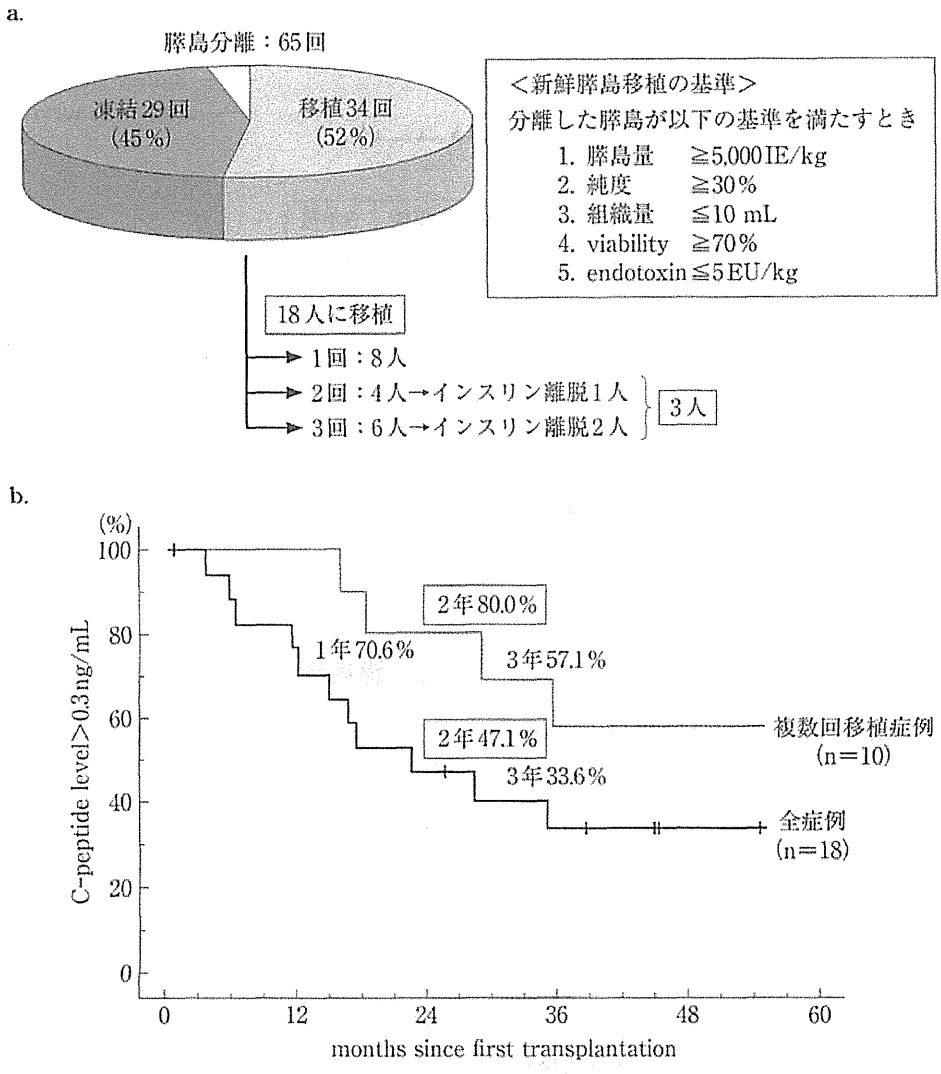

図2 我が国の膵島分離・移植回数(a)，膵島生着率(b)

否かについて一定の基準を設けている．我が国では，膵島分離後にレシピエント体重あたり5,000 IE/kg以上の収量があり，純度30％以上，組織量10 mL以下，viability 70％以上，エンドトキシン5 EU/kg以下，グラム染色陰性などの基準を膵島分離の結果が満たした場合に膵島移植が行われる．我が国では，2004年に初めての臨床膵島移植が実施され，以降2007年3月までに65回の膵島分離が行われ，このうち34回で移植の条件を満たしていたため18症例に対して膵島移植が行われた[9]．1例の脳死ドナーを除く64回は心停止ドナーからの提供であった．前述の'エドモントン・プロトコール'に準じて行われ，移植回数は1回8人，2回4人，3回6人であった．これらの症例のうち，2回移植の1例と3回移植の2例の計3症例で一時的にインスリンを離脱しえた(**図2-a**)．初回移植後1年，2年，3年時における膵島生着率はそれぞれ70.6％，47.1％，33.6％であったが，複数回移植症例10例のみに限ると，初回移植後1年，2年，3年時における膵島生着率はそれぞれ100％，80％，57.1％であった(**図2-b**)．

膵島移植の適応基準は，①内因性インスリン分泌が著しく低下し，インスリン治療を必要とする状態で，②糖尿病専門医の治療努力によっても血糖コントロールが困難な，③75歳以下の患者，としている．糖尿病性腎症に関しては，膵島単独移植の場合はIII A期までを適

応とし，腎移植後膵島移植症例では，移植後6カ月以上経過し，クレアチニン1.8 mg/dL以下で直近6カ月の血清クレアチニンの上昇が0.2以下，などの基準を満たす症例を移植の対象としている．2011年9月末の時点でレシピエント候補者として124人が待機中である．

3 膵臓・膵島移植の問題点と今後の展開

我が国の膵臓移植はいわゆる'マージナルドナー'が多い．マージナルドナーの定義を，45歳以上，不安定な血行動態による高用量カテコールアミンの使用，心停止ドナーとすると，マージナルドナーにより移植となった症例は前述61例中45例と，マージナルドナーを移植に供しない欧米に比して極めて高率である[3]．このような場合，移植手術，術後管理には高度な技術と経験が必要となるため，日本膵・膵島移植研究会において支援体制を構築し，臓器摘出，移植手術，術後管理まで必要な支援を行い，良好な成績を維持している．

膵島移植の主な問題点として，膵臓移植に比して長期のインスリン離脱が難しいことと，1人のレシピエントに複数のドナーを必要とすることが挙げられる．欧米では抗胸腺細胞グロブリン，抗TNFα抗体による導入療法に続いて，低用量タクロリムス，シロリムス主体の維持療法を行う方法により比較的長期のインスリン離脱が期待されており[10]，北米を中心に多施設共同第III相臨床試験が行われている．我が国でもこの方法を踏襲したプロトコールを作成し，多施設共同臨床試験の実施体制を整えた(UMIN試験ID: UMIN000003977)．このプロトコールは，膵島に対する自己免疫反応の抑制，拒絶反応の予防，移植直後におけるカルシニューリン阻害剤の減量，制御性T細胞の誘導，移植膵島に対する非特異的免疫反応の抑制などにより，移植膵島の生着率を向上させることを目的としている．また，膵島移植は，2010年11月に第3項先進医療(高度医療)の承認を受けており(先進医療技術名：重症低血糖発作を伴うインスリン依存性糖尿病に対する心停止ドナーからの膵島移植)，膵島移植を高度医療評価制度の下で実施できる体制にある．

おわりに

本稿では，1型糖尿病に対する移植治療として存在する膵臓移植と膵島移植の現状について紹介した．世界共通あるいは我が国特有の問題を解決しつつ，今後も治療成績の向上を目指した試みが継続されることが望まれる．

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Results of Islet Isolation and Their Relationship to the Clinical Outcome of Kidney Transplantation in Cases Where Both Grafts Are Harvested From the Same Non-Heart-Beating Donor

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Grafts from non-heart-beating donors (NHBDs) are used because of the limited availability of heart-beating brain-dead donors. These grafts sustain ischemic damage, and the severity of this damage varies among different areas of an organ. This study determined whether the results of islet isolation were correlated with the clinical outcomes of kidney transplantations in cases where both grafts were harvested from the same NHBD. Islets we isolated from the pancreata of 23 NHBDs between February 2004 and March 2007. Forty-six kidneys were also harvested from these NHBDs. The recipients of kidney transplants were divided into the successful isolation ($n = 14$) and failed isolation ($n = 32$) groups depending on the results of islet isolation. The clinical outcomes of kidney transplantation were compared between the recipients in these two groups. The immediate graft function rate and the 1-year graft survival rate after kidney transplantation in both groups were similar. Hemodialysis after transplantation was required for 6.0 days (SD, 5.2 days) in the successful isolation group and for 12.7 days (13.1 days) in the failed isolation group ($p < 0.05$). The serum creatinine concentrations at 1, 3, 6, and 12 months after transplantation were elevated in the failed isolation group ($p < 0.05$). The islet yield was inversely correlated with the requirement of hemodialysis (days) and the serum creatinine level at 1 month after transplantation. However, hemodialysis was required for only 7 days in the recipients of six kidneys that were obtained from NHBDs from whom <40,000 IEQ were obtained (extreme failure of islet isolation). The results of islet isolation were found to correlate with the kidney function after transplantation when both grafts are harvested from the same NHBD. However, the marginal conditions of NHBDs affect the results of islet isolation more than they do the posttransplantation kidney function.

Key words: Islet isolation; Kidney transplantation; Non-heart-beating donor; Correlation

INTRODUCTION

Grafts from non-heart-beating donors (NHBDs) are now used for kidney, pancreas, liver, and even lung transplantation because of the limited availability of heart-beating brain-dead donors (3). Grafts from NHBDs sustain ischemic damage before and after cardiac death, and the severity of this damage may vary among the different areas of an organ. Islets are usually not harvested from heart-beating brain-dead donors in Japan. NHBDs are the only source of islets for transplantation. The technique of procuring pancreatic grafts and isolating islets from damaged pancreatic grafts has been improved (5) and has yielded promising results: 7 of 23 isolations (30.4%) were successful. The results of islet transplantation were compared with the clinical outcomes of kidney transplantation in cases where both grafts were obtained from the same donor. This study was undertaken to determine whether the clinical outcomes of kidney transplantation could be predicted on the basis of the results of islet isolation.

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MATERIALS AND METHODS

This study retrospectively analyzed the isolation islets from 23 pancreata and the transplantation of 46 kidneys harvested from the same NHBDs between February 2004 and March 2007. All the NHBDs had sustained serious brain injuries such as intracranial hemorrhage, trauma, and hypoxia, but did not meet the diagnostic criteria for brain death or the criteria for organ donation upon brain death (in Japan, donation from brain-dead donors is strictly regulated by law). The aorta was cannulated before the occurrence of cardiac arrest in 13 donors. Withdrawal of ventilatory support prior to cardiac arrest was performed in only two donors; cardiac arrest occurred prior to the withdrawal of ventilatory support in the case of the other donors. The abdominal aorta was perfused with cold University of Wisconsin (UW) solution or Euro Collins (EC) solution after the declaration of death if the cannulation had been performed before death. If not, the donor was transferred to the operating room, and closed chest massage was performed until the operation was initiated. A laparotomy was then performed, and the abdominal aorta was cannulated from the iliac artery and perfused with cold UW solution or EC solution. Crushed Ringer ice was placed around the kidney and into the omental sac to facilitate local cooling of the kidney and pancreas. The pancreatectomy was performed after the removal of both kidneys. The warm ischemic time (WIT) was defined as the interval from cardiac arrest to the start of cold perfusion. The cold ischemic time (CIT) of the kidney was defined as the time from the start of cold perfusion to reperfusion with an arterial flow in the recipient. The CIT of the pancreas was defined as the time from the start of cold perfusion to the injection of collagenase into the pancreatic duct.

Seventeen pancreata were preserved using a two-layer method (6,8), and six pancreata were preserved by simple storage in cold UW solution. Islets were isolated in the cell-processing center in accordance with the Edmonton protocol (10). In brief, the pancreatic duct was cannulated and perfused with cold Liberase solution (Liberase HI; Roche Diagnostics, IN, USA). The pancreas was enzymatically and mechanically dissociated and purified in Euro-Ficoll solution with a discontinuous gradient by using a refrigerated COBE 2991 Cell Processor. The standard islet diameter was assumed to be 150 μm, and the islets were counted twice (9). Islet isolation was considered successful if more than 200,000 islet equivalents (IEQ) with a purity of >30% were recovered after islet purification.

Furthermore, 46 kidneys were harvested from the 23 NHBDs from whom the pancreata were collected using the procedure described above. The recipients of the kidneys were administered immunosuppressive therapy of a calcineurin inhibitor (cyclosporin or tacrolimus), antimetabolites (azathioprine or mycophenolate mofetil), and corticosteroids along with induction therapy involving anti-CD25 monoclonal antibody (basiliximab). The immediate kidney function after transplantation was defined as whether or not there was a need to perform hemodialysis after transplantation. All clinical data related to the kidney transplantations were deposited with the Japan Organ Transplantation Network.

The Institutional Review Board at NHO Chiba-East National Hospital approved all protocols.

The two-sided unpaired *t*-test or the Mann-Whitney test was used to compare the groups, as appropriate. Pearson's correlation coefficient test was used to evaluate any correlations among the factors.

RESULTS

Donor Characteristics

The median age of the 23 NHBDs was 44.0 years (range 14–69 years). A total of 10 donors (43.5%) died of cerebrovascular disease; seven of brain edema after hypoxia; five of trauma; and one of brain tumor. Cardiac arrest occurred before arrival at the hospital in eight donors. The median serum creatinine concentration was 122 μmol/L (range 53–896 μmol/L), and the median hospital stay was 9 days (range 2–84 days).

Islet Isolation

The median WIT and CIT of the pancreata were 5 min (range 1–30 min) and 309 min (range 211–540 min), respectively. The median weight of the pancreas was 92 g (range 37–134 g). The islet yield and purity were 126,740 IEQ (range 0–369,427 IEQ) and 40% (range 1–80%), respectively. Islet isolation was successful in seven cases, and the harvested islets were used for transplantation in six of these seven cases. Islets could not be harvested, had to be discarded, or were cryopreserved in the remaining 16 isolations. The recipients of kidney transplants were divided into the successful isolation (SI) and failed isolation (FI) groups depending on the results of islet isolation (Table 1).

Clinical Outcomes of Kidney Transplantation

The overall graft survival at 12 months after transplantation was 87.0%, that in the SI group was 85.6%, and that in the FI group was 87.5%. The difference between the groups was not significant. The immediate graft function rate was 21.4% in the SI group and 6.25% in the FI group (N.S.). Hemodialysis was required for 6.0 days (SD 5.2 days) in the SI group and 12.7 days (13.1 days) in the FI group ($p < 0.05$).

The mean serum creatinine concentrations in patients with functioning grafts at 1, 3, 6, and 12 months after

Table 1. Islet Isolation Characteristics

Variables	Success Group ($n = 7$)	Failed Group ($n = 13$)	p-Value
WIT (min)	8.3 ± 7.4	10.6 ± 9.6	N.S.
CIT (min)	282.9 ± 60.0	329.8 ± 90.7	N.S.
S-creatinine (mmol/L)	276.0 ± 349.2	193.1 ± 149.9	N.S.
Hospital stay (day)	12.7 ± 5.8	14.3 ± 20.0	N.S.
Episode of cardiac arrest	1 (14.3%)	7 (43.8%)	N.S.
Two-layer method	7 (100%)	10 (62.5%)	N.S.
Graft weight (g)	94.7 ± 27.2	82.6 ± 23.4	N.S.
Yield (IEQ)	324,430 ± 93,138	87,757 ± 64,049	$p < 0.0001$
Yield (IEQ/g)	3,575 ± 1,135	1,158 ± 866	$p < 0.0001$
Purity (%)	50.0 ± 15.3	31.6 ± 23.1	N.S.

WIT, warm ischemic time; CIT, cold ischemic time; IEQ, islet equivalents. N.S., not significant.

transplantation were elevated in the FI group in comparison with the SI group ($p < 0.05$). These values were 92.8, 95.5, 98.1, and 93.7 μmol/L in the SI group and 205.1, 146.7, 149.4, and 147.0 μmol/L in the FI group (Fig. 1).

Correlation Between the Islet Yield and Clinical Outcome of Kidney Transplantation

The islet yield (IEQ) was inversely correlated with the number of days for which hemodialysis was required (Fig. 2A) and with the serum creatinine concentration at 1 month after kidney transplantation (Fig. 2B).

Discrepancy Between the Islet Yield and Clinical Outcome of Kidney Transplantation

Hemodialysis was required for only 7 days in six recipients of kidneys that were obtained from NHBDs from whom <40,000 IEQ were obtained (extreme failure of islet isolation). The conditions of the donors from which these grafts had been harvested were extremely poor. These donors had undergone cardiopulmonary resuscitation, received high doses of catecholamines, or had high serum creatinine concentrations (Table 2).

DISCUSSION

This study presents the relationship between the results of islet isolation and the clinical outcomes of kidney transplantation in cases where both the islet grafts and kidneys are harvested from the same NHBD. The antemortem conditions of the NHBDs varied. Even in the case of heart-beating brain-dead donors, the results of islet isolation depend on several factors (1). The condition of grafts obtained from NHBDs is worse than that of grafts obtained from heart-beating brain-dead donors because of severe warm ischemic damage. Nevertheless, pancreata harvested from NHBDs are now used for islet transplantation owing to the improvement in islet procurement and isolation techniques (7). NHBDs are an important source of kidneys for transplantation. Some large series of kidney transplants recovered from NHBDs have been reported (2,11,12). However, this is the first report to describe the relationship between the results of islet isolation and the clinical outcomes of kidney transplantation in cases where both grafts are obtained from the same donor.

In general, patients who receive kidneys from NHBDs experience long periods of oliguria and require prolonged hemodialysis (2). A large-scale single-center study reported that recipients of grafts obtained from NHBDs experience significantly higher rates of delayed graft function than the recipients of grafts obtained from heart-beating brain-dead donors. Further, the serum creatinine levels in the former recipients are elevated for 7 days after transplantation. However, no measurable difference is observed between the recipients of grafts from NHBDs and those of grafts from heart-beating brain-dead donors by the end of the first postoperative month (12). In this study, patients in the FI group required hemodialysis for longer periods than those in the SI group. Furthermore, the islet yield was inversely correlated with the number of days for which hemodialysis was required. The serum creatinine concentration after kidney transplantation was better in the recipients in the SI group than those in the FI group. Therefore, good results of islet isolation predict a good clinical outcome of kidney transplantation in cases where the kidneys and pancreas are harvested from the same donor. However, in some cases, the results of islet isolation and the clinical outcomes of kidney transplantation were discrepant. Even though the results of islet isolation were very poor in three cases (<40,000 IEQ harvested), the recipients of kidney transplants required hemodialysis for only 7 days. The condition of the donors of these grafts was

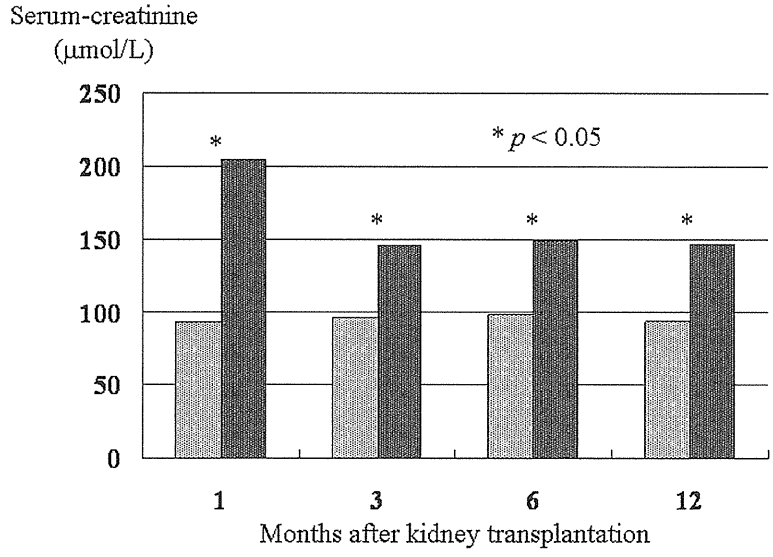
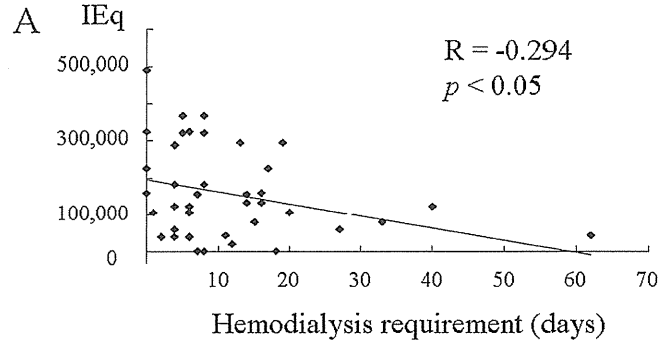


Figure 1. Serum creatinine concentration after kidney transplantation. Light columns: successful isolation group; dark columns: failed isolation group.



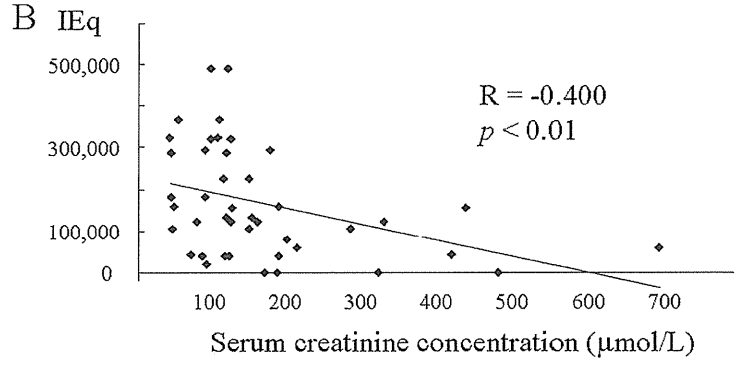


Figure 2. Relationship between the islet yield (IEQ) and hemodialysis requirement (A), and serum creatinine concentration 1 month after kidney transplantation (B).

Table 2. Discrepancy Between the Islet Yield and Clinical Outcome of Kidney Transplantation

Case	Islet Yield (IEQ)	Hemodialysis Requirement (Days)	1 Month *S*-Creatinine (μmol/L)	Donor Factors				
				Cause of Death	CPR	Anuria (h)	Catecholamine	*S*-Creatinie (μmol/L)
1	37,840	2	85.7	choke	yes	0	10 γ	114.9
		6	187.4					
2	38,560	6	120.2	choke	yes	36	20 γ	N/A
		4	114.9					
3	0 (discard)	7	184.8	SAH	no	N/A	N/A	896.4
		7	321.8					

CPR, cardiopulmonary resuscitation; SAH, subarachnoid hemorrhage.

extremely poor. These donors had undergone cardiopulmonary resuscitation and received high doses of catecholamines. Many think that stringent criteria must be used for the selection of NHBDs for pancreata (4). The pancreatic function is thought to be more severely affected by warm ischemic damage than the kidney function.

In conclusion, the results of islet isolation were observed to correlate with the outcomes of kidney transplantation in cases where both grafts are harvested from the same NHBD. However, the results of islet isolation are more severely affected by the marginal conditions of NHBDs than are the functions of transplanted kidneys. It is therefore difficult to predict the clinical outcomes of kidney transplantation based only on the results of islet isolation.

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Comparison of Ulinastatin, Gabexate Mesilate, and Nafamostat Mesilate in Preservation Solution for Islet Isolation

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For islet transplantation, maintaining organ viability after pancreas procurement is critically important for optimal graft function and survival. We recently reported that islet yield was significantly higher in the modified ET-Kyoto (MK) solution, which includes a trypsin inhibitor (ulinastatin), compared with the UW solution, and that the advantages of MK solution are trypsin inhibition and less collagenase inhibition. In this study, we compared ulinastatin with other trypsin inhibitors, gabexate mesilate, and nafamostat mesilate, in preservation solution for islet isolation. Ulinastatin was easily dissolved in ET-Kyoto solution, while ET-Kyoto with gabexate mesilate and nafamostat mesilate became cloudy immediately after addition. Although there were no significant differences in islet yield among the three groups, viability was significantly higher for the MK group than for the GK group or the NK group. The stimulation index was significantly higher for the MK group than for the GK group. In summary, there are no other trypsin inhibitors that are more effective than ulinastatin. Based on these data, we now use ET-Kyoto solution with ulinastatin for clinical islet transplantation.

Key words: Islet transplantation; Islet isolation; Modified ET-Kyoto (MK) solution; Trypsin inhibitor; Preservation solution

INTRODUCTION

The solution for organ preservation is critically important for maintaining its function, reducing ischemia-reperfusion injury, and, therefore, increasing transplant efficiency. For islet transplantation, the storage conditions of the donor pancreas may influence the deleterious consequences of isolated islets during transplantation, which remains a major source of current issues in clinical practice. Donor pancreata for islet transplantation are usually preserved with University of Wisconsin (UW) solution. 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 (30). Extracellular-type trehalose-containing Kyoto (ET-Kyoto) solution was recently developed for organ preservation solution and its effectiveness in cold lung storage has been demonstrated in clinical lung transplantation (4,23). It also is effective for skin flap storage and its clinical application is beginning in this field (31).

We previously showed that pancreas preservation with modified ET-Kyoto (MK) solution significantly

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improved islet yields, compared with UW preservation (21). Although high potassium in UW solution causes insulin release from pancreatic β-cells (6), ET-Kyoto solution has a high sodium/low potassium composition. Moreover, UW solution inhibits the activity of collagenase, an enzyme blend for pancreatic digestion (5,27), but MK solution inhibits collagenase to a lesser extent (21). 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 that using ET-Kyoto solution without the trypsin inhibitor in a rat model (21).

In this study, we compared ulinastatin with other trypsin inhibitors (gabexate mesilate, and nafamostat mesilate) in preservation solution for islet isolation.

MATERIALS AND METHODS

Preservation Solution

We used ET-Kyoto solution (4,23) with ulinastatin (Miraclid®, Mochida Pharmaceutical, Tokyo, Japan; named MK solution), gabexate mesilate (FOY®, Ono Pharmaceutical, Osaka, Japan; named GK solution), and nafamostat mesilate (FUTHAN®, Torii Pharmaceutical, Tokyo, Japan; named NK 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, GK, and NK solutions and ET-Kyoto solution without trypsin inhibitors (control), 3 ml of 0.3 mM *N*-benzoyl-L-arginine ethylester reagent (BAEE; Sigma, Tokyo, Japan) was 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 (12). Absorbance was measured at every minute up to 6 min. A BAEE unit was defined as a change in optical density of 0.001/min.

Table 1. Composition Preservation Solutions

	MK	GK	NK
Na (mmol/L)	100	100	100
K (mmol/L)	43.5	43.5	43.5
Gluconate (mmol/L)	100	100	100
Phosphate (mmol/L)	25	25	25
Trehalose (mmol/L)	120	120	120
Hydroxyethyl starch (g/L)	30	30	30
Ulinastatin ($\times 10^3$ U/L)	100	—	—
Gabexate mesilate (mg/L)	—	1000	—
Nafamostat mesilate (mg/L)	—	—	20

Porcine Islet Isolation

Porcine pancreata were obtained at a local slaughterhouse. About 10 min after the cessation of heart beating, the operation was started. After removing the pancreas, we immediately inserted a cannula into the main pancreatic duct, infused each preservation solution for ductal protection (9,20), and put the pancreas into each two-layer [preservation solution/perfluorochemical (PFC)] preservation container. 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 as previously described (9,10,15,22) in the standard Ricordi technique (26) with modifications introduced in the Edmonton protocol (11,24,28). 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 7–9 pieces, placed in a 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 confirmed to be complete, RPMI-1640 medium (Gibco, Carlsbad, CA) was introduced into the system. Then 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 (Optiprep®, Sigma-Aldrich, St. Louis, MO)-Kyoto (IK) solution in an apheresis system (COBE 2991 Cell Processor, Gambro Laboratories, Denver, CO). Since iodixanol has low viscosity, it needs less force during centrifugation, compared with Ficoll. For the solution, low-density (density 1.077) and high-density (density 1.100–1.125) IK solutions were produced by changing the volumetric ratio of iodixanol and Kyoto solution (9,14,15).

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 (IEQ; diameter standardizing to 150 μm) (25). 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) (11,25). Each parameter was graded from zero to 2 with zero equaling the worst and 2 the best score, so that the worst islet preparations were given a cumulative score of zero 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 islet cells simultaneously (3,11,25). Fifty islets were inspected and their individual viability was determined visually, followed by calculation of their average viability (11).

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 (28). Briefly, 1,200 IEQ were incubated with either 2.8 or 25 mM glucose in RPMI-1640 for 2 h at 37°C and 5% CO_2. The supernatant was 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 by total proteins 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 adenosine diphosphate/adenosine triphosphate (ADP/ATP) ratio correlated with transplantation outcome (7). The ADP/ATP ratio was measured to evaluate the energy status of cultured islets, using the ApoGlow™ kit (Cambrex Bio Science Nottingham Ltd., Nottingham, UK). In brief, 80 IEQ of islets 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, and 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

Severe combined immunodeficient mice (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 2,000 IEQ pig islets obtained from each group were transplanted into the renal subcapsular space of the left kidney of a diabetic SCID mice as previously described (16,17,20). 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 three groups of mice. Mouse studies were approved by the Institutional Animal Research Committees of Kyoto University and Fujita Health University.

Statistics

The differences between each group were considered significant if the value was $p < 0.05$ using an unpaired Student's *t*-test with Bonferroni correction or the Kaplan-Meier log-rank test.

RESULTS

Dissolution of Trypsin Inhibitors in ET-Kyoto Solution

Ulinastatin, gabexate mesilate, and nafamostat mesilate were used in this study. When these trypsin inhibitors were added in ET-Kyoto solution, ulinastatin was easily dissolved in ET-Kyoto solution, while ET-Kyoto with gabexate mesilate and nafamostat mesilate became cloudy immediately after addition (Fig. 1A). After 30 min, gabexate mesilate and nafamostat mesilate were dissolved in ET-Kyoto solution.

Inhibition of Trypsin Activity

Previous reports show that trypsin inhibition with TLM preservation improves islet yields (12,21). We examined whether MK, GK, and NK solutions inhibited trypsin activity. All three solutions significantly inhibited trypsin activity (Fig. 1B), compared with ET-Kyoto (control). These observations suggest that these solutions could reduce trypsin activity during pancreas preservation.

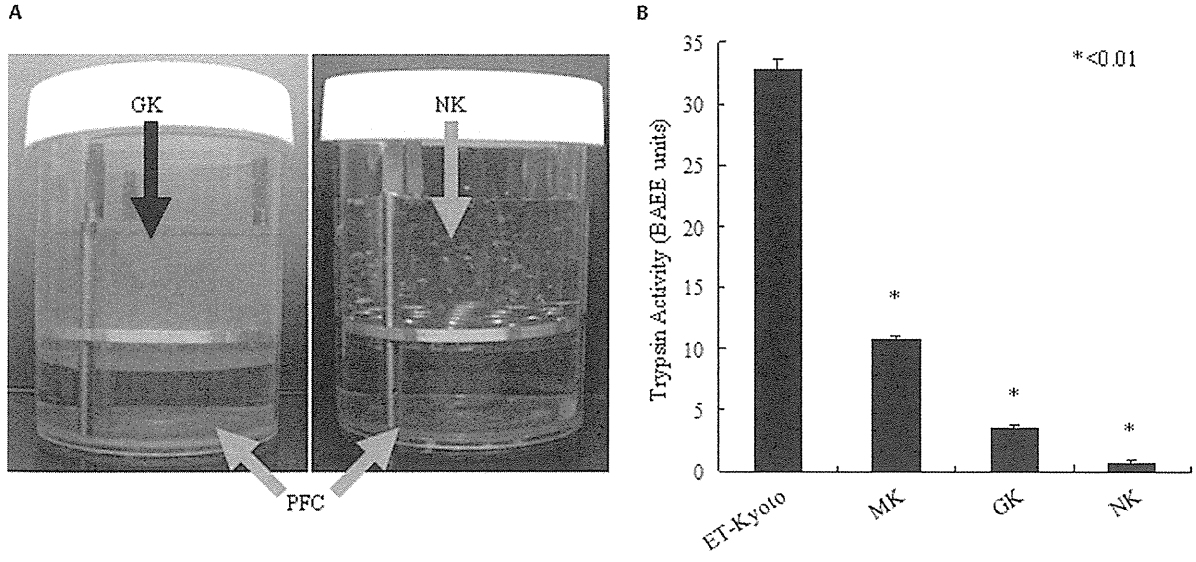

Figure 1. Dissolution of trypsin inhibitors in ET-Kyoto solution. (A) Extracellular-type trehalose-containing Kyoto (ET-Kyoto) with gabexate mesilate (GK) and nafamostat mesilate (NK) became cloudy immediately after addition. (B) Impact of MK (Kyoto with ulinastatin), GK, or NK solution on trypsin activity. *N*-benzoyl-L-arginine ethylester reagent (BAEE) was incubated for 5 min at 25°C and then trypsin along with MK solution ($n = 5$), GK solution ($n = 5$), NK solution ($n = 5$), or ET-Kyoto (control; $n = 5$) 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. Data are expressed as the mean ± SE. PFC, perfluorochemical.

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 among the three groups. Phase I and phase II periods were also similar for the three groups.

Islet yield before purification was higher, but not significantly so, in the MK group ($n = 9$), compared with the GK group ($n = 3$) or NK group ($n = 3$) (MK: 9063 ±

Table 2. Pig Islet Isolation Characteristics

	MK ($n = 9$)	GK ($n = 3$)	NK ($n = 3$)
Pancreas size (g)	109.0 ± 5.4	113.3 ± 4.1	96.7 ± 10.5
Operation time (min)	7.2 ± 0.7	6.7 ± 0.3	5.7 ± 1.7
Warm ischemic time (min)	26.1 ± 1.1	26.0 ± 1.0	23.7 ± 1.2
Cold ischemic time (min)	122.9 ± 0.9	125.7 ± 1.8	127.7 ± 3.3
Phase I period (min)	8.8 ± 1.2	7.3 ± 1.3	8.0 ± 3.1
Phase II period (min)	32.4 ± 3.3	36.3 ± 1.8	32.0 ± 1.0
Viability (%)	97.9 ± 0.4†	91.4 ± 1.8	93.7 ± 1.8
Score	9.4 ± 0.2	8.7 ± 0.7	8.7 ± 0.9
Purity (%)	76.1 ± 5.4	65.8 ± 11.2	70.0 ± 11.5
Postpurification recovery (%)*	75.7 ± 5.6	84.2 ± 8.6	93.4 ± 10.5
Stimulation index	2.39 ± 0.19‡	1.46 ± 0.16	2.12 ± 0.33

Data are expressed as mean ± SE. PFC, perfluorochemical.
*Islet recovery (%) = IEQ after purification/IEQ before purification × 100.
†Viability was higher for the MK/PFC group than for the GK/PFC group or the NK/PFC group ($p < 0.01$).
‡Stimulation index was higher for the MK/PFC group than for the GK/PFC group ($p < 0.025$).

640 IEQ/g, GK: 8378 ± 642 IEQ/g, NK: 6058 ± 2116 IEQ/g) (Fig. 2A). Islet yield after purification was higher, but not significantly so, in the MK group, compared with the NK group. There were no significant differences between the MK group and the GK group (MK: 6864 ± 727 IEQ/g, GK: 6967 ± 439 IEQ/g, NK: 5645 ± 2268 IEQ/g) (Fig. 2B). Other porcine islet characteristics are shown in Table 2. Viability was significantly higher for the MK group than for the GK group or the NK group ($p < 0.01$). The stimulation index was significantly higher for the MK group than for the GK group ($p < 0.025$). There were no other significantly different characteristics among each group.

Assessment of Islet Function In Vitro and In Vivo

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 each group (data not shown). To assess the islet graft function of each group in vivo, 2,000 IEQ islets of each group were then transplanted below the kidney capsule of STZ-induced diabetic SCID mice. The blood glucose levels of 8 of the 10 mice (80.0%) receiving islets of the MK group, 7 of the 9 mice (77.8%) of the GK group, and 7 of the 10 mice (70.0%) of the NK group decreased gradually and reached normoglycemia. There was no significant difference between the groups with respect to the attainability of posttransplantation normoglycemia (Fig. 3). Morphologic 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).

In summary, there are no other trypsin inhibitors more effective than ulinastatin.

DISCUSSION

In this study, we showed that MK solution was superior to GK or NK solutions, although the trypsin inhibition is greater in GK and NK than in MK. It may be due to differences of 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 TNF mRNA, the activation of endothelial cells, and the expression of ICAM-1 induced by endotoxin in vitro (1,2,13). It has been shown that administration of ulinastatin decreased the ischemia-reperfusion injury (29) or attenuated the elevation in the concentrations of inflammatory cytokines and C-reactive protein, a marker of inflammation (32) in transplanted small intestine. The superiority of ulinastatin may also be due to less collagenase inhibition than other trypsin inhibitors. We previously reported that MK solution was superior to modified Celsior solution (Celsior solution with hydroxyethyl starch and nafamostat mesilate; HNC). HNC solution including nafamostat mesilate inhibited collagenase activity (18). Although different preservation solutions were used in the report, it seems

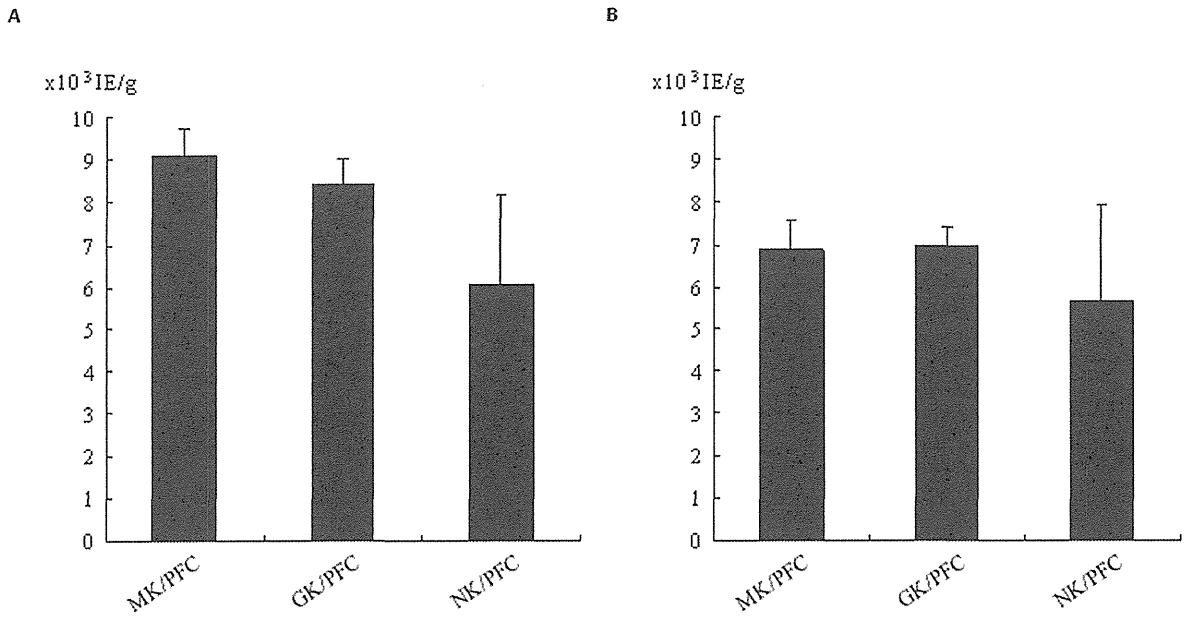

Figure 2. Islet yield before purification (A) and after purification (B). Data are expressed as the mean ± SE. IE, islet equivalents.

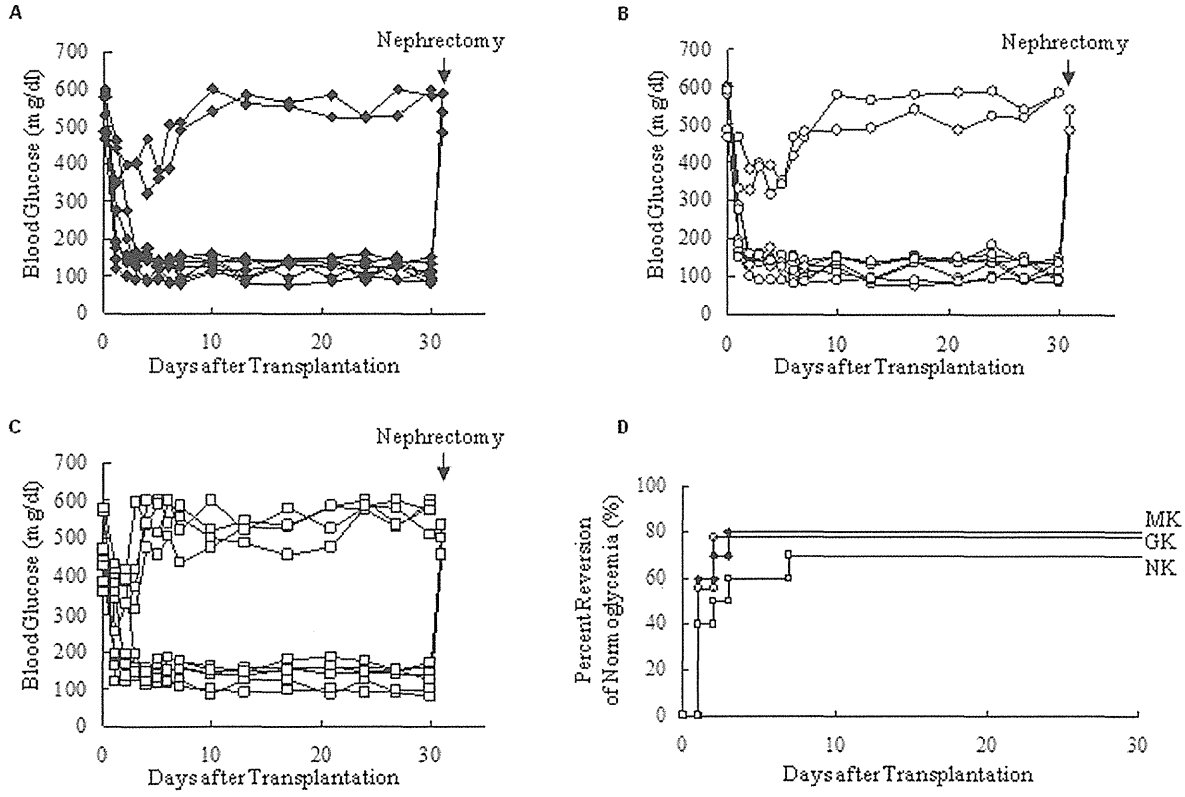

Figure 3. Evaluation of islet quality of each group in vivo. 2,000 IEQ islets of each group were then transplanted below the kidney capsule of streptozotocin (STZ)-induced diabetic severe combined immunodeficient (SCID) mice. (A–C) Nonfasting blood glucose levels of mice that were transplanted with islets of MK group (A), of GK group (B), or NK group (C). (D) Percentage of normoglycemia of STZ-induced diabetic mice after islet transplantation. Normoglycemia was defined as two consecutive posttransplant blood glucose levels showing less than 200 mg/dl. MK, $n = 10$; GK, $n = 9$; NK, $n = 10$.

that collagenase inhibition by nafamostat mesilate is higher than ulinasatatin.

Previously, we demonstrated that MK preservation including ulinastatin eliminated trypsin activity during pancreas preservation and ET-Kyoto preservation without ulinastatin resulted in lower islet yields (21). We also reported that ET-Kyoto with ulinastatin was the better combination for pancreas preservation than ET-Kyoto with Pefabloc (19). Other studies have shown that trypsin inhibition during human pancreas digestion improves islet yield (8,12). Since trypsin from pancreatic acinar cells destroys islets and ductal cells, trypsin inhibition could reduce degradation of the ductules and secure the delivery of collagenase solution to the immediate vicinity of the islets.

In conclusion, we showed that ET-Kyoto with ulinastatin was the best combination for pancreas preservation. Based on these data, we now use the ET-Kyoto solution with ulinastatin for clinical islet transplantation from brain-dead donor pancreata in US and non-heart-beating donor pancreata in Japan. MK preservation makes it feasible to use marginal donors for efficient islet transplantation into type 1 diabetes patients.

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Attenuation of Murine Graft-Versus-Host Disease by a Tea Polyphenol

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Since donor T-cells' allorecognition of host antigens is a prerequisite for the onset of graft-versus-host disease (GVHD), blocking their cellular signaling pathways can decrease the severity of GVHD. We hypothesized that epigallocatechin-3-gallate (EGCG), due to its strong affinity to macromolecules, would adhere to surface molecules of donor T cells, inhibit their allorecognition, and attenuate GVHD in the recipient. We tested the hypothesis by treating donor splenocytes with EGCG in both in vitro and in vivo murine GVHD models. EGCG treatment decreased the proliferation of donor cells in MLR cultures and secretion of IL-2 and INF-γ. It also reduced the epitope detection of CD3ε, CD4, and CD28 but did not downregulate the protein expression of these molecules, suggesting blockage of cell surface stimulatory signals. Similarly, EGCG treatment did not decrease mRNA expression for some of these molecules but decreased mitogen-induced cell proliferation, indicating that EGCG did not interfere the transcription of these genes but affected cell proliferation pathways. Furthermore, EGCG-treated donor splenocytes, when transplanted into immunocompromized recipient mice, decreased of proliferation, and the treatment extended the recipients' survival at least during the early stage of GVHD. These results strongly suggest that EGCG attenuates GVHD by both blocking specific cell surface molecules and affecting the donor T-cell proliferation pathways.

Key words: Graft-versus-host disease (GVHD); Epigallocatechin-3-gallate (EGCG); T cells; Tea polyphenol

INTRODUCTION

Graft-versus-host disease (GVHD), in which immunocompetent donor lymphocytes attack recipient tissues after allogenic transplantation, often causes significant morbidity and mortality, particularly in immunocompromised recipients. An ordinary therapeutic remedy for GVHD is the application of immunosuppressive drugs that inhibit donor T-cell activation, which underlies this disease (4,5). Although these pharmacological agents prevent the disease by inhibiting T-cell signaling and activation, they sometimes cause significant side effects on the recipients (2,17,25).

Studies on transplantation medicine have shown that certain plant-derived chemicals can decrease the severity of transplantation-associated problems such as GVHD and graft rejections (8,26,27). Among these phytochemicals, a green tea polyphenol, epigallocatechin-3-gallate (EGCG), has been found to protect certain tissue transplants from allograft rejection. In fact, immersing peripheral nerve tissues in an EGCG-containing solution before transplanting them prevented graft rejection in recipient rats (11).

Because EGCG has a strong affinity to cell surface macromolecules (19) and a potential to impair receptor–ligand interactions (1), it is hypothesized to adhere to the surface molecules of donor T cells and inhibit allorecognition and GVHD in transplant recipients. In our previous study, we found in an in vitro graft rejection model that EGCG treatment of donor splenocytes in mixed lymphocyte cultures significantly reduced recipient T-cell proliferation response (12). In the present

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study, we tested the immunocamouflage hypothesis by using both in vivo and in vitro murine GVHD models and further examined the influence of EGCG on various parameters of allorecognition. The results demonstrate that EGCG treatment of donor T cells inhibits allorecognition and attenuates the severity of an early stage GVHD in a murine allograft context.

MATERIALS AND METHODS

Mice

For both in vitro and in vivo studies, female BALB/c (H-2K^d) and C57BL/6 (H-2K^b) mice (Japan SLC, Shizuoka, Japan) between 5 and 11 weeks of age were maintained under specific pathogen-free (SPF) conditions. All experiments were approved by the local review board of Kyoto University and were conducted in accordance with national and international guidelines of laboratory animal care.

EGCG Treatment of Splenocytes

Spleens were aseptically removed from mice, and cell suspensions were prepared by dissecting the spleens with forceps and filtering splenocytes through 50-μm nylon filter mesh in Hanks balanced salt solution [HBSS; +1% fetal calf serum (FCS)]. The cells were centrifuged at 300 × *g* for 5 min, resuspended in 10 ml RPMI-1640 (Sigma Aldrich, Tokyo, Japan) containing 0, 200, or 400 μM EGCG/10^7 cells, and incubated at 4°C for 1 h. The cells were washed twice with RPMI-1640 (10% FCS) before being used in experiments.

Mixed Lymphocyte Reaction (MLR)

Splenocytes derived from BALB/c mice were used as "stimulator" cells and those from C57BL/6 as "responder" cells. In both one-way and two-way MLRs, prior to coculture, the responder cells were treated with EGCG as described earlier. In one-way MLR, cells from BALB/c mice were treated with 20 μg/ml mitomycin C (MMC)(MP Biomedicals, Aurora, OH) in RPMI-1640 (10% FCS) at 37°C for 30 min to arrest their proliferation before EGCG treatments. In two-way MLR, both stimulator and responder populations were left capable of responding. Each cell population was resuspended in RPMI-1640 (10% FCS, 100 U/ml penicillin, and 100 μg streptomycin) to a final concentration of 5.0 × 10^6 cells/ml. The stimulator and responder cells were mixed at a 1:1 ratio (100 μl each) and incubated in triplicate in 96-well plates at 5% CO_2 and 37°C for 72 h. Cell proliferation was estimated by counting a small aliquot of cells from each MLR well with a trypan blue exclusion method. After the period of MLR incubation, supernatants from the cultures were assayed for interleukin-2 (IL-2) and interferon-γ (INF-γ) production using cytokine quantification ELISA kits (eBioscience, San Diego, CA) according to the manufacturer's protocol. To examine the toxicity of EGCG on unstimulated cells, C57BL/6 splenocytes were treated with EGCG before culturing in 96-well plates for 72 h, and the proportion of live cells was determined for small samples taken every 24 h.

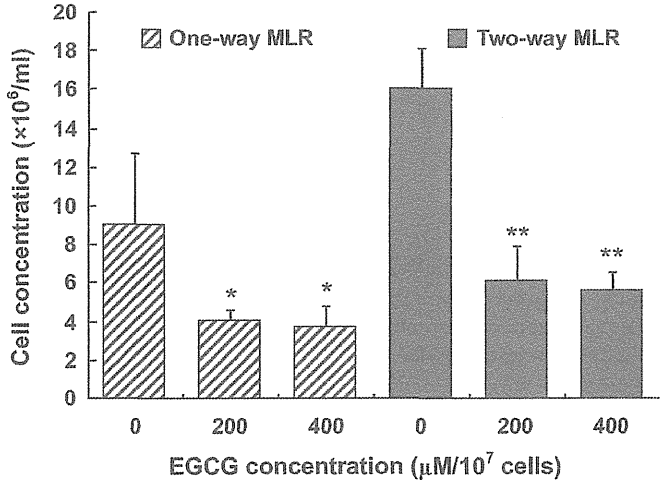

Figure 1. Effects of epigallocatechin-3-gallate (EGCG) treatment on T-cell proliferation. Data are the mean ± SD cell concentrations for triplicate samples in one-way (hatched columns) and two-way (solid columns) mixed lymphocyte reaction (MLR) cultures. Differences were significant between untreated (0 μM) and treated groups (200 or 400 μM) (*$p < 0.01$; **$p < 0.0001$).

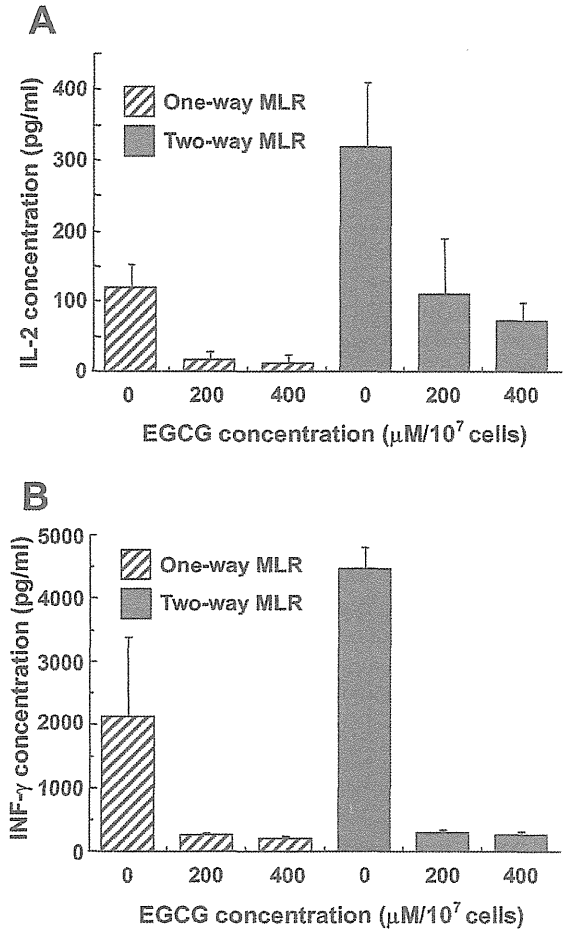

Figure 2. Effects of EGCG on interleukin-2 (IL-2) (A) and interferon-γ (INF-γ) (B) production. Data are mean ± SD cytokine concentrations for triplicate samples in one-way (hatched columns) and two-way (solid columns) MLR cultures. Differences were significant between untreated (0 μM) and treated (200 or 400 μM) groups for both cytokines in either one-way or two-way MLR cultures ($p < 0.0001$).

Flow Cytometric Analysis of Cell Surface Molecules

Flow cytometric analyses of cell surface molecules were performed using a series of fluorescence-labeled monoclonal anti-mouse antibodies and corresponding isotype-matched control antibodies. These antibodies are to detect cluster of differentiation 2 (CD2), CD3ε, CD4, CD11a, CD 28, CD49d (eBioscience) and T-cell receptor (TCR)αβ (BD Biosciences Pharmingen, San Diego, CA). The splenocytes were treated with red blood cell lysis buffer (eBioscience) for 5 min, and the remaining mononuclear cells were treated with EGCG and washed with RPMI (10% FCS) and then with a staining buffer (eBioscience) at 4°C. Each splenocyte sample was reconstituted in a 50-μl aliquot in the staining buffer and subjected to antibody labeling for 15 min at 4°C. After washing the samples twice with the staining buffer, a minimum of 10,000 events per sample was collected and analyzed on a FACScan flow cytometer (Becton Dickinson, Flanklin Lakes, NJ).

Immunoblot Analysis

To examine how EGCG treatment influences the production of cell surface proteins, immunoblot analyses were performed. Splenocytes of C57BL/6 were treated with EGCG in PBS at 4°C for 1 h. The cells were washed once with PBS and incubated in RPMI-1640 (10% FCS, 100 U/ml penicillin, and 100 μg streptomycin) at 37°C for 24 h. Harvested cells were washed once in cold PBS and lysed in RIPA buffer (BD Biosciences Pharmingen) to prepare protein extracts from three treatment groups (i.e., cells treated with 0, 200, or 400 μM EGCG/10^7 cells). The samples were electrophoresed on a 4–20% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel for 1 h (60 V, 15 mA) and transferred to a nitrocellulose membrane for 1 h (4 V, 20 mA). After the membrane was blocked with a blocking solution (Blocking One, Nacalai Tesque, Japan) at room temperature (RT) for 1 h, primary antibodies against mouse TCRαβ (Santa Cruz Biotechnology, Santa Cruz, CA), CD3ε (BD Biosciences Pharmingen), CD28 (eBioscience), and CD49d (Cedarlane Laboratories, Ontario, Canada) were applied on the membrane and incubated at RT for 1 h on a swirl shaker. The membrane was washed three times with TBS and 0.5% Tween 20 for 10 min and treated with horseradish peroxidase-conjugated secondary antibodies for 1 h at RT. The three times washed membrane was then treated with Chemi-lumi One Western blotting kit (Nacalai Tesque), and banding images were developed on X-ray films.

RNA Isolation and Semiquantitative RT-PCR

C57Bl/6 splenocytes (5.0×10^6 cells) were treated with 0, 200, and 400 μM EGCG at 4°C for 1 h. After washing with PBS once, total RNA was extracted from the cells by RNeasy Mini Kit (Qiagen, Inc.), and DNA contaminants were removed by RNase-free DNase I Set (Qiagen, Inc.). The RNA (50 ng) was reverse transcribed using Super Script III (Invitrogen, Inc.) with random primers (Invitrogen), and synthesized cDNAs were treated with RNase H (Wako Chemicals Co.) to remove RNA contaminants. PCR was performed with 50 ng cDNA with temperature cycling conditions: one cycle of 95°C for 2 min, followed by 28–40 cycles of 94°C for 30 s, 45–59°C for 30 s, and 72°C for 45 s. The number of cycles and annealing temperatures were adjusted for detecting the amount of amplicons that increased linearly. Primer pairs used to detect murine genes were:

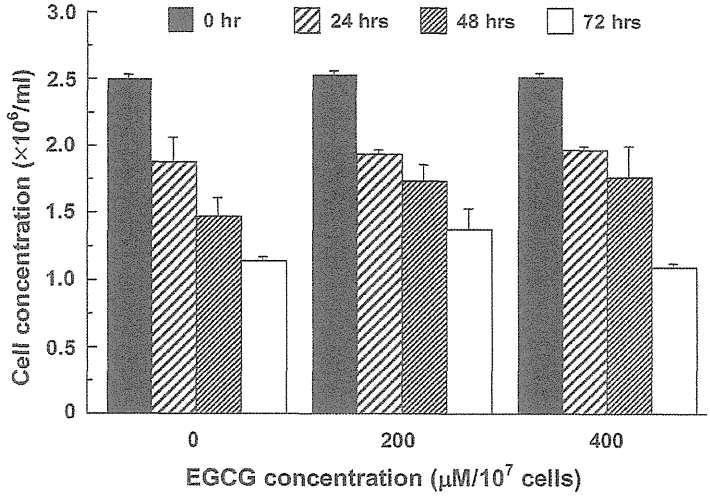

Figure 3. Effects of EGCG on the viability of splenocytes. Splenocytes from C57Bl/6 mice were untreated (0 μM) or treated with 200 or 400 μM EGCG, and the number of live cells was examined after 0, 24, 48, and 72 h of culture (mean ± SD for triplicate samples). The cell viability was significantly higher for 200 μM EGCG than the untreated control (two-way ANOVA, effect of EGCG concentration: $p = 0.0038$). There was no significant differences between 400 μM EGCG and the untreated control or 200 μM EGCG ($p > 0.05$).

CD3ε (GenBank accession No. NM_007648, amplicon size 235 bp, forward primer, GAT GCG GTG GAA CAC CT, reverse primer, ACT GTC CTC GAC TTC CGA GA); *CD4* (GenBank accession No. NM_013488, amplicon size 222 bp, forward primer, AGG AAG TGA ACC TGG TGG TG, reverse primer, TCC TGG AGT CCA TCT TGA CC); *CD28* (GenBank accession No. NM_007642, amplicon size 166 bp, forward primer, CGG GAA TGG GAA TTT TAC CT, reverse primer, GTA AGG CGG AGG GTA CAT GA); *GAPDH* (GenBank accession No. NM_008084.2, amplicon size 223 bp, forward primer, AAC TTT GGC ATT GTG GAA GG, reverse primer, ACA CAT TGG GGG TAG GAA CA). Amplified PCR products were analyzed by gel electrophoresis with 2% agarose gel and 0.001% ethidium bromide.

Mitogen Stimulation

C57Bl/6 splenocytes were treated with EGCG as described earlier, and triplicate cultures of the splenocytes containing 3.0×10^5 cells/ml were incubated for 72 h at 37°C in the presence of phytohemagglutinin (PHA; 0.5 μg/ml; Sigma-Aldrich) or a combination of phorbol 12-myristate 13-acetate (PMA; 50 μg/ml; Sigma-Aldrich) and ionomycin (2 μg/ml; Sigma-Aldrich). Cell proliferation was assessed by counting cells with a trypan blue exclusion method.

In Vivo Murine Experiments

BALB/c mice (5–6 weeks old) and C57BL/6 mice (8–9 weeks old) were used for in vivo experiments. To make immunocompromized animals, BALB/c mice were lethally irradiated (6.3 Gy) by a ^{137}cesium irradiator. These immunocompromized animals were housed in

Table 1. Effect of EGCG Treatment on Detection of Cell Surface Epitopes

	EGCG Concentration (/10^7 Cells)		
Epitope	0 μM	200 μM	400 μM
TCRαα	9.1	8.6 (5.5)	8.7 (4.4)
CD2	24.5	13.3 (45.7)*	16.3 (33.5)*
CD3ε	6.8	3.5 (48.5)*	2.3 (66.2)*
CD4	19.9	8.9 (55.3)*	12.8 (35.7)*
CD11a	26.7	22.4 (16.1)*	21.8 (18.4)*
CD28	13.5	2.9 (78.5)*	2.8 (79.3)*
CD49d	7.5	5.6 (25.3)*	5.6 (25.3)*

Values indicate the mean fluorescence intensity ratio (mean fluorescence of test antibody/mean fluorescence of isotype antibody). Values in parentheses for EGCG-treated triplicate samples (200 and 400 μM/10^7 cells) represent the average percent reduction in the intensity ratio relative to the untreated control samples (0 μM/10^7 cells). Differences were significant (*$p < 0.01$) or not significant ($p > 0.05$) compared with the untreated controls. EGCG, epigallocatechin-3-gallate; TCR, T-cell receptor; CD, cluster of differentiation.