patients, including two cases of single transplantation, showed a positive serum C-peptide level (0.4–0.8 ng/ml) immediately after transplantation. Although insulin independency was not achieved, all patients experienced stabilization of their blood glucose levels, a reduction in the amount of insulin required, and the disappearance of hypoglycemic unawareness. Hemoglobin A1C levels were significantly decreased from 9.4 ± 3.1% to 6.4 ± 0.6% at 4 months after transplantation. Although stomatitis and diarrhea, side effects of sirolimus, were observed in 2 patients, severe complications did not occur. In patient #1, serum C-peptide levels decreased gradually after transplantation (Fig. 3). Blood glucose levels were, however, again stabilized after the second islet transplantation (Fig. 4).

Discussion

Encouraged by the successful results of the Edmonton Protocol, a Working Group began preparations for a clinical islet transplantation program in Japan, and the first human islet isolation was performed at our institution on September 12, 2003. The first human islet transplantation for a type 1 diabetic patient was performed by the Kyoto group [9–11].

In Japan, pancreata from non-heart-beating donors are used for islet isolation since the pancreata from brain-dead donors are usually used for pancreas or pancreas/kidney transplantation. A respirator is rarely withdrawn from a patient, even though the donor is diagnosed as brain dead. Moreover, the donors usually are not examined to diagnose brain death; therefore, cannulation into the abdominal aorta with a double balloon catheter via the femoral artery and systemic heparinization are not permitted before a cardiac arrest. Under the present conditions in Japan, viability of the pancreata may decrease before procurement, thus often resulting in a poor yield and a low purity of the islet isolation.

In our experience, the results of islet isolation from non-heart-beating donors (yield and purity) were extremely varied. We analyzed the outcomes of 23 human islet isolations performed in our CPC and compared the factors of successful isolation (fulfilling the fresh islet transplantation criteria) and unsuccessful isolation. Donor age, gender, warm ischemic time, and in situ perfusion solution were not different between the two isolation groups. Only the cold ischemic time was significantly shorter in the successful isolations than that in the unsuccessful isolations. Moreover, the use of the two-layer method improved the outcome of islet isolations in comparison to the use of UW solution, though not to a significant degree. The two-layer method is considered to be advantageous for the preservation of the pancreata for islet isolation and transplantation [12–18]. However, Caballero-Corbalán et al. recently reported that the two-layer method had no beneficial effect in comparison to the use of UW solution on human islet isolation and transplantation [30].

Six isolations were successful and were used for fresh islet transplantation, even from non-heart-beating donors. These results demonstrate that the Edmonton Protocol is indicated for islet isolation from a damaged pancreas with some modifications. We used a two-step digestion technique as previously reported. Our previous data using a two-step digestion technique demonstrated that the islets were protected from digestion damage, resulting in a higher yield and purity using both porcine and human pancreas [24]. Further improvement of islet isolation is needed, especially from the pancreata of non-heart-beating donors in Japan. The Kyoto group developed a modified two-layer preservation method and improved the islet yields in human and porcine islet isolation [31, 32].

The major advantage of islet transplantation is that it is a safe procedure in comparison to pancreas transplantation. The complications often caused by the organ transplantation procedure were not observed in the six islet transplantations in our institution. Blood pressure and portal pressure, which was monitored during transplantation showed no significant change in any of the patients. The infusion of the islet suspension required only 15–20 min. Stomatitis and diarrhea, side effects of sirolimus, occurred in two patients and were the only complications observed after transplantation. Both patients recovered from these complications with a reduction of the sirolimus dose (patient #1) or a change from sirolimus to mycophenolate mofetil (patient #2).

The islets isolated from the pancreata of non-heart-beating donors functioned immediately after transplantation in all four patients. These patients were free from hypoglycemic unawareness and obtained stabilized blood glucose levels. All patients showed a positive serum C-peptide level and a reduced insulin requirement. These data demonstrate that the islets isolated from damaged pancreas with ischemia functioned as well as those from brain-dead donors.

Many problems remain to be solved in clinical islet transplantation. The most essential problem is the difficulty in maintaining persistent islet graft function. In particular, long-term insulin independency after islet transplantation is rarely observed, both in international trials of the Edmonton Protocol [20] and in the Japanese trial. A number of factors are considered to cause declining islet function over time. Selection of the immunosuppressive regimen is an important key for islet graft survival. A steroid-free immunosuppressive regimen was introduced for islet

transplantation because of the β-cell toxicity and the diabetogenic effect of this agent [5]. A steroid-free immunosuppressive regimen was also recently indicated for kidney and liver transplantation [33–35]. This steroid-free immunosuppressive regimen was also effective for islets after kidney transplantation [36, 37]. However, an mTOR inhibitor, sirolimus, which is used as the main agent in the steroid-free immunosuppressive regimen of the Edmonton Protocol, has a number of side effects including renal toxicity and severe ulceration of the small bowel [38, 39]. Further improvements in the immunosuppressive regimen after islet transplantation are needed.

Another important factor for persistent islet graft function is the promotion of islet engraftment after transplantation. We reported the efficacy of nicotinamide and 15-deoxyspergualin on the engraftment of mice islet isografts [40]. In addition, in a clinical study, 15-deoxyspergualin promoted the engraftment of unpurified islet transplantation [41]. A recent report by Yasunami et al. demonstrated the efficacy of inhibition of proinflammatory cytokines on islet engraftment in mice [42]. This agent may have a promising role to play in clinical islet transplantation in Japan.

From the data of the Japanese trial of islet transplantation promoted by the Working Group, the effectiveness of islet transplantation was shown, even when using pancreata from non-heart-beating donors. The patients showed positive C-peptide levels and stabilization of blood glucose levels. In particular, the disappearance of hypoglycemic unawareness led to a prominent improvement in the patient's quality of life. Although there are a number of problems to be solved, including further improvement of islet isolation, transplantation technique, and long-term graft survival, we can state that the start of clinical islet transplantation provides a reason to hope for type 1 diabetic patients.

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Living donor pancreas transplantation in Japan

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Abstract
Background/purpose Living-donor pancreas transplants (LDPs) were introduced at Chiba-East National Hospital in 2004, and 12 LDPs have been performed at this institution to date. Based on the outcome of these 12 LDPs, the efficacy and safety of LDPs are herein discussed.
Methods Twelve diabetic patients underwent LDPs; ten had simultaneous pancreas and kidney transplants from living donors, one had pancreas transplant after a kidney transplant from a living donor, and one had a pancreas transplant alone from a living donor. The donors were parents or brothers and the ABO blood types were incompatible in three LDPs. The procedures for the donor and recipient operations were performed according to the technique established by the University of Minnesota. Bladder drainage was used in 11 recipients and enteric drainage was used in one patient. Tacrolimus, basiliximab, mycophenolate mofetil, and prednisone were used for induction and immunosuppressive treatment. A splenectomy, double-filtered plasmapheresis, and plasma exchange were added in the ABO-incompatible LDPs.
Results No complications were observed in the donors during hospitalization. The 1-year survivals of the patients, kidney grafts, and pancreas grafts were 100, 100, and 100%, respectively. The 3-year survivals were 91.7, 90, and 91.7%, respectively. Three patients developed leakage of pancreatic juice and one patient required a surgical procedure. Cytomegalovirus antigenemia was detected in five patients (42%).
Conclusions Based on the excellent outcome of the LDPs at this institution, LDPs is therefore expected to become a promising option for the treatment of patients with severe diabetes.

Keywords Living-donor pancreas transplantation · Safety for the donor · ABO-incompatible · Insulin independence

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Introduction

Since the first pancreas transplantation was performed at the University of Minnesota by Kelly et al. [1], more than 23,000 diabetic patients have undergone pancreas transplantation [2]. The outcome of pancreas transplantation has improved so that it is almost equivalent to that of kidney transplantation, due to improvements in the surgical techniques and the introduction of an improved immunosuppressive protocol [3]. Pancreas transplantation has now become the most successful physiological treatment for type 1 diabetic patients.

In Japan, the first pancreas transplantation was performed at Tsukuba University in 1984 by Fukao et al. [4] from a brain-dead donor. In Japan, the availability of brain-dead donors for pancreas or other organ transplantations is, however, extremely limited because of the social

circumstances in this country. Therefore, until 1994, most pancreas transplantations were from obtained non-heart-beating donors. The procurement of organs for transplantation from brain-dead donors, however, was officially permitted in 1997 according to the establishment of the law in 1997 in Japan. Subsequently, pancreas transplantation from a brain dead donor was begun again at Osaka University, by Ito et al. [5] and, as of December 2007, 52 patients had undergone pancreas transplants from brain dead donors. Although the outcome of pancreas transplantation from brain-dead donors in this country was excellent, even when using marginal donors [6], 19 patients on the waiting list for pancreas transplantation died of diabetic complications, including hypoglycemic episodes and cardiovascular disease, between 2000 and 2007.

The first extrarenal organ to be successfully transplanted using living donors was the pancreas. The first pancreas transplantation using a living donor was performed on 20 June 1979, at the University of Minnesota [7, 8]. Furthermore, simultaneous pancreas and kidney transplantation from a living donor was introduced in 1994, also at the University of Minnesota [9]. The outcome of the living-donor transplantations performed at the University of Minnesota demonstrated the segmental pancreas to be able to normalize plasma glucose levels and provide insulin independence to patients with severe diabetes. The outcome of the donors was considered to be acceptable when stringent donor criteria concerning endocrine function were used [10].

Based on the severe shortage of deceased donors in Japan and the satisfactory outcome of living-donor pancreas transplantations at the University of Minnesota, living-donor pancreas transplantation was introduced in this country on 7 January 2004 [11]. In Japan, 15 living-donor pancreas transplantations have so far been performed at three institutions (Chiba-East National Hospital, 12; Niigata University Hospital, 2; Osaka University Hospital, 1).

The present article describes the outcomes of both the donors and the recipients of living-donor pancreas transplants (LDPs) at Chiba-East National Hospital, because 80% of LDPs in this country were performed at this institution. In addition, the efficacy and the safety of this procedure is also discussed.

Patients and methods

Recipients

Twelve type 1 diabetic patients underwent living donor pancreas transplantations at Chiba-East National Hospital from January 2004 to June 2008. Ten patients (83%) underwent a simultaneous pancreas and kidney transplant from living donors (LDSPK) because of end-stage renal disease (ESRD). One patient (8.3%) underwent a pancreas transplant after a kidney transplant from a living donor (LDPAK) and the other patient (8.3%) underwent a pancreas transplant alone from a living donor (LDPTA).

Table 1 Characteristics of the recipients and donors of pancreas transplantation from a living donor (Department of Surgery, Chiba-East National Hospital, 2004–2008)

Recipients	
Patient number	12
Age, in years (range)	34.6 ± 5.1 (30–46)
Gender (male/female)	6/6
Age at onset of DM in years (range)	12.8 ± 5.9 (0.9–19)
Duration of DM in years (range)	22.0 ± 4.5 (15–30)
Amount of insulin (units/day)	41.6 ± 19.2 (four times daily)
Anti GAD or IA-2 Abs	Positive 6, negative 6
M value	68.2 ± 14.5
ESRD	Yes 10 (HD 8, preemptive 2), no 2
Donors	
Patient number	12
Age in years	58.8 ± 10.6 (28–72)
Gender (male/female)	4 (3 fathers, 1 brother)/8 mothers
ABO compatibility	Identical, 9; incompatible, 3
75-g-OGTT	Normal pattern
IV-GTT (δCPR, 0–5 min)	6.8 ± 1.7 ng/ml per 5 min
Body mass index	22.9 + 1.87

DM diabetes mellitus, *Ab* antibodies, *ESRD* endstage renal disease, *HD* hemodialysis, *75-g-OGTT* 75-g oral glucose tolerance test, *CPR* C-peptide release

The characteristics of the recipients are shown in Table 1. All patients had type 1 diabetes and the onset of diabetes had been rapid due to diabetic ketoacidosis. All patients showed frequent hypoglycemic episodes despite carrying out four courses of potent insulin injection therapy based on their self-measured plasma glucose levels. Unstable plasma glucose levels resulted in a high *M* value [12] (68.2 ± 14.5). Serum C-peptide levels were undetectable (<0.03 ng/ml) in nine patients and levels were less than 0.1 ng/ml in the other three patients. The peripheral nerve conduction velocity was decreased in 11 of the 12 patients and a head-up tilt test demonstrated an autonomic disturbance by diabetes in 11 patients. No retinopathy was observed in any patients at the time of transplantation.

Donors

The donors were the mothers for eight recipients, fathers for three recipients, and a brother for one of the recipients. The donors' ages ranged from 28 to 72 years. ABO blood

type compatibilities to the recipients were identical in nine donors and incompatible in the other three donors. A potential donor first must undergo an interview with the doctors, nurses, transplant coordinators, and medical social workers and must provide voluntary consent. Subsequently, the donor must show negative findings on a flow cross-match examination between the donor's T lymphocytes and the recipient's serum. All donors must fulfill the criteria for living-pancreas transplantation donors, as shown in Table 2.

The evaluation of pancreatic endocrine function includes a normal 75-g oral glucose tolerance test (75 g-OGTT), normal CS1 (first-phase C-peptide secretion calculated from the sum of the C-peptide secretion rates from 0 to 5 min after intravenous glucose tolerance test (IV-GTT) [13] and a normal level of hemoglobin (Hb) A1C. In addition, islet cell autoantibodies (anti-GAD and anti-IA2 antibodies) must be absent, and body mass index (BMI) must be less than 25.

Table 2 Criteria for donors for living pancreas transplantation (Department of Diabetology, Chiba-East National Hospital, 2004)

Age ≤65 years (desirable)
No family history except for the recipient
Normal endocrine function
75-g-OGTT: normal pattern (all plasma glucose levels <180 mg/dl)
IVGTT: normal CS1
HbA1C: ≤5.5%
Negative anti-GAD and IA-2 antibodies
BMI <25
Contraindications
Active infectious disease
HIV (+), HTLV-1 (+), HBs antigen (+), HCV antibody (+)
Malignancy
Abnormal anatomy of the pancreas
Alcoholism

CS1 the first phase C-peptide secretion calculated from the sum of C-peptide secretion rates from 0 to 5 min after glucose injection, *BMI* body mass index

Eleven of the 12 donors completely fulfilled our criteria. One donor did not fulfill the criteria because she was 72 years old. But because her pancreatic endocrine function was excellent and she qualified as a potential donor for LDPTA, the committee for living donor pancreas transplantation and the Institutional Review Board (IRB) at Chiba-East National Hospital finally approved her as a donor after obtaining sufficient informed consent from her and her family members.

The contraindications were almost the same as those for other transplantations. For the safe procedure of the donor operation, the blood vessels of the pancreas and the kidney were evaluated using three-dimensional angiography from dynamic computed tomography (CT Fig. 1). In order to evaluate segmental function of the donor pancreas, ^{11}C-methionine positron emission tomography (PET) was also performed [14] (Fig. 2).

Operative methods and postoperative care

Donors

Procurement of the distal pancreas from the living donor was performed according to the procedure of the Minnesota Group as previously reported [7]. In LDSPK, under an open laparotomy, the left kidney was first excised, followed by a distal pancreatectomy with a splenectomy (Fig. 3). In two recent donors, however, hand-assisted laparoscopic surgery (HALS) was introduced as a less invasive procedure for simultaneous nephrectomy and distal pancreatectomy.

Antibiotics were administrated intravenously for 7 days after the surgery. Gabexate mesilate (600 mg/day) was given for 7 days in order to inhibit the occurrence of residual pancreatitis. Oral intake was resumed 6 days after the surgery. Before being discharged from the hospital, the donors underwent a CT scan to rule out the formation of a pancreatic cyst or abscess. To assess the exocrine and endocrine function of the residual pancreas, serum

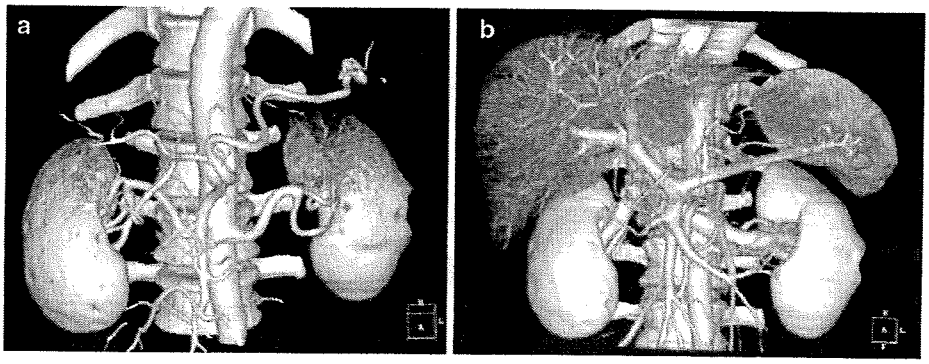

Fig. 1 **a**, **b** Images of the blood vessels of pancreas and kidneys of a donor; reconstructed from three-dimensional angiography from a dynamic computed tomography (CT) scan. Department of Radiology, Chiba-East National Hospital

amylase, lipase, trypsin, and plasma glucose levels were determined daily. In addition, the serum C-peptide levels were determined once a week. After discharge, the donors were monitored at the outpatient clinic and their plasma glucose levels, HbA1C, and serum C-peptide levels were measured at 1 and 3 months and every year after the operation. A 75-g-OGTT was performed at 6 months, and then every year after the surgery.

Recipients

In the recipients, the kidney transplantation was performed in the standard fashion as previously described in detail, with vascular anastomoses to the left external iliac vessels and an ureterocystostomy. The segmental pancreatic graft was revascularized by anastomosing the donor splenic artery and vein to the right iliac artery and vein of the recipient. A pancreaticocystostomy was performed using a two-layer technique including the anastomosis between the pancreatic duct and the mucosa of the urinary bladder to drain the pancreatic juice of the graft.

Induction of immunosuppression was achieved by quadruple therapy using tacrolimus, basiliximab, mycophenolate mofetil (MMF), and prednisone. Immunosuppression was maintained with triple therapy consisting of tacrolimus, MMF, and prednisone. Desensitization for the patients receiving transplants from ABO-incompatible donors was achieved according to a protocol for ABO-incompatible kidney transplantation, which included the administration of MMF for 4 weeks before transplantation; a splenectomy at −14 days; double-filtered plasmapheresis (DFPP) at −6, −4, and −2 days; and plasma exchange (PE) at −1 day.

Anticoagulation therapy was started at the time of the operation, using heparin (200 units/h), and 10,000–20,000 units was continuously given intravenously for 10 days after transplantation. Gabexate mesilate (600 mg) was continuously administrated for 7 days to inhibit graft pancreatitis and 100 units of octreotide was given every 12 h for 5 days to inhibit the secretion of pancreatic juice from the graft. In addition, we also administered antibacterial prophylaxis with piperacillin for a week, antifungal prophylaxis with fluconazole for a week, and anti-cytomegalovirus (CMV) prophylaxis with gancyclovir for 10 days. Insulin was continuously given intravenously to maintain plasma glucose levels of 100–150 mg/dl.

Oral intake was started at 7 days after transplantation. During the hospitalization, each recipient was monitored daily for pre- and postprandial serum glucose and amylase, blood cell count, electrolytes, creatinine, and urinary

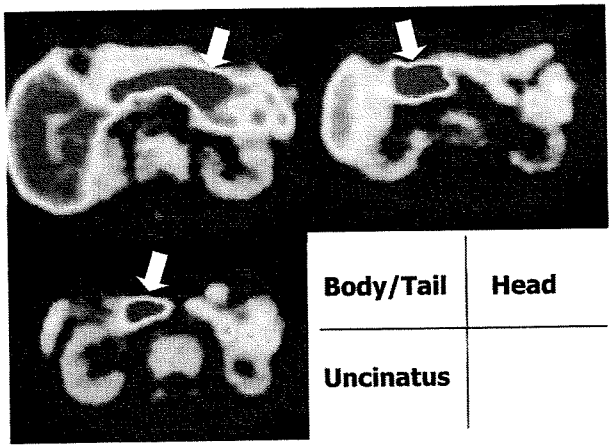

Fig. 2 Pancreatic function of donor evaluated by ^{11}C-methionine positron emission tomography (^{11}C-Met PET). The segmental pancreatic function was evaluated from the images (*arrows*) of the head and the body/tail

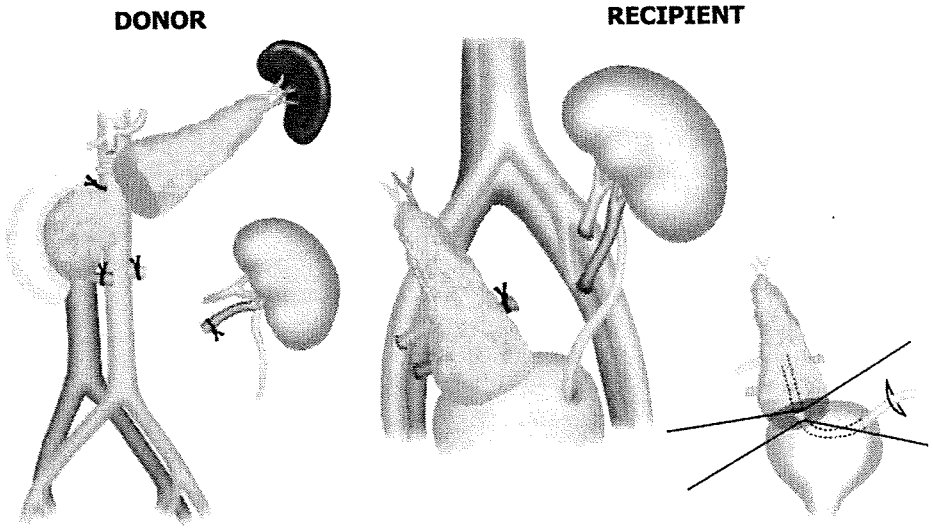

Fig. 3 Surgical method of simultaneous pancreas and kidney transplant from living donor (LDSPK). In the donor operation, left nephrectomy was performed, followed by distal pancreatectomy with splenectomy. The body/tail of the pancreas was excised at the left edge of the portal vein and procured as the pancreatic graft with the spleen. In the recipient operation, the kidney was transplanted into the left iliac fossa, followed by pancreas transplantation into the right iliac fossa, using a pancreaticocystostomy

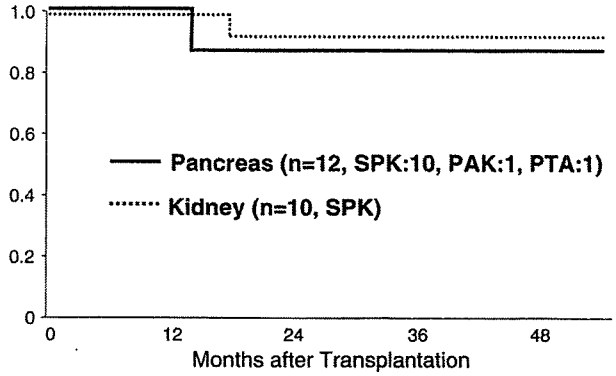

Fig. 4 Survival rates of pancreas and kidney grafts after living donor pancreas transplantation at Chiba-East National Hospital. The 1-year survivals of the kidney graft (LDSPK) and the pancreas graft were 100 and 100%. The 3-year survivals of the recipients, kidney grafts, and pancreas grafts were 91.7, 90, and 91.7%. *SPK* simultaneous pancreas and kidney transplantation, *PAK* pancreas after kidney transplantation, *PTA* pancreas transplant alone

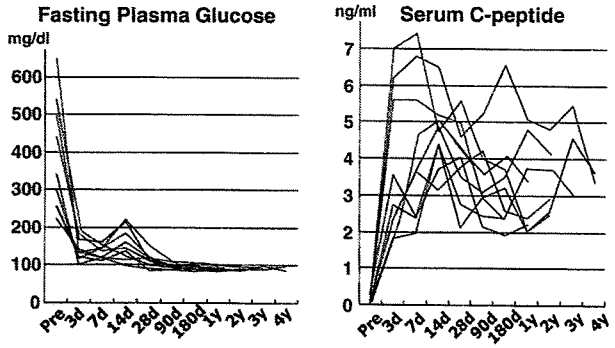

Fig. 5 Fasting plasma glucose levels and serum C-peptide levels in the patients who underwent living pancreas transplantation, excluding the patient with primary nonfunction of the graft. *Pre* before the operation, *d* day, *y* year

amylase excretion. All the above parameters were subsequently monitored continually at the outpatient clinic.

Results

Donor outcomes

No complications, including the formation of a pancreatic fistula or an intraabdominal abscess, were observed during hospitalization. The donors were discharged from the hospital at 23.3 ±5.2 days after surgery and immediately returned to their normal life. Only one donor (donor 6) developed pancreatic pseudocysts, at 6 months after surgery with minor symptoms. The cyst was punctured from the stomach using gastro-fiberscopy and the cyst completely disappeared. The development of diabetes has not been observed in any donors during an observation period ranging from 6 months to 5 years.

Recipient outcomes

One patient, who underwent LDPAK, died of a cerebral hemorrhage at 13 months after transplantation with a functioning pancreas graft. The pancreas and kidney graft survivals of the recipients are shown in Fig. 4. The 1-year survivals of the patients, kidney grafts (LDSPK), and pancreas grafts were 100, 100, and 100%, respectively. The 3-year survivals were 91.7, 90, and 91.7%, respectively. Insulin independence was obtained in 11 patients immediately after transplantation. However, one patient, who underwent LDPTA, required a small amount of exogenous insulin injection from 3 months after transplantation even though his serum C-peptide was positive. Another patient, who underwent LDSPK, showed primary nonfunction of the pancreas graft. Her kidney graft functioned well and serum creatinine level had decreased to 0.8 mg/dl within 3 days after transplantation. Although neither arterial nor venous thromboses were observed, the serum C-peptide levels decreased to 0.05 ng/ml after transplantation. Except in the patient with primary nonfunction of the pancreas graft, the levels of HbA1C decreased to less than 6.0% within 3 months after transplantation. The fasting plasma glucose levels stabilized at under 100 mg/dl, and positive C-peptide levels ranging from 1.8 to 7.5 ng/ml were maintained (Fig. 5). The 75-g-OGTT performed at 6 months and at 1, 2, and 3 years after transplantation showed a normal pattern.

Although a biopsy-proven acute cellular rejection (ACR) was observed in two patients (16.7%), steroid pulse rescue therapy completely resolved the ACR in both patients. In addition, no antibody-mediated rejection (AMR) occurred in the three patients who had undergone LDSPKs from ABO-incompatible donors. The leakage of pancreatic juice was observed in three patients. One of them developed bleeding from the external iliac artery and required an additional operation to stop the bleeding. Cytomegalovirus antigenemia was detected in five patients (41.7%) from 30 to 56 days after transplantation and three patients required the intravenous administration of gancyclovir. The recipients were discharged from 37 to 148 days after transplantation and thereafter returned to their normal lives.

Discussion

In Japan, the number of diabetic patients has increased every year and has now reached more than eight million. Although type 1 diabetes is less frequent in this country

than in the United States and Europe, the quality of life in type 1 diabetic patients with ESRD is extremely low and the prognosis is very poor. Pancreas transplantation using brain-dead donors was begun again in 2000 for such patients. However, only 52 pancreas transplants have been performed over the past 8 years because of the severe shortage of deceased donors in this country. So far, 19 patients on the waiting list for pancreas transplantation have died due to diabetic complications such as hypoglycemic episodes and cardiovascular disease.

Living donor pancreas transplantation was introduced at the University of Minnesota in 1979 [8]. Initially, they performed living donor pancreas transplants only in recipients without uremia (LDPTA) or recipients who had received a kidney graft from the same donor (LDPAK) [15]. Subsequently, they performed the first successful LDSPK in March 1994 [9], and 20 LDSPKs had been done by March 1997 [10]. The 1-year survivals of the patients, kidney grafts, and pancreas grafts at that time were 100, 100, and 78%, respectively, which were higher than those of pancreas transplants from brain-dead donors at that time. An analysis in 2001 of 32 recipients of LDSPK showed that the 1-year survival of the pancreas graft had improved to 87% [15, 16]. Those results clearly demonstrated that the segmental pancreas was able to normalize the glucose metabolism in patients with severe diabetes.

Based on the shortage of deceased donors in Japan and the excellent outcome obtained at the University of Minnesota, the first LDSPK in this country was performed on 7 January, 2004, for a type 1 diabetic patient with ESRD for whom the donor was the father [11]. Donor safety has been the most important consideration in the conduct of LDSPK. The donor criteria for pancreas transplantation, as shown in Table 2, were determined by transplant surgeons, diabetologists, nephrologists, nurses, and transplant coordinators. The stringent Minnesota criteria were applied and were modified according to the lower ability among the Japanese to secrete insulin from the islets. As a tool for endocrinological evaluation, CS1, which is calculated by the sum of the C-peptide secretion rates from 0 to 5 min after glucose injection, is used in the IV-GTT. Tokuyama et al. [13] demonstrated that the sum of the C-peptide secretion rates was directly correlated with β-cell function and they showed that the CS1 expressed the first phase of insulin release.

The outcome of the 12 living pancreas transplants performed at this institution was excellent. The 1-year survivals of the patients, kidney grafts (LDSPKs), and pancreas grafts were 100, 100, and 100%, respectively, and the 3-year survivals were 91.7, 90, and 91.7%, respectively. The plasma glucose levels started to decrease at the time of the operation in almost all patients. Although exogenous insulin administration was required from 1 to 30 days after transplantation because of the administration of a high dose of steroid and hyperalimentation, all but one of the patients achieved insulin independence. One patient who underwent LDSPK showed primary nonfunction of the pancreas graft. Blood flow was recognized in both the pancreas and kidney grafts, using an ultrasonography power Doppler technique. Her kidney graft functioned immediately and the serum creatinine levels had decreased below 1.0 mg/dl at 3 days after transplantation. One-hour biopsy of the pancreas graft showed normal structure of both the exocrine tissue and islets. Although the mechanism of the development of primary nonfunction in this patient is unknown, an autoimmune response may be one of the possible explanations, because her anti-GAD antibody was extremely high, at 2,940 U/ml. The other patients who underwent LDSPK achieved both insulin independence and hemodialysis independence and showed a marked change in their outlook on life.

The leakage of pancreatic juice was the most frequent problem as a surgical complication after living pancreas transplant. Although a surgical procedure was required to treat the bleeding in one patient, three patients who developed leakage of the pancreatic juice maintained the function of the pancreatic graft. No other surgical complication was observed in the 12 patients, which demonstrated that the safety seemed to be almost the same as that of kidney transplantation. The frequency of acute rejection, including ACR and AMR, was also almost the same as that seen with kidney transplantation under standard immunosuppression.

In the series of living pancreas transplants conducted at, Chiba-East National Hospital, excellent outcomes were observed in both the recipients and the donors. Further consideration is needed in order to establish this procedure in this country as one of the therapies for patients with severe diabetes. The major issue must be the safety of the donor, especially of the donor for LDSPK. Hand-assisted laparoscopic surgery (HALS) was introduced for simultaneous nephrectomy and distal pancreatectomy in two recent donors for LDSPK. The operation was performed according to a previously reported technique [17]. The pancreas, however, was dissected directly from the open 7-cm wound using the Multiflap Gate (MD49611, Sumitomo Bakelite Co. Ltd., Tokyo). The two donors who underwent a HALS operation rapidly recovered after the operation and no analgesics were needed apart from the epidural administration of local anesthetics. The introduction of this procedure may therefore help to improve donor safety. In addition, long-term maintenance of the metabolism in the donor is still an important problem. In our present experience, the donors have not developed diabetes during an observation period of up to 5 years. Increased levels of HbA1C were, however, observed in two donors (6.0,

6.1%). Therefore, all donors are followed up by the diabetologist and a nephrologist, in addition to the transplant surgeons, at our hospital.

As previously reported, evaluation of the quality of life of LDSPK recipients, using a short-form 36 version 2 showed rapid increases in both the physical and mental summary scores in patients after LDSPK. In addition, the donors maintained their presurgical levels of both scores after the surgery [18].

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Regular Article

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

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Full text of this paper is available at http://www.jstage.jst.go.jp/browse/dmpk

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], $r^2 = 0.992$).

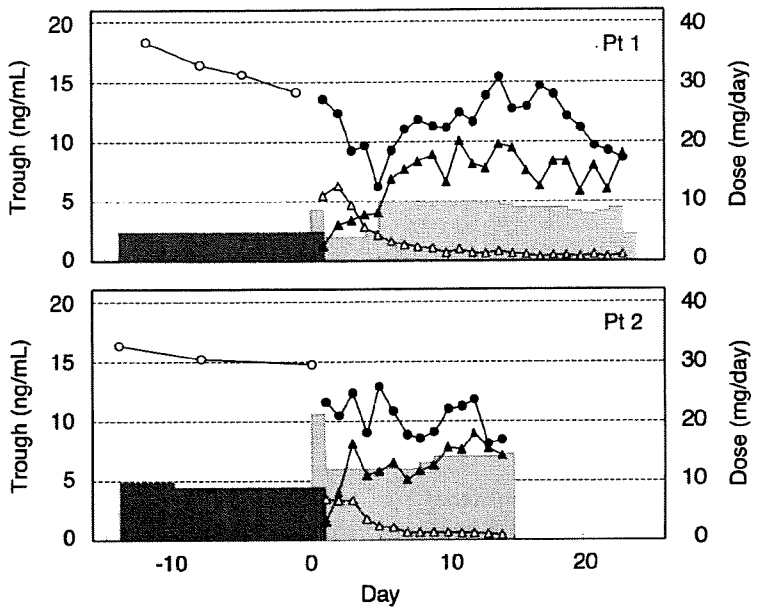

Fig. 1. Trough blood concentrations of sirolimus measured by HPLC-UV (open circles) and LC-MS (open triangles) and those of everolimus measured by FPIA (closed circles) and LC-MS (closed triangles) are plotted for each patient
Dark and light shaded areas show daily dosages of sirolimus and everolimus,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

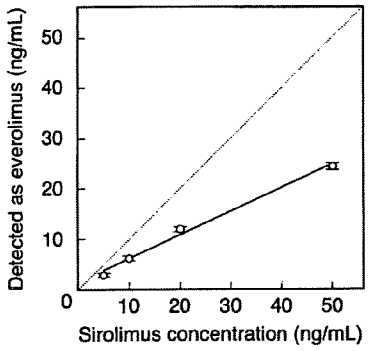

Fig. 2. Sirolimus blood concentrations measured by the FPIA method for everolimus. Each point represents the mean ± SD (n = 3)
The solid line shows the fitting line. The dotted line represents the line of identity (*i.e.*, slope = 1).

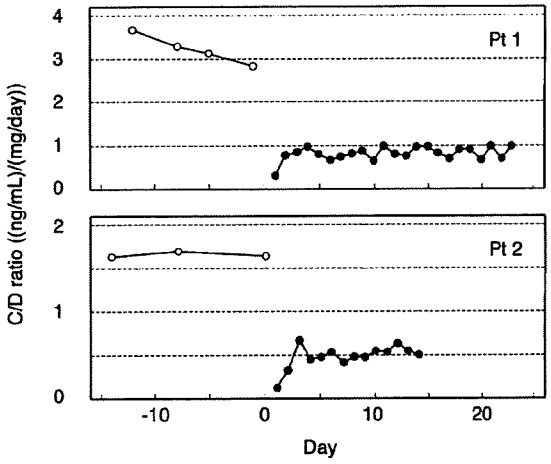

Fig. 3. The trough concentration per dose (C/D) ratios of sirolimus (open circles) and everolimus (closed circles) were plotted for each patient

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

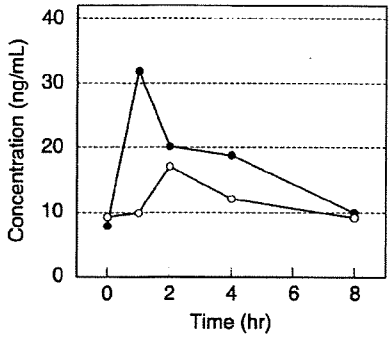

Fig. 4. Everolimus blood concentration profiles after oral administration in the two patients
Open and closed circles show everolimus concentration of patient 1 and patient 2, respectively.

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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Comparison of Trypsin Inhibitors in Preservation Solution for Islet Isolation

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Islet transplantation has recently emerged as an effective therapy and potential cure for type 1 diabetes mellitus. Recent reports show that the two-layer method (TLM), which employs oxygenated perfluorochemical (PFC) and University of Wisconsin (UW) solution, is superior to simple cold storage in UW for pancreas preservation in islet transplantation. Moreover, we recently reported that islet yield was significantly higher in the ET-Kyoto solution with ulinastatin (MK)/PFC preservation solution compared with the UW/PFC preservation solution in the porcine model and that the advantages of MK solution are trypsin inhibition and less collagenase inhibition. In this study, we compared ulinastatin with another trypsin inhibitor, Pefabloc, in preservation solution for islet isolation. Islet yield before purification was higher in the MK/PFC group compared with the ET-Kyoto with Pefabloc (PK)/PFC group. The stimulation index was higher for the MK/PFC group than for the PK/PFC group. These data suggest that ET-Kyoto with ulinastatin was the better combination for pancreas preservation than ET-Kyoto with Pefabloc. Based on these data, we now use ET-Kyoto solution with ulinastatin for clinical islet transplantation.

Key words: Islet transplantation; Islet isolation; MK solution; Trypsin inhibitor; Preservation solution

INTRODUCTION

Since the report of the Edmonton protocol (37), islet transplantation has advanced significantly and more than 600 type 1 diabetes patients in more than 50 institutions have undergone islet transplantation to cure their disease. The cadaveric pancreas is injured due to brain death, hypotension, and vasopressor therapy, and subsequently from warm ischemia after donor cross-clamping and cold ischemic storage. There is a clear relationship between these injuries and the reduced success of subsequent islet isolation (4,12). In Japan, pancreatic islets are isolated from non-heart-beating donors (NHBDs) for clinical islet transplantation because donations from heart-beating brain-dead donors are only two to five cases per year and most of their pancreata are used for pancreas organ transplantation. We therefore need to develop an efficient isolation technique for NHBD pancreata.

We have recently demonstrated that islet isolation and transplantation with NHBDs using the modified Ricordi method (Kyoto islet isolation method) effectively cures type 1 diabetes (23). The transplantation rate (transplantation number/isolation number) is more than 80%, higher than recently published data using brain-dead heart-beating donors (14,21,31). The isolation method includes an in situ cooling system for pancreas procurement (19), ductal injection (28), the modified two-layer method (MK/PFC) (27,30), and iodixanol-based purification (14). We previously showed that MK/PFC preservation significantly improved islet yields, compared with UW/PFC preservation (30). MK solution includes a trypsin inhibitor (ulinastatin), which is one of the advantages of this solution. Indeed, pancreas preservation using MK solution was superior to preservation with ET-Kyoto solution without the trypsin inhibitor in a rat model (30).

In this study, we compared ulinastatin with another trypsin inhibitor, Pefabloc, in preservation solution for islet isolation.

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MATERIALS AND METHODS

Preservation Solution

We used ET-Kyoto solution (5,32) with ulinastatin (Miraclid®, Mochida Pharmaceutical, Tokyo, Japan; ET-Kyoto + ulinastatin = "MK solution") or Pefabloc (Roche Applied Science, Germany; ET-Kyoto + Pefa bloc = "PK solution"). The components of the solutions are shown in Table 1.

Measurement of Trypsin Inhibition Ability of Solutions

In order to assess the trypsin inhibition of MK solution, PK solution, and ET-Kyoto solution without trypsin inhibitors (control), 3 ml of 0.3 mM *N*-benzoyl-L-arginine ethylester reagent (BAEE; Sigma, Tokyo, Japan) were incubated for 5 min at 25°C and then 5 µl of 1 mg/ml trypsin and 45 µl of each solution were added. Trypsin activity was measured by absorption spectrophotometry (λ253 nm) using BAEE for the trypsin substrate, according to a previous report (17). Absorbance was measured every minute for 6 min. A BAEE unit was defined as a change in optical density of 0.001/min.

Porcine Islet Isolation

Porcine pancreata were obtained at a local slaughterhouse. The operation was started about 10 min after the cessation of heart beating. After removing the pancreas, we immediately inserted a cannula into the main pancreatic duct, infused each preservation solution for ductal protection, and put the pancreas into a two-layer preservation container that had one of the preservation solutions (preservation solution/PFC). Operation time was defined as the time elapsed between the start of operation and removal of the pancreas. Warm ischemic time (WIT) was defined as the time elapsed between cessation of heart beating and placement of the pancreas into the preservation solution. Cold ischemic time (CIT) was defined as the time elapsed between placement of the pancreas into the preservation solution and the start of islet isolation.

Islet isolation was conducted in accordance with the method described in the Edmonton protocol (15,16,33, 34,37). In brief, after decontamination of the pancreas, the ducts were perfused in a controlled fashion with a cold enzyme blend of Liberase HI (1.4 mg/ml; Roche Molecular Biochemicals, Indianapolis, IN). The distended pancreas was then cut into nine pieces, placed in a sterilized Ricordi chamber, and shaken gently. While the pancreas was being digested by recirculating the enzyme solution through the Ricordi chamber at 37°C, we monitored the extent of digestion with dithizone staining by taking small samples from the system. Once digestion was completed, RPMI-1640 medium (Gibco, Carlsbad, CA) was introduced into the system, and the system was cooled to stop further digestive activity. The digested tissue was collected and washed with fresh medium to remove the enzyme. The phase I period was defined as the time between placement of the pancreas in the Ricordi chamber and the start of collecting the digested pancreas. The phase II period was defined as the time between the start and end of collection.

Islets were purified with a continuous density gradient with Iodixanol-Kyoto solution in an apheresis system (COBE 2991 cell processor, Gambro Laboratories, Denver, CO). Because Iodixanol has a lower viscosity than Ficoll, it needs less force during centrifugation, which causes less damage to islets. For the solution, low-density (density: 1.077) and high-density (density: 1.100–1.125) Iodixanol-Kyoto solutions were produced by changing the volumetric ratio of Iodixanol and Kyoto solution.

Islet Evaluation

The crude number of islets in each diameter class was determined by counting islets after dithizone staining (3 mg/ml, final concentration) (Sigma Chemical Co., St. Louis, MO) using an optical graticule. The crude number of islets was then converted to the standard number of islet equivalents (IE; diameter standardizing to 150 µm) (35). Gross morphology was qualitatively assessed by two independent investigators scoring the islets for shape (flat vs. spherical), border (irregular vs. well-rounded), integrity (fragmented vs. solid/compact), uniformity of staining (not uniform vs. perfectly uniform), and diameter (least desirable: all cells <100 µm/most desirable: more than 10% of the cells >200 µm) (16,35). Each parameter was graded from 0 to 2 with 0 equaling the worst and 2 the best score, so that the worst islet preparations were given a cumulative score of 0 and the best a score of 10. Spherical, well-rounded, solid/compact, uniformly stained, and large islets were characterized as the best islets.

Islet viability after purification was assessed using acridine orange (10 µmol/L) and propidium iodide (15 µmol/L) (AO/PI) staining to visualize living and dead

Table 1. Composition of Each Preservation Solution

	MK	PK
Na (mmol/L)	100	100
K (mmol/L)	43.5	43.5
Gluconate (mmol/L)	100	100
Phosphate (mmol/L)	25	25
Trehalose (mmol/L)	120	120
Hydroxyethyl starch (g/L)	30	30
Ulinastatin ($\times 10^3$ U/L)	100	—
Pefabloc (mg/L)	—	1000

islet cells simultaneously (3,16,35). Fifty islets were inspected and their individual viability was determined visually, followed by calculation of their average viability (16).

In Vitro Assessment of Islet Function

Islet function was assessed by monitoring the insulin secretory response of the purified islets during glucose stimulation according to a procedure described by Shapiro and colleagues (37). Briefly, 1200 IE were incubated with either 2.8 or 25 mM glucose in RPMI-1640 for 2 h at 37°C and 5% CO_2. The supernatants were collected and insulin levels were determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (ALPCO Insulin ELISA kit; ALPCO Diagnostics, Windham, NH). The stimulation index was calculated by determining the ratio of insulin released from islets in high glucose concentration to the insulin released in a low concentration. The data were normalized to total protein from the cell lysate. All assessments were made in triplicate and the data (mean ± SE) were expressed as a percentage of the control values in each experiment to eliminate variables caused by differences among donor pancreata.

Recently, Goto et al. showed that the measurement of the ADP/ATP ratio correlated with transplantation outcome (8). The ADP/ATP ratio was measured to evaluate the energy status of cultured islets, using the Apo Glow™ kit (Cambrex Bio Science Nottingham Ltd., Nottingham, UK). In brief, 80 IEs were washed in PBS and then mixed with 100 µl of nucleotide-releasing reagent for 10 min at room temperature. Thereafter, 20 µl of nucleotide-monitoring reagent was added to the solution. The ATP levels were measured using a luminometer (FB 12 Luminometer, Berthold Detection Systems GmbH, Pforzheim, Germany) and expressed as the number of relative light units (RLU). After 10 min, the ADP in the solution was converted to ATP by adding 20 µl ADP converting reagent and then measured as the number of RLU. Subsequently, the ADP/ATP ratio of the islets was calculated.

In Vivo Assessment of Islet Function

Mice with severe combined immunodeficiency disease (SCID; CLEA Japan, Inc., Meguro, Tokyo) were used for the experiments. The recipients were rendered diabetic by a single injection of streptozotocin (STZ) at a dose of 220 mg/kg. Hyperglycemia was defined as a glucose level of >350 mg/dl detected twice consecutively after STZ injection. The 2000 IE pig islets obtained from each group were transplanted into the renal subcapsular space of the left kidney of diabetic SCID mice. During the 30-day posttransplantation period, the nonfasting blood glucose levels were monitored three times per week. Normoglycemia was defined when two consecutive blood glucose level measurements showed less than 200 mg/dl. No statistical differences in either pretransplantation blood glucose levels or pretransplantation body weight were observed among the four groups of mice. Mouse studies were approved by the Institutional Animal Research Committees of Kyoto University, Nagoya University, and Fujita Health University.

Statistical Analysis

Values for the data represent the mean ± SE. Two or three groups were compared by Student's *t*-test with Bonferroni correction.

RESULTS

Inhibition of Trypsin Activity

Previous reports show that trypsin inhibition with TLM preservation improves islet yields (17,30). We examined whether MK and PK solutions inhibited trypsin activity. Both solutions inhibited trypsin activity (Fig. 1) compared with ET-Kyoto solution (control) ($p < 0.01$), suggesting that these solutions could be useful in reducing trypsin activity during pancreas preservation.

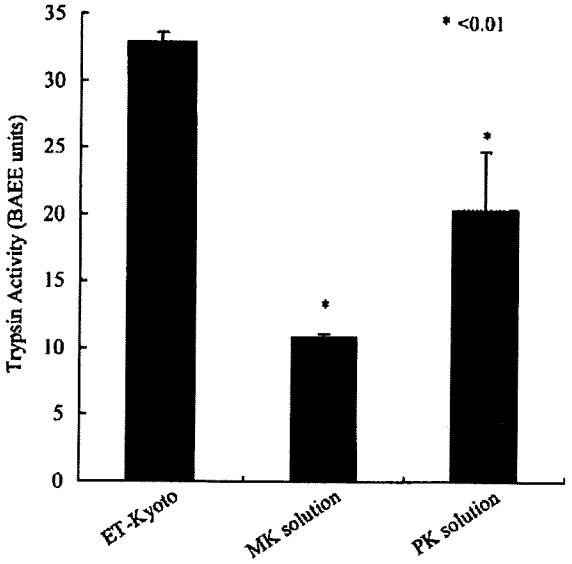

Figure 1. Impact of MK or PK solution on trypsin activity. BAEE was incubated for 5 min at 25°C and then trypsin along with MK solution ($n = 7$), PK solution ($n = 7$), or ET-Kyoto (control; $n = 7$) was added. Trypsin activity was measured by absorption spectrophotometry (λ253 nm) using BAEE reagent. Absorbance was measured every minute for 6 min. A BAEE unit was defined as a change in optical density of 0.001/min. MK and PK solutions inhibited trypsin activity significantly more than ET-Kyoto ($p < 0.01$). Data are expressed as the mean ± SE.

Table 2. Pig Islet Isolation Characteristics

	MK	PK
Pancreas size (g)	105.0 ± 8.9	87.3 ± 3.4
Operation time (min)	8.0 ± 1.0	4.3 ± 0.9
Warm ischemic time (min)	27.2 ± 1.7	25.7 ± 2.8
Cold ischemic time (min)	123.6 ± 1.6	122.3 ± 1.2
Phase I period (min)	10.2 ± 1.8	7.7 ± 1.2
Phase II period (min)	35.0 ± 3.0	29.7 ± 3.2

Data are expressed as mean ± SE.

Table 3. Pig Islet Characteristics

	MK	PK
Viability (%)	97.5 ± 0.5	96.5 ± 1.6
Score	9.2 ± 0.3	8.7 ± 0.2
Purity (%)	66.0 ± 6.8	70.0 ± 5.8
Recovery rate (%)	68.1 ± 8.4	70.5 ± 13.6
Stimulation index	2.50 ± 0.21*	1.37 ± 0.18

Data are expressed as mean ± SE.
*Stimulation index was higher for the MK/PFC group than for the PK/PFC group ($p < 0.05$).

Porcine Islet Isolation Characteristics

The characteristics of porcine islet isolation protocols are shown in Table 2. There were no significant differences in pancreas size, operation time, WIT, or CIT between the two groups. Phase I and phase II periods were also similar for the two groups.

Islet yield before purification was significantly higher in the MK/PFC group ($n = 5$) than the PK/PFC group ($n = 3$) (MK/PFC; 9676 ± 635 IE/g, PK/PFC; 6999 ± 844 IE/g, $p < 0.05$) (Fig. 2A). The islet yield after purification for the MK/PFC group was higher than the PK/PFC group (MK/PFC; 6608 ± 927 IE/g, PK/PFC; 4964 ± 1153 IE/g) but not significantly so (Fig. 2B). Other porcine islet characteristics are shown in Table 3. The stimulation index was higher for the MK/PFC group than for the PK/PFC group ($p < 0.05$). There were no other significant differences in characteristics between the two groups.

Assessment of Islet Function In Vitro and In Vivo

Recently, Goto et al. showed that the measurement of the ADP/ATP ratio correlated with transplantation outcome (8). To assess the islet graft function of each group in vitro, the ADP/ATP ratio was measured. There was no significant difference in ADP/ATP ratio between the groups (data not shown).

To assess the islet graft function of each group in vivo, 2000 IE of each group were then transplanted below the kidney capsule of STZ-induced diabetic SCID mice. There was no significant difference between the groups with respect to the attainability of posttransplantation normoglycemia (data not shown). Morphologic

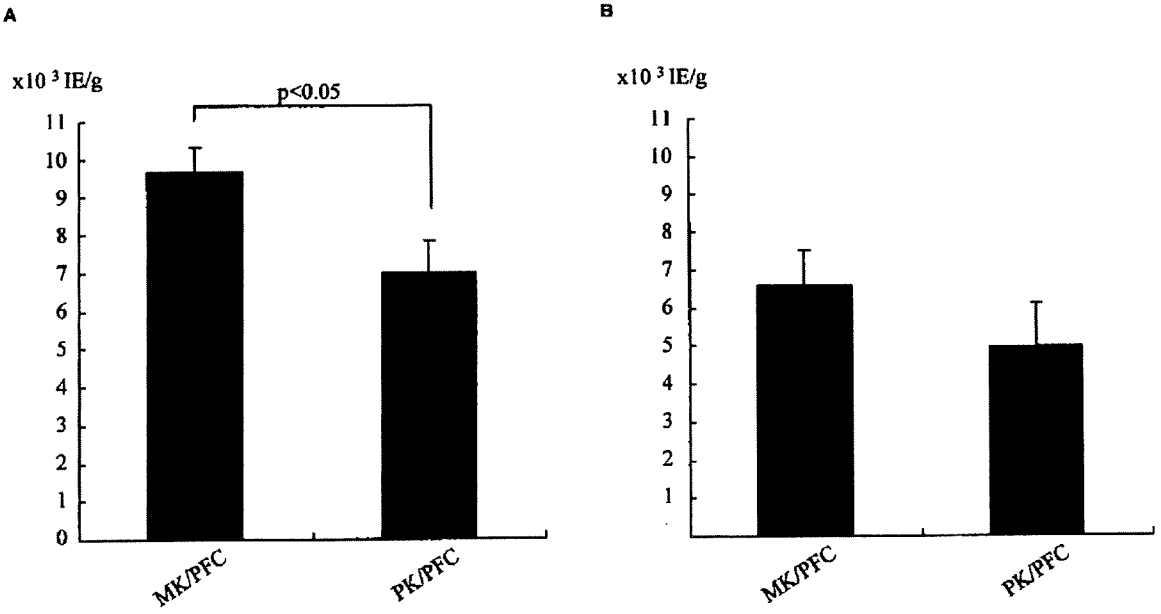

Figure 2. Islet yield before purification (A) and after purification (B). The MK/PFC group had significantly better islet yield before purification than the PK/PFC group ($p < 0.05$). Data are expressed as the mean ± SE.

studies showed the presence of islets under the kidney capsule of all SCID mice 30 days after transplantation. The islet grafts of each group in the normoglycemic mice showed intense insulin staining (data not shown).

Taken together, these data suggest that MK/PFC preservation is superior to PK/PFC preservation.

DISCUSSION

Islet allotransplantation can achieve insulin independence in patients with type 1 diabetes (37). Since the Edmonton protocol was announced, islet transplantations from brain-dead donors (9,10) as well as from non-heart-beating donors (13,14,21) and living donors (15) have been performed. These advances were based on advanced pancreas transport systems (10,16,17,30), revised immunosuppressant protocols (24,25), improved islet isolation methods (17), and enhanced islet engraftment (26). Although experiments of β-cell regeneration from stem cells have proceeded (20,22,29), there is still no reliable method for producing β-cells. Until a new method to generate β-cells is developed, improving the efficacy of islet transplantation seems the most realistic and prudent method to cure diabetes.

Donor pancreata are usually preserved with University of Wisconsin (UW) solution. Recent reports have shown that the two-layer method (TLM), which employs oxygenated perfluorochemical (PFC) and UW solution, is superior to simple cold storage in UW to preserve not only the whole pancreas but also individual islets for transplantation (16,17). However, UW solution has several disadvantages: it is chemically unstable, it must be cold stored until use, and its short shelf life makes it expensive. It is also highly viscous, which may complicate the initial organ flush (39). Recently, our university developed a new preservation solution, ET-Kyoto solution, and its effectiveness in cold lung storage has been demonstrated in clinical lung transplantation (5,32). It also is effective for skin flap storage and its clinical application is beginning in this field (40). Although high potassium in UW solution causes insulin release from pancreatic β-cells (7), ET-Kyoto solution has a high sodium/low potassium composition. Moreover, UW solution inhibits the activity of Liberase, an enzyme blend for pancreatic digestion (6,36), but ET-Kyoto solution with ulinastatin inhibits Liberase less (30).

Trypsin from pancreatic acinar cells destroys islets. Previous studies have shown that trypsin inhibition by Pefabloc during human pancreas digestion improves islet yield and reduces the fraction of embedded islets (11,17), suggesting that trypsin may degrade the ductules and thus reduce the delivery of collagenase solution to the immediate neighborhood of the islets. Previously, we demonstrated that modifying TLM preservation, by including ulinastatin, eliminated trypsin activity during pancreas preservation, and ET-Kyoto/PFC preservation without ulinastatin resulted in lower islet yields (30). In this study, we showed that MK solution was synthetically superior to PK solutions. It may be due to differences in inhibitory effects of cytokines. Ulinastatin has been shown to inhibit not only trypsin activity but also the release of neutrophil elastase. It also downregulates transcription of tumor necrosis factor mRNA, the activation of endothelial cells, and the expression of ICAM-1 induced by endotoxin in vitro (1,2,18). It has been shown that administration of ulinastatin decreased the ischemia-reperfusion injury (38) and attenuated the elevation of inflammatory cytokines and C-reactive protein, a marker of inflammation (41) in transplanted small intestine.

In conclusion, we show that ET-Kyoto with ulinastatin is a better combination for pancreas preservation than ET-Kyoto with Pefabloc. Based on these data, we now use the ET-Kyoto solution with ulinastatin for clinical islet transplantation from NHBD pancreata. MK/PFC preservation makes it feasible to use NHBDs for efficient islet transplantation into type 1 diabetes patients.

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