was no significant difference in immune function before and after conversion; this result suggested that conversion also did not affect immune function. In addition, it was important that none of the recipients showed adverse effects, rejection, or severe infection and none had to increase the dose of Advagraf, while five of 21 recipients (24%) were even able to reduce the dose of Advagraf during this study. In our policy of immunosuppression, especially in long-term cases, we reduce and maintain the dose of immunosuppressant as long as possible, keeping the lowest level of tacrolimus needed to prevent rejection. According to the results of this study, Advagraf might be a feasible treatment for avoiding an overdose of tacrolimus.

Conclusions

This study suggested that the conversion of Advagraf can be safely and effectively applied to stable LDLT recipients without affecting liver, renal, and immune function.

Disclosure

The authors have no conflicts of interest or funding to disclose.

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ELSEVIER

Clinical Outcome of Pancreas Transplantation From Marginal Donors in Japan

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ABSTRACT

In Japan, absolute shortage of donors still continues even after the law allowing organ transplantation from deceased donors came into force in 1997. With the passage of the waiting period after registration for pancreas transplantation (PTx), both deaths and serious cases of diabetic complications necessitating withdrawal of the registration have undoubtedly increased. Therefore, so-called "marginal donor" (MD) has been considered as a potential solution for shortage of donors in Japan. The aim of the present study is to evaluate feasibility of MD in terms of post-PTx outcomes using data from Japan Organ Transplantation Network. A total of 148 PTx were performed from deceased donors in Japan from 2000 to 2012. MD was defined as follows: (1) >45 years old; (2) hemodynamically unstable at harvest using a high-dose dopamine or more than 2 vasopressors; or (3) non–heart-beating status. Postoperative outcomes after PTx were compared between the MD group and the non-MD group. Among the 148 PTx donors, 108 donors (73.0%) satisfied the criteria of MD. Early graft loss of pancreas graft during 3 months post-transplant was observed in 15 patients (10.1%), and the marginality (MD vs non-MD) was not significantly correlated with the early loss of pancreas graft. The overall patient survival of the MD group (1, 3, 5 years: 94.7%, 94.7%, 94.7%) was not significantly different from that of the non-MD group (1, 3, 5 years: 95.0%, 95.0%, 95.0%). Pancreas graft survival in the MD group (1, 3, 5 years: 80.9%, 73.2%, 66.0%) seemed to be slightly lower than that in the non-MD group (1, 3, 5 years: 92.5%, 85.2%, 77.4%), but no statistically significant differences were found between the 2 groups. These results suggest the feasibility of the use of MD for PTx.

PANCREAS TRANSPLANTATION (PTx) is an established treatment for type 1 diabetes [1–3]. It is the only effective therapeutic option to restore normal glucose metabolism, to improve quality of life of the patients, and to even reduce chronic complications of the diabetes. Although its outcome was not satisfactory previously, graft survival has much improved during the last 30 years because of development in immunosuppressants, surgical techniques, and postoperative management.

In Japan, the Organ Transplant Law was enforced on October 1997, and it was revised on July 2010. Since the revision, the number of donation is increasing. However, absolute shortage of donors still continues even after the revision. With the passage of the waiting period after registration for PTx, both deaths and serious cases of diabetic complications necessitating withdrawal of the registration have undoubtedly increased. Accordingly, we have had to depend on the so-called "marginal donor" (MD). To date, however, the feasibility of PTx from MD has not yet investigated well. In this regard, the present study was performed to evaluate its feasibility in terms of postoperative outcomes using data from Japan Organ Transplantation Network.

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PATIENTS AND METHODS

Patients

Between April 2000 and December 2012, a total of 148 PTx were performed for type 1 diabetes from deceased donors in Japan. Among the 148 cases of PTx, 146 cases were from brain-dead donors and the remaining 2 were non–heart-beating donors. In Japan, PTx is performed in 17 approved institutions. Characteristics of the 148 patients are shown in Table 1.

Criteria of Marginal Donor

The criteria of MD for PTx of Kapur et al were used in this study; donors of 45 years of age and more, hemodynamically unstable donors at the time of harvest (with dopamine dose > 10 μg/k/min, or 2 or more vasopressors), or non–heart-beating donors [4]. Based on these criteria, the donors were divided into 2 groups: the MD group and the non-MD group.

Graft Failure

Pancreas graft failure was defined as return to insulin-dependence or serum C-peptide level < 0.3 ng/mL. Kidney graft failure was defined as return to dialysis. Death with a functioning graft was also considered be a graft failure. Early graft loss was defined as that within 3 months post-PTx in this study.

Statistical Analysis

Survival was calculated according to the Kaplan–Meier method and compared using the log-rank test. Statistical analysis was performed using StatView (version 5.0; SAS Institute Inc., Cary, NC, United States). A P value <.05 was considered statistically significant.

Table 1. Characteristics of 148 PTx Patients (n = 148)

Factors	
Donor-related factors	
Age (≤45 y/>45 y)	74/74
Gender (male/female)	80/68
Body mass index (kg/m^2) (<25/≥25)	115/33
Cause of death (CVA/trauma/others)	87/28/33
Type of death (brain-dead/non–heart-beating)	146/2
Hemodynamics (stable/unstable)	87/61
Cardiopulmonary resuscitation (−/+)	86/62
Marginality (MD/non-MD)	108/40
Recipient-related factors	
Age (≤50 y/>50 y)	123/25
Gender (male/female)	56/92
Duration of diabetes (<30 y/≥30 y)	90/58
Duration of dialysis (<10 y/≥10 y)	72/47
PTx-related factors	
TCIT (<12 h/≥12 h)	86/62
Type of PTx (SPK/PAK/PTA)	119/20/9
Duct management (bladder drainage/enteric drainage)	30/118
GDA reconstruction (−/+)	35/87
Immunosuppressive regimen	
CNI (TAC/CyA)	144/4
Antibody (−/+)	7/141

Abbreviations: PTx, pancreas transplantation; CVA, cerebrovascular accident; MD, marginal donor; TCIT, total cold ischemic time; SPK, simultaneous pancreas and kidney transplantation; PAK, pancreas transplantation after kidney transplantation; PTA, pancreas transplantation alone; GDA, gastroduodenal artery; CNI, calcineurin inhibitor; TAC, tacrolimus; CyA, cyclosporine.

RESULTS

Ratio of Marginal Donors

Among the 148 donors at the PTx, 74 were 45 or more years old. Sixty-one donors were hemodynamically unstable at the time of harvest. Two donors were non–heart-beating donors. In total, 108 donors (73.0%) of the 148 donors satisfied the criteria of MD and categorized into the MD group, and the remaining 40 donors (27.0%) were categorized into the non-MD group. Characteristics of the 148 patients are shown in Table 1.

Risk Factors for Early Loss of Pancreas Graft

Among the 148 PTx cases, early graft loss of pancreas graft was observed in 15 patients (10.1%). Thrombosis was the most frequent cause of the graft loss (8/15, 53%). The other causes were as follows: sepsis in 3, rejection in 2, duodenal perforation in 1, and cardiogenic shock in 1.

To investigate whether the marginality (MD vs non-MD) is a risk factor for the early loss of pancreas graft, as well as to identify factors that significantly correlate with the early graft loss, donor-related factors were compared between cases with the early graft loss and without the early graft loss (Table 2). The incidence of the early graft loss was significantly higher in donors with total cold ischemic time (TCIT) ≥12 hours (P = .05), and the marginality (MD vs non-MD) was not significantly correlated with the graft loss.

Long-Term Outcome After Pancreas Transplantation

We examined long-term outcomes of PTx in terms of overall patient survival, pancreas graft survival, and kidney graft survival (SPK cases). As shown in Table 3, in all the 148 cases, postoperative mortality was found in 5 patients in the MD group (4.6%) and in 3 patients in the non-MD group (7.5%). The incidence was not significantly different between the 2 groups (P = .45). Overall patient survival in the 148 cases was 94.8%, 94.8%, and 94.8% at 1, 3, and 5 years, respectively. The overall patient survival of the MD group (1, 3, 5 years: 94.7%, 94.7%, 94.7%) was not significantly different from those of the non-MD group (1, 3, 5 years: 95.0%, 95.0%, 95.0%; P = .42, Fig 1A). Twenty-four pancreas grafts were lost during the observation period

Table 2. Correlation of Donor-Related Factors With Early Loss of Pancreas Graft in the 148 PTx Cases

Factor	Early Graft Loss (−) (n = 133)	Early Graft Loss (+) (n = 15)	P Value
Age (≤45 y/<45 y)	66/67	8/7	.79
Gender (male/female)	70/63	10/5	.41
Body mass index (kg/m^2) (<25/≥25)	103/30	12/3	.56
Cause of death (CVA/others)	78/55	10/5	.59
Hemodynamics (stable/unstable)	80/53	7/8	.41
Cardiopulmonary resuscitation (−/+)	78/55	8/7	.78
TCIT (<12 h/≥12 h)	81/52	5/10	.05
Marginality (MD/non-MD)	96/37	12/3	.76

Abbreviations: PTx, pancreas transplantation; CVA, cerebrovascular accident; MD, marginal donor; TCIT, total cold ischemic time.

Table 3. Incidence of Mortality and Graft Failures in MD Group and Non-MD Group

	MD Group	Non-MD Group	*P* Value
Mortality	5/108 (4.6%)	3/40 (7.5%)	.45
Cardiogenic	1	2	
Cerebral bleeding	1	0	
Sepsis	2	1	
GVHD	1	0	
Pancreas graft failure	24/108 (22.2%)	4/40 (10.0%)	.08
Thrombosis	7	1	
Duodenal perforation/ bleeding	2	0	
Pancreatitis	1	0	
Recurrent diabetes	2	0	
Rejection	12	3	
Kidney graft failure	8/88 (9.1%)	1/31 (3.2%)	.44
Thrombosis	0	0	
Primary nonfunction	1	0	
Rejection	7	1	

Abbreviations: MD, marginal donor; GVHD, graft-versus-host disease.

among the 108 cases in the MD group, and 4 pancreas grafts were lost in the 40 cases in the non-MD group (Table 3). The incidence of the pancreas graft failure in the MD group tended to be higher than the non-MD group ($P = .08$, Table 3). Especially, thrombosis and rejection were frequently observed as a cause of the graft failure in the MD group. Pancreas graft survival in all the 148 cases was 84.8%, 76.4%, and 68.9% at 1, 3, and 5 years, respectively. Pancreas graft survival in the MD group and the non-MD group was 80.9% and 92.5%, 73.2% and 85.2%, and 66.0% and 77.4% at 1, 3, and 5 years post-PTx, respectively, and there was no significant difference between the 2 groups ($P = .35$, Fig 1B). Incidence of kidney graft failure in 119 SPK cases was also compared. The incidence was not significantly different between the 2 groups ($P = .44$, Table 3). Kidney graft survival in the SPK cases was 84.8%, 76.4%, and 68.9% at 1, 3, and 5 years, respectively. Kidney graft survival of the MD group (1, 3, 5 years: 89.1%, 89.1%, 86.0%) was not significantly different from that of the non-MD group (1, 3, 5 years: 93.5%, 93.5%, 84.2%; $P = .77$, Fig 1C).

DISCUSSION

The present study first showed that MD has been mostly utilized for PTx in Japan, compared with the condition of PTx donors in the United States [2,3]. However, the patient survival and graft survival were not significantly different from that in the United States. In case of simultaneous liver harvest in Japan, the reconstruction of gastroduodenal artery in pancreas graft has been done as much as possible (71.3%) to increase the blood flow in pancreas head region [5]. It remains unknown whether this procedure will have an effect on the early graft outcome.

The present study also demonstrated that there are no statistically significant differences in long-term outcomes after PTx between the MD group and the non-MD group. Furthermore, we investigated risk factors for the early loss of pancreatic graft and found that the marginality (MD vs non-MD) is not statistically significantly correlated with the early loss. These findings suggested the possibility that PTx from MDs is feasible in terms of postoperative outcomes. We also showed that the incidence of the early pancreatic graft loss within 3 months posttransplant is significantly increased when TCIT is over 12 hours. On the other hand, in the United States, it has been reported that preservation time of pancreatic graft >20 hours is significantly associated with post-PTx complications [6,7]. In this regard, a permissive range of the preservation time is likely to be narrow in Japan as compared to the United States where non-MDs are mostly available.

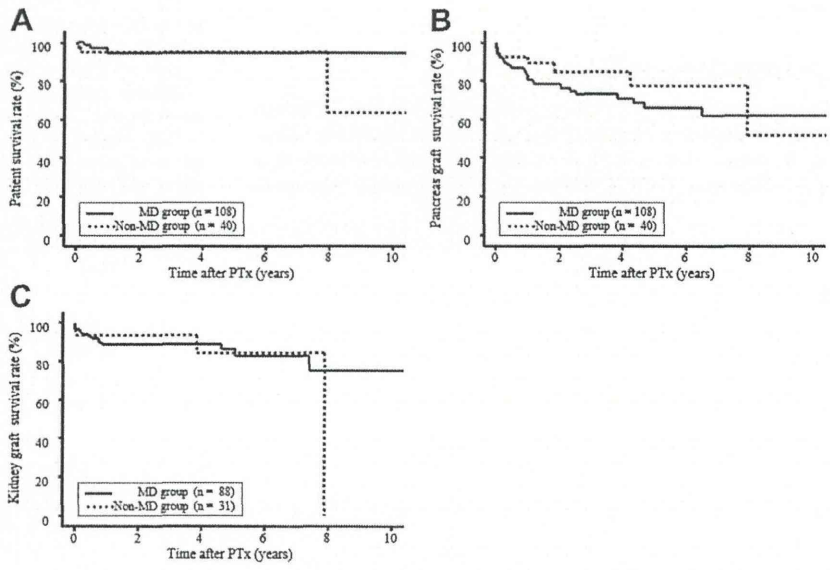

Fig 1. Long-term outcome after pancreas transplantation. Overall patient survival (**A**), pancreas graft survival (**B**), and kidney graft survival (**C**) were compared between the MD group (solid lines) and the non-MD group (dotted lines). Overall patient survival and pancreas graft survival were calculated in all the 148 PTx cases, and kidney graft survival was calculated in 119 simultaneous pancreas and kidney transplantation cases. Survival was not significantly different between the 2 groups. MD, marginal donor; PTx, pancreas transplantation.

In addition to the preservation time of the graft, to date, many donor-related risk factors have been considered as key determinants of outcomes after PTx such as donor age, obesity, donation after cardiac death, and cause of death. Especially, donor age is one of the most common risk factors. In general, aging affects nearly all the kinds of cells that play roles in outcomes of PTx including insulin-producing islet cells and endothelial cells of blood vessels, potentially affecting formation of thrombus. Salvalaggio et al reported from the United States data that old donors (>45 years) result in poorer long-term outcome in comparison to younger donors [8]. European data suggest equivalent outcomes [9]. Furthermore, donor age has been recognized as one of the factors composing scoring index for assessment of donor risk [10,11].

Indeed, the results of the present study may help expand the donor pool and resolve the donor shortage by using pancreas from MD. However, based on these previous reports, there seems to be another possibility that the current study enrolled too few cases to find statistically significant differences in post-PTx outcomes between the MD group and the non-MD group. Actually, the incidence of the pancreas graft failure in the MD group tended to be higher than the non-MD group, though the difference of the incidence was not statistically significant. To allow any conclusion on whether usage of grafts from MD is an acceptable option at PTx, studies with larger PTx numbers will be needed. If the outcome of PTx from MDs is judged to be worse than those from non-MDs, further investigations may be also necessary to clarify factors that contribute to better outcomes in MDs.

In summary, the current study suggested that PTx from MDs is feasible in terms of postoperative outcomes based on data obtained so far from a nationwide database in Japan. At the same time, considering the small number of PTx in Japan compared to other countries, the finding should be validated in studies with a larger number of PTx cases.

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ELSEVIER

Current Status of In-Hospital Donation Coordinators in Japan: Nationwide Survey

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ABSTRACT

Objectives. When the Japanese Organ Transplantation Act was issued, the Japanese Organ Transplantation Network (JOT) was established in 1997. JOT lists recipients, assesses and manages organ donors, and educates publics and headquarters for organ donations. JOT procurement transplant coordinators (PTC) play roles in obtaining consent from relatives for organ donation, donor evaluation and management, organ recovery management, organ transport, and care of donor families during and after donation. Every prefecture has at least one PTC who is mainly working in public education and hospital development. They also help the JOT PTC at the time of organ procurement. Most prefectures commission hospital staff in the procurement hospital to be an in-hospital PTC (In-Hp PTC), who make their hospital staff aware of organ donation and support organ procurement. Although the Act was revised in 2010 with brain-dead organ donation increased from 13 to 44 cases yearly, the number was still extremely smaller than other developed countries. In these circumstances, In-Hp PTC may play greater roles to increase donation and smooth procurement procedures Our primary aim was to describe the current status of In-Hp PTC in Japan.

Materials and methods. Between December 15, 2011, and January 31, 2012, we invited 1889 In-Hp PTC to complete a letter survey using a self-designed questionnaire. In all, 56 In-Hp PTC (40%) completed and returned it.

Results. The occupation of the respondents was nurse (66%), physician (18%), or other (16%). Although 52% of respondents belonged to the hospital, which was designated for brain-death organ donation by the government, only 46% had any experience with a cadaveric donor. Only 2% were full-time In-Hp PTC. They mainly played a role in preparing their own manual for organ procurement (57%), providing in-hospital lectures (44%) or their own simulation exercise (29%), as well as coordinating donation cases. Although 77% had attended seminar about organ donation provided by JOT or the prefecture PTC, 93% wanted more professional education. However, it was difficult for them to attend these activities, to manage a rare and sudden donation case, and to find time to learn about organ donation because they had another post. The topics that they wanted to learn were donor family care (72%), overall organ/tissue donation procedures (65%), the role of In-Hp PTC (67%), simulations of donation (65%), legislation and social system of organ donation (61%), medical indications for donation (61%), current status of donation and transplantation in Japan (57%), donor management (56%), and case studies (49%). There were significant variations in the topics of interest among the occupations.

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As they had another post, they could find only a short period (1 or 2 days) to take professional education, such as lectures. Therefore, it was difficult for them to attend practical on-the-job training.

Conclusions. To establish an organ procurement system and increase organ donation, In-Hp PTC have important roles in Japan. However, none is a full-time In-Hp PTC. Most In-Hp PTC require more professional education. A systematic education program for each occupation must be established soon.

SINCE 1978, the donation of kidneys after cardiac death (DCD) has been legally accepted in Japan after family consent. Small children had been able to donate their kidneys after cardiac death. The Japanese Organ Transplantation Act for brain-death (BD) donation was issued in October 1997. The Act required a living written consent for BD declaration and organ donation; it did not allow BD donation from children younger than 15 years. For these reasons, only 81 BD organ donations were performed in Japan over 13 years since the Act was issued in October 1997.

The Japanese Organ Transplant Act issued in 1997, established the Japanese Organ Transplantation Network (JOT), which lists recipients, assesses and manages organ donors, and educates publics and headquarters of organ donations. JOT procurement transplant coordinators (PTC) play roles in obtaining consent for organ donation from relatives, donor evaluation and management, management of organ recovery, organ transport, and care of donor families during and after donation.

Every prefecture has at least one of their own 1 PTC who is mainly working on public education and hospital development. They also collaborate with the JOT PTC at the time of organ procurement. Most prefectures commission staffs in procurement hospitals to be in-hospital PTC (In-Hp PTC), who make their hospital staffs aware of organ donation and support organ procurement (Fig 1).[1,2]

Finally the Act was revised on July 17 2010.[1–3] Renewal of the Act allowed organs to be donated after BD with family consent if not previously denied before the event. Although the Act was revised in 2010 and BD organ donation increased from 13 to 44 cases in a year, the number was still extremely smaller than that in other developed countries. The revised Act accepts organ donation from BD children younger than 15 years. However, only 158/504 (42.4%) procurement hospitals where BD organ donation is allowed by the Government have established procurement systems from children. In these circumstances, In-Hp PTC may play a great role to increase organ donation and smooth procurement procedures.

The Department of Coordinators and the JOT coordinator committee play the main roles in educating these PTC. JOT has prepared guideline manuals of standard roles and procedures of PTC during organ procurement from BD and DCD donors. Although the JOT has prepared a textbook for In-Hp PTC and held several educational programs for In-Hp PTC, they have been educated mainly by prefectural PTC or their own hospital. Therefore, educational systems for In-Hp PTC should be modified to establish an effective tool once the current status and needs of In-Hp PTC are clarified. Therefore, the primary aim of this study was to describe the current status of In-Hp PTC in Japan based upon a national survey.

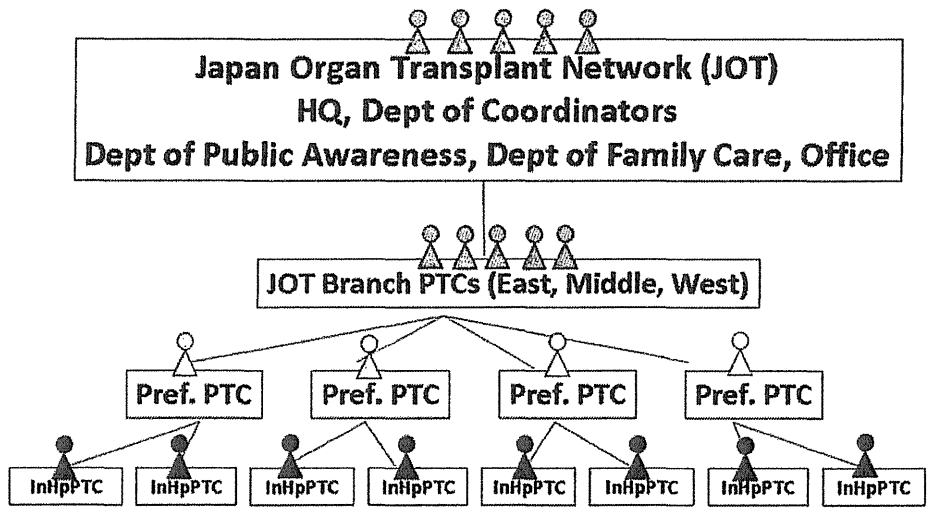

Fig 1. Organ transplant network in Japan. PTC, procurement transplant coordinator; In HpPTC, in-hospital PTC.

MATERIALS AND METHODS

We developed a 28-item self-completed questionnaire that queried: (1) occupation and status; (2) activities and issues (daily and at organ donation); (3) education and experiences of organ donation; (4) needs for learning about organ donation.

Survey letters were sent to 389 donor hospitals and 1889 In-Hp PTC. In 40 prefectures where the In-Hp PTC was delegated by the prefectural government, survey letters were directly or indirectly sent to the delegated In-Hp PTC. In 4 prefectures (Tokyo, Chiba, Saitama, and Osaka) where In-Hp PTC are not delegated, survey letters were sent to the BD donor hospitals. In all, 756 In-Hp PTC (40%) completed and returned the survey. The survey period was December 15, 2011, to March 31, 2012.

RESULTS

Among 1679 letters sent to In-Hp PTC in 40 prefectures where an In-Hp PTC was delegated by the prefectural government, there were 739 (44%) In-Hp PTC responses. But only 17 In-Hp PTC (8%) responded among 210 letters sent to In-Hp PTC in 4 prefectures that had not delegated the In-Hp PTC.

Hospital Where In-Hp PTC Were Working

Overall, 52% of respondents worked in donor hospitals accepted to undergo BD organ donation; 38% in hospitals accepted only to undergo donation after cardiac death (Fig 2a). Only 46% worked in donor hospitals where cadaveric organ donations had been performed in the past (Fig 2b). However, 63% worked in a donor hospital where committees were established for cadaveric organ donation (Fig 2c).

Occupation and Status of In-Hp PTC

The occupations of the respondents were nurse in (66%), physician (18%) or other (16%; Fig 3). Although only 2% of individuals was a full-time In-Hp PTC; 83% had been delegated by the prefectural government and 49% were supported from an advisory committees in their hospital (Fig 4).

Activities and Issued of In-Hp PTC

Their main roles was to prepare a manual for organ procurement (57%), to provide awareness of organ donation for patients and medical staff (56%), to arrange in-hospital seminars (44%), to consult about organ transplantation and donation (24%), to organize simulations of organ donation (29%), as well as to coordinate donation cases (Fig 5).

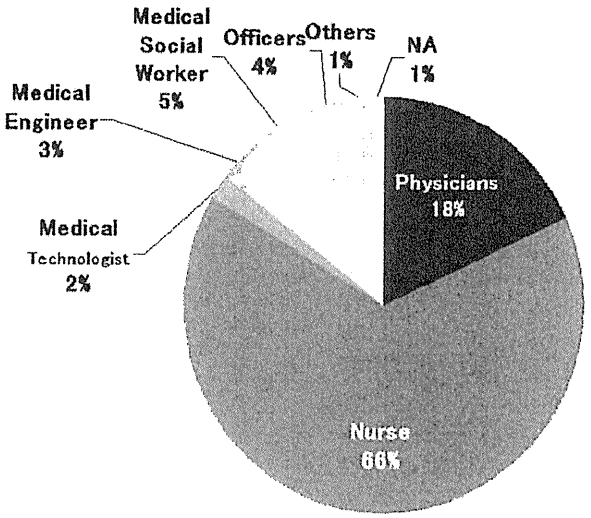

Fig 3. Occupation of in-hospital procurement transplant coordinator.

Among the 345 respondents who had experienced a cadaveric donor procedure, 77% had coordinated the in-hospital staff and arranged organ procurement; 59% communicated with prefectural and JOT PTC; 58% had cared for the donor family and 35% had obtained informed consent for donation accompanied by a prefectural or JOT PTC (Fig 6).

However, it was difficult for them to do these activities, namely, manage a rare, sudden donation case and to learn organ donation, because they had another post with regard to daily issues, they answered "hard to work as In-Hp PTC due to part time activity" (42%) "no daily time to work as In-Hp PTC" (31%), and "no support by hospital administration and other medical staffs" (29%; Fig 7). With regard to issues at organ donation, they answered no knowledge of their activities at organ donation (77%), "cannot preferentially

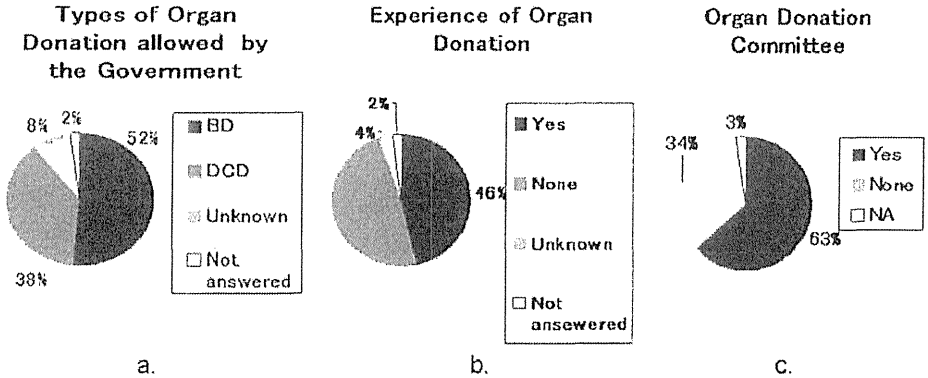

Fig 2. Hospital where in-hospital transplant coordinated works. BD, brain dead; DCD, donation after cardiac death; NA, not applicable.

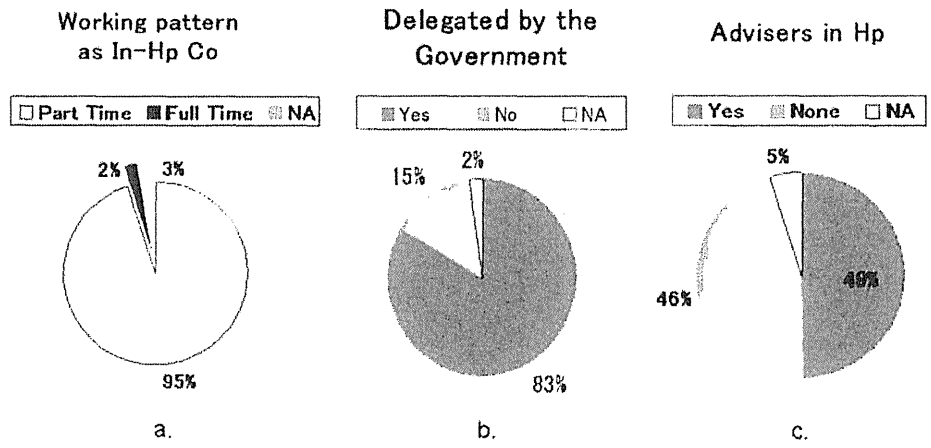

Fig 4. Status of in-hospital procurement transplant coordinator. In-Hp Co, in- hospital coordinator; Hp, hospital; NA, not available.

involve in donation" (66%), "annoying their partners because they cannot do their daily jobs" (65%), no knowledge of donor family care (49%), and no knowledge of organ donation process (36%; Fig 8).

Educational Experiences of Organ Donation

Although 77% have attended seminars about organ donation provided by JOT or the prefecture PTC (Fig 5), 93% still wanted to obtain more professional education. Nurses and medical social workers were more likely to desire more professional education than physicians or medical examiners.

The topics that they desired to learn were donor family care (72%), overall procedures of organ/tissue donation (65%), role of In-Hp PTC (67%), simulations of organ donation (65%), legislation and social system of organ donation (61%), medical indications for donation (61%), current status in Japan (57%), donor management (56%), and case studies (49%; Fig 9). There were significant variations in interested topics among the occupations.

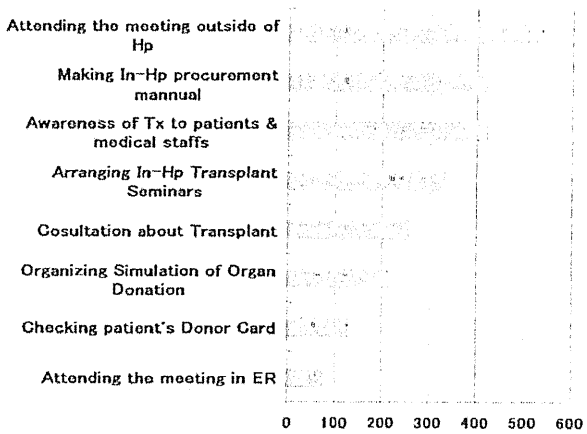

Fig 5. Daily activities as in-hospital procurement transplant coordinator; Hp, hospital; Tx, transplant; ER, emergency room.

DISCUSSION

Although the Transplantation Act was revised in 2010 and BD organ donation increased from 13 to 44 cases in a year, the number is still extremely smaller than that in other developed countries. In these circumstances, In-Hp PTC may play great roles to increase organ donation and smoothing procurement procedures.

Currently, JOT PTC and prefectural PTC conduct classroom lectures for In-Hp PTC and other medical staff in each donor hospital. The topics include organ transplantation/donation legislation in Japan, current status of DCD and BD donation in Japan, the roles of medical staffs and the donation processes: namely, initial actions, family consent, donor evaluation. Indeed, although 77% of respondents attended these seminars, 93% still wanted more professional education. Nurses and medical social workers were more likely than physicians and medical examiners to desire more professional education.

As most of In-Hp PTC had another post, they could find only a short period (1 or 2 days) to take professional education such as lectures. Even at organ donation, they could not be preferentially involved in organ donation

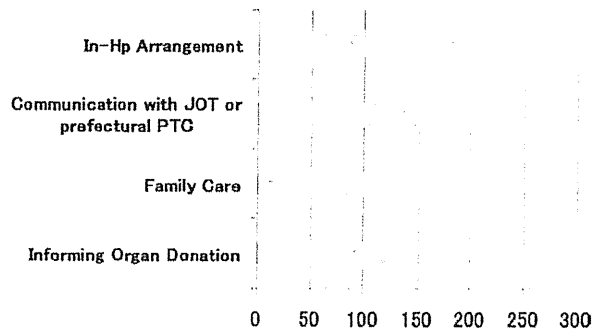

Fig 6. Activities at organ procurement. In-Hp, in-hospital; JOT, PTC, procurement transplant coordinator.

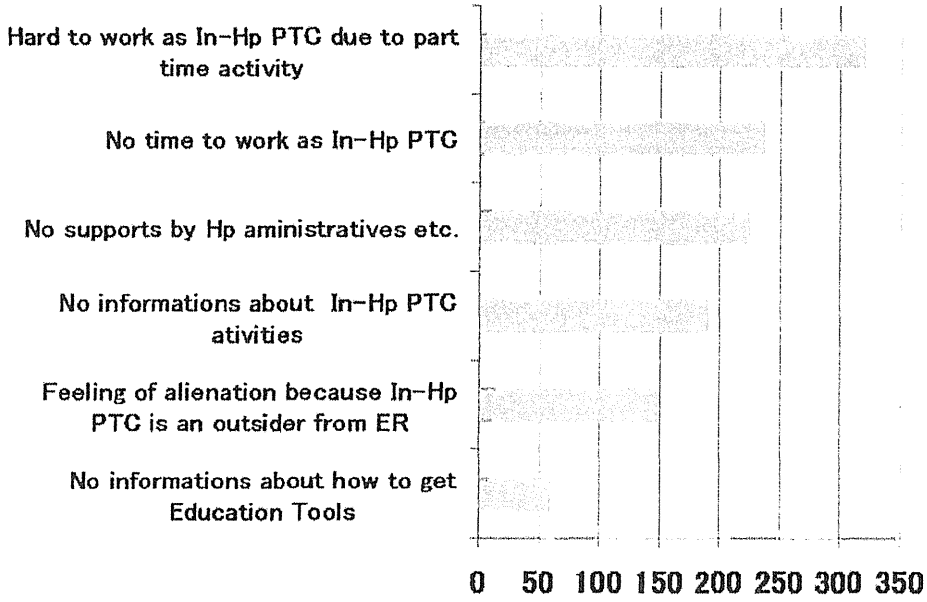

Fig 7. Issues of daily activities as In-Hp PTC. Hp, hospital; PTC, procurement transplant coordinator; ER, emergency room.

because they felt sorry for their partners if they did not do their daily jobs. Therefore, it was difficult for them to find time to obtain practical on-the-job training.

Of course, it may be effective to establish full time In-Hp PTC in every donor hospital. However, it is hard to establish such a post in every hospital, because the number of organ donations has been extremely small in Japan. Therefore, we need to establish special educational programs for In-Hp PTC.

The results of the present study led our department to hold a special educational seminar and establish a program for In-Hp PTC in Japan: namely, 2 lectures per day for 10 days from May to October 2012. Topics of this seminar were the history of transplantation, current status of organ transplantation and donation in Japan, legislation and network system of organ transplantation in Japan, outcomes of organ transplantation in Japan, nursing care of transplant recipients, pathophysiology and diagnosis of BD, the role of PTC, process of organ donation, the visit of JOT office, family care and informed consent for organ donation, survey of donor family, donor evaluation and management, role of In-Hp PTCs, case studies and simulations of organ donation, pediatric organ donation, determination of child abuse, informing organ donation, and care of the family of

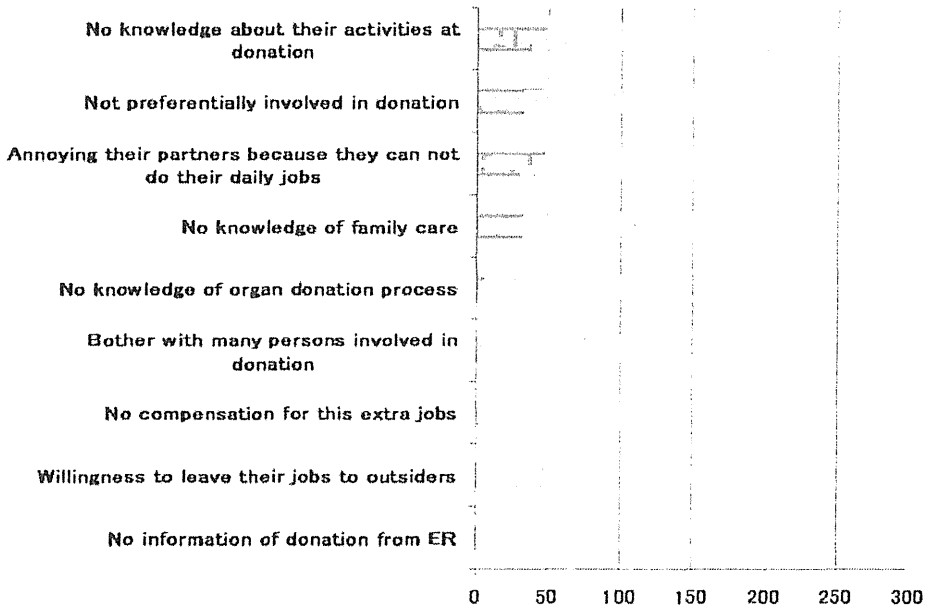

Fig 8. Issues at organ donation. ER, emergency room.

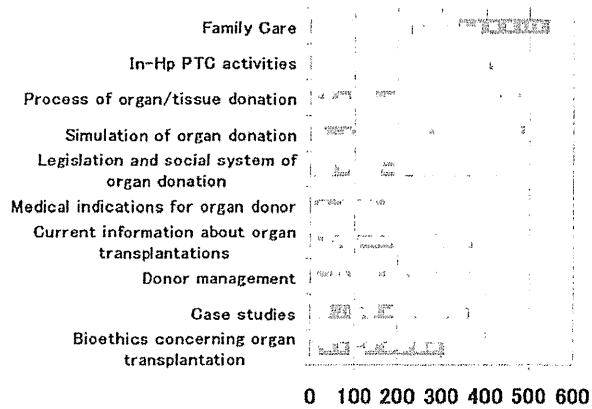

Fig 9. Top-10 subjects desired to learn. Hp, hospital; PTC, procurement transplant coordinator.

an end-life patient. Survey of the 16 participants showed all of them to be satisfied with the program, but they still wanted to have on-the-job training.

Although we may be able to establish an education curriculum for In-Hp PTC in the near future, national support is essential to provide these programs to all In-Hp PTC in Japan.

In conclusion, to establish the organ procurement system and increase donation, In-Hp PTCs have great roles in Japan. However, few of them have full-time positions and most of them require more professional education. Each In-Hp PTC has several problems, seeking to study the organ donation process and their role in BD the donations under the revised Transplant Act. We need to make a special national educational program for in-Hp PTCs.

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Prevention of GVHD and graft rejection by a new S1P receptor agonist, W-061, in rat small bowel transplantation

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ABSTRACT

Background: In small bowel transplantation (SBTx), inhibition of both graft-versus-host disease (GVHD) and allograft rejection is necessary.
Methods: We investigated the potency of a new sphingosine-1-phosphate receptor agonist, W-061, for these two immune responses in SBTx. W-061 has a completely different molecular structure from FTY720. Heterotopic SBTx was performed from Wistar-Furth (WF) into (WF×ACI) F1 rats as a GVHD model or F1 to WF rats as a rejection model. Recipients were orally given 3 mg/kg/day W-061 for 14 days after SBTx. Recipient survival, body weight, histopathology, lymphocyte subpopulations, and the cytokine profile were evaluated.
Results: W-061 treatment significantly prolonged graft survival over 100 days in four out of six recipients in the GVHD group and over 60 days in three out of six recipients in the rejection group. W-061 strongly inhibited GVHD and rejection as seen histopathologically in comparison with untreated control rats. W-061 caused a significant reduction in donor-derived T cells in target organs and infiltrating T cells in allografts by promoting these cells to home into the secondary lymphoid tissues and sequestrating those cells there. W-061 significantly decreased production of interferon-γ in target organs and allografts.
Conclusion: Therefore, these data suggest that W-061 has considerable potential as a new therapeutic immunosuppressant in patients with SBTx.

1. Introduction

Small bowel transplantation (SBTx) is a developing therapeutic option for patients with irreversible intestinal failure. The allogeneic immune response is more difficult to control in SBTx than in other solid organ transplantations [1]. It is well known that SBTx can induce two types of immunological reactions in graft recipients: host-versus-graft disease (HVGD) and graft-versus-host disease (GVHD) [2]. Once a severe immune response occurs, the graft will be unable to fully recover despite any kind of immunosuppressive therapy. According to the intestinal transplant registry report of 2009, the overall short-term results of SBTx have improved in recent years, but long-term graft survivals are suboptimal. One-, 3-, and 5-year graft survival rates were 71%, 55%, and 45%, respectively [3]. Therefore, further improvements in immunosuppressive therapy are mandatory.

A novel immunosuppressive drug, FTY720 (FTY), is a metabolite of the ascomycete, *Isaria sinclairii*, and has been shown to be potent as single or combined therapy, not only in organ transplantation models using rodents, canines, and primates, but also in autoimmune disease models [4–7]. FTY has been reported to accelerate the homing of naive lymphocytes from peripheral blood (PB) and spleen into secondary lymphoid tissues, such as mesenteric lymph nodes (MLN), Peyer's patches (PP), and peripheral lymph nodes [8,9]. Recent studies have revealed that cell motility is mediated by the interaction between sphingosine, a structural analog of FTY, and sphingosine 1-phosphate receptor 1 ($S1P_1$) [10]. It is likely that FTY acts as an agonist for $S1P_1$. Some reports have shown that FTY is effectively phosphorylated *in vivo* and works through S1P receptor signaling pathways to modulate chemotactic responses [10,11]. Stimulation of these receptors is the most likely mechanism by which this drug leads to migration and sequestration of lymphocytes into secondary lymphoid tissues, thus preventing movement into inflammatory lesions.

It has been reported that FTY treatment induces significant reductions in infiltrating donor-derived T cells into target organs and recipient T cells into the graft in intestinal GVHD and allograft rejection models, respectively [12,13]. Recently, the new S1P agonist W-061,

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which has a completely different molecular structure than FTY, has been developed as a prototype of ONO-4641, with which a multicenter double-blind, placebo-controlled study is now ongoing in patients with relapsing-remitting multiple sclerosis [14].

2. Objective

In the present study, heterotopic SBTx was performed from Wistar-Furth (WF) into (WF×ACI) F1 rats as a GVHD model or F1 to WF rats as a rejection model. Recipients were orally given 3 mg/kg/day W-061 for 14 days after SBTx. Recipient survival, body weight, histopathology, lymphocyte subpopulations, and the cytokine profile were evaluated. Our present study was performed to investigate the potency of W-061 in treating allografts in SBTx.

3. Materials and methods

3.1. Cell culture

CHO-K1 cells stably expressing human $S1P_1$ ($hS1P_1$), human $S1P_2$ ($hS1P_2$), human $S1P_3$ ($hS1P_3$), human $S1P_4$ ($hS1P_4$), or human $S1P_5$ ($hS1P_5$) were cultured in Ham's F12 medium supplemented with 10% bovine serum (Sigma-Aldrich, St. Louis, MO) and 0.25 mg/mL G418 sulfate (Invitrogen, Carlsbad, CA) in 5% CO_2/95% air at 37 °C.

3.2. Membrane binding assay

Membranes were prepared from CHO-K1 cells stably expressing each of the human S1P receptors based on the methods of Mandala et al. [10]. Briefly, cells were washed in PBS, suspended in 10 mM Tris–HCl (pH 7.5), 5 mM EDTA, and 1× Complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), and disrupted on ice using a polytron homogenizer. Following centrifugation at 80,000×*g* for 40 min at 4 °C, the pellet was resuspended in 10 mM Tris–HCl (pH 7.5), 10% glycerol, and 1× Complete protease inhibitor cocktail and stored at −80 °C. [^{33}P]-S1P for $S1P_1$, $S1P_2$, $S1P_3$, and $S1P_5$, and [^{3}H]-S1P for $S1P_4$ (American Radiolabeled Chemicals, St. Louis, MO) were used as radio-labeled ligands. S1P, phosphorylated FTY720 (FTY-P), and W-061 in assay buffer were added to a 96-well plate. Radioligands and membranes were added to a final volume of 200 μl. Binding was performed for 60 min at room temperature and terminated by collecting the membranes onto unifilter GF/B plates (Perkin Elmer, Boston, MA) with a UniFilter96 Harvester (Perkin Elmer). After drying the filter plates for 30 min, filter-bound radionuclide was measured on a TopCount NXT microplate scintillation counter (Perkin Elmer). Specific binding was calculated by subtracting nonspecific radioactivity that remained in the presence of 1000-fold excess of unlabeled S1P.

3.3. Intracellular calcium measurement

CHO-K1 cells stably expressing $hS1P_1$ or $hS1P_3$ were plated at 2×10^4 cells/well in a 96-well plate and incubated for 2 days at 37 °C in 5% CO_2/95% air. The cells were loaded with 5 μM Fura2-AM and 20 mM HEPES (pH 7.4) in Ham's F12 medium at 37 °C for 1 h. After loading, the cells were washed with Hanks' solution containing 20 mM HEPES (pH 7.4) and stimulated with S1P, FTY-P, and W-061. Fluorescence intensity was measured by the ratio of emission fluorescence at 500 nm to excitation at 340 and 380 nm using a Fluorescence Drug Screening System (FDSS-6000, Hamamatsu Photonics K.K., Shizuoka, Japan).

3.4. Animals

Male Wistar-Furth (WF) rats ($RT1^u$), ACI rats ($RT1^a$), and male (WF×ACI) F_1 ($RT1^{a/u}$) hybrid rats weighing 200–300 g were obtained from Japan SLC (Shizuoka, Japan) and the Animal Center of Osaka University (Osaka, Japan). Rats were housed in a specific pathogen-free animal facility and provided with *ad libitum* access to standard rat chow and tap water. The experimental protocol was approved by the Ethical Review Committee for Animal Experiments at Osaka University (Osaka, Japan).

3.5. Operative technique

Heterotopic SBTx in rats was performed by interposing the graft using the cuff technique [15,16]. Donor WF or F1 rats were not fed for 12 h before surgery. All surgical procedures were performed in a sterile field under Sevoflurane anesthesia. Briefly, we harvested an approximately 70-cm segment of distal intestine with the attached vascular pedicles, consisting of the portal vein and superior mesenteric artery with aortic cuff. Intraluminal irrigation of the graft intestine was performed with 10 ml cold (4 °C) normal saline solution. End-to-side anastomosis was performed with 9-0 nylon between the donor superior mesenteric artery and recipient aorta, followed by cuffed anastomosis of the donor portal vein to the recipient left renal vein after removal of the left kidney. A Thiry-Vella loop was placed in the left abdominal flank. Animals' surviving <4 days were considered technical failures and were excluded from data analysis.

3.6. Experimental design

To separately evaluate the effects of W-061 on GVHD and allograft rejection in SBTx, a parent and F1 semi-allogeneic combination was utilized. The experimental SBTx protocol was comprised of four groups: Group A, untreated F1 rats with WF grafts as a control GVHD model (n=6); Group B, F1 recipients treated with 3 mg/kg W-061 (n=6); Group C, untreated WF rats with F1 grafts as a control allograft rejection model (n=6); Group D, WF recipients treated with 3 mg/kg W-061 (n=6).

W-061 was donated by Ono Pharmaceutical Co., Ltd., (Osaka, Japan) in a dry powder form. It was dissolved in sterile 0.5% methylcellulose (Wako Pure Chemicals, Osaka, Japan) solution and administered to recipients at 3 mg/kg by oral gavage. Recipients were weighed every day after transplantation and measured once every week from day 28 post-transplantation. GVHD was diagnosed when there was evidence of weight loss, reddening of the skin, hair loss, and hunched posture. The day of recipient death was regarded as the end point of GVHD. Acute rejection was manifested by progressive ischemia of the stoma, an abdominal mass, a poor general appearance, and anorexia leading to death. For the following experiments, recipient rats were sacrificed on day 14 post-transplantation. The number and percentage of each subtype of lymphocytes in host target tissues and allografts were examined in three independent experiments. Recipient survival, pathology, and cytokine production in target organs were also assessed.

3.7. Preparation of lymphocytes from target tissues and allografts

Lymphocytes were isolated from PB, liver, native MLN, PP, and lamina propria (LP) of GVHD models (Groups A & B) and from allograft MLN, PP, and LP of rejection models (Groups C & D) on day 14 post-transplantation. PB was spun on a density separation medium (Ficoll-Paque Plus; Pharmacia Biotech AB, Uppsala, Sweden). Single-cell suspensions of MLN were prepared using a standard mechanical disruption procedure. All MLN were taken along the ileocecal artery, and all PP were removed from whole small intestine. PP and LP lymphocytes were prepared using an enzymatic dissociation method using collagenase [17,18]. Briefly, after the removal of fat and mesentery, intestinal tissues were flushed with 20 ml phosphate-buffered saline (PBS) and cut into small pieces. Fragments were stirred in RPMI 1640 medium (Sigma, Ayrshire, UK) supplemented with 10% fetal calf serum (FCS) (Gibco, Tokyo, Japan) and 90 U/ml collagenase

Table 1
Binding affinity of S1P, FTY720-P, and W-061 on S1P receptors.

	$hS1P_1$	$hS1P_2$	$hS1P_3$	$hS1P_4$	$hS1P_5$
S1P	0.131	0.439	0.0782	7.60	0.372
FTY720-P	0.160	4090	3.74	2.16	1.09
W-061	4.11	>43,800	1710	65.4	10.1

Data are shown as Ki values. FTY720-P: FTY720 phosphate.

(Type C-2139; Sigma) for 90 min. The remaining cell suspension was centrifuged, and the pellet was resuspended in 40% Percoll and spun in 75% Percoll at 600×g for 20 min at room temperature. Intestinal LP lymphocytes were harvested from the interface and washed twice in RPMI 1640 medium.

Liver lymphocytes were prepared as described [19]. Briefly, liver was minced, pressed through a mesh, and suspended in RPMI-1640 medium. To remove hepatic parenchymal cells, the cells were resuspended in 35% Percoll containing 100 U/ml heparin and centrifuged at 800×g for 20 min at room temperature. The pellet was resuspended in red blood cell lysing solution and washed three times in RPMI-1640 medium.

3.8. Flow cytometry analysis

Cell suspensions were prepared in PBS containing 1% FCS and 0.05% sodium azide. Each cell suspension (5×10^5 cells in 100 µl) was incubated with fluorescein isothio-cyanate (FITC) and/or phycoerythin (PE)-conjugated mAbs for 30 min. Stained cells were washed twice, resuspended, and analyzed using a FACScan (Becton Dickinson, Mountain View, CA, USA). FITC-conjugated anti-rat $RT1A^{ab}$ (C3) and PE-conjugated T-cell receptor (TCR) γδ (V65) were purchased from Pharmingen (San Diego, CA). PE-conjugated anti-rat CD4 (W3/25), CD8 (OX-8), and TCRαβ (R73) were obtained from Serotec (Oxford, UK). Channel numbers for analysis were chosen based on the staining pattern of normal splenocytes. Results were analyzed using Cell quest software (Becton Dickinson). Staining of normal F_1 and ACI splenocytes with anti-major histocompatibility complex (MHC) ($RT1^{ab}$) reagents resulted in a unimodal positive profile when compared with negative WF controls. When donor (WF)-derived cells were detected in GVHD rats, the cells were seen as subpopulations that were clearly negative (equivalent to nonspecific findings) for F_1-specific MHC ($RT1^a$).

3.9. Cytokine production in culture supernatants

Host lymphocytes from the liver, MLN, PP, and LP of F1 rats and allograft lymphocytes from the MLN, PP, and LP of WF rats were cultured for 48 h in 24-well plates that had been coated with carbonate buffer (pH 9.6) containing 10 µg/ml mouse anti-rat CD3 mAb (clone 1F4; Serocet, Oxford, UK). Supernatants were harvested and frozen at −20 °C. Supernatants from culture plates were assayed for interleukin (IL)-2, IL-4, IL-10 and interferon (IFN)-γ with a solid-phase sandwich ELISA kit (Biosource International, Camarillo, CA). Optical densities were measured on a microplate reader (Model-680; Bio-Rad, Hercules, CA) at 450 nm. Data were analyzed using Microplate Manager Version 5.2 software (Bio-Rad) by preparing standard curves and then automatically calculating the concentrations of samples.

3.10. Histology

Tissues harvested from rats were fixed in 4% buffered formalin. Fixed tissues were embedded in paraffin, sectioned at 5 µm thickness, and stained with hematoxylin and eosin.

3.11. Statistical analysis

Results are expressed as the means ± SD. Mean values were compared between groups using a two-tailed Student's *t*-test. Survival data were plotted using Kaplan–Meier methods and analyzed using the long-rank test. Values of $p<0.05$ were considered significant.

Table 2
Host survival and clinical GVHD or rejection in a unidirectional, semi-allogeneic rat model of small bowel transplantation.

Group	Treatment [a]	n	Survival (days)	Mean ± S.D.(days)	p [b]	GVHD	Rejection
A: WF-F1	No	6	13,16,16,17,17,18	16 ± 1.7		6/6	
B: WF-F1	W-061(3 mg/kg)	6	39[c],62[c], >100×4	83 ± 21.5	<0.001 versus A	1/6[d]	
C: F1-WF	No	6	14,19,21,22,24,26	21 ± 4.1			6/6
D: F1-WF	W-061(3 mg/kg)	6	39[e],43[e],46[e], >60×3	51 ± 9.7	<0.001 versus D		3/6

[a] W-061 was orally administered for 14 days from the day of transplantation.
[b] Generalized savage (Mantel-Cox) value comparing survival times to that of untreated control.
[c] Represents day of animal death due to small bowel ileus.
[d] Transient GVHD was seen around day 44 in 1 of 6 recipients.
[e] Transient rejection was seen around day 40 in 3 of 6 recipients.

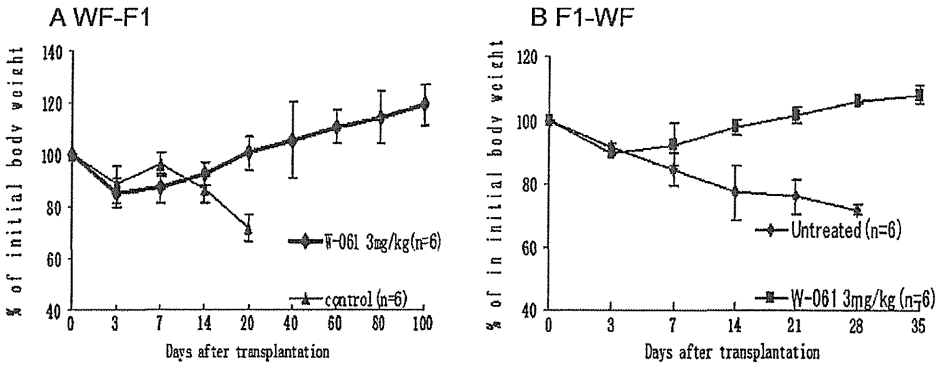

Fig. 1. Postoperative body weight changes following small bowel transplantation in rats. Semi-allogeneic parent-to-F1 model of GVHD (A) and F1-to-parent model of rejection (B). W-061 (3 mg/kg)-treated recipients show progressive weight gain while the untreated group shows weight loss and death. Data are the means ± SD.

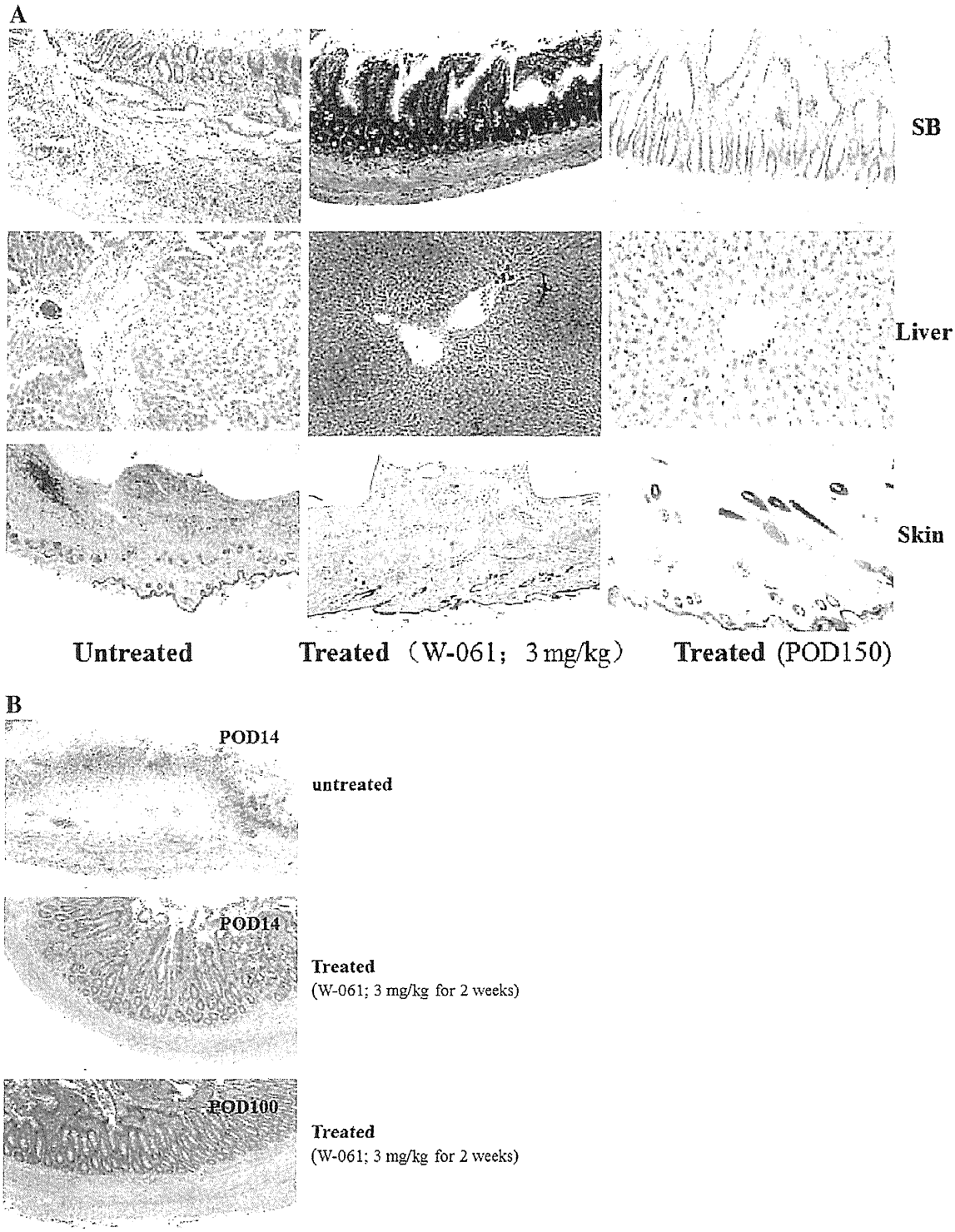

Fig. 2. W-061 treatment of rats with GVHD and rejection inhibited histopathology in target organs and allografts. (A) On day 14, small bowel, hepatic, and skin samples from SBTx recipients were obtained and analyzed microscopically in the left and middle column. Untreated GVHD rats showed severe intestinal histopathology, including mucosal erosion, villous blunting, and cellular infiltration in the lamina propria, as well as prominent lymphocyte infiltration in the periportal area of the liver. Skin histopathology showed prominent cellular infiltration and atrophy of skin appendages. W-061 treatment inhibited histopathological changes in the small bowel, liver, and skin (original magnification; ×100, small bowel and liver; ×40, skin). On day 150, all target organs showed almost normal architecture by W-061 treatment (original magnification; ×100, small bowel, liver and skin). (B) Acute rejection of an intestinal allograft showing marked lymphocyte infiltration, goblet cell loss, and destruction of intestinal architecture. Intestinal allograft of rats treated with W-061 showed no signs of acute and chronic rejection and intact mucosal architecture (original magnification ×100).

4. Results

4.1. Affinity and agonistic activities of S1P

To compare S1P and FTY-P, the binding affinity of S1P receptors and the agonistic activity of W-061 were evaluated by examining Ca^{2+} mobilization in CHO-K1 cells stably expressing human S1P receptors. W-061 bound to all types of human S1P receptors except for $hS1P_2$ (Table 1). The binding affinity of S1P receptors was lower than that of FTY-P. Also, W-061 had a lower agonistic activity on $S1P_1$ than FTY-P, but W-061 had higher specific agonistic activity on $S1P_1$ compared to $S1P_3$ (Supplement Fig. 1).

4.2. Effect of W-061 on recipient survival after SBTx

All untreated F1 recipients died of GVHD with a mean survival time (MST) of 16±1.7 days. GVHD clinical features were observed beginning at day 10 post-transplantation (Group A; Table 2). In contrast, W-061-treated F1 recipients displayed significantly higher survival rates of up to 83±21.5 days (Group B, $p<0.01$) when a 3 mg/kg dose of W-061 was administered for 2 weeks. In the long-term survivors, no chronic GVHD was observed. Furthermore, all untreated WF recipients died of rejection with a MST of 21±4.1 days (Group C). In contrast, W-061-treated WF recipients showed significantly higher survival rates of up to 51±9.7 days (Group D, $p<0.01$).

The recipient rats with GVHD and rejection gradually lost weight, whereas W-061-treated recipients displayed progressive weight gain (Fig. 1).

4.3. Histopathological findings of recipient target organs treated with W-061

On day 14 after SBTx, target organs in Group A demonstrated typical characteristics of GVHD, such as mucosal erosion, villous blunting, and cellular infiltration in the LP of the intestine, as well as prominent lymphocyte infiltration in the periportal area of the liver. Skin histopathology also showed prominent cellular infiltration and atrophy of skin appendages. In contrast, the W-061-treated Group B demonstrated substantial inhibition of histopathological changes in the small bowel, liver, and skin, resulting in almost intact tissue architecture (Fig. 2A). Further, in a long-term survivor of Group B on day 150 posttransplantation, the target organs demonstrated almost normal histological findings (Fig. 2A).

The allografts in Group C demonstrated typical signs of rejection, such as villous sloughing, extensive mononuclear infiltration, crypt cell necrosis, and capillary

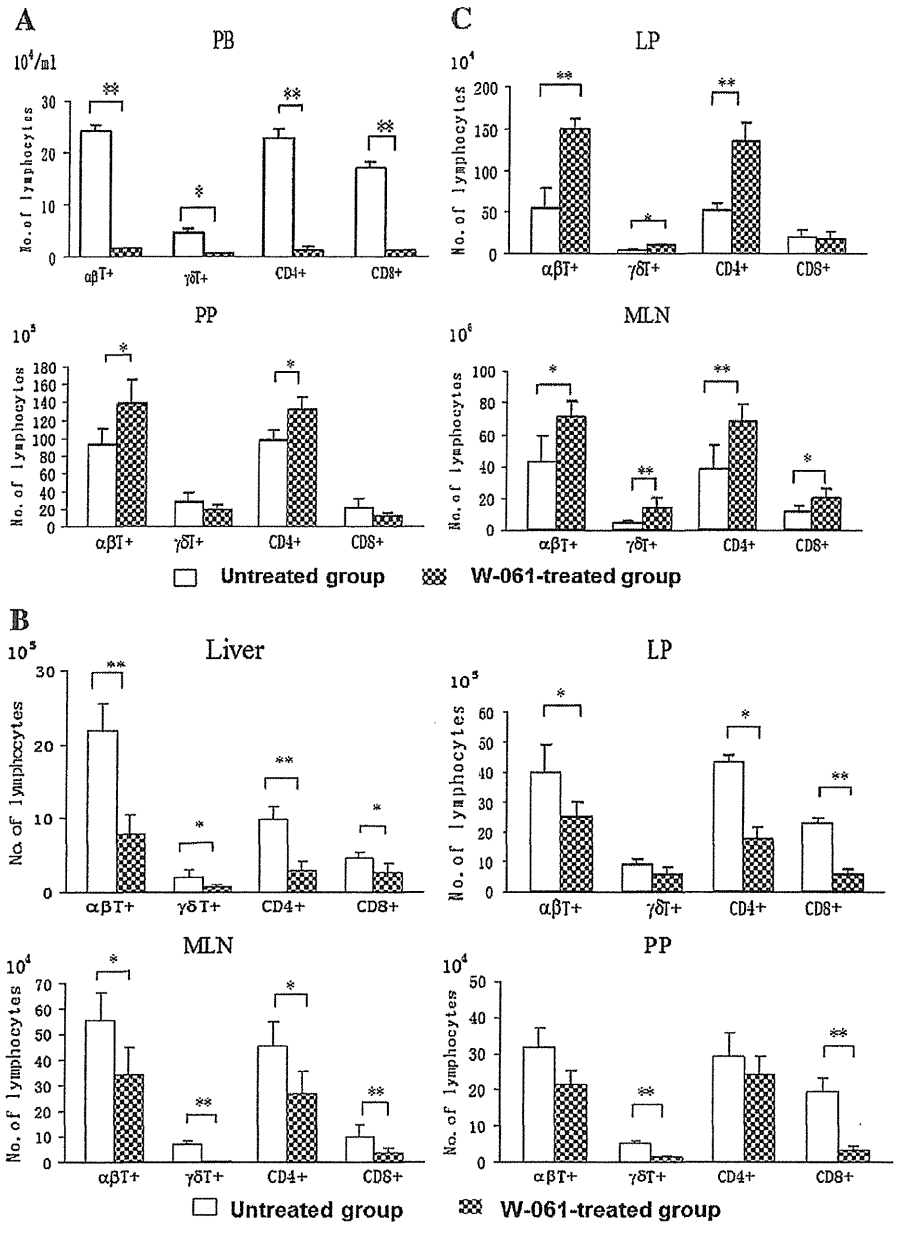

Fig. 3. Effect of W-061 treatment on the numbers of donor-derived T cells in the GVHD model. Lymphocytes in PB (A), target organs of the host (B), and graft (C) were prepared from acute GVHD and W-061-treated rats. Donor-derived T cells were determined by multiplying the total number of cells by the fraction of $RT1A^{ab}$-negative and TCRαβ-, TCRγδ-, CD4-, and CD8-positive cells. Five rats were analyzed from each group. Data are the means±SD. *$p<0.05$; **$p<0.01$. αβT, TCRαβ T cell; γδT, TCR γδ T cell.

thrombosis. In contrast, none of the allografts in the W-061-treated Group D showed signs of acute and chronic rejection, and the intestinal architecture remained intact (Fig. 2B).

4.4. Effect of W-061 on the numbers of donor-derived T-cell subpopulations in target organs and allografts in the GVHD condition

In PB, the numbers of donor-derived TCRαβ$^+$ T cells, CD4$^+$ T cells, and CD8$^+$ T cells were significantly increased during GVHD (Fig. 3A). In the host target organs, donor-derived TCRαβ$^+$ T cells, CD4$^+$ T cells, and CD8$^+$ T cells in the liver, MLN, and LP, in addition to CD8$^+$ T cells in PP, were increased during GVHD, but were significantly reduced by W-061 treatment (Fig. 3B). Although the total numbers of TCRγδ$^+$ T cells were relatively low during GVHD, the number was also significantly decreased in the liver, LP, and PP in the W-061-treated group (Fig. 3B).

In the graft, however, the numbers of donor-derived TCRαβ$^+$ T cells and CD4$^+$ T cells were significantly increased in the MLN, PP, and LP of the graft in W-061-treated rats. Significantly higher numbers of donor-derived CD8$^+$ T cells were also observed in the MLN of the graft compared to untreated controls, but no significant differences in the PP of the graft were identified. Obviously higher numbers of donor-derived TCRγδ$^+$ T cells were seen in the MLN and LP with W-061 treatment (Fig. 3C).

4.5. Effect of W-061 on the numbers of infiltrating T-cell subpopulations in allografts ongoing rejection

In PB, the numbers of TCRαβ$^+$ T cells, TCRγδ$^+$ T cells, CD4$^+$ T cells, and CD8$^+$ T cells were significantly increased during rejection (Fig. 4-A). With W-061 treatment, however, the numbers of TCRαβ$^+$ T cells, TCRγδ$^+$ T cells, CD4$^+$ T cells, and CD8$^+$ T cells were remarkably reduced in the LP of allografts and in PB. In contrast, the numbers of TCRαβ$^+$ T cells, CD4$^+$ T cells, and CD8$^+$ T cells were significantly increased in the MLN and PP of allografts, in addition to TCRγδ$^+$ T cells in PP during rejection (Fig. 4).

4.6. Cytokine production in target organs of GVHD and allografts undergoing rejection in recipients treated with W-061

IFN-γ levels were significantly higher in untreated recipients compared with naive F1 or WF rats (Fig. 5A, B). After administration of W-061, IFN-γ production in all target organs and allografts was significantly diminished. IL-2 production in all target organs of GVHD recipients was significantly decreased after administration of W-061, but that in allografts was not significantly decreased except for MLN (Fig. 5A, B). In PP and the liver of GVHD recipients, the production of IL-4 was significantly reduced by W-061 treatment (Fig. 5A). Also, in MLN, PP, and liver of GVHD recipients, the production of IL-10 was significantly reduced by W-061 treatment (Fig. 5A). However, no significant differences in the production of Il-4 and IL-10 were observed between W-061-treated and untreated rats when examining allografts (Fig. 5B).

5. Discussion

With the recent advances in immunosuppressive therapy, the outcome of SBTx has improved, but still remains unsatisfactory. To further improve the results following transplantation, overcoming major immunological obstacles in SBTx is required. The small intestine itself is more immunogenic as compared to other organs, such as the liver, kidney, and heart. The small intestine seems to be susceptible to allograft rejection, and establishing transplantation tolerance is difficult. Further, GVHD will sometimes occur because intestinal grafts include a lot of lymphoid tissues. The prognosis is very poor once GVHD develops [20]. Clinically, both immunological responses GVHD and allograft rejection coexist in small intestinal grafts. Therefore, we may not be able to discriminate one response from the other in the graft. However, we can separately analyze each response using a unidirectional and semi-allogeneic rat combination.

Unlike conventional immunosuppressants, S1P receptor agonists have two advantages in organ transplantation: anti-tumor effects [21,22] and protective modality of opportunistic infections [23]. Thus, S1P receptor agonists are expected to be ideal immunosuppressants in SBTx in which stronger immunosuppression is necessary than other organ transplantations.

Recently, the S1P receptor agonists FTY and KRP-203 have been shown to inhibit acute rejection and GVHD in some animal experimental models [12,24,25]. Our previous study also demonstrated the effectiveness of FTY in GVHD of the SBTx model using a unidirectional and semi-allogeneic rat combination [13]. FTY inhibits lethality and histopathological changes in target organs when administered at 0.5 mg/kg. FTY effectively reduces recirculation of activated donor-derived T cells and their recruitment to target organs in GVHD.

The step-by-step molecular basis for the action of FTY has recently been elucidated. Similar to S1P, FTY is phosphorylated *in vivo* by sphingosine kinase to become FTY-phosphate (FTY-P), which mediates its effects through five G protein-coupled receptors (S1P receptors; $S1P_{1-5}$) on the surface of some cells [10]. Specific roles of S1P receptor subtypes have been reported. $S1P_1$ is highly expressed in T and B cells, whereas $S1P_3$ is expressed exclusively on the atrium [26]. In the case of antigen presentation in the LN, activated T cells transiently downregulate $S1P_1$ by aberrant internalization, rendering these T cells unresponsive to the obligatory egress signal by S1P, resulting in proliferation in the LN.

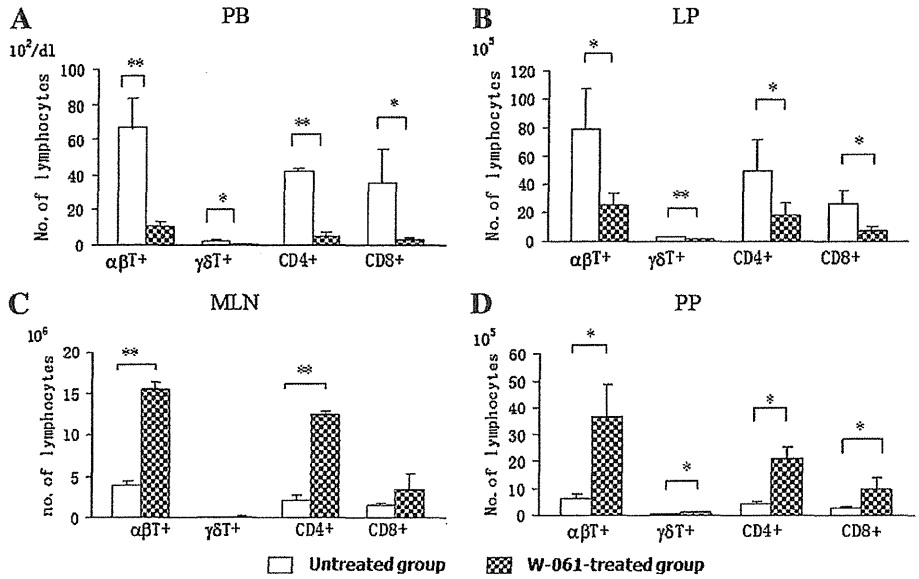

Fig. 4. Effect of W-061 treatment on the numbers of infiltrating lymphocytes in the rejection model. Lymphocytes in PB (A), LP (B), MLN (C), and PP (D) in the allograft were prepared from acute rejection and W-061-treated rats. Infiltrating T cells were determined by multiplying the total number of cells by the fraction of TCRαβ-, TCRγδ-, CD4-, and CD8-positive cells. Five rats were analyzed from each group. Data are the means ± SD. *$p<0.05$; **$p<0.01$. αβT, TCRαβ T cell; γδT, TCR γδ T cell.

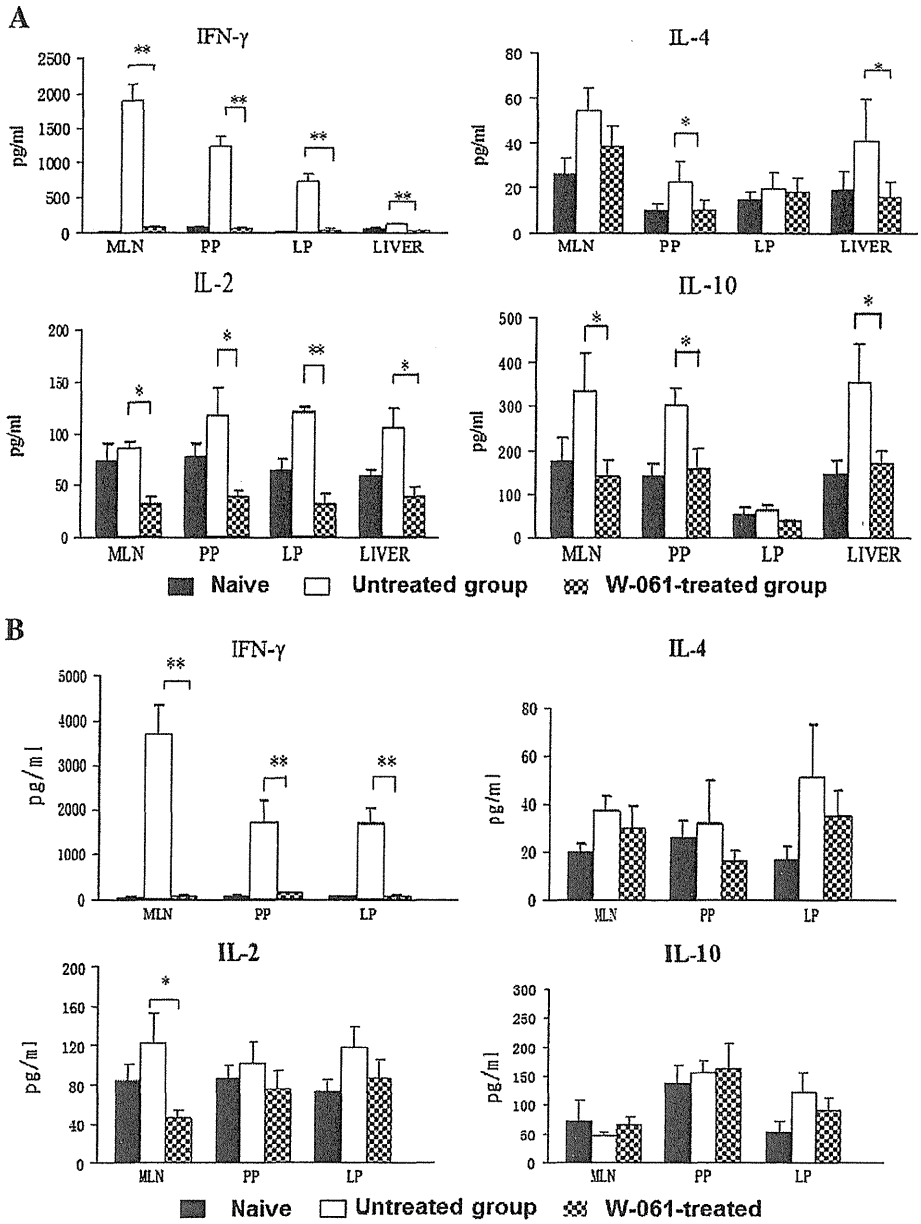

Fig. 5. Cytokine profile of lymphocytes in target organs and allograft after daily administration of 3 mg/kg W-061 for 14 days. In the treated group, IFN-γ production in target tissues of the GVHD model (A) and the rejection model (B) was significantly diminished. However, no significant differences were noted in production of IL-4 and IL-10 by the target tissue lymphocytes. Data are the means ± SD. Four rats were analyzed from each group. *$p<0.05$; **$p<0.01$.

At the end of proliferation, activated T cells upregulate $S1P_1$ and egress from LNs in a $S1P/S1P_1$-dependent step [27]. FTY-P, which is phosphorylated *in vivo*, acts as a $S1P_1$ agonist on naive and activated T cells, thereby inducing aberrant internalization of $S1P_1$. This phenomenon renders all T cells unresponsive to obligatory egress signal by S1P. As a result, both naive and activated T cells are "sequestrated" in LNs and are unable to recirculate and be recruited to peripheral tissues such as inflammatory sites.

The new S1P receptor agonist, W-061, has a molecular structure that is completely different from FTY or KRP-203. Unlike FTY, W-061 is active *in vivo* without being phosphorylated. From *in vitro* results using S1P receptors, W-061 showed a relatively lower affinity for $S1P_1$ than FTY, but had more agonistic activity on $S1P_1$ as compared with that on $S1P_3$. Therefore, W-061 will be expected not to induce transient bradycardia, which is caused by interaction of FTY with $S1P_3$ on the atrium. In our present study, we investigated the effect of W-061 both on GVHD and allograft rejection in the rat SBTx model using unidirectional, semi-allogeneic combinations. In the parent-to-F1 combination, W-061 remarkably inhibited GVHD and completely abrogated recipient mortality with 3 mg/kg W-061 alone. In the F1-to-parent rejection model, W-061 significantly prolonged recipient survival, although three out of six recipients died of rejection with the same dose.

On day 14 post-transplantation, up to 2.5×10^5–2.0×10^6 donor-derived T cells were surprisingly detected in PB and target organs during GVHD, which may be explained as follows. Lymphatic vessels in small bowel grafts are completely detached by the transplantation procedure. It has been reported that 4–8 weeks are required for full lymphatic regeneration after SBTx [28]. However, the cut edge is probably open to the abdominal cavity until lymphatic regeneration is fully established. Thus, donor lymphocytes from lymphatic vessels are absorbed through the peritoneum and recirculate into the blood stream. In our present study, few donor-derived T cells were detected

in PB, and they were significantly decreased in target organs in animals treated with W-061. Instead, donor T cells were sequestrated selectively into the LP as well as the MLN and PP of the graft and were unable to recirculate and be recruited to target organs with W-061 treatment. These features were not observed in the secondary lymphoid tissues of the recipient. As compared with the graft, the number of infiltrating donor-derived T cells in the MLN, PP, and LP of target organs was approximately 0.5%, 1.4%, and 17%, respectively, when calculated from the results from Fig. 4B and C. In contrast, the numbers of infiltrating TCR$\alpha\beta^+$ T cells, TCR$\gamma\delta^+$ T cells, $CD4^+$ T cells, and $CD8^+$ T cells were remarkably reduced in the LP of allografts. These activated anti-donor T cells were sequestrated into the secondary lymphoid tissues, such as the MLN and PP of allografts and were unable to recirculate and be recruited to LP. The results were very similar with FTY-treated recipients after SBTx [13].

According to results from human and murine GVHD, donor $CD4^+$ T cells initiate a Th1-type immune response against host class II MHC antigens. Dominant secretion of Th1-type cytokines, IFN-γ in particular, occurs in murine models during GVHD [29,30]. Administration of W-061 prevented trafficking of not only donor $CD4^+$ T cells but also $CD8^+$ T cells *in vivo*, resulting in a significant reduction in IFN-γ and IL-2 without affecting levels of the Th2-type cytokine IL-4 and IL-10. In contrast, IFN-γ production is detected early during rejection in the absence of clinical symptoms, and its levels progressively increase with time [31]. IFN-γ may also be an important immune mediator of graft rejection in SBTx [32]. The most prominent findings in our present study were that treatment with W-061 significantly downregulated the production of IFN-γ and IL-2 but not IL-4 or IL-10, compared with untreated recipients.

In conclusion, our present results support the feasibility of including W-061 into the immunosuppressive regimen to control the immune reaction and to prolong survival of recipients. The mechanism is similar to that of FTY regarding accelerated sequestration of circulating activated T cells into secondary lymphoid tissues, resulting in the reduction of activated donor T-cell migration into target organs and LP in allografts. As a result, W-061 inhibits Th1 immune responses with a significant decrease in IFN-γ production during acute GVHD and rejection.

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