Table 1. Recipient Characteristics and Islet Infusion Characteristics

	Patient No.					
	1	2	3	4	5	6
Age (years), male/female	36F	39M	39F	47F	58M	36F
Body weight (kg)	60	71	58	51	72	51
BMI	20.3	21.9	21.2	20.2	22.0	20.1
Autoantibodies*	+	++	++	−	−	+++
Daily insulin (U/kg)	0.67	0.70	0.67	0.88	0.49	0.58
Islet transplantation						
IEQ of first Tx	354,384	406,082	621,551	465,598	479,656	262,319
IEQ of second Tx	474,234	380,726	498,928	357,259	869,826	—
IEQ of third Tx	—	393,588	—	447,931	297,720	—
Total IEQ	828,618	1,180,396	1,120,479	1,270,788	1,647,202	261,329
IEQ/kg	13,810	16,626	19,319	24,917	23,832	5,103
Daily insulin (U/kg)	0	0.28	0.38	0	0	0.39

−: negative, +: single positive, ++: double positive, +++: triple positive.
*Autoantibodies: anti-GAD antibody, anti-insulin antibody, anti-IA-2 antibody.

no autoantibody (no or single autoantibody: 15.2 ± 2.4, $n = 6$; double or triple autoantibodies: 8.4 ± 0.8, $n = 5$) (Table 4). The increase of the SUIT index in patients with 5 or 6 (full) mismatched HLA was significantly lower than in patients with 2–4 mismatched HLA (mismatch 2–5: 15.4 ± 2.3, $n = 6$; mismatch 5 or 6: 8.2 ± 0.7, $n = 5$) (Tables 4 and 5). Multivariate analysis including the variables of culture, autoantibody, and HLA mismatch identified only culture as significantly contributing to the variations in the SUIT index ($p = 0.03$).

Patients #1, #4, and #5 became insulin independent and the mean of their final SUIT was 33.8 ± 3.0. The other patients had positive C-peptide with reduced insulin 1 month after each transplantation but did not achieve insulin independence and their mean SUIT was 8.4 ± 2.3, significantly lower than the independent group ($p < 0.01$). Data from patient #4 showed the lowest SUIT value among insulin-independent patients, 28 of SUIT index, thus suggesting that 28% β-cell mass is required to become insulin independent.

EIR Index

The EIR index was used to evaluate the rate of islet engraftment. EIR shows the percentage of engrafted islets per transplanted islets, assuming that a normal subject has 1 million IEQ. As shown in Table 3, the percentage of engrafted islets for one transplantation is about 30%. The EIR index in patients who received cultured islets was significantly lower than in patients who received fresh islets (fresh: 28.9 ± 4.3, $n = 11$; cultured: 9.7 ± 6.3, $n = 3$) (Table 4). Because IEQ decreased after overnight culture by about 30% and the EIR index in patients who received cultured islets was significantly lower, cultured islets were less effective than fresh islets in islet transplantation from NHBDs. With the transplantation of fresh islets, the EIR index in patients with dou-

Table 2. Increase of SUIT Index in Each Islet Transplantation

	Patient No.					
	1	2	3	4	5	6
SUIT index before Tx	0	0	0	0	0	0
ΔSUIT after first Tx	16.4	8.2*	1.3*†	2.2†	18.4	8.9
ΔSUIT after second Tx	22.1	9.0*	5.6	8.0†	7.9	—
ΔSUIT after third Tx	—	10.1	—	18.0	8.4	—
SUIT index after final Tx	38.5	10.1	5.6	28.2	34.7	8.9

ΔSUIT: increase of SUIT index, Tx: Transplantation.
*SUIT index decreased and become 0 before additional transplantation.
†Cultured islets.

Table 3. EIR Index in Each Islet Transplantation

	Patient No.					
	1	2	3	4	5	6
First transplant	46.3	20.2	2.1*	4.7*	38.3	34.0
Second transplant	46.6	23.6	11.2	22.4*	9.1	—
Third transplant	—	25.6	—	40.2	28.2	—

*Cultured islets.

ble or triple autoantibodies was lower, but not significantly, than in patients with a single or no autoantibody (no or single autoantibody: 34.8 ± 5.9, $n = 6$; double autoantibodies: 22.9 ± 3.7, $n = 5$) (Table 4). The EIR in patients with 5 or 6 (full) mismatched HLA was significantly lower than in patients with 2–4 mismatched HLA (mismatched 2–5: 38.9 ± 2.9, $n = 6$; mismatched 5 or 6: 18.0 ± 3.3, $n = 5$ (Tables 4 and 5). Multivariate analysis including the variables of culture, autoantibody, and HLA mismatch identified only culture as barely significantly contributing to the variation of the EIR index ($p = 0.05$). There was no significance in other factors such as WIT, CIT, donor age, amylase level (Table 4), AST/ALT level, BUN/Cr level, or BMI (data not shown).

In summary, a statistically significant contribution of SUIT and EIR indexes in multivariate analysis was found in culture. However, the number of subjects evaluated was not sufficient to conclude that fresh islets are better than cultured islets.

DISCUSSION

Allogenic islet transplantation was effectively performed from NHBDs using the Kyoto Islet Isolation Method for the treatment of type 1 diabetes. Fasting blood glucose and C-peptide levels was examined in seven posttransplantation stages of four patients and found a linear relationship between F-BG and F-CPR in each stage (15,42). The Miami group also showed that the C-peptide to glucose ratio was constant from 1 week to 30 days and the ratio after the second islet transplantation doubled compared to the first islet transplantation (5). The SUIT index is correlated with the acute insulin response to glucagon after islet transplantation (42). The Edmonton group showed that the functional capacity of transplanted islets in insulin-independent patients was estimated to correspond to only about 20–40% of that in nondiabetic subjects (34,35), which corresponded with the SUIT index of our insulin-independent patients (SUIT index 28.2–38.5). Also, in general, diabetes after

Table 4. Evaluation of Islet Transplantation From NHBDs Using SUIT and EIR Indexes

	Increase of SUIT Index	p-Value	EIR Index	p-Value
Culture				
(–) ($n = 11$)	12.1 ± 1.7		28.9 ± 4.3	
(+) ($n = 3$)	3.8 ± 2.1	$p = 0.03$	9.7 ± 6.3	$p = 0.03$
Fresh islet transplantation				
HLA				
Mismatch <5 ($n = 6$)	15.4 ± 2.3		38.9 ± 2.9	
Mismatch ≥5 ($n = 5$)	8.2 ± 0.7	$p = 0.02$	18.0 ± 3.3	$p = 0.001$
Autoantibody				
(–) or (+) ($n = 6$)	15.2 ± 2.4		34.8 ± 5.9	
(++) or (+++) ($n = 5$)	8.4 ± 0.8	$p = 0.03$	22.9 ± 3.7	$p = 0.14$
WIT				
≥5 min ($n = 5$)	12.2 ± 2.2		29.5 ± 6.3	
<5 min ($n = 6$)	12.0 ± 2.7	$p = 0.94$	29.3 ± 5.4	$p = 0.98$
CIT				
≥240 min ($n = 6$)	9.5 ± 1.5		24.4 ± 5.8	
<240 min ($n = 5$)	15.2 ± 2.7	$p = 0.09$	35.4 ± 4.2	$p = 0.17$
Donor age				
≥50 years ($n = 7$)	12.2 ± 2.2		27.8 ± 6.0	
≤50 years ($n = 4$)	11.9 ± 3.4	$p = 0.93$	31.7 ± 7.8	$p = 0.70$
Amylase				
≥150 ($n = 5$)	11.1 ± 2.8		25.5 ± 6.1	
<150 ($n = 6$)	12.9 ± 2.2	$p = 0.62$	32.6 ± 5.1	$p = 0.40$

Table 5. HLA Typings of the Patients and Islet Donors

	HLA			
	A	B	DR	Mismatch
Patient #1	**2, 11**	**34, 54**	**4, -**	
Donor 1	1, 24	37, **54**	**4**, 10	4
Donor 2	**2**, -	51, 61	**4**, 9	3
Patient #2	**2, 24**	**7, 54**	**1, 4**	
Donor 1	11, 26	61, 67	9, 16	6
Donor 2	26, 31	51, 61	8, 9	6
Donor 3	33, -	44, -	13, -	6
Patient #3	**2, 33**	**44, 61**	**9, 13**	
Donor 1*	26, -	54, 62	8, **9**	5
Donor 1'*	**2**, 24	48, 49	8, **9**	4
Donor 2	**2**, 26	35, 51	1, 15	5
Patient #4	**2, 33**	**44, 54**	**4, 13**	
Donor 1*	**24, -**	**52, 54**	14, 15	5
Donor 2*	**2**, 24	52, -	5, -	5
Donor 3	11, **33**	**44, 54**	9, **13**	2
Patient #5	**11, 33**	**44, 54**	**4, 11**	
Donor 1	2, 31	48, 55	**4, 11**	4
Donor 2	24, 26	51, 61	**4**, 9	5
Donor 3	24, **33**	**44**, 60	**11**, 13	3
Patient #6	**26, -**	**61, -**	**9, -**	
Donor 1	**26**, 31	51, **61**	4, 9	3

*Cultured islets.

a pancreatectomy usually occurs when the remnant pancreas is less than 30%, which corresponds with the lowest SUIT value among insulin-independent patients, 28, which might indicate that 28% β-cell mass is required to become insulin independent. Therefore, the SUIT index could predict the efficacy of islet transplantation.

The EIR shows the ratio of ΔSUIT/transplanted islet mass × 1,000,000, which might correlate with the rate of engrafted islets from transplanted islets. It was shown that a normal person has about 0.5–1 million IEQ (9,37). Assuming that a normal subject has 1 million IEQ, a SUIT index of 100 also means 1 million IEQ for that person and SUIT × 10,000 shows the number of engrafted IEQ. Therefore, the EIR also shows the percentage of engrafted islets per transplanted islets, assuming that a normal subject has 1 million IEQ. These data show that the percentage of engrafted islets from one transplantation is about 30%. In experimental islet transplantation, it is generally accepted that 50–70% of transplanted islets will be lost in the immediate posttransplantation period (3,4), although these experiments were based on multi-islet grafts implanted beneath the kidney capsule of immunocompromised recipients. These data also show that only a small fraction of transplanted islets are successfully engrafted.

It is known that the results of human islet isolation depend on the quality of the donor pancreata (10,16), so it is not easy to isolate suitable human islets successfully from NHBDs. This study suggests that it should be possible to cure type 1 diabetes by means of islet transplantation using the current islet isolation method with NHBDs. However, patients #2 and #3, who received over 15,000 IEQ/kg patient body weight of islets, did not become insulin independent and patients #4 and #5 needed over 20,000 IEQ/kg of islets to become insulin independent, although patients who received over 10,000 IEQ/kg of islets from brain-dead donors usually became insulin independent (39). This may be due to the quality of islets. Indeed, the SUIT index in a patient who received living-donor islet transplantation was 38.5 and the EIR was over 90, while the average SUIT and EIR in islet transplantation from NHBDs were about 10 and less than 30, respectively. Therefore, the islet quality strongly affects the engrafted islet rate.

SUIT indexes in patients #2 and #3 from 3 months after the first transplantation to the next transplantation dramatically decreased and the indexes became 0 (C-peptide <0.1 ng/ml) before the next transplantation, although their EIR within 3 months after the first transplantation was not significantly different, in comparison to the EIR of patients #1, #4, and #5. This may be due to autoimmunity and/or alloimmunity, and glucotoxicity. The interval between transplantations in patients #2 and #3 was relatively long (20); therefore, these patients may have suffered autoimmunity and/or alloimmunity and glucotoxicity over a long period. The SUIT index could also be used to evaluate long-term graft survival.

Recently, it has been shown that fresh islets are more effective than cultured islets at reversing hyperglycemia in a mouse model (8,31). Moreover, the Edmonton group showed that the clinical outcome of cultured islet transplantation is worse than that of fresh (noncultured) islet transplantation (presented at IPITA meeting, 2005). The present data show that increases in the SUIT and EIR indexes in patients who received fresh islets are significantly higher than in patients who received cultured islets, although the number of cultured islet transplants performed was low. Although some groups have shown the effectiveness of using cultured islets (6,7), the effect seems due to the pretreatment of patients and improvement of islet purity (and therefore decreased tissue volume) rather than improved islet quality. In addition, some groups have transplanted cultured islets from brain-dead donors but the present study transplanted islets from NHBDs, which received greater stress than those from brain-dead donors. Based on these data, islets without culture have been used for transplantation. However, the number of subjects evaluated was insufficient to

conclude that fresh islets are better than cultured islets and this study yielded no data concerning cultured islets from brain-dead donors.

The SUIT index resembles that in the computer-solved model of pancreatic β-cell function, HOMA-β, which is 20 × insulin (mU/L)/(F-BG − 3.5) (17). However, HOMA-β cannot be used to assess βcell function in those taking exogenous insulin (41), due to the inability of insulin assays to differentiate endogenous insulin from exogenous insulin. The SUIT index is independent of the amount of exogenous insulin. Therefore, it is possible to determine the β-cell function after the injection of long-acting insulin.

In conclusion, this method makes it feasible to use tissue harvested from NHBDs efficiently for islet transplant into type 1 diabetic patients. The SUIT and EIR indexes are useful to evaluate single islet transplantation because two or three islet transplantations are required to achieve insulin independence.

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Analysis of Donor- and Isolation-Related Variables From Non-Heart-Beating Donors (NHBDs) Using the Kyoto Islet Isolation Method

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Recently, we demonstrated that islet transplantation from non-heart-beating donors (NHBDs) using the Kyoto islet isolation method (KIIM) successfully reversed patients' diabetes state. In this study, we evaluated the effects of donor- and isolation-related variables on islet isolation results from NHBDs by KIIM. Twenty-one islet preparations from the pancreata of NHBDs were isolated by KIIM. Islet preparations that met transplantation criteria and achieved improved patient diabetes control after transplantation were defined as successful isolations. Potential risk factors deemed to affect islet isolation results, such as age, gender, body mass index, hospital stay, donors' blood biochemical tests, a modified pancreata procurement method, and isolation and purification procedure-related variables, were analyzed. Seventeen out of 21 islet isolations (81%) were successful isolations. Postpurification islet yield was 447,639 ± 39,902 islet equivalents (IE) in the successful isolation group and 108,007 ± 31,532 IE in the failure group. Donor age was significantly younger in the success group (41.9 ± 4.0 years old in the success group vs. 57.5 ± 2.2 years old in the failure group, $p = 0.003$). Chronic pancreatitis significantly decreased islet yields ($p = 0.006$). Phase I time was significantly shorter ($p = 0.010$) and undigested tissue volume was significantly smaller ($p = 0.020$) in the success group. Purity was in positive correlation to postpurification islet yield, while donor age was in reverse correlation to postpurification islet yield. KIIM enables us to perform islet transplantation from NHBDs; however, the decision to use pancreata from older donors or those with chronic pancreatitis requires careful consideration.

Key words: Non-heart-beating donor; Kyoto islet isolation method; Pancreatic islet transplantation; Type 1 diabetes

INTRODUCTION

After the Edmonton group showed that transplantation of human islets of Langerhans could be a novel treatment for patients with type 1 diabetes mellitus (22), the problem of donor shortage became prominent in islet transplantation. A non-heart-beating donor (NHBD) pool might be a beneficial supplement to alleviate donor shortage in the islet transplantation field (5); however, few islet teams prefer manipulating such a pancreas because of its notorious difficulty in islet isolation and purification (9). In Japan, pancreatic islet transplantation is categorized as tissue transplantation and heart-beating brain-dead donors are not allowed. This leaves the potential donor pool to come from only non-heart-beating donors (NHBDs) (10,11,14) and living donors (12,15). Previously, we initiated the Kyoto Islet Isolation Method (KIIM), which was modified for NHBDs (11,19) and manipulated 21 islet preparations from the pancreata of NHBDs. It was encouraging that 17 out of 21 (81%) islet preparations from NHBDs met the transplantation criteria, and were transplanted into eight patients. All patients achieved improved blood glucose levels after transplantation with no hypoglycemia episodes, reduced

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insulin requirement, and three instances of insulin independence (11).

We were curious to identify the risk factors from NHBDs using the KIIM in order to gain a higher success rate. In this study, we review our experience of islet isolation and purification with KIIM from NHBDs, and analyze possible donor- and procedure-related variables affecting islet yield.

MATERIALS AND METHODS

Donor Background

Twenty-one pancreata from NHBDs were procured through the Japan Organ Transplantation Network between April 7, 2004 and January 21, 2006. The study was approved by the Ethics Committee of the Kyoto University Graduate School and Faculty of Medicine. The average donor age was 44.9 ± 3.5 years old, body weight was 59.4 ± 2.7 kg, body mass index (BMI, weight in kg/height in m^2) was 21.9 ± 0.7 kg/m^2, and pancreas weight was 87.9 ± 5.8 g. Average warm ischemic time (WIT, time from donor death to chilling solution perfusion) was 7.9 ± 2.2 min and cold ischemic time (CIT, time from chilling solution perfusion to initiation of isolation) was 253.1 ± 16.9 min.

Pancreata Procurement, Islet Isolation, and Purification

All 21 islet preparations were manipulated according to GMP (Good Manufacturing Practice) at the Center for Cell and Molecular Therapy of Kyoto University Hospital. Islet isolation was performed according to KIIM, which is based on the Ricordi method with some modifications (11). The key procedures are described as follows. We procure pancreata in conjunction with kidneys taken by the kidney procurement team. This is done by inserting double-balloon catheters into the iliac vessels before cardiac arrest and after confirmation of brain death and informed consent. We start regional organ chilling after cardiac arrest using an in situ regional organ cooling (ISRC) system (17). The pancreas is protected during kidney procurement and is removed after the kidney. We remove the duodenum and spleen from the pancreas at the procurement site. A cannula is immediately inserted into the procured pancreas through the main pancreatic duct from the direction of the pancreatic head and M-Kyoto solution is administered intraductally (11,18). M-Kyoto solution is 100,000 U/L of Ulinastatin (Mochida Pharmaceutical Co Ltd, Tokyo, Japan) in ET-Kyoto solution (Kyoto Biomedical Science, Kyoto, Japan). For pancreas preservation during transportation, we use an oxygenated perfluorocarbon/M-Kyoto solution-based two-layer method (11,18) instead of UW solution. We check the density of the isolation aggregates with a test tube density adjustment method as follows. Six density test tubes were prepared with 5 ml of purification solution of different densities (1.085, 1.090, 1.095, 1.100, 1.105, and 1.110 g/cm^3) and covered by 1 ml cap solution (dithizone, Sigma Chemical Co., St. Louis, MO) (0.5 mg/ml, in Hanks' balanced salt solution). The different densities were achieved by adjusting the ratio of iodixanol (OptiPrep, Axis-Shield PoC AS, Norway) to M-Kyoto solution. Samples were taken from the isolation aggregates and added to the discontinuous density test tubes. The tubes were spun at 1000 rpm for 5 min and the densities of aggregates were determined by whether they pelleted or floated in the different density solutions. Islets were purified with a continuous density gradient of iodixanol/M-Kyoto solution (Ulinastatin 50,000 U/L in ET-Kyoto solution) in an apheresis system (COBE 2991 cell processor, Gambro Laboratories, Denver, CO) (11). The heavy density solution was chosen according to the test tube density adjustment method described above and the gradient was achieved by varying the ratio of iodixanol to M-Kyoto solution.

Islet Evaluation

Islet evaluation was independently judged by two investigators. Islet yield was determined with dithizone staining (2 mg/ml, Sigma Chemical Co.) under optical graticule and converted into a standard number of islet equivalents (IE, diameter standardizing to 150 μm) (13,21). Purity was assessed by comparing the relative quantity of dithizone-stained tissue to unstained exocrine tissue. Mantled islets were defined as more than 50% of the islet surface being covered by exocrine tissue (13). Islet viability was evaluated using acridine orange (10 μmol/L) and propidium iodide (15 μmol/L) (AO/PI) staining to visualize living and dead cells simultaneously (13,21). Islet function was evaluated by the insulin secretory response of the purified islets during a glucose stimulation test (13,21).

Grouping

Islet preparations that met transplantation criteria (11,22) and improved patient diabetes control after transplantation were included in the success group, and those that did not, in the failure group. Features of improved patients' diabetes include stable blood glucose control without hypoglycemic unawareness, improved hemoglobin A_{1C} (HbA_{1C}) levels with positive C-peptide, and reduced exogenous insulin requirement.

Donor- and isolation-related factors were analyzed, including: age, gender, BMI, pancreatic status (healthy vs. chronic pancreatitis), donors' hospital stays, donors' blood biochemistry tests [peak transaminase levels, peak creatinine levels, peak amylase, peak blood urea nitrogen (BUN) levels, and peak blood glucose levels], car-

diac arrest or norepinephrine administration, ISRC system, perfusion time and volume, WIT, CIT, phase I time (time from placement in the Ricordi chamber to the start of collection), phase II time (time of collection), undigested tissue volume, and prepurification islet yield (IE, and IE/g).

Statistical Analysis

Differences between means were compared with the independent *t*-test. Differences between ratios were analyzed with Fisher's exact test. Logistic regression analysis was performed to determine factors affecting islet yield. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS 10.0, SPSS, Chicago, IL). Data were expressed as the average ± SEM and $p < 0.05$ was considered significant.

RESULTS

Overall Isolation Results

In all cases after islet transplantation, HbA_{1C} levels were improved (Fig. 1) and there was no hypoglycemic unawareness. Therefore, all preparations that met transplantation criteria were included in the success group. Thus, 17 out of 21 islet preparations achieved success (a success rate of 81%). Average islet yield (from both the success and failure groups) before purification was 508,622 ± 59,473 IE (6,010 ± 665 IE/g), and after purification was 382,945 ± 44,146 IE (4,589 ± 504 IE/g). Purity after purification was 46.8 ± 3.3%. Viability assessed by AO/PI after purification was 96.2 ± 0.7%, and all samples were above 85%. The average percentage of mantled islets was 30.9 ± 5.2%. The average insulin stimulation index was 4.2 ± 1.8.

Donor Characteristics

Donor characteristics were analyzed based on success and failure groups (Table 1). The average postpurification islet yield was 447,639 ± 39,902 IE in the success group and 108,007 ± 31,532 IE in the failure group. There were no significant differences between the two groups in gender, BMI, hospital stay, peak creatinine level, peak BUN level, peak blood glucose level, peak amylase level, peak transaminase level, or norepinephrine administration.

Significant differences were found in donors' ages and pancreata status between the two groups. The donor age in the success group was 41.9 ± 4.0 years old and in the failure group 57.5 ± 2.2 years old ($p = 0.003$). All of the 21 pancreata underwent histological examination with hematoxylin and eosin staining (H&E stain). Pancreata with diffuse fibrosis were defined as chronic pancreatitis ($n = 7$) and those without evidence of inflammation were defined as healthy pancreata ($n = 14$). In the success group, 3 out of 17 pancreata had chronic pancreatitis (CP); while in the failure group, all four cases had CP ($p = 0.006$) (Table 1).

Isolation Characteristics

Isolation characteristics were also analyzed based on success and failure groups (Table 2). The success group had a significantly shorter phase I time ($p = 0.010$) and significantly smaller undigested tissue volume ($p = 0.020$). Although there was no significant difference, the suc-

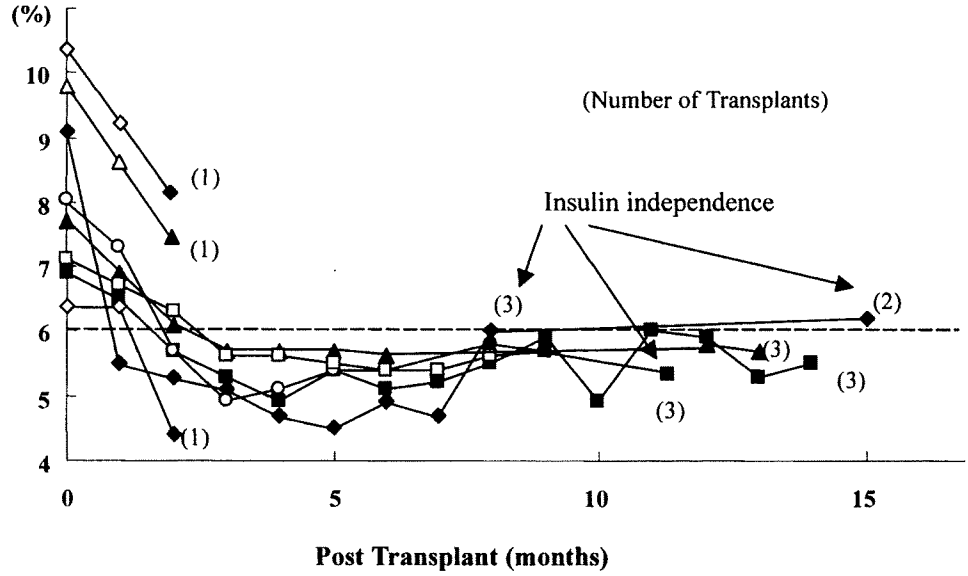

Figure 1. Eight patients' HbA_{1C} levels before and after transplantation of islets from NHBDs.

Table 1. Donor Characteristics

Variables	Success Group ($n = 17$)	Failure Group ($n = 4$)	p-Value
Sex (male/female)	12/5	3/1	0.684
Age (years)	41.9 ± 4.0	57.5 ± 2.2	0.003*
Body mass index (kg/m^2)	21.9 ± 0.8	21.5 ± 2.2	0.845
Pancreas weight (g)	84.1 ± 5.5	104.0 ± 19.5	0.183
Hospital stay (days)	10.2 ± 8.9	5.5 ± 1.6	0.323
Peak transaminase (U/L)	248.2 ± 105.7	56.5 ± 11.6	0.399
Peak creatinine (mg/dl)	3.2 ± 0.6	4.5 ± 2.0	0.372
Peak amylase (IU/L)	343.1 ± 118.9	54.0 ± 28.0	0.331
Peak blood urea nitrogen (mg/dl)	57.9 ± 12.8	52.3 ± 10.6	0.840
Norepinephrine administration or cardiac arrest (No./total)	13/17	2/4	0.081
In situ regional organ cooling (No./total)	16/17	2/3	0.284
Pancreata status (healthy/chronic pancreatitis)	14/3	0/4	0.006*

The two groups were statistically compared using the independent t-test and Fisher's exact test.
*$p < 0.05$ was considered significant.

cess group had about a twofold higher percentage of mantled islets. This was due to our modified purification method using the density adjustment method before purification, which enhanced the recovery of mantled islets in all cases. Obviously, the prepurification islet yield was the most striking factor for success.

Effect of Pancreata Status

Because CP adversely affected the isolation success rate (Table 1), we further analyzed the effect of pancreata status on islet isolation (Table 3).

The donor age was significantly older in the CP group ($p = 0.002$), suggesting that aging is correlated with CP onset. The phase I time was significantly longer ($p = 0.001$) and the undigested tissue volume was significantly larger ($p = 0.016$) in the CP group, which suggests that pancreatitis inhibits pancreas digestion and leads to low islet yield and low purity.

Relationship Between Postpurification Islet Yields and Other Factors

Purity was positively correlated with postpurification islet yield ($R = 0.619$, $p = 0.003$) (Fig. 2A). Donor age was inversely correlated with the postpurification yield ($R = -0.616$, $p = 0.003$) (Fig. 2B). The insulin stimulation index and islet viability showed no correlation with postpurification islet yield (IE/g) ($R = 0.014$, $p = 0.953$ and $R = 0.318$, $p = 0.161$, respectively) (Fig. 2C, D).

DISCUSSION

Previous studies reported several factors that had significant impact on islet isolation using brain-dead heart-

Table 2. Isolation Characteristics

Variables	Success Group ($n = 17$)	Failure Group ($n = 4$)	p-Value
Warm ischemic time (min)	6.4 ± 2.0	14.3 ± 7.6	0.158
Perfusion time (min)	67.1 ± 16.2	84.3 ± 26.7	0.587
Perfusion volume (L)	24.2 ± 6.1	27.3 ± 6.0	0.725
Cold ischemic time (min)	241.1 ± 16.0	304.25 ± 56.1	0.147
Phase I time (min)	19.2 ± 1.3	27.8 ± 2.2	0.010*
Phase II time (min)	53.7 ± 3.0	55.5 ± 8.5	0.810
Undigested tissue volume (g)	11.2 ± 1.1	18.3 ± 3.8	0.020*
Percentage of mantled islets (%)	33.9 ± 6.2	18.5 ± 3.8	0.258
Prepurification islet yield (IE)	590,364 ± 55,238	154,466 ± 51,807	0.002*
Prepurification islet yield (IE/g)	7,099 ± 540	1,382 ± 204	<0.001*

The two groups were statistically compared using the independent t-test.
*$p < 0.05$ was considered significant.

Table 3. Effect of Pancreas State on Islet Yield

Variable	Healthy Pancreata ($n = 14$)	Chronic Pancreatitis ($n = 7$)	p-Value
Age (years)	38.9 ± 4.4	56.7 ± 1.7	0.002*
Phase I time (min)	17.9 ± 1.3	26.7 ± 1.8	0.001*
Phase II time (min)	52.4 ± 3.4	57.3 ± 5.1	0.431
Undigested tissue volume (g)	10.6 ± 1.2	16.6 ± 2.3	0.016*
Prepurification islet yield (IE/g)	7,375 ± 597	3,282 ± 1,007	0.001*
Postpurification islet yield (IE/g)	5,470 ± 458	2,825 ± 922	0.009*
Purity (%)	53.6 ± 3.0	33.3 ± 4.7	0.001*

The two groups were statistically compared using the independent t-test.
*$p < 0.05$ was considered significant.

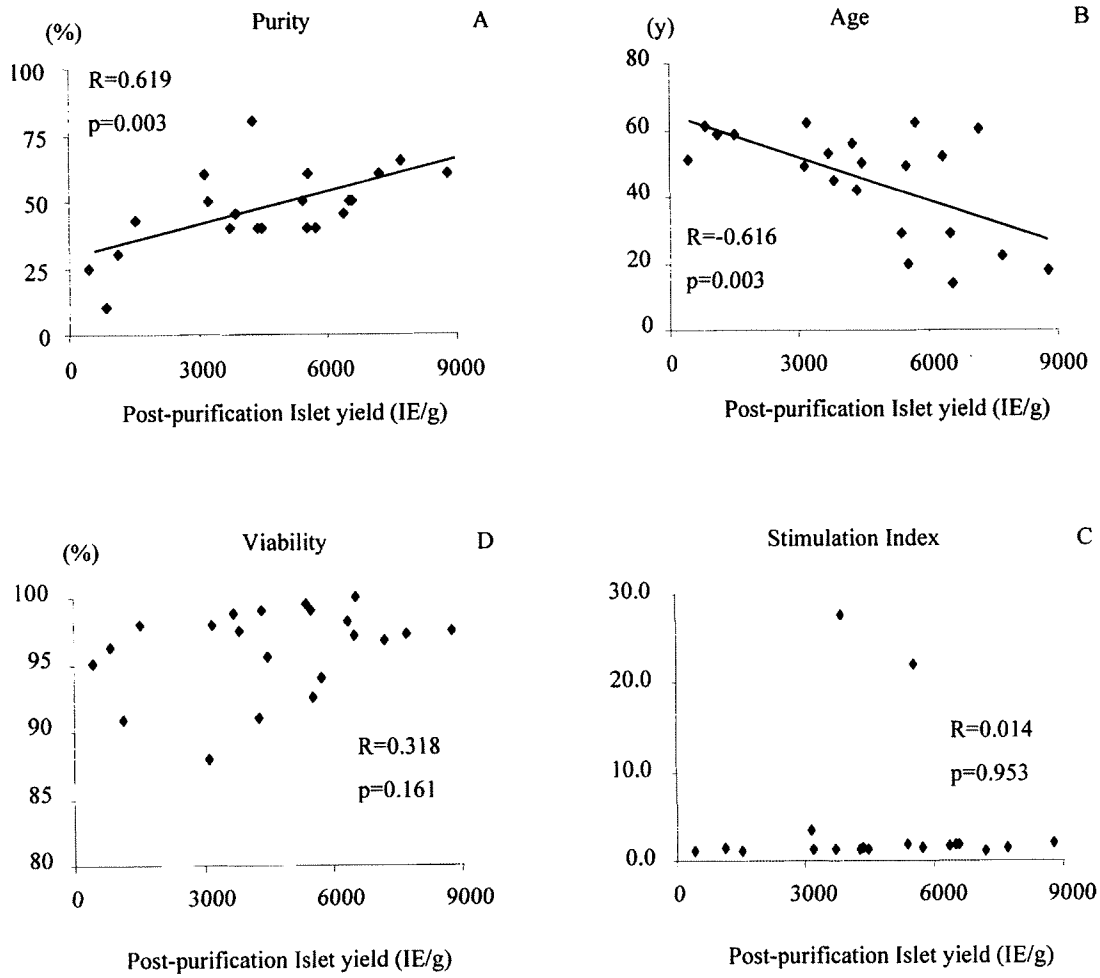

Figure 2. Relationships between postpurification islet yield (IE/g) and purity (A), age (B), insulin stimulation index (C), and viability (D). Postpurification islet yield (IE/g) was strongly correlated with purity (A). Postpurification islet yield (IE/g) was inversely correlated with donor age (B). Islet viability and insulin stimulation index showed no correlation with postpurification islet yield (IE/g) (C, D).

beating donors (2,4,8,16,25); however, this is the first time that factors relating to the use of NHBDs for actual clinical islet transplantation have been reported. Although our data set is small, the results are interesting and should be helpful for assessing pancreata from NHBDs before islet processing.

In terms of donor factors, although overweight older donors have been considered more suitable for islet isolation than younger donors (4,8), our study highlighted the benefit of using pancreata from young donors for islet preparations. In our study, the donor age in the success group was quite young. Additionally, donor age showed an inverse correlation with postpurification islet yield. The main obstacle to gaining high postpurification islet yields from young donors lies in the higher percentage of mantled islets, which are embedded in acinar tissue (20). Due to anatomical and physiological differences of the pancreata from young donors, it is difficult for islets to be separated from acinar tissue without overdigestion and islet fragmentation (8). To avoid overdigestion, mantled islets were accepted and collected after isolation. However, because they were surrounded by exocrine tissue, the density of mantled islets differed from that of isolated islets and resulted in difficulty in extraction from the exocrine tissues during purification. In the case of mantled islets from young donors, the standard islet isolation method with fixed purification density can achieve a large quantity of IE before purification but cannot recover mantled islets after purification.

Socci et al. (23) discovered that the ducts in young pancreata collapse, which hampers the enzyme solution from thoroughly infusing and uniformly distributing throughout the parenchyma, leading to mantled islets. In KIIM, we immediately insert a cannula into the main pancreatic duct on the duodenal terminus after procurement and ductally administer M-Kyoto solution (11,18). Two key features of this approach are: 1) Ulinastatin, a trypsin inhibitor in M-Kyoto solution, helps protect the main duct wall from trypsin injury, and 2) we fill the pancreatic duct system with M-Kyoto solution to maintain the three-dimensional duct system and prevent its collapse. Also, M-Kyoto solution shows a lower Liberase inhibition than UW solution (18), which contributes to sufficient digestion of the pancreas and decreases the amount of mantled islets. Efforts were taken to improve the infusion efficiency of the enzyme and thus increase the level of whole pancreas digestion (to lower the number of mantled islets). Even with this effort, we still collected some mantled islets after isolation. In order to recover this fraction of islets, we individualized the density of the high-density purification solution for each islet preparation before purification based on the results of the test tube density adjustment method. By using an optimal density gradient range, we successfully collected mantled islets. In fact, in our success group, more than 30% of all mantled islets might have been lost if we had not utilized this technique. Although exocrine tissue may hinder the ability of nutrition to diffuse to the islets (6), the detachment of islets from exocrine tissue triggered an apoptotic signal and resulted in islet death (24). Investigators have confirmed the advantages of islets from young donors, both in vitro in terms of insulin secretory function (7) and in vivo after transplanting to diabetic mice (1). We believe that if we can only recover mantled islets from young donors, the islet preparations should be of high quality both in function and in islet number.

The pancreatic status of donors, especially those with CP, could severely affect the isolation results. To analyze this, we further investigated the effect of the pancreata status on islet isolation and transplantation (Table 3). In our study population, CP is defined as diffuse fibrosis after H&E histological staining. Phase I time (digestion time) was significantly longer and undigested tissue volume was significantly larger in the CP group. These were due to the difficulty in digesting a pancreas with CP and led to failed islet isolation. In addition, purity was significantly lower in the CP group, suggesting that, even by sacrificing purity, it was still hard to obtain a high islet yield from pancreata with CP. Because donor age was significantly older in the CP group (Table 3) and older pancreata are more prone to CP, this is another reason to seek younger donors. Based on our data, older NHBDs with chronic pancreatitis are an apparent risk factor for islet isolation. A multicenter, large-scale, randomized trial will be necessary to verify this. Importantly, all cases within the healthy pancreata group (14/14) resulted in successful islet isolation and transplantation. This was especially important for confirming that KIIM was a reliable islet isolation method with normal pancreata. It allowed us to perform living donor islet transplantation successfully with KIIM (12). A reliable islet isolation method is of crucial importance when performing living donor islet transplantation.

Other donor-related factors such as BMI, pancreas weight, length of hospital stay, peak transaminase level, peak creatinine level, peak amylase level, peak BUN level, norepinephrine administration, or cardiac arrest showed no significant effects on islet preparation results. This might be due to our modified procedures in KIIM, which could overcome these obstacles. Another possible reason is the limited size of our study population. For instance, BMI is a decisive indicator of a subject's nutritional status and lean donors (with BMI <24 kg/m^2) had significantly lower islet yields (3,8). However, in our study population, most of the donors were of low BMI (range 14.8–27.2 kg/m^2). It is hard to tell based on our

data whether BMI affects success, but our study justified the utilization of donors with lower BMI for clinical islet transplantation.

In terms of isolation-related factors, isolation characteristics revealed that the phase I time was significantly shorter and the undigested tissue volume was significantly smaller in the success group, suggesting that quick and complete digestion of the donor pancreas was important for the success of islet isolation with NHBDs. Both WIT and CIT were shorter in the success group; however, there were no significant differences because we minimized WIT and CIT in all cases.

Finally, we analyzed the relationship between islet yield and other factors. Unexpectedly, purity was positively correlated with islet yield. We obtained maximum islet yield, including mantled islets, using the density adjusted purification method. This technique enhanced islet yield but it sacrificed purity. Perfect islet isolation should result in high islet yield with a minimum of mantled islets, which is usually shown as high islet yield with high purity. This might be the reason that purity was still positively correlated with islet yields even when attempting to sacrifice some purity to attain higher islet yields.

To summarize, KIIM enables us to perform islet transplantation using NHBDs with a high success rate; however, the use of older donors and those with chronic pancreatitis requires prudent judgment.

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Larger Dosage Required for Everolimus than Sirolimus to Maintain Same Blood Concentration in Two Pancreatic Islet Transplant Patients with Tacrolimus

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Summary: We attempted a switch of mammalian target of rapamycin (mTOR) inhibitors from sirolimus to everolimus, a derivative of sirolimus and now on the market in Japan, in two pancreatic islet transplant patients. Both patients were administered tacrolimus with sirolimus or everolimus. They had been administered 5 or 9 mg sirolimus once a day and had maintained a trough concentration of about 15 ng/mL as measured by high performance liquid chromatography with ultraviolet detection. After the switch from sirolimus to everolimus, they were given 10 or 12 mg/day of everolimus twice a day to maintain a trough concentration of 12-15 ng/mL as measured by a fluorescence polarization immunoassay (FPIA) method. Afterward, the blood concentrations of everolimus and sirolimus after the conversion were measured by high performance liquid chromatography with mass spectrometry and everolimus concentrations were found to be 5-10 ng/mL. These data show that a larger dosage is needed for everolimus than sirolimus to maintain the same trough blood concentration. Data obtained by the FPIA for everolimus should be carefully evaluated after switching from sirolimus to everolimus because of the cross-reactivity of the antibody with sirolimus.

Keywords: everolimus; sirolimus; tacrolimus; pancreatic islet transplantation

Introduction

Pancreatic islet transplantation is a critical treatment for type 1 diabetes when it is difficult to control blood glucose levels despite an optimal insulin regimen and less invasive than pancreatic transplantation. With the Edmonton protocol,[1)] results of pancreatic islet transplantation improved markedly. According to the Edmonton protocol, Kyoto University Hospital performed 17 transplantations from non-heart-beating donors for 9 patients as of the end of 2006. The first successful living-donor islet transplantation was carried out on January 19, 2005.[2)]

The Edmonton protocol consists of high-dose sirolimus (rapamycin) and low-dose tacrolimus for immunosuppression.[1)] Sirolimus suppresses the proliferation of lymphocytes by blocking growth factor-driven signal transduction through the inhibition of mammalian target of rapamycin (mTOR).[3)] In Japan, however, sirolimus is not approved by the Japanese government as an immunosuppressant. Everolimus, a derivative of sirolimus, has a shorter elimination half-life than sirolimus,[4–6)] and is expected to achieve a steady-state more quickly and adjust blood concentrations more easily. Everolimus has already been approved as an immunosuppressant in Europe and in March 2007, was approved as an immunosuppressant for heart transplant patients in Japan. Hence, we conducted a switch of mTOR inhibitors from sirolimus to everolimus in pancreatic islet transplant patients. Generally, clinical studies on everolimus in organ transplant patients have been performed with the concomitant administration of cyclosporine and steroids. There are a few reports on everolimus using tacrolimus.

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Since everolimus as well as cyclosporine and tacrolimus are metabolized by cytochrome P450 (CYP) 3A and also transported via P-glycoprotein,[7-9] pharmacokinetic interactions may vary between everolimus and tacrolimus or cyclosporine.

Here, we report pharmacokinetic differences between sirolimus and everolimus in two pancreatic islet transplant patients concomitantly administered tacrolimus. The blood concentration of everolimus was measured by fluorescence polarization immunoassay (FPIA) method as well as high performance liquid chromatography with mass spectrometry (LC/MS).

Methods

Ethics: These studies were conducted in accordance with the Declaration of Helsinki and its amendments and were approved by the Kyoto University Graduate School and Faculty of Medicine Ethics Committee. Written informed consent was obtained from each patient.

Monitoring of blood concentrations for immunosuppressants: Whole blood concentrations of sirolimus (Rapamune®, Wyeth, Madison, NJ) were measured by high performance liquid chromatography with ultraviolet detection (HPLC-UV) as described previously.[10] The whole blood concentration of everolimus (Certican®, Novartis Pharma AG, Basel, Switzerland) was determined by a FPIA (Innofluor® Certican® Assay, Seradyn, Inc., Indianapolis, IN) using a TDxFLx® analyzer (Abbott Japan Co. Ltd., Tokyo, Japan).

Remnant blood samples after measurement of everolimus by FPIA were stored at −80°C. Everolimus and sirolimus whole blood concentrations were determined by a liquid-liquid extraction procedure and analysis of the extract by LC/MS in selected ion monitoring mode using atmospheric pressure chemical ionization as an interface at the laboratory of Novartis Pharma S. A. S. (Rueil Malmaison, France). Assay quantification limits were 0.3 ng/mL for everolimus and 0.5 ng/mL for sirolimus.

Cross-reactivity of sirolimus with the antibody for everolimus: To evaluate the cross-reactivity of sirolimus with the antibody for everolimus used in the assay, sirolimus was spiked in control human whole blood and sirolimus concentration was measured using FPIA for everolimus. Sirolimus concentrations were prepared at 5, 10, 20 and 50 ng/mL and tested in triplicate.

Time course study of everolimus in islet transplant patients: On the day immediately before the discharge of each patient, a time course study of everolimus was conducted. Blood samples were collected just before and 1, 2, 4, and 8 hrs after the morning administration. Whole blood concentrations of everolimus were determined using LC/MS at the laboratory of Novartis.

Results

Case report: Patient 1, a 48-year-old Japanese woman, had been treated with sirolimus and tacrolimus (Prograf®, Astellas Pharma Inc., Tokyo, Japan) after islet transplantation, according to the Edmonton protocol.[1] Thirty-six days after the transplantation, the mTOR inhibitor was converted. We called the day of conversion day 0. Both everolimus and sirolimus were administered on day 0 and only everolimus was administered after that. She kept taking tacrolimus as before (3–4 mg/day). Sirolimus was administered once a day. Everolimus and tacrolimus were administered twice daily. Blood sampling was performed once a day in the morning before the next administration of drugs. Before day 0, the whole blood concentration of sirolimus was quantified by HPLC-UV to adjust the trough concentration of sirolimus to 12–15 ng/mL. After day 0, the dosage of everolimus was adjusted to achieve a target trough blood concentration of 12–15 ng/mL as determined by FPIA. On day 0, the administration of everolimus was started at 4 mg/day, which was less than the dosage of sirolimus on day −1 (5 mg/day). Since the trough concentration of everolimus gradually decreased, the everolimus dosage was increased to 10 mg/day and the blood concentration reached the target level (**Fig. 1**, upper panel).

Patient 2, a 41-year-old Japanese woman, started the administration of everolimus 63 days after transplantation. Based on experience with patient 1, from the start, she was administered 12 mg/day of everolimus, this being greater than the dosage of sirolimus on day −1 (9 mg/day). As a result she did not experience a remarkable fall in the trough concentration of everolimus (**Fig. 1**, lower panel). During the switch from sirolimus to everolimus, she was concomitantly administered 4–6 mg/day of tacrolimus.

Neither patient showed remarkable change in tacrolimus trough concentration, which remained at 3–6 ng/mL, or had clinical complications during the study period. Neither patient was treated with potent inducers or inhibitors of CYP3A and P-glycoprotein.

Pharmacokinetic analysis: Whole blood concentrations of everolimus and sirolimus after the conversion were determined using LC/MS. After discontinuance of administration, sirolimus remained in the blood for several days (**Fig. 1**). The concentration of everolimus measured by FPIA was greater than that obtained by LC/MS, especially immediately after the conversion. To evaluate the cross-reactivity of sirolimus with the antibody for everolimus in the assay, we measured concentrations of sirolimus spiked in control human whole blood using FPIA for everolimus. As shown in **Figure 2**, the antibody for everolimus showed extensive cross-reactivity with sirolimus ([Detected as everolimus] = 1.43 + 0.47 × [Sirolimus concentration], $r^2 = 0.992$).

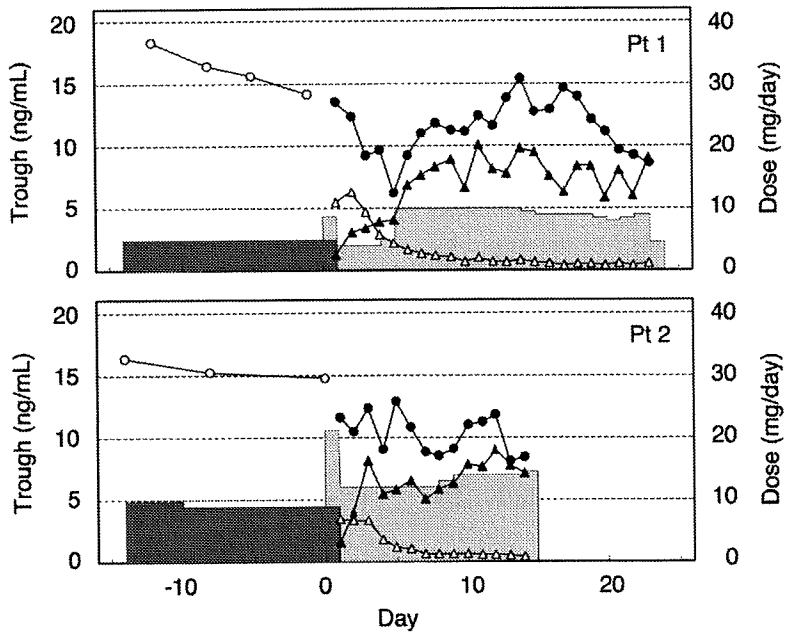

Fig. 1. Trough blood concentrations of sirolimus measured by HPLC-UV (open circles) and LC-MS (open triangles) and those of everolimus measured by FPIA (closed circles) and LC-MS (closed triangles) are plotted for each patient
Dark and light shaded areas show daily dosages of sirolimus and everolimus, respectively.

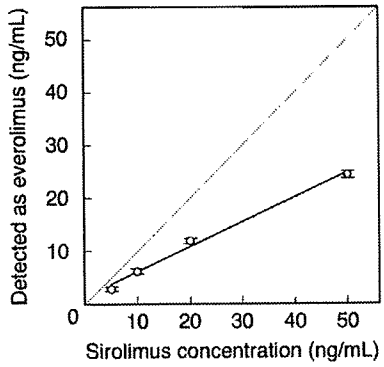

Fig. 2. Sirolimus blood concentrations measured by the FPIA method for everolimus. Each point represents the mean ± SD (n = 3)
The solid line shows the fitting line. The dotted line represents the line of identity (*i.e.*, slope = 1).

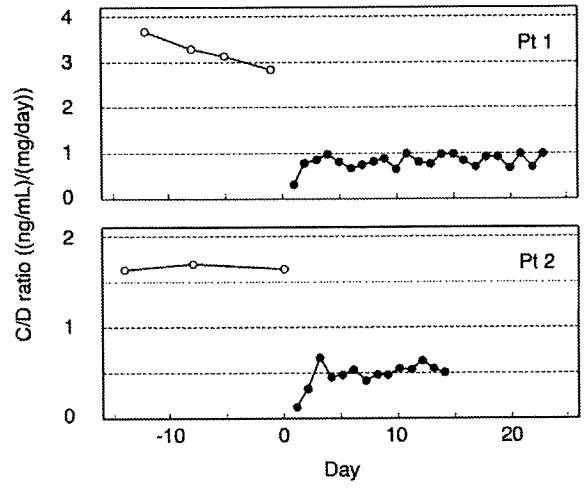

Fig. 3. The trough concentration per dose (C/D) ratios of sirolimus (open circles) and everolimus (closed circles) were plotted for each patient

Figure 3 shows the trough concentration per dose (C/D) ratio profiles of sirolimus and everolimus. C/D ratios of everolimus were calculated from concentrations determined by LC/MS and the dosage administered on the previous day. In patient 1, C/D ratios of sirolimus and everolimus were 3.26 ± 0.35 (ng/mL)/(mg/day) (mean ± standard deviation, n = 4) and 0.87 ± 0.12 (n = 22, except day 1), respectively. In patient 2, the ratios were 1.67 ± 0.03 (n = 3) and 0.52 ± 0.09 (n = 13, except day 1), respectively. In each patient, the C/D ratio of everolimus was approximately three times less that of sirolimus. C/D ratios of everolimus and sirolimus in patient 1 were twice those in patient 2.

We performed a time course study on everolimus. On day 23 for patient 1 and day 13 for patient 2. Everolimus concentration profiles measured by LC/MS are shown in **Figure 4**. Patient 1 was administered 4.5 mg everolimus and the peak concentration (17.1 ng/mL) was obtained at

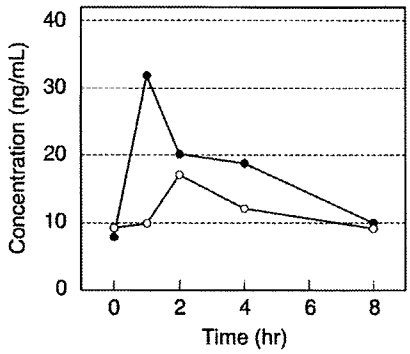

Fig. 4. Everolimus blood concentration profiles after oral administration in the two patients
Open and closed circles show everolimus concentration of patient 1 and patient 2, respectively.

2 h after the administration. Patient 2 was administered 7 mg everolimus and the peak concentration (31.8 ng/mL) was obtained at 1 h. The areas under the concentratioin-time curve from 0 to 8 h (AUC_{0-8}) calculated by the trapezoidal method were 94 and 142 ng·hr/mL in patient 1 and patient 2, respectively, while the concentrations at pre-dose and 8 h in patient 1 were nearly the same as those in patient 2, respectively.

Discussion

As shown in **Figure 1**, our patients were administered 8–14 mg/day of everolimus (with tacrolimus), and achieved trough concentrations of 5–10 ng/mL as measured by the LC/MS. Compared with other reports in which 1.5 or 3 mg/day of everolimus with cyclosporine were administered to renal transplant patients to maintain trough concentrations in a similar range,[11,12] our doses were quite large. We consider that this discrepancy mainly resulted from the difference in calcineurin inhibitor used, namely tacrolimus or cyclosporine. Everolimus as well as tacrolimus and cyclosporine are substrates of CYP3A and P-glycoprotein,[7-9] but lower blood concentrations of tacrolimus than cyclosporine in the clinical situation compared with each affinity value may have little influence on the pharmacokinetics of everolimus. Recently, Kovarik *et al.*[13] reported that the level of exposure to everolimus was 2.5 fold higher with cyclosporine than tacrolimus. It has been reported that average everolimus predose blood concentrations were significantly lower by 2.9 fold in the absence compared with the presence of cyclosporine.[12] The trough concentrations of sirolimus with cyclosporine are reported to be 1.42 times higher than those with tacrolimus.[14] Taking these findings into consideration, cyclosporine has a more profound effect on everolimus than sirolimus pharmacokinetics and our patients may need a considerably larger dosage of everolimus due to the lack of pharmacokinetic interaction with tacrolimus.

Interestingly, the C/D ratio of everolimus was three times smaller than that of sirolimus in the same patients (**Fig. 3**). Coadministration of inhibitors or inducers of CYP3A or P-glycoprorein would be expected to alter sirolimus or everolimus pharmacokinetics, but comedications in the two patients did not change during the study period. Hepatic impairment would decrease the oral clearance of sirolimus,[15] but neither patient had clinical complications such as hepatic dysfunction. Actually, the trough concentrations of tacrolimus, also metabolized by CYP3A and transported via P-glycoprotein, remained in a similar range during the conversion from sirolimus to everolimus in these patients. Therefore, we consider that a larger dosage is needed for everolimus than sirolimus to maintain the same trough blood concentration in the same patients with tacrolimus. As discussed in the previous paragraph, in the case of concomitant administration of cyclosporine, dosage of everolimus might not be so different from that of sirolimus, because of the more profound pharmacokinetic interaction of cyclosporine with everolimus compared to sirolimus. Pharmacokinetic differences between sirolimus and everolimus with cyclosporine in the same patient should be clarified in future study.

Everolimus has been reported to have a large inter-individual variability in the pharmacokinetics,[16] as also found in our cases. In the time course study, the trough concentrations of everolimus in patients 1 and 2 were similar and peak concentrations and AUC_{0-8} in patient 2 were approximately twice those in patient 1 at dosage of 7 mg and 4.5 mg, respectively (**Fig. 4**). Apparent clearance of everolimus approximately estimated by the dose-normalized AUC_{0-8} seems similar in these patients. In contrast, dose-normalized trough concentrations for everolimus and sirolimus were different as also shown in **Figure 3**. One possible reason for these findings is that the patients had different absorption profiles. In general, the recommended therapeutic range for everolimus is reported as a trough concentration of 3 to 8 ng/mL[17] and the clinical significance of AUC monitoring for everolimus remains to be elucidated.

FPIA is easy and convenient to determine whole blood concentrations of everolimus, but it is known to overestimate everolimus concentrations due to cross-reactivity of the antibody with metabolites of everolimus.[18] Actually, the everolimus concentration measured by FPIA was greater than that obtained by LC/MS over the study period (**Fig. 1**). This finding is consistent with a report using samples from renal transplant recipients.[19] In a recent report,[20] FPIA gave a positive bias of 1.2 ng/mL compared with HPLC-UV. The antibody for everolimus may cross-react with sirolimus because of the similarity in chemical structure between everolimus and sirolimus. Immediately after switching of the mTOR inhibitors, it was considered that few metabolites of everolimus were present in blood, but the values obtained were greater

with FPIA than LC/MS (**Fig. 1**). We consider the difference between the two methods to be caused by cross-reactivity with sirolimus and clarified the cross-reactivity of sirolimus with the antibody used in FPIA for everolimus (**Fig. 2**), as consistent with recent reports.[19,20] However, since the values measured by FPIA exceeded the sum of everolimus and sirolimus concentrations measured by LC/MS immediately after the conversion (**Fig. 1**), we consider that metabolites of sirolimus may also cross-react with the antibody of FPIA. These results indicate that the values of everolimus by the FPIA method should be carefully evaluated especially when transplant patients are switched from sirolimus to everolimus.

In conclusion, we report two cases of changing mTOR inhibitors from sirolimus to everolimus with tacrolimus after pancreatic islet transplantation. Each patient needed a considerably larger dosage of everolimus compared to sirolimus to maintain the same trough blood concentrations, which may be explained by lack of pharmacokinetic interaction between tacrolimus and mTOR inhibitors. The concentrations of everolimus measured by FPIA were considerably greater than those by LC/MS. These findings should provide useful information regarding the replacement of sirolimus with everolimus in transplant patients.

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Development of a novel neodymium compound for *in vivo* fluorescence imaging

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ABSTRACT: We developed a novel fluorescent probe that contains the neodymium(III) complex moiety and fluorescein moiety. This probe can emit long-lived near-infrared luminescence derived from a Nd ion through excitation of the fluorescein moiety with visible light (λ_{ex} = 488 nm, λ_{em} = 880 nm, lifetime = 2.3 μs). These results indicate the possibility of the probe as a candidate for *in vivo* fluorescence molecular imaging. Copyright © 2007 John Wiley & Sons, Ltd.

KEYWORDS: neodymium; near-infrared; fluorescein; energy transfer; long-lived luminescence

INTRODUCTION

Molecular imaging is a rapidly emerging biomedical research field that may be defined as the visual representation, characterization and quantification of biological processes at the cellular and subcellular levels within a living organism (1–3). As a technique of molecular imaging, fluorescence imaging attracts great interest.

In vivo fluorescence imaging, however, has several points that need to be considered, such as the permeability of the emission light, the background from scattered and reflected excitation light and self-fluorescence from tissues. To overcome such problems, the development of novel *in vivo* fluorescent probes has been required.

Near-infrared (NIR) light (700–1000 nm) has a potential for *in vivo* imaging. Provided that NIR light is used as emission light, it can permeate the body without intense absorbance and scatter by tissues (4) to offer a solution to problems such as permeability and background. In addition it has another advantage, that most biological compounds in living systems have no self-fluorescence in the NIR region.

Scattered and reflected light derived from excitation light are other obstacles, increasing background noise. Fortunately, background noise from excitation light can be removed by appropriate filters, provided that the difference between the excitation and emission wavelengths of the probe (Stoke's shift) is large enough; therefore, fluorescent probes with a large Stoke's shift have the potential to increase the S/N ratio in *in vivo* fluorescent imaging. However, most previously reported NIR probes (5–7) had an organic fluorescence centre with a small Stoke's shift ineffective for removing noise.

Consequently, we planned to develop a new fluorescent probe by chelating a neodymium (Nd) ion in its fluorescent centre for *in vivo* fluorescence imaging. Nd complexes have luminescence originating from $^4F_{3/2}$ to $^4I_{9/2}$ transition in the NIR region of 880 nm (8–10), unlike other lanthanide ions (e.g. Eu and Tb), and their Stoke's shift exceeds 200 nm, which is nearly 10 times larger than typical fluorescent dyes (fluorescein and rhodamine have Stoke's shifts of ~25 nm and ~20 nm, respectively). Such a favourable characteristic can increase the S/N ratio with the use of appropriate filters. In addition, the lifetime of lanthanide luminescence reaches the microsecond (μs) or millisecond (ms) order in contrast to the nanosecond (ns) order of organic fluorophores, making it possible to further cut down noise by time-resolved fluorescence imaging (4, 11).

As a chelating moiety, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) was selected, because DOTA and a Nd^{3+} ion form a highly stable complex [the log K value of Nd^{3+} and $DOTA^{4-}$ is 25.69 (12)] due to the tight coordination of DOTA with eight positions at maximum. This implies that the complex is not susceptible to metabolic degradation in living systems (13).

To excite a Nd ion, an antenna moiety, a sensitizing chromophore, is required in the structure because of the low absorbance of lanthanide f–f transitions (14). Thus, we selected fluorescein as the antenna moiety because of its long, less harmful excitation wavelength (~500 nm) and previous success in transferring energy to Nd (14). Moreover, it is also important that fluorescein is widely used in various fields, is highly soluble in water and can be easily modified for use in follow-up studies (15, 16).

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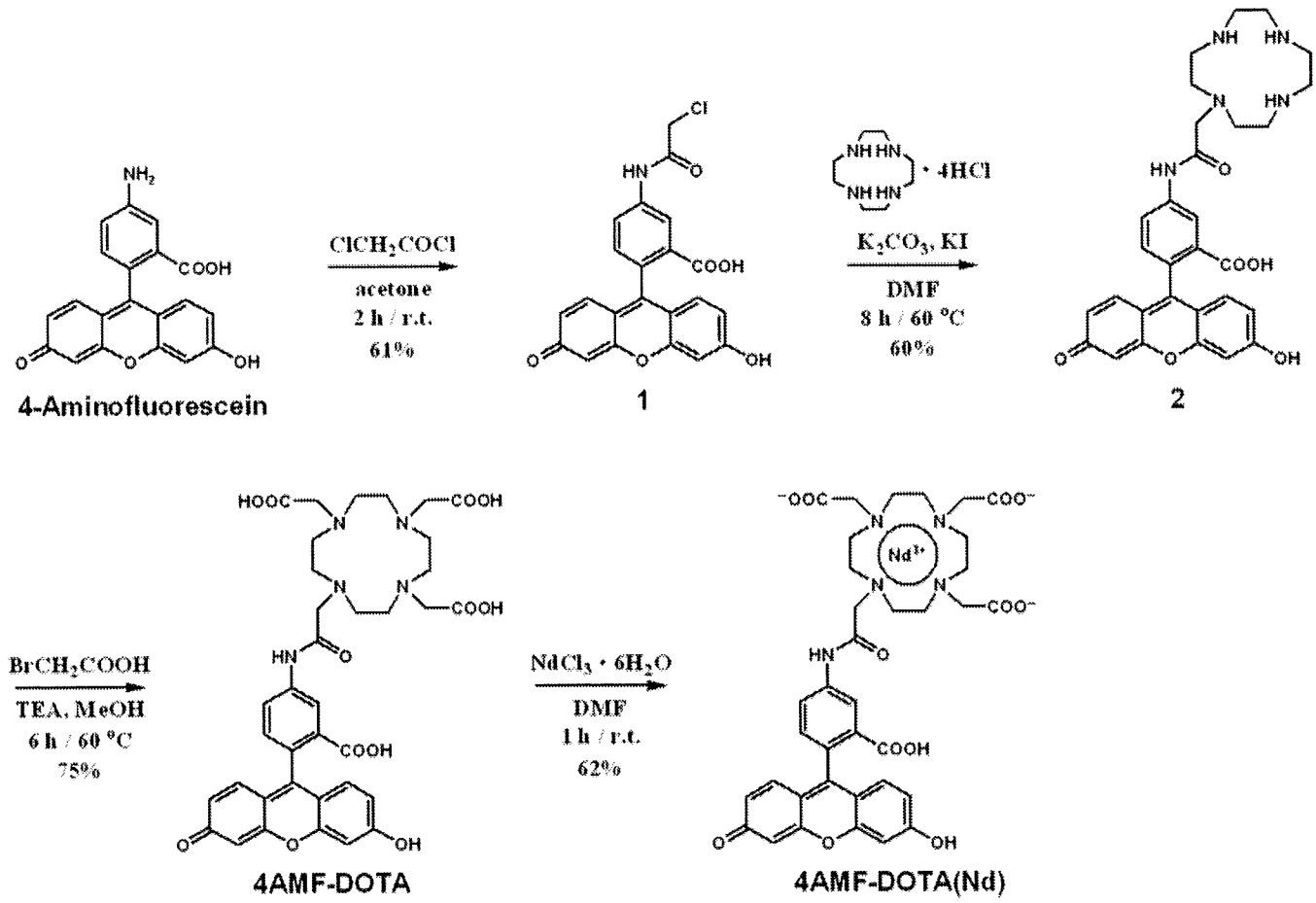

Scheme 1. Synthetic reaction scheme for 4AMF-DOTA(Nd).

In this study, we synthesized 4AMF-DOTA(Nd) including the Nd-DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-triacetic acid) complex and fluorescein as a NIR fluorescent probe (Scheme 1), and investigated its chemical and physical features.

MATERIALS AND METHODS

Materials

All chemicals used in this study were commercial products of the highest purity and were further purified by standard methods if necessary.

Instruments

FT-IR spectra were recorded using a Jasco FT/IR-4100 (Nihon Bunko Inc., Tokyo, Japan). UV-vis spectra were measured using a Hitachi U2001 (Hitachi High-Tech Manufacturing & Service Corp., Ibaraki, Japan). Electrospray mass spectral (ESI–MS) measurements were performed on a SHIMADZU LC-MS2010 EV (Shimadzu Corp., Kyoto, Japan). ^{1}H-NMR spectra were recorded on a JEOL JNM-AL400 (JEOL Ltd, Tokyo, Japan). Fluorescence spectroscopy was performed with a Fluorolog-3 (Horiba Jobin Yvon Inc., Kyoto, Japan). The slit width was 10 nm for both excitation and emission. Time-resolved fluorescence spectra were recorded on a Fluorolog-3 with Phosphorescence (Horiba Jobin Yvon). The slit width was 12 nm for both excitation and emission. In both fluorescence spectra measurements, the photomultiplier voltage was 1450 V.

Fluorescence emission and excitation spectral measurements

The fluorescence emission spectra of 4AMF-DOTA(Nd) (10 μmol/L) without delay time were measured in 10 mmol/L Tris–HCl buffer, pH 8.0, 10 mmol/L Britton–Robinson buffer, pH 2–11, MeOH, EtOH and DMSO (each organic solvent contained 0.1% v/v Et_3N) at 25°C, following excitation at 488, 498, 504 and 523 nm in the buffers, MeOH, EtOH and DMSO, respectively. Excitation spectra were obtained at an emission wavelength of 870 nm.