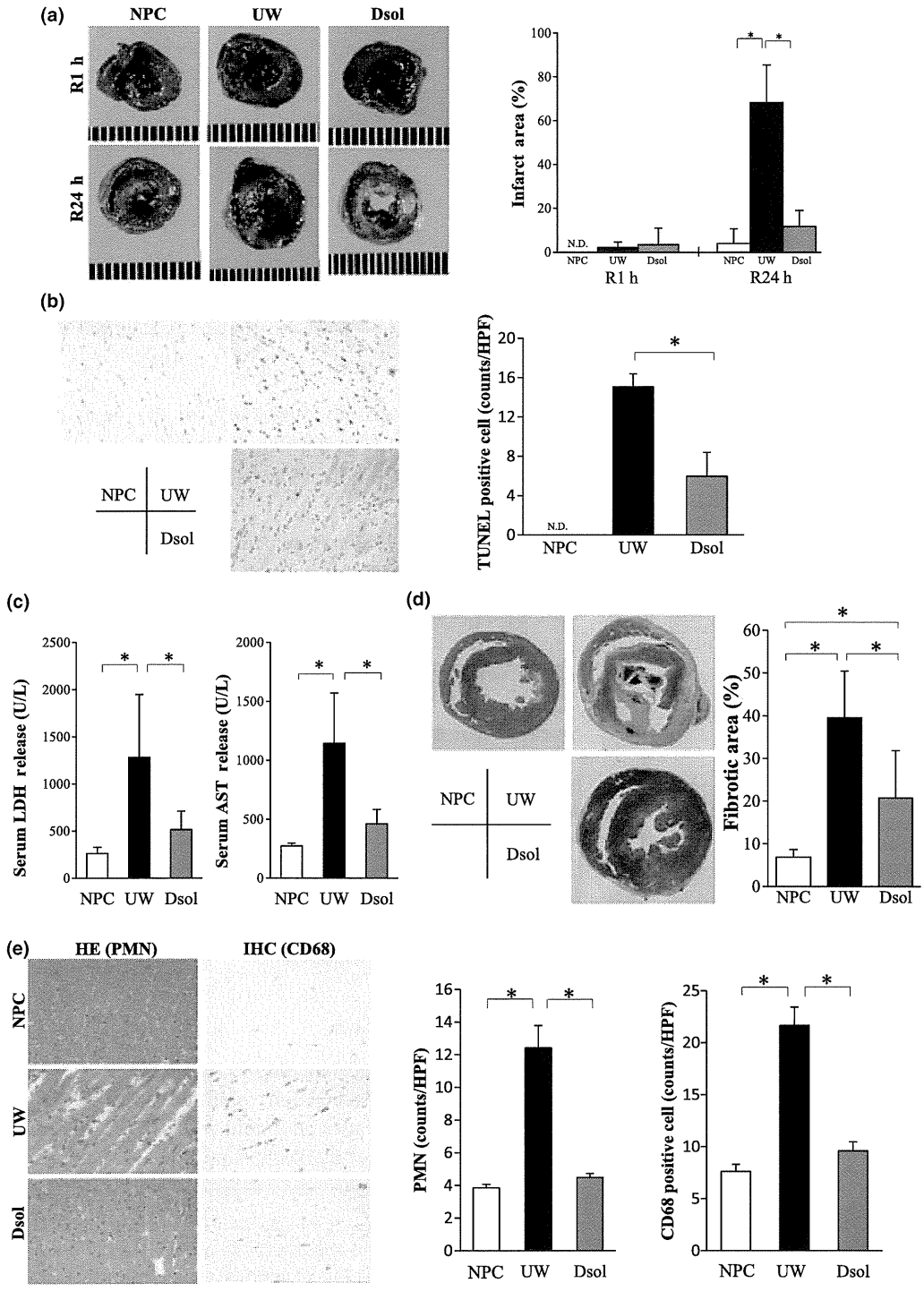
Dsol ameliorated graft survival

All hearts in the non-preservation control group (NPC) survived for 7 days (Fig. 1). In the 24-h cold preservation experiment, the rate of 7-day graft survival in the Dsol group was 100% (5/5), versus 80% (four of five) in the UW group. In the 36-h preservation experiment, the rate of 7-day graft survival was 75% (six of eight) in the Dsol group, whereas it was only 14% (one of seven) in the UW group ($P < 0.05$; Dsol36 vs. UW36).

Dsol decreased graft infarction, apoptosis, LDH and AST release

Graft infarction at 1 h after reperfusion (R1h) was not evident in all groups, and ranged from 0% to 3.4% of the total LV area. At R24h, the infarct area was 67.8% ± 16.5% of the total LV area in the UW group, whereas it was 11.7% ± 7.3% in the Dsol group ($P < 0.05$; Dsol vs. UW; Fig. 2a).

TUNEL-positive cells, i.e. apoptotic cardiomyocytes, were not found in the NPC group at R24h. The number of TUNEL-positive cardiomyocytes at R24h was signifi-



cantly smaller in the Dsol group (5.97 ± 2.44 counts/HPF) than in the UW group (15.1 ± 1.30 counts/HPF, Fig. 2b).

Serum LDH and AST levels in the UW group (1282 ± 667 and 1144 ± 427 IU/l, respectively) were significantly higher than those in the Dsol group (516 ± 195 and 463 ± 120 IU/l, respectively) at R24h (Fig. 2c).

Dsol reduced graft fibrosis

The fibrotic area at R7d was significantly larger in the UW group (39.5% ± 11.0%) than in the Dsol group (20.7% ± 11.1%) or NPC group (6.9% ± 1.8%, $P < 0.05$ for UW versus NPC and for UW versus Dsol, Fig. 2d).

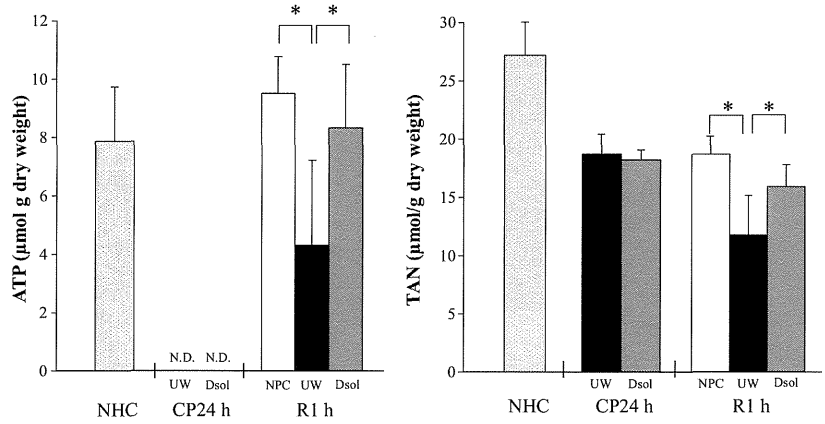


Figure 3 Graft ATP and total adenine nucleotide contents of the normal heart controls, after 24 h of cold preservation, and 1 h after reperfusion were measured by HPLC. Dsol was associated with significantly faster recovery of ATP and TAN content at 1 h after reperfusion. Values represent the mean ± SD, $n = 4$ each group, $*P < 0.05$, Fischer's PLSD *post hoc* test. N.D., not detected; NHC, normal heart control; NPC, non-preservation control.

Dsol suppressed the infiltration of inflammatory cells

The number of polymorphonuclear neutrophils (PMNs) in the interstitium at R24h was significantly higher in the UW group (12.4 ± 1.37 counts/HPF) than in the Dsol group (4.5 ± 0.24 counts/HPF). The number of CD68-positive monocytes/macrophages at R24h was significantly higher in the UW group (21.7 ± 1.76 counts/HPF) than in the Dsol group (9.6 ± 0.87 counts/HPF, Fig. 2e).

Dsol improved the restoration of high energy phosphates after reperfusion

ATP content in the normal heart was 7.87 ± 1.86 (μmol/g dw), whereas ATP was not detected at the end of the 24-h cold preservation in either group. At R1h, it was significantly higher in the Dsol group (8.34 ± 2.16 μmol/g dw) than in the UW group (4.32 ± 2.90 μmol/g dw, Fig. 3). TAN was also significantly higher in the Dsol group (15.94 ± 1.89 μmol/g dw) than in the UW group (11.77 ± 3.39 μmol/g dw).

Dsol inhibited cold preservation-induced Ca^{2+} overload *in vitro*

After 24-h cold preservation, MFI_{535} increased to as much as 376% of the basal level in the UW group, whereas it increased to only 140% of the basal level in the Dsol group ($P < 0.0001$). Therefore, Dsol inhibited Ca^{2+} overload during cold preservation (Fig. 4a).

Dsol inhibited calpain and caspase-3 activation

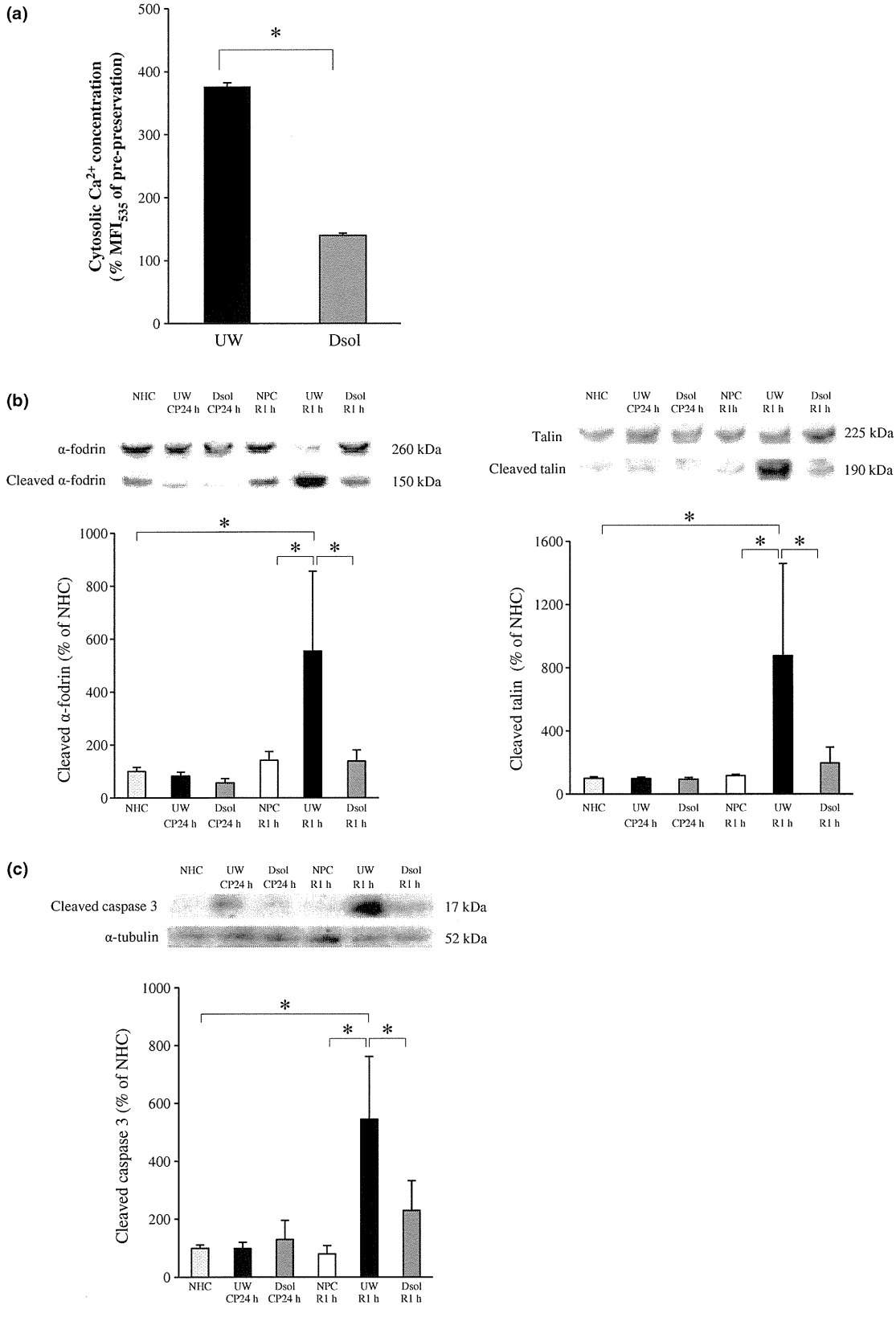
The calpain-specific substrates, talin and α-fodrin, were not cleaved at the end of the 24-h cold preservation period in either the UW or Dsol group (Fig. 4b). At R1h, they showed a significantly greater amount of cleavage in the UW group compared to the normal heart control group (NHC). Calpain-mediated cleavage was significantly suppressed in the Dsol group ($P < 0.05$ vs. UW, Fig. 4b).

The activations of caspase 3 by cleavage were assessed. The active cleaved fragments of caspase 3 (17 kDa) were significantly increased at R1h in the UW group compared to the NHC group ($P < 0.05$, vs. NHC), whereas they were significantly suppressed in the Dsol group ($P < 0.05$, vs. UW, Fig. 4c).

Discussion

In the current study, we demonstrated that the novel organ preservation solution Dsol improved cardiac graft survival after 36-h cold preservation. After 24-h preservation, Dsol markedly suppressed necrosis and apoptosis as

Figure 2 Graft injury after 24-h cold preservation and reperfusion. (a) Graft infarction at 1 h and 24 h after reperfusion as determined by TTC staining. Representative TTC-stained sections from grafts (Upper, R1hr; Lower, R24hr) and infarct size as measured by planimetry ($n = 6$ each group). Each point on the scale represents 1 mm. (b) Apoptosis of cardiomyocytes after 24 h of reperfusion as determined by TUNEL staining. Representative TUNEL-stained sections and TUNEL-positive myocardial cell counts in each section are shown ($n = 6$ each group). TUNEL-positive nuclei appear dark brown. Magnification ×400. (c) Serum LDH and AST release at 24 h after reperfusion (NPC: $n = 6$; UW: $n = 5$; Dsol: $n = 5$). (d) Graft fibrosis at 7 days after reperfusion as determined by Masson's trichrome staining. The fibrotic area stains blue, and the viable area stains red. Representative sections (original magnification: ×20) are shown, and the fibrotic area was calculated (NPC: $n = 6$; UW: $n = 4$; Dsol; $n = 5$). (e) Histological and immunohistochemical examination of graft-infiltrating PMNs and monocytes after 24 h of preservation and 24 h after reperfusion. Representative photographs of HE staining and immunohistochemical staining by anti-CD68 antibody (magnification ×400). CD68-positive cells appear brown. PMNs and CD68-positive cells were counted in HE and IHC, respectively ($n = 6$ each group). Dsol diminished graft injury significantly, as revealed by the lower levels of infarction, apoptosis, serum LDH and AST release, graft fibrosis and infiltration of inflammatory cells after reperfusion. Data are presented as the mean ± SD, $*P < 0.05$ by the Tukey–Kramer *post hoc* test. NPC, non-preservation control; N.D., not detected.



compared to UW solution. Dsol also enabled rapid restoration of the high energy phosphate that had been exhausted from the grafts during the preservation period. Dsol was clearly shown to prevent the elevation of cytosolic Ca^{2+} concentration during cold preservation *in vitro*, and inhibited Ca^{2+} -dependent activation of calpain and subsequent activation of caspases-3, compared to UW solution *in vivo*. These data clearly demonstrated the advantage of Dsol over UW, with the former showing excellent inhibition of cardiac graft injury after prolonged simple cold static preservation and subsequent cardiac transplantation in rats.

In previous reports using the same model, the graft function of the UW-preserved rat hearts after transplantation was recovered in the 12-h preservation group [33], whereas it was impaired in the 18-h preservation group [34]. Further, 24-h preservation in UW raised the possibility of graft loss due to the critical ischemia/reperfusion injury [5]. Infarction of grafts after prolonged cold preservation presents a risk of graft loss. To avoid graft loss in such cases, previous reports have suggested the importance of suppressing graft infarction to below 15% of the total area of individual grafts after reperfusion [35,36]. In the present study, Dsol suppressed the graft infarction in just 11% of total area of grafts, and prevented graft loss completely. On the other hand, UW induced 68% graft infarction and resulted in graft loss in 20% of grafts after 24-h cold preservation. In addition, the surviving grafts in the UW-preserved group tended to beat more weakly than the Dsol-preserved grafts. However, we could not evaluate graft function in this study because we employed a non-functional model. Functional assessment using a functional model remains a challenge for future studies. However, the present results do indicate that Dsol has a more powerful protective effect than UW solution, although this protective effect appeared more evident after 36-h preservation.

Necrosis at the center of the infarction and apoptosis around the necrotic area, the so-called area at risk (AAR), are closely related to graft survival and contractile function [37]. After prolonged cold preservation and reperfusion, cardiomyocytes fell into necrosis for various reasons, including hypercontracture, insufficient blood flow due to vascular failure, and activation of necrosis-inducing proteases [14,38,39]. In the present study, UW could not prevent necrotic cell death, as demonstrated by TTC staining, AST and LDH release, and eventual graft fibrosis, which was consistent with a previous report [35], whereas Dsol achieved nearly complete inhibition. Necrotic cardiomyocytes induced infiltration of inflammatory cells in the UW group but not in the Dsol group. These cells, in turn, damage viable cardiomyocytes by secreting inflammatory mediators [40]. Therefore, the prevention of necrosis also has important implications in terms of stopping this harmful cycle. Cardiomyocytes that manage to just avoid necrosis often fall into apoptotic cell death within the AAR [37]. We demonstrated that abundant TUNEL-positive apoptotic myocardia were found at the AAR in UW-preserved hearts, whereas they were significantly suppressed in the Dsol group. Dsol prevented cell death not only by preventing necrosis but also by preventing apoptosis.

Cytosolic Ca^{2+} overload during prolonged cold preservation and Ca^{2+} -dependent activation of calpain and caspases after reperfusion play a central role in cellular necrosis and/or apoptosis. Calpain is activated by cytosolic Ca^{2+} overload, and activated calpain, in turn, induces necrosis by cleavage of cytoskeletal proteins such as α-fodrin and talin [39]. Calpain also triggers apoptosis by caspase-12 activation [41], and Bid [42] and Bax cleavage [43], followed by caspase 3 activation. Among the many unique properties of D_2O, such as stabilization of the microtubules [18], actin cytoskeleton [19], plasma membrane [20], and membrane-bound proteins [21], we focused on the ability of D_2O to suppress the elevation of cytosolic Ca^{2+} concentration [17]. D_2O is reported to inhibit calcium influx via the plasma membrane L-type Ca^{2+} channel [44] as well as calcium efflux from the sarcoplasmic reticulum (SR) to the cytosol [45]. Our present *in vitro* study demonstrated that cytosolic Ca^{2+} concentration was elevated up to 3.8-fold after 24-h cold preservation in the UW group. Elevated cytosolic Ca^{2+} at the end of the cold preservation period in turn leads to the activation of Ca^{2+} -dependent proteases, and thereby protease-induced necrosis and apoptosis of cardiomyocytes after reperfusion. In this study, the major source of aug-

Figure 4 (a) The cytosolic Ca^{2+} concentration of H9c2 cardiomyocytes after 24-h cold preservation was assessed by using a Premo Cameleon Calcium Sensor™. After 24-h cold preservation, MFI_{535} increased to as much as 376% of the baseline level in the UW group, versus 140% of the baseline level in the Dsol group. Values represent the mean ± SD, $n = 6$ each group. $^*P < 0.0001$ by Fischer's PLSD *post hoc* test. (b and c) Western blotting analyses of calpain and caspase-3 activity in the cardiac grafts after 24 h of cold preservation and 1 h of reperfusion. (b) Activated calpain mediated the cleavage of α-fodrin and talin. Representative Western blots of cleavage of intact α-fodrin (260 kDa) to a cleaved fragment (150 kDa), and intact talin (225 kDa) to a cleaved fragment (190 kDa) are shown. Semi-quantitative analyses are shown below. (c) Representative Western blots of cleavage of caspase-3 to the active fragments of caspase-3 (17 kDa). The results of the semi-quantitative analyses are shown below. Dsol significantly inhibited calpain and caspase-3 activation after reperfusion. All values are expressed as the mean ± SD, $n = 3$, $^*P < 0.05$, Turkey–Kramer *post hoc* test. NHC, normal heart control; NPC, non-preservation control.

mented cytosolic Ca^{2+} during preservation should be the efflux from SR, because both UW and Dsol are Ca^{2+} -free solutions. The D_2O present in the Dsol could inhibit Ca^{2+} release from SR and suppressed the elevation of cytosolic Ca^{2+} concentration during cold preservation. Accordingly, Dsol dramatically suppressed the activation of these degradative Ca^{2+} -dependent proteases thereafter. This property of D_2O should be a key mechanism of the graft protection with Dsol.

In addition to cellular death, the energy state, which is established mainly by mitochondrial oxidative ATP production, is closely related to the cardiac kinetics after transplantation. Flameng *et al.* reported that the impairment of ATP restoration after reperfusion, even if the ATP content was maintained at the end of 24-h cold static preservation, causes cardiac contractile dysfunction after transplantation [12]. Although Dsol failed to preserve ATP content during cold preservation in the present study, rapid recovery of ATP content was clearly shown at 1 h after reperfusion. Meanwhile, UW failed to recover ATP synthesis, even though graft infarction was not evident.

Although the intracellular-type component and HES adopted by UW can potently prevent cellular swelling during cold preservation, they tend to induce graft infarction as a result of coronary endothelial injury [13,14]. Therefore, we adopted the extracellular-type component without HES for Dsol. In this respect, the concept of Dsol is similar to that of Celsior [46], which showed better preservation than UW within a relatively short period [47], but not after extended cold preservation [48,49]. The reasons for the potent protection by Dsol even after a prolonged period could be the modified impermeants and D_2O, which could compensate for the demerits of the extracellular-type composition. Modified impermeants such as mannitol and sucrose, which *per se* have cytoprotective [15] and anti-oxidative effects [16], could reduce organ swelling. Other properties of D_2O, in addition to the inhibition of Ca^{2+} -overload, such as stabilization of the microtubules [18], actin cytoskeleton [19], plasma membrane [20], and membrane-bound proteins [21], could help Dsol to inhibit graft injury.

In conclusion, Ca^{2+} overload initiated during cold preservation induces the activation of harmful proteases, and subsequent apoptosis and necrosis of cardiomyocytes after reperfusion, finally leading to graft loss. A novel organ preservation solution, Dsol, was shown to be superior to UW solution at inhibiting myocardial injury during extended cold preservation and subsequent syngeneic transplantation of rat hearts by inhibiting Ca^{2+} overload during cold preservation and subsequent activation of proteases. This solution could reduce the mortality of heart transplantation. Moreover, the protective effect of this solution could prolong the safe preservation time of cardiac grafts and increase the opportunities for organ distribution.

Authorship

KW, MF, KY and ST: designed the experiments. KW and MF: wrote the article. KW, MF, TK, GH, SS and DF: contributed to the acquisition of data and analysis. SH, TS, MT, TS and HF: provided expertise. MF and MS: provided new reagents. KW, MF, KY, TK and ST: interpreted the data.

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HEPATOLOGY

Interleukin-28B single nucleotide polymorphism of donors and recipients can predict viral response to pegylated interferon/ribavirin therapy in patients with recurrent hepatitis C after living donor liver transplantation

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Key words
core, hepatitis C virus, interferon sensitivity-determining region, interleukin-28B, liver transplantation.

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Abstract

Background and Aim: Interleukin-28B (*IL28B*) single nucleotide polymorphism (SNP) influences viral response (VR) to interferon (IFN) therapy in patients with hepatitis C. We studied the relationship between VR and the *IL28B* polymorphism (rs8099917) in patients on long-term pegylated IFN plus ribavirin (PEGIFN/RBV) therapy for recurrent hepatitis C after living-donor liver transplantation (LDLT).

Methods: Thirty-five patients with recurrent hepatitis C after LDLT were treated with PEGIFN/RBV. We evaluated the effect of *IL28B* SNP on the outcome in 20 patients infected with hepatitis C virus genotype 1 who completed IFN therapy.

Results: The sustained VR (SVR) rate was 54% (19/35) for all patients; 46% (13/28) for genotype 1. The SVR rate of donors' TT group (major genotype) was higher than that of donors' TG + GG group (minor genotype) (73% *vs* 20%), while that of recipients' TT group was similar to that of recipients' TG + GG group (64% *vs* 50%). With regard to the combined effect of donors' and recipients' *IL28B* SNP, the SVR rates of TT : TT (donors' : recipients'), TT : TG + GG, TG + GG : any group were 81%, 50%, and 20%, respectively. The VR rate of TT : TT, TT : TG + GG and TG + GG : any group at 12 weeks were 28%, 0%, and 0%; those at 48 weeks were 70%, 50%, 20%, and those at the end of treatment were 100%, 50%, 20%, respectively. The multivariate analysis identified *IL28B* of donors : recipients (TT : TT) as the only independent determinant of SVR (odds ratio 15.0, $P = 0.035$).

Conclusion: Measurement of donors' and recipients' *IL28B* SNP can predict the response to PEGIFN/RBV therapy, and the donors' *IL28B* SNP might be a more significant predictor than that of the recipients.

Introduction

Hepatitis C virus (HCV) has infected 170 million people worldwide, and such infection sometimes progresses to liver cirrhosis and/or hepatocellular carcinoma.[1] The current treatment for patients infected with HCV genotype 1 (HCV-1) is the combination of pegylated interferon-α and ribavirin (PEGIFN/RBV) for 48 weeks.[2] However, this treatment results in sustained viral response (SVR) in only approximately 50% of patients with HCV-1 infection.

In a recent genome-wide association study, a single nucleotide polymorphism (SNP) upstream of the interleukin (IL)-28B (*IL28B*) gene on chromosome 19, coding for IFN-λ-3, was found to be strongly associated with SVR rate in treatment-adherent HCV-1 patients.[3–8] The G nucleotide of rs8099917 was associated with a poor response to treatment (minor allele), whereas a T nucleotide was found to be associated with a fair response to treatment (major allele) in Japanese patients.

HCV-related end-stage liver disease is currently the leading indication for liver transplantation (LT). However, the outcome of LT for patients with HCV-related liver disease has been less satisfactory than those with HCV-negative liver disease.[9–15] HCV recurrence is universal after LT with accelerated progression of liver fibrosis. Approximately 20–25% of HCV-positive

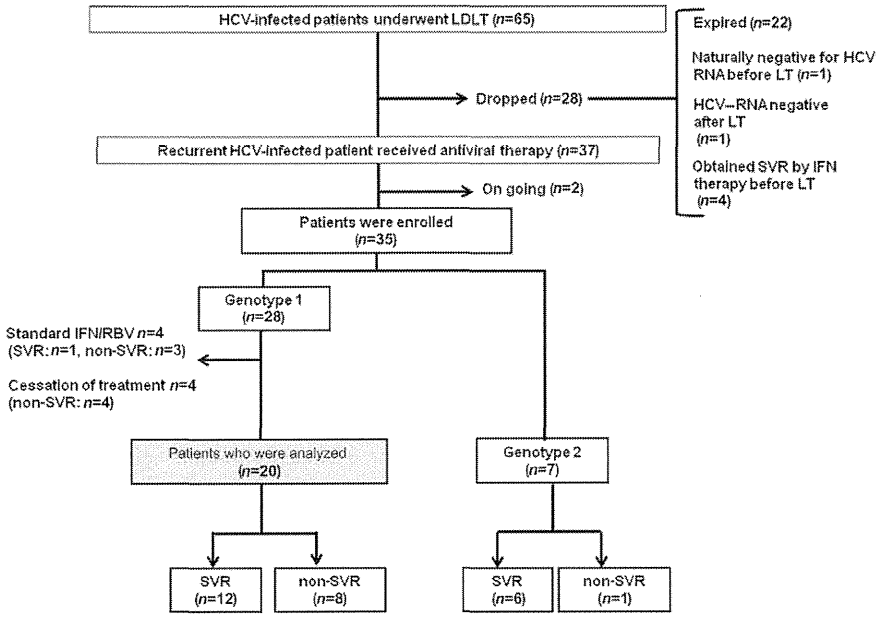

Figure 1 Flow diagram of patient recruitment. HCV, hepatitis C virus; IFN, interferon; LDLT, living-donor liver transplantation; LT, liver transplantation; RBV, ribavirin; SVR, sustained viral response.

patients develop cirrhosis within 5 years after LT, and approximately 50% within 10 years.[13,16,17] LT recipients with recurrent HCV are treated with a combination of PEGIFN/RBV for 48 weeks. However, eradication with IFN therapy after LT is hampered by the use of immunosuppressive agents, anemia, frequent side-effects, and the need to discontinue or reduce therapy. The outcome of PEGIFN/RBV antiviral therapy after LT is poor, with the SVR rate ranging from 10% to 30% for HCV-1-infected patients.[18–24]

However, Fukuhara *et al.*[8] reported that in patients with recurrent HCV infection after LT, combination analyses of SNP of *IL28B* in both the donor and recipient tissues and mutations in HCV–RNA allow the prediction of SVR to PEGIFN/RBV therapy.

We reported previously the effectiveness of the treatment of recipients with PEGIFN/RBV until HCV–RNA reaches undetectable levels, followed by continuation of treatment for at least 48 weeks (i.e. long-term IFN therapy).[25] Others also reported SVR rates of 34% and 50% under the same treatment, respectively.[26,27]

In the present study, we analyzed the viral response to long-term PEGIFN/RBV therapy in patients according to the major and minor genotypes of the polymorphic *IL28B* gene.

Methods

Patients. Sixty-five patients underwent living-donor LT (LDLT) for HCV-related end-stage liver disease between 2000 and January 2011. Among them, 22 patients died before the start of therapy, one was naturally negative for HCV–RNA before LT, one did not become positive for HCV–RNA after LDLT, and four obtained SVR by IFN therapy before LT, thus leaving 37 patients treated with IFN therapy at our institution. Of these, two patients are currently continuing antiviral therapy. A total of 35 patients were enrolled in this retrospective study.

There were 28 patients with HCV-1, and seven with HCV-2. The data of eight of the 28 patients with HCV-1 were excluded from the analysis due to the use of standard IFN/RBV in four patients, and cessation due to side-effects in four patients. Thus, the study included 20 patients with HCV-1 (Fig. 1).

Protocol of antiviral therapy. Patients received PEGIFN-α-2b subcutaneously once weekly combined with RBV (200 mg/day). The dose of the latter was increased to 800 mg/day in a stepwise manner, according to individual tolerance within the first 12 weeks of therapy. The combination PEGIFN/RBV therapy was continued for more than 48 weeks after the disappearance of serum HCV–RNA. At the end of the active treatment, patients were followed for another 24 weeks without treatment. In patients who remained positive for HCV–RNA in spite of treatment for more than 48 weeks, PEGIFN was switched to PEGIFN-α-2a, and treatment was continued as described earlier.

The study was conducted in accordance with the Declaration of Helsinki, and was approved by the local ethics committees of all participating centers. Written, informed consent was obtained from all participating patients.

Assessment of therapy efficacy. HCV–RNA levels were measured using one of several reverse transcription–polymerase chain reaction (RT–PCR)-based methods (*Taq*Man RT–PCR test) at weeks 4, 8, and 12, and thereafter every 4 weeks of treatment, and at 24 weeks after the cessation of therapy.

SNP genotyping and quality control. Because the two reported significant *IL28B* SNP (rs8099917 and rs12979860) are in strong linkage disequilibrium, we examined only rs8099917 in this study. Some samples obtained from patients with HCV-1 were determined using the Illumina HumanHap610-Quad Genotyping BeadChip (San Diego, CA, USA), whereas the remaining samples were genotyped using the Invader assay (Third Wave Technologies, Madison, WI, USA), as described previously.[28,29]

Table 1 Characteristics of 20 patients with recurrent hepatitis C genotype 1 after living-donor liver transplantation

Age (years)†	58 (44–70)
Sex (male/female)	15/5
Body mass index (kg/m²)†	24.3 (18.8–42.2)
Viral load at therapy (LogIU/mL)†	6.6 (4.9–7.8)
Time from transplantation to therapy (months)†	4 (1–41)
No. mutations in the ISDR (0–1/2–5)	12/8
HCV core70 region (mutant/wild)	12/8
HCV core 91 region (mutant/wild)	10/10
Donors' *IL28B* genotype TT/TG + GG	15/5
Recipients' *IL28B* genotype TT/TG + GG	14/6
Combination of donors' and recipients' *IL28B* genotype (TT : TT/TT : TG + GG/TG + GG : TT/TG + GG : TG + GG)	11/4/3/2
Immunosuppression (tacrolimus/cyclosporine)	16/4
Adherence to PEGIFN ≥ 70/< 70 (%)†	11/9
Adherence to RBV ≥ 50/< 50 (%)†	8/12

†Values are median (range). HCV, hepatitis C virus; *IL28B*, interleukin-28B; ISDR, interferon sensitivity-determining region; PEGIFN, pegylated interferon; RBV, ribavirin.

Analysis of the nucleotide sequences of the core and non-structural 5A regions. The amino acid (aa) substitutions at aa 70 and aa 91 of the HCV core region and mutation at the IFN sensitivity-determining region were analyzed in the non-structural 5A region of HCV by the direct sequencing method, as described previously by our group.[25,30,31] Samples after LT were used.

Statistical analysis. Non-parametric tests (χ^2-test and Fisher's exact probability tests) were used to compare the characteristics of the groups. Univariate logistic regression analysis was used to determine those factors that significantly contributed to early viral dynamics. The odds ratios and 95% confidence intervals were also calculated. All P-values < 0.05 using two-tailed tests were considered significant. Variables that achieved statistical significance ($P < 0.05$) or marginal significance ($P < 0.10$) in the univariate analysis were entered into multiple logistic regression analysis to identify significant independent predictive factors. Statistical analyses were performed using PASW 18 statistical software (SPSS, Chicago, IL, USA).

Results

Patient characteristics. Table 1 shows the baseline characteristics of the 20 patients with recurrent hepatitis C after LT who completed PEGIFN/RBV treatment. The median age of the patients (15 males and 5 females) was 58 years, and the median body mass index was 24.3. The median latency between transplantation and the initiation of antiviral therapy was 4 months. The median pretreatment serum HCV–RNA viral load was 6.6 LogIU/mL. The *IL28B* genotype (rs8099917) of the donors was TT in 15 patients, and TG + GG in five patients, whereas that of the recipients was TT in 14, and TG + GG in six. Immunosuppressive therapy included tacrolimus in 16, and cyclosporine in four.

Efficacy and tolerance of IFN therapy and side-effects. Figure 1 shows the effects of IFN therapy according to genotype. The SVR rate was 54.2% (19/35) for all patients. Among the patients infected with HCV-1, one of eight patients who were treated with mono-IFN/RBV or ceased treatment had SVR. Twelve of 20 patients with HCV-1 who completed IFN therapy achieved SVR. Thus, the SVR rate was 46.4% (13/28) for those with HCV-1, and 85.7% (6/7) with HCV-2. In patients with HCV-1, four ceased IFN therapy due to adverse effects. These included general fatigue in one, rejection in two, and cerebral hemorrhage in one patient.

Relationship between IL28B and viral response in patients infected with HCV genotype 1. Data or eight of 28 patients with HCV-1 were excluded from the analysis due to standard-IFN plus RBV in four patients, and the cessation of IFN therapy due to adverse effects in four patients. Thus, the data of 20 patients with HCV-1 were available for the analysis of *IL28B*.

In the donors, the SVR rate of the TT group (73.3% [$n = 11/15$]) was higher than that of the TG + GG group (20% [$n = 1/5$], $P = 0.053$, Fig. 2a). In the recipients, the SVR rate of the TT group (64.2% [$n = 9/14$]) was similar to that of the TG + GG group (50% [$n = 3/6$]) (Fig. 2b). The SVR rate of the TT : TT group (donors' *IL28B* : recipients' *IL28B*) was 81.8% ($n = 9/11$), which was higher than the SVR rate of the TT : TG + GG group (50% [$n = 2/4$], Fig. 2c). The SVR rate of the TG + GG : any group (donors' *IL28B* : recipients' *IL28B* of either TT or TG + GG) was 20% ($n = 1/5$), which was lowest among the three groups. There was significant difference between the SVR of the TT : TT group and TG + GG : any group ($P = 0.036$). We also analyzed the viral response (VR) rate according to the combination of donors' and recipients' *IL28B*. The VR rates of TT : TT, TT : TG + GG, TG + GG : any group at 12 weeks were 28%, 0%, and 0%; those at 48 weeks were 70%, 50%, and 20%; and those at the end of treatment were 100%, 50%, and 20%, respectively. The VR rate of the TT : TT group was 63.6% ($n = 7/11$), which was higher than the VR rate of the TG + GG : any group (0% [$n = 0/5$]) at 24 weeks. The VR rate of the TT : TT group was 100% ($n = 11/11$), which was higher than the VR rate of the TG + GG : any group (20% [$n = 1/5$]) at the end of treatment. The SVR rate of the TT : TT group was 100% ($n = 11/11$), which was higher than the SVR rate of the TG + GG : any group (20%, $n = 1/5$) at 24 weeks at the end of treatment (Fig. 3).

Analysis of factors associated with SVR in HCV-1 patients with recurrent hepatitis C. The univariate analysis identified three parameters that correlated with SVR either significantly or marginally: the combination of donors' and recipients' *IL28B* (TT : TT $P = 0.037$), donors' *IL28B* (TT genotype; $P = 0.053$), and adherence to RBV therapy (≥ 50; $P = 0.076$, Table 2). The combination of donors' and recipients' *IL28B* (TT : TT genotype) and adherence to RBV (> 50; $P = 0.076$) were entered into the multiple logistic regression analysis to identify significant independent predictive factors. The multivariate analysis identified the combination of donors' and recipients' *IL28B* (TT : TT) as the only significant and independent factor that influenced the SVR: (odds ratio: 15.0, 95% CI: 1.2–185.1, $P = 0.035$).

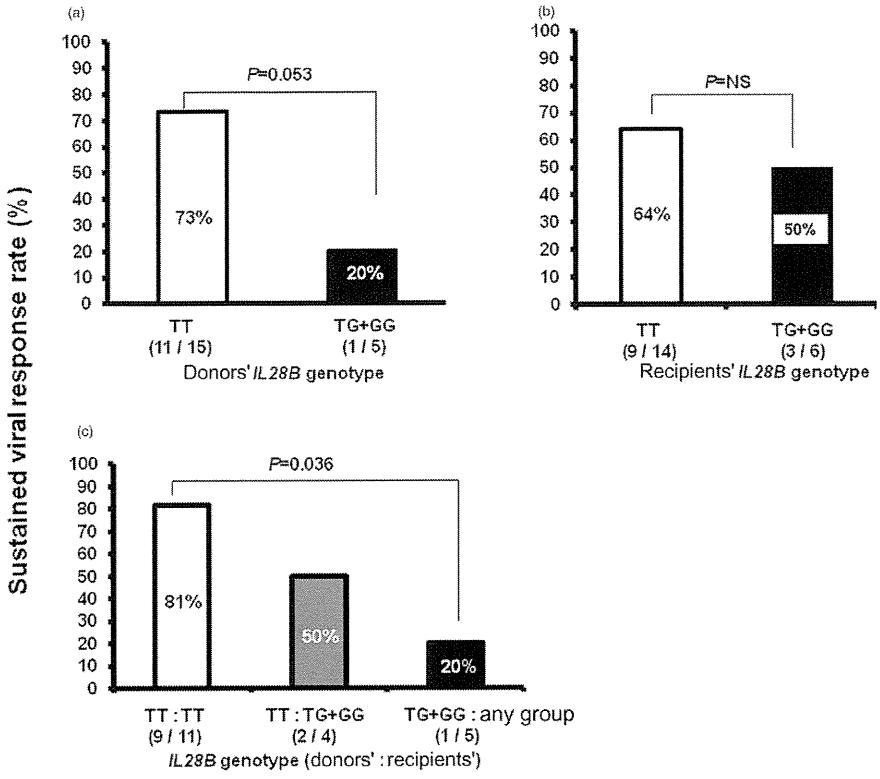

Figure 2 Sustained viral response rates according to (a) donors' interleukin-28B (*IL28B*), (b) recipients' *IL28B*, and (c) donors' and recipients' *IL28B* in patients infected with hepatitis C virus genotype 1. TT : TT group (donors' *IL28B* TT: recipients' *IL28B* TT), TT : TG + GG group (donors' *IL28B* TT: recipients' *IL28B* TG + GG), TG + GG : any group (donors' *IL28B* TG + GG: recipients' *IL28B* either TT or TG + GG). NS, not significant.

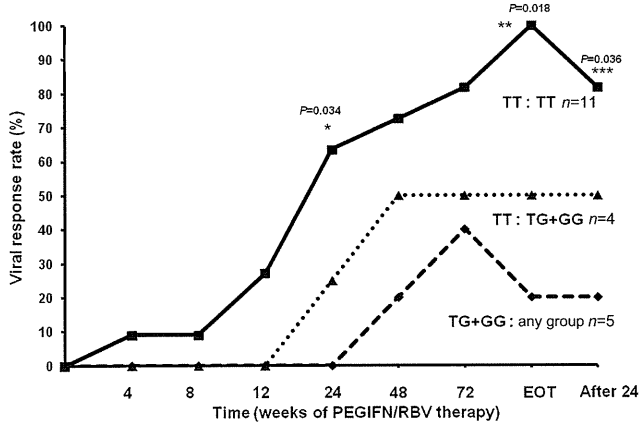

Figure 3 Viral response rates according to donors' and recipients' interleukin-28B (*IL28B*) genotyping. TT : TT group (donors' *IL28B* TT: recipients' *IL28B* TT), TT : TG + GG group (donors' *IL28B* TT: recipients' *IL28B* TG + GG), TG + GG : any group (donors' *IL28B* TG + GG: recipients' *IL28B* either TT or TG + GG). *Viral rate (VR) of the TT : TT group was 63.6% ($n = 7/11$), which was higher than the VR rate of the TG + GG : any group (0%, $n = 0/5$) at 24 weeks. **VR rate of the TT : TT group was 100% ($n = 11/11$), which was higher than the VR rate of the TG + GG : any group (20%, $n = 1/5$) at the end of treatment (EOT). ***Sustained VR (SVR) rate of the TT : TT group was 100% ($n = 11/11$), which was higher than the SVR rate of the TG + GG : any group (20%, $n = 1/5$) at 24 weeks at the EOT. PEGIFN, pegylated interferon; RBV, ribavirin.

Discussion

The SVR rate has improved since the introduction of PEGIFN/RBV for patients who undergo LT for HCV-related end-stage liver disease. The current estimated SVR rate for LT patients with a history of HCV-1 infection is 30–50%.[21–24,26,27] These results are much better than those reported in the 1990s and early 2000s; however, more than half of recipients still suffer from recurrent chronic hepatitis C.

Although many studies have determined the predictive factors of the viral response for PEGIFN/RBV among patients with chronic hepatitis C, recent molecular biological analyses and genome-wide analyses of the human genome have identified genetic variations of *IL28B* and amino-acid substitution of HCV core 70 as the most significant predictive factors for IFN response.[3–5,32,33] *IL28B* encodes a cytokine distantly related to type I IFN and the IL-10 family. It has been reported that the expression level of the *IL28* gene in peripheral blood mononuclear cells is significantly lower in individuals with minor alleles than in individuals with major alleles.[5]

Several studies have determined the predictive factors for the viral response to PEGIFN/RBV in patients with recurrent post-LT hepatitis C viral infection, and recent molecular and genome wide analyses of the human genome have demonstrated that genetic variation of *IL28B* is the most significant predictive factor of the response to IFN.[8,34–37] In the present study, we examined whether the same factors can also predict the response to PEGIFN/RBV in LT recipients. Several groups have reported that recipients' and donors' *IL28B* influenced the SVR to PEGIFN/RBV in patients with recurrent hepatitis C after LT.[8,36,37] Furthermore, others

Table 2 Univariate analysis of factors associated with sustained viralresponse (SVR) during interferon therapy in genotype 1 patients with recurrent hepatitis C

	SVR ($n = 12$)	Non-SVR ($n = 8$)	*P*-value
Age (years)†	60 (44–69)	57 (47–65)	0.48
Sex (male/female)	10/2	5/3	0.3
Body mass index (kg/m^2)†	24.1 (21.4–26.5)	24.2 (18.9–42.2)	0.4
Viral load at therapy (LogIU/mL)†	6.3 (5.8–6.6)	6.6 (5.9–7.2)	0.52
Time from transplantation to therapy (months)†	4 (1–41)	3 (1–6)	1.7
No. mutations in the ISDR (0–1/2–5)	7/5	5/3	1.0
HCV core70 region (mutant/wild)	7/5	5/3	1.0
HCV core 91 region (mutant/wild)	7/5	3/5	0.6
Donors' *IL28B* genotype TT/TG + GG	11/1	4/4	0.053
Recipients' *IL28B* genotype TT/TG + GG	9/3	5/3	0.6
Donors' and recipients' *IL28B* genotype TT : TT/others	9/3	2/6	0.037
Immunosuppression (tacrolimus/cyclosporine)	9/3	7/1	1.0
Adherence to PEGIFN ≥ 70/< 70 (%)†	8/4	3/5	0.3
Adherence to RBV ≥ 50/< 50 (%)†	7/5	1/7	0.076

†Values are median (range). HCV, hepatitis C virus; *IL28B*, interleukin-28B; ISDR, interferon sensitivity-determining region; PEGIFN, pegylated interferon; RBV, ribavirin.

reported that donors' *IL28B* influenced the SVR in patients treated with PEGIFN/RBV for recurrent hepatitis C after LT,[34] and that recipients' *IL28B* influenced the SVR to PEGIFN/RBV in patients with recurrent post-LT hepatitis C.[35,36]

The results of the present study indicate that both donors' and recipients' *IL28B* influence the SVR to PEGIFN/RBV in patients with recurrent post-LT hepatitis C. Both recipients' and donors' *IL28B* influenced the SVR to PEGIFN/RBV in recurrent hepatitis C after LT; however it is not clear whether the recipients' or donors' *IL28B* influenced the SVR to PEGIFN/RBV.

However, the donors' *IL28B* might have influenced the SVR to PEGIFN/RBV in patients with recurrent post-LT hepatitis C more than the recipients' *IL28B*. This conclusion is based on the following results: although the SVR rate of the TT group (64.2%) was similar to that of the TG + GG group (50%), according to the recipients' *IL28B*, the SVR rate of the TT group (73.3%) was higher than that of the TG + GG group (20%), according to the donors' *IL28B*. Furthermore, the VR rates of TT : TT, TT : TG + GG, TG + GG : any group at 12 weeks were 28%, 0%, and 0%; those at 48 weeks were 70%, 50%, and 20%; and those at the end of treatment were 100%, 50%, and 20%, respectively. That is, the time to VR of the TG + GG : any group was the latest among the three groups. Lange *et al.* reported that donors' *IL28B* influenced the SVR in patients treated with PEGIFN/RBV for recurrent hepatitis C after LT.[34] In this regard, Hiraga *et al.*[38] reported that IFN-stimulated gene expression levels in mice livers measured at 2 weeks after IFN treatment were significantly higher in mice transplanted with donor human hepatocytes (*IL28B*; TT) than from donor (*IL28B*; TG + GG) mice. Furthermore, previous studies reported that the expression level of IFN- λ-3, coded for the *IL28B* gene, was higher in hepatocytes than hematopoietic cells.[39]

However, we demonstrated the feasibility of treatment of LT recipients with PEGIFN/RBV until HCV–RNA reached undetectable levels, followed by the continuation of treatment for at least 48 weeks (i.e. long-term IFN therapy). In fact, the SVR rate (50%) of the recipients' *IL28B* TG + GG group was higher than that reported by others[8] (SVR rate: 11%). Furthermore, the SVR rate (81%) of the combination of donors' and recipients' *IL28B* (TT : TT) group was higher than that reported by Fukuhara *et al.*[8] (SVR rate: 56%). However, the SVR rate of the donors' *IL28B* TG + GG group (SVR rate: 20%) was similar to that reported by Fukuhara *et al.*[8] (SVR rate: 9%). We believe that the treatment of LT recipients with PEGIFN/RBV until HCV–RNA reaches undetectable levels, followed by the continuation of treatment for at least 48 weeks, is not useful for donors with *IL28B* TG + GG.

In Japan, LDLT is more common than orthotopic LT. In finding a suitable donor, it is better to select a donor with TT of the *IL28B* gene than a TG or GG donor. In conclusion, our results demonstrated the suitability of donors with the TT *IL28B* genotype, and that long-term PEGIFN/RBV therapy seems useful for recipients of LDLT who develop recurrent hepatitis C after transplantation.

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Case Report

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Achievement of Sustained Viral Response after Switching Treatment from Pegylated Interferon α-2b to α-2a and Ribavirin in Patients with Recurrence of Hepatitis C Virus Genotype 1 Infection after Liver Transplantation: A Case Report

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Key Words
PEG-IFN α-2a · PEG-IFN α-2b · *IL28B* · Hepatitis C virus · Liver transplantation

Abstract
We report a case in which sustained viral response was achieved after switching treatment from pegylated interferon (PEG-IFN) α-2b to α-2a and ribavirin (RBV) in patients with recurrence of hepatitis C virus (HCV) infection after living donor liver transplantation. The patient was a 62-year-old man with liver cirrhosis due to HCV genotype 1b infection. The patient had 8 amino acid (aa) substitutions in the interferon sensitivity-determining region, and had substitutions for mutant and wild-type at aa70 and aa91, respectively, in the core region. The patient had minor genotype (GG) *IL28B* single nucleotide polymorphisms (rs8099917). He had initially received interferon α-2b and RBV for 2 years, and later developed hepatocellular carcinoma (HCC). After surgical resection of HCC, he subsequently received PEG-IFN α-2b and RBV for 1.5 years, without undetectable viremia during the treatment course. Due to recurrence of HCC, the patient received a living donor liver transplantation. Later on, hepatitis C relapsed. For the management of relapse, he received another course of PEG-IFN α-2b and RBV. However, breakthrough viremia occurred. PEG-IFN was thus switched from α-2b to α-2a and RBV for another 17 months. The patient eventually achieved a sustained viral response.

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Introduction

Currently, pegylated interferon (PEG-IFN) α and ribavirin (RBV) are used as standard therapy for the treatment of patients with hepatitis C virus (HCV) infection; successful outcomes with PEG-IFN and RBV have been achieved in approximately 60% of the treated cases [1]. However, about 50% of the patients treated with this therapy have been reported to show an increase in the viral load and/or serum alanine aminotransferase (ALT) level during therapy [2, 3]. The event of increase in the viral load is called 'breakthrough viremia'. No treatment regimens have been established for patients who develop breakthrough viremia during treatment with or relapse following PEG-IFN and RBV. In some reports, PEG-IFN α-2a and RBV has been reported to result in a higher sustained viral response (SVR) than that achieved by PEG-IFN α-2b and RBV [4]. Moreover, PEG-IFN α-2a and RBV was reported to be effective for treatment of some HCV patients who experienced relapse after PEG-IFN α-2b and RBV [5]. Although there are reports about interferon (IFN) therapy in patients with HCV genotype 1 infection after liver transplantation (LT) [6–8], there are no reported cases where SVR was achieved by switching treatment with PEG-IFN from α-2b to α-2a and RBV in patients with HCV genotype 1 infection after LT.

We report a case in whom SVR was achieved after switching treatment from PEG-IFN α-2b to α-2a and RBV in a patient with recurrence of HCV genotype 1 infection after LT.

Case Report

The patient was a 62-year-old man with liver cirrhosis due to HCV genotype 1b infection. The patient's height was 168 cm, weight 70.4 kg, and body mass index 24.9.

HCV RNA was 1,200 kIU/ml. The patient had undergone IFN therapy with conventional IFN α-2b (6 MU) plus RBV (800 mg) for 24 months since 2002. He was administered IFN α-2b (6 MU) for thrombopenia, but the therapy was stopped since he showed no response to the therapy.

The patient developed hepatocellular carcinoma (HCC) and was treated by hepatic resection and had stage F3 fibrosis in September 2005. After that, IFN therapy was started with PEG-IFN α-2b (60 μg) and RBV (200 mg) in December 2005. At that time, HCV RNA was 2,400 kIU/ml. However, RBV was stopped since the patient developed itching. However, HCV RNA never reached undetectable levels. After that, HCC recurred. Therefore, the patient underwent splenectomy, and hepatectomy for HCC recurrence in August 2006. At the time, the patient showed stage F3 fibrosis. After that, IFN therapy was restarted.

Table 1. Laboratory data at the start of IFN therapy after LT

CBC	
WBC/μl	3,160
RBC/μl	4.50×10^6
Hb, g/dl	13.0
Ht, %	37.4
Plt/μl	257×10^3
Blood coagulation test	
PT, %	118
Blood chemistry	
T-bil, mg/dl	2.4
AST, IU/l	89
ALT, IU/l	45
LDH, IU/l	269
ALP, IU/l	497
γGTP, IU/l	377
TP, g/dl	6.9
Alb, g/dl	3.1
TC, mg/dl	129
TTT, U	5
ZTT, U	12
BUN, mg/dl	13
Cr, mg/dl	1.17
CRP, mg/dl	<0.2
FBS, mg/dl	267
HbA_{1c}, %	6.6
NH_3, μg/ml	47
Tumor marker	
AFP, ng/ml	27.1
HCV virus marker	
HCV RNA, kIU/ml	27,000
MELD score	6
Child-Pugh	A
aa substitution in ISDR	eight
aa70 in the core region	mutant
aa91 in the core region	wild
IL28B, genotype	GG

AFP = α-Fetoprotein; Alb = albumin; ALP = alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate aminotransferase; Cr = creatinine; CRP = C-reactive protein; FBS = fasting blood sugar level; Hb = hemoglobin; LDH = lactate dehydrogenase; Plt = platelets; PT = prothrombin time; RBC = red blood cells; T-bil = total bilirubin; TC = total cholesterol; TTT = thymol turbidity test; WBC = white blood cells; ZTT = zinc sulfate turbidity test; aa substitution in ISDR = amino acid substitutions in the IFN sensitivity-determining region.

Tumor stage was stage III [9]. Treatment with curative intent was not possible owing to the presence of multiple HCC lesions. Although the MELD score was 6 and Child-Pugh score A, his sister wished to be the donor for LT; LT was performed with informed consent in June 2007. At the time, the patient showed stage F4 fibrosis.