clinical islet transplantation are currently not fully understood, this decline may reflect chronic toxicity of immunosuppressive drugs on islet β cells.

Rapamycin is widely used both as an induction and maintenance immunosuppressant in islet transplantation as part of the original Edmonton protocol.[1] Rapamycin may have deleterious effects on islet β cells. The immunosuppressive mechanism of rapamycin is based on blockade of mammalian target of rapamycin (mTOR), a molecule with a pivotal role in cell cycle progression from late G1 into S phase in response to T-cell growth factor stimulation.[3] The mTOR, which is ubiquitously expressed in various cell types, is a serine/threonine protein kinase that regulates important cellular process including growth, proliferation, motility, survival, protein synthesis, and transcription.[4] Furthermore, mTOR activity inhibits autophagy in cells ranging from yeast to human.[5] Accordingly, the ability of rapamycin to inhibit mTOR activity may induce autophagy.

Autophagy, meaning to eat oneself, is one of the main mechanisms for maintaining cellular homeostasis. Although this pathway is not directly a death pathway, it is a self-cannabalistic pathway. Mediated via lysosomal degradation, autophagy is responsible for destroying cellular proteins and degrading cellular organelles, recycling them to ensure cell survival. Although altered autophagy has been observed in various diseases, including neurodegenerative diseases, cancers, and cardiac myopathies,[6–8] its role is not known; the crux of the problem is whether the response is cell protective or a mechanism of death.

The relative contributions of autophagy are poorly understood in transplanted islets. The objective of the present study was to evaluate the effects of rapamycin on islet β-cells, including autophagy induction, viability, and insulin secretion, factors that may strongly contribute to progressive dysfunction of transplanted islets.

MATERIALS AND METHODS

Isolation of Pancreatic Islets

Anesthetized male BL6 mice underwent bile duct cannulation with pancreatic inflation using 3 mL of extracellular-type trehalose-containing Kyoto (ET-Kyoto) solution containing 1 mg/mL of collagenase. The inflated pancreas was excised; cleaned of lymph nodes, fat, and bile duct; and digested with collagenase VIII, followed by purification using a discontinuous Ficoll gradient. Isolated islets were cultured in RPMI (Roswell Park Memorial Institute) 1640 medium.

Western Blot Analysis

Western blot analysis was performed to detect the accumulation of LC3-II, an LC3-phosphorylated conjugate, which is an early marker of autophagy. Fresh islets (30 per well) were incubated for 24 hours in culture medium in the absence or presence of either 1 or 10 ng/mL of rapamycin. Protein samples from lysed cells underwent electrophoresis with 15% sodium dodecylsulfate–polyacrylamide gel and were transferred to polyvinylidene fluoride membranes. Lysate LC3-II was recognized by immunoblotting with an anti-LC3 monoclonal antibody (MBL International Corp, Woburn, Massachusetts).[9] As the loading control for the samples, we also detected protein expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Protein expression levels of both LC3-II and GAPDH, expressed in arbitrary units, were quantified using an image analyzer (Fluor-Chem; Bio-Rad Laboratories, Inc, Hercules, California). The relative protein expression of LC3-II in islets was normalized to that of GAPDH and expressed as the ratio of LC3-II to GAPDH.

Islet Viability Assay

Thirty cells of fresh mice islets were cultured for 24 hours with complete medium in the absence or presence of either 1 or 10 ng/mL of rapamycin. Subsequently, islet viability was determined using the colorimetric methyl tetrazolium salt (MTS) Cell Titer 96 Aqueous One Solution cell proliferation assay (Promega Corp, Madison, Wisconsin).[10] The colorimetric reagent was added to each well and incubated for 2 hours before absorbance values were read at 490 nm.

Blocking Assay of Autophagic Signaling

To determine whether rapamycin-treated islets recovered their viability, they were assessed using the MTS assay in the absence or presence of 10 mmol/L of 3-methyladenine (3-MA), an inhibitor of class 3 phosphatidylinositol 3-kinase, an inhibitor of autophagy.[11]

Glucose-Stimulated Insulin Release and Stimulation Index

To further determine the in vitro potency of rapamycin-treated islets, static glucose challenge was performed in the absence or presence of 10 mmol/L of 3-MA.[12] After overnight culture, islets were incubated with either 2.8 or 20 mmol/L of glucose in culture medium for 2 hours at 37°C. The collected supernate was stored at −80°C for measurement of insulin with an enzyme-linked immunosorbent assay. Glucose-stimulated insulin release was expressed as the stimulation index, that is, the ratio of insulin release during exposure to high glucose (20 mmol/L) incubation compared with low glucose (2.8 mmol/L) incubation.

Generation of GFP-LC3 Transgenic Mice

For ex vivo studies to monitor autophagy in rapamycin-treated islets, transgenic mice expressing GFP-LC3 under the control of the constitutive CAG (chicken β-actin) promoter were purchased from RIKEN BioResource Center, Wako, Japan.[13] Fresh mouse islets, isolated as described above, were incubated for 24 hours in culture medium in the absence or presence of 1 ng/mL of rapamycin. In addition, rapamycin-treated islets were incubated in the presence of 10 mmol/L of 3-MA. Either untreated control islets, rapamycin-treated islets, or rapamycin plus 3-MA–treated islets were directly observed using a fluorescence microscope (Biozero; Keyence Corp, Osaka, Japan) to detect GFP-LC3 dots.

RESULTS

Endogenous LC3-II Markedly Accumulates in Islets Treated With Rapamycin

Endogenous LC3-II protein was detected in control islets (Table 1). From the results for islets treated with either 1 or 10 ng/mL of rapamycin, the expression level of endogenous LC3-II in 1 ng/mL of rapamycin-treated islets was similar to that in control islets. However, the amount of endogenous LC3-II was doubled in 10 ng/mL of rapamycin-treated islets

Table 1. LC3-II Accumulation in Rapamycin-Treated Islets

Assay	Untreated Control Islets	Rapamycin Treated Islets		Blocking Assay With 3-MA	
		1 ng/mL	10 ng/mL	1 ng/mL of Rapamycin Plus 10 mmol/L of 3-MA	10 ng/mL of Rapamycin Plus 10 mmol/L of 3-MA
LC3-II expression at Western blot analysis (LC3-II/GAPDH ratio)	0.50	0.46	1.08	1.04	0.73
Absorbance by MTS assay (islet viability, recovery of viability), %	100 (control)	56.8 (14.1)	49.0 (2.0)	68.5 (0.5)	75.8 (25.9)

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LC3-II, light chain 3, membrane bound; 3-MA, 3-methyladenine; MTS, methyl tetrazolium salt.

(Table 1). Under blocking conditions of autophagic signaling by 3-MA, there was an approximately 32% reduction in the amount of LC3-II observed in rapamycin-treated islets in the presence of 10 mmol/L of 3-MA as judged by the LC3-II/GAPDH ratio (Table 1).

Rapamycin Treatment of Islets Results in Reduced Islet Viability

To assess the direct effects of rapamycin on islet viability, we performed the MTS assay. On the basis of treatment with rapamycin, there were approximately 43% and 51% reductions in viability with treatment with 1 and 10 ng/mL of rapamycin, respectively (Table 1). In contrast, the viability of rapamycin-treated islets markedly recovered in the presence of 3-MA. Approximately 69% and 76% islet viability was noted after treatment with 1 ng/mL of rapamycin plus 10 mmol/L of 3-MA and with 10 ng/mL of rapamycin plus 10 mmol/L of 3-MA, respectively (Table 1).

Rapamycin Strongly Affects In Vitro Islet Function

Islet potency was assessed using a static glucose challenge in vitro. The stimulation index (SI) of untreated control islets was 1.38 ± 0.16 (Fig 1). However, treatment of islets with rapamycin dramatically reduced the SI. The SI was 1.11 ± 0.01 with 1 ng/mL of rapamycin, and no insulin output occurred with 10 ng/mL of rapamycin treatment (Fig 1). In contrast, the SI dramatically improved with the addition of 3-MA. Islets treated with 1 ng/mL of rapamycin plus 10 mmol/L of 3-MA, completely recovered compared with untreated control islets (Fig 1). These results indicate that rapamycin treatment of isolated islets elicited not only autophagy induction but also reduced islet viability and potency.

GFP-LC3 Signal is Strong in Rapamycin-Treated Islets

Autophagy in response to rapamycin treatment was seen on fluorescence photomicrographs of islet samples prepared from GFP-LC3 transgenic mice (Fig 2). In untreated control islets, the GFP-LC3 signal was detected diffusely in islets with few punctuate dots (Fig 2A). After 24 hours of incubation with 1 ng/mL of rapamycin, the number of GFP-LC3 dots markedly increased; most were detected as cup- or ring-shaped structures (Fig 2B). In contrast, the level of GFP-LC3 signals of rapamycin-treated islets in the presence of 10 mmol/L of 3-MA was diffuse and returned to the base level of control islets (Fig 2C).

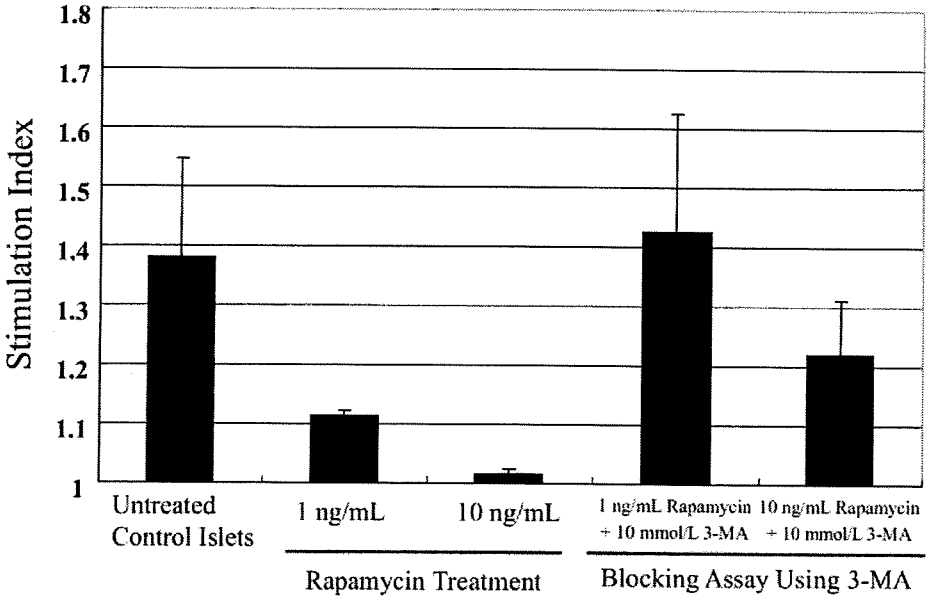

Fig 1. In vitro potency assessments of rapamycin-treated islets. Islet potency was assessed using static glucose challenge. Islet potency, expressed as stimulation index, was markedly reduced by rapamycin treatment. However, islet potency dramatically recovered with rapamycin plus 3-MA treatment. Values are expressed as the mean (SD) from three independent islets preparations.

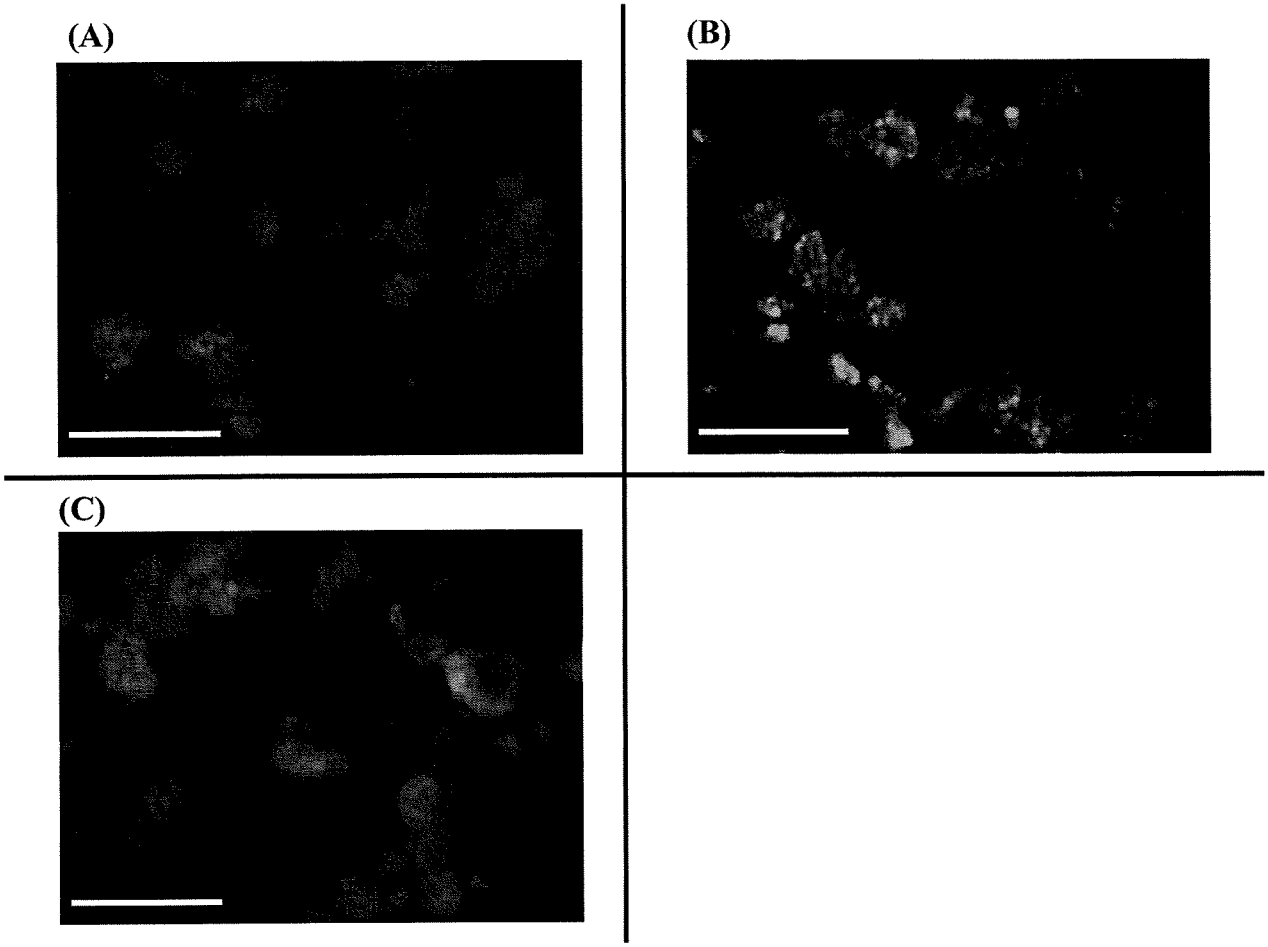

Fig 2. Islet autophagy in response to rapamycin treatment. Islet samples were prepared from GFP-LC3 transgenic mice. A, Untreated control islets. B, Islets treated with 1 ng/mL of rapamycin. C, Islets treated with rapamycin plus 3-MA. Bars indicate 100 μm.

DISCUSSION

Rapamycin, which is a natural bacterial product that inhibits mTOR by association with an intracellular receptor FKBP12,[14] is widely used as the central immunosuppressant in islet transplantation. As is well known, mTOR is a kinase that regulates important cellular processes such as inhibition of autophagy. Our results demonstrate that rapamycin treatment of isolated islets induced autophagy. This phenomenon impaired both islet viability and potency. These deleterious effects of rapamycin on islet β cells were markedly improved by the addition of 3-MA, which is an inhibitor of autophagy. Accordingly, therapeutically targeting this novel pathway may yield significant benefits, preventing the progressive islet graft dysfunction observed in transplant recipients.

REFERENCES

1. Shapiro AM, Lakey JR, Ryan EA, et al: Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med 343:230, 2000

2. Ryan EA, Paty BW, Senior PA, et al: Five-year follow-up after clinical islet transplantation. Diabetes 54:2060, 2005

3. Kirken RA, Wang YL: Molecular actions of sirolimus: sirolimus and mTor. Transplant Proc 35(3 suppl):227S, 2003

4. Hay N, Sonenberg N: Upstream and downstream of mTOR. Genes Dev 18:1926, 2004

5. Schmelzle T, Hall MN: TOR, a central controller of cell growth. Cell 103:253, 2000

6. Ravikumar B, Vacher C, Berger Z, et al: Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. Nat Genet 36:585, 2004

7. Paglin S, Hollister T, Delohery T, et al: A novel response of cancer cells to radiation involves autophagy and formation of acidic vesicles. Cancer Res 61:439, 2001

8. Nakai A, Yamaguchi O, Takeda T, et al: The role of autophagy in cardiomyocytes in the basal state and in response to hemodynamic stress. Nat Med 13:619, 2007

9. Tanida I, Minematsu-Ikeguchi N, Ueno T, et al: Lysosomal turnover, but not a cellular level, of endogenous LC3 is a marker for autophagy. Autophagy 1:84, 2005

10. Woods DC, Alvarez C, Johnson AL: Cisplatin-mediated sensitivity to TRAIL-induced cell death in human granulosa tumor cells. Gynecol Oncol 108:632, 2008

11. Kim KW, Mutter RW, Cao C, et al: Autophagy for cancer therapy through inhibition of pro-apoptotic proteins and mammalian target of rapamycin signaling. J Biol Chem 281:36883, 2006
12. Ranuncoli A, Cautero N, Ricordi C, et al: Islet cell transplantation: in vivo and in vitro functional assessment of nonhuman primate pancreatic islets. Cell Transplant 9:409, 2000
13. Mizushima N, Yamamoto A, Matsui M, et al: In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker. Mol Biol Cell 15:1101, 2004
14. Huang S, Bjornsti MA, Houghton PJ: Rapamycins: mechanism of action and cellular resistance. Cancer Biol Ther 2:222, 2003

ELSEVIER

Intracellular and Extracellular Remodeling Effectively Prevents Human CD8$^+$Cytotoxic T Lymphocyte-Mediated Xenocytotoxicity by Coexpression of Membrane-Bound Human FasL and Pig c-FLIP$_L$ in Pig Endothelial Cells

M. Tanemura, A. Saga, K. Kawamoto, T. Machida, T. Deguchi, T. Nishida, Y. Sawa, Y. Doki, M. Mori, and T. Ito

ABSTRACT

Human CD8$^+$ cytotoxic T lymphocyte (CTL)–mediated cytotoxicity, which participates in xenograft rejection, is mediated mainly by the Fas/FasL apoptotic pathway. We previously developed methods to inhibit human CTL xenocytotoxicity by extracellular remodeling using overexpression of membrane-bound human FasL on pig xenograft cells, and by intracellular blockade of death receptor-mediated apoptotic signals, such as the Fas/FasL pathway using the pig c-FLIP$_L$ molecule. To investigate the cooperative effects of both membrane-bound FasL and pig c-FLIP$_L$, we cotransfected both genes into pig endothelial cells (PEC). The double remodeling with these molecules effectively prevented CD8$^+$ CTL killing. Although double transfectants and single high transfectants of either membrane-bound FasL or c-FLIP$_L$ gene displayed similar inhibition of CTL cytotoxicity, the expression levels of these 2 molecules in double transfectants were almost half the expression levels of single transfectants. Furthermore, to show in vivo prolongation of xenograft survival, we transplanted PEC transfectants under the rat kidney capsule. Prolonged survival was displayed by PEC double transfectant xenografts whereas those from either parental PEC or MOCK (vehicle control) were completely rejected by day 5 posttransplantation. These data suggested that intracellular and extracellular remodeling by coexpression of membrane-bound FasL and pig c-FLIP$_L$ in xenograft cells may prevent an innate cellular response to xenografts. The gene compatibility of these molecules to generate transgenic pigs may be sufficient to create a window of opportunity to facilitate long-term xenograft survival.

OVERCOMING hyperacute rejection (HAR) by α1, 3-galactosyltransferase gene knockout animals (GT-KO) that bear deletion of the major xenoantigen, α-gal epitopes (Galα1-3Galβ1-4GlcNAc-R), has been a significant step toward successful pig-to-primate organ xenotransplantation.[1,2] However, long-term xenograft survival has yet to be achieved because a xenograft may be rejected directly by cell-mediated immunity, including natural killer (NK) cells, macrophages, and CD8$^+$ cytotoxic T lymphocytes (CTLs).[3–5] Therefore, overcoming cell-mediated immunity, especially mediated by human CD8$^+$ CTLs, is an important strategy for long-term successful xenograft survival. Our previous studies have reported that the highly detrimental cytotoxic activity of human CD8$^+$ CTLs against pig endothelial cells (PEC) is mediated in major part by the Fas/FasL apoptotic pathway.[5] To inhibit this strong CTL killing, we have exploited the weapon of extracellular overexpression of a membrane-bound human FasL that carries a deletion at the metalloproteinase cleavage site.[5] In addition, we have demonstrated that intercellular overexpression of pig c-FLIP$_{long}$ (c-FLIP$_L$), which is a potent inhibitor of death receptor-mediated pro-apoptotic signals

From the Osaka University Graduate School of Medicine, Osaka, Japan.

Address reprint requests to Masahiro Tanemura, MD, PhD, Osaka University Graduate School of Medicine, Department of Surgery; Gastroenterological Surgery, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan. E-mail: mtanemura@gesurg.med.osakau.ac.jp

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360 Park Avenue South, New York, NY 10010-1710

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protects PEC from human CTL-mediated killing[6] by virtue of blocking the signaling pathway more upstream (before caspase-8 activation and release).

The present study addressed the question regarding human CD8$^+$ CTL-mediated xenocytotoxicity—will double overexpression of both membrane-bound human FasL and pig c-FLIP$_L$ on pig xenograft cells display cooperative effects to prevent CTL-mediated xenocytotoxicity? Furthermore, we examined the in vivo prolongation effects of xenograft survival by double remodeling of these molecules, using transplantation studies.

MATERIALS AND METHODS

Cell Culture

A PEC line, MYP-30,[7] was maintained in DMEM (Sigma-Aldrich, St Louis, Mo United States) supplemented with 10% FBS (Sigma-Aldrich), 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.1 mmol/L nonessential amino acids (Invitrogen, Carlsbad, Calif, United States).

Gene Construction

Complementary DNA (cDNA) encording the membrane-bound human FasL, which cannot be cleaved with metalloproteinase, was subcloned into the site of pEF-BOS expression vector, which carried the promoter of the human elongation factor 1α chromosomal gene.[5] cDNA of pig c-FLIP$_L$ was subcloned into the EcoRI site of pCR3.1 expression vector, which carried a cytomegalovirus (CMV) promoter.[8]

Transfection of Plasmids

Each 20 μg of these plasmids was cotransfected into the PEC line (MYP-30) using lipofectamine (Invitrogen), according to the manufacture's instructions. PEC that had been stably transfected with pEF-BOS and pCR3.1 expression vectors, which lack the cDNA fragments of either membrane-bound FasL or pig c-FLIP$_L$, was also established (ie, MOCK) as a vehicle control. The expression of membrane-bound human FasL on the PEC surface was assessed using FACS analysis, as previously described.[5] The intracellular protein expression of pig c-FLIP$_L$ was detected using Western blot analysis, as previously described.[6] Protein expression levels of pig c-FLIP$_L$ in PEC transfectants were quantified using Fluor-chem image analyzer (BioRad) as expressed by arbitary units. As the loading control for each sample, protein expression of pig GAPDH in either parental, MOCK, or PEC transfectants was detected using a goat anti-pig GAPDH monoclonal antibody (mAb; Santa Cruz Biotechnology, Santa Cruz, Calif, United States). The relative protein expression of pig c-FLIP$_L$ in PEC transfectants was normalized to that of pig GAPDH as expressed by the c-FLIP$_L$/GAPDH ratio.

Preparation of Human CD8$^+$ CTL

To generate human CD8$^+$ CTLs, 10 to 15 × 10^6 separated PBMCs were cocultured for 14 days with irradiated PEC as stimulator cells in the presence of recombinant human interleukin (IL)-2, as previously described.[5,6] Subsequently, human CD8$^+$ CTLs positively isolated by magnetic beads (Dynal, Oslo, Norway) coated with anti-human CD8 mAb (RPA-T8, BD Biosciences Pharmingen, San Jose, Calif, United States) were examined using an in vitro cytotoxicity assay.

In Vitro Cytotoxicity Assay

The cytotoxic activity of human CD8$^+$ CTLs incubated under various conditions was assessed using a ^{51}Cr release assay as previously described.[5,6] Parental PEC, MOCK, and PEC transfectants with either membrane-bound FasL, pig c-FLIP$_L$, or both genes were plated at 5000 cells/well in 96 well plates as target cells. After labeling with ^{51}Cr for target cells, human CTLs isolated using magnetic beads were added to the wells. ^{51}Cr released from the dead cells was measured in the supernates. The cooperative effects of FasL and pig c-FLIP$_L$ double expression on human CTL-mediated xenocytotoxicity were determined by comparisons with PEC single transfectants and parental PEC.

Transplantation Studies and Immunohistochemical Analysis

Lewis rats (8 to 10 weeks old) purchased from Oriental Yeast (Tokyo, Japan) were distributed randomly between experimental groups (n = 5 rats per group) to receive either parental PEC, MOCK, or PEC transfectants. Rats immunized 3 times intraperitoneally with pig kidney membranes (250 mg) with a 1-week interval between injections were used as recipients. In each case, 2.5 × 10^6 cells of either parental PEC, MOCK, or PEC transfectants were transplanted under the kidney capsule of rats in the absence of immunosuppression. Transplanted rats were monitored until the time of harvest at day 2, day 3, or day 5 posttransplantation. Each grafted kidney was analyzed using immunohistochemistry. Kidney specimens cut into small blocks and fixed in formalin were embedded in a single paraffin block. After quenching endogenous peroxidase activity by exposure to 3% H_2O_2/methanol, paraffin sections were stained with a rabbit anti-human Von Willebrand Factor (vWF) polyclonal antibody (DAKO) to specifically detect endothelial cells. The rinsed sections were then incubated with link antibody, followed by incubation of horseradish peroxidase-conjugated streptavidin. Immunostaining was visualized with 0.02% diaminobenzidine (DAB, Sigma-Aldrich) as the chromogen. The specificity for the primary vWF antibody was verified by control sections in which we omitted the primary antibody.

RESULTS

Establishment of PEC Transfectants Overexpressed Either Membrane-Bound FasL, Pig c-FLIP$_L$, or Both Genes

Two single positive clones were isolated: one had a high expression of membrane-bound human FasL, and other had a high expression of pig c-FLIP$_L$. Additionally, we also established three double-positive clones. Of the double transfectants, double-1 had a high expression of membrane-bound FasL and a low level of pig c-FLIP$_L$ protein; whereas, double-2 had a low expression of FasL and a moderate protein expression of pig c-FLIP$_L$, and double-3 showed moderate expression levels of both molecules with almost half of the levels compared with the single high expression clones of each molecule, respectively (Table 1).

Double-Overexpression of FasL and c-FLIP$_L$ Effectively Prevents CD8$^+$ CTL-Mediated Cytotoxicity Against PEC

Human CD8$^+$ CTLs generated by in vitro culture displayed strong killing against parental PEC and MOCK, namely, a lysis of >80% at an effector to target ratio of 50:1 (Fig 1A).

Table 1. Changes in the Expression Levels of Either Membrane-Bound FasL or c-FLIP$_L$ Molecules

Cells	Protein Expression in PEC Transfectants	
	Membrane-Bound FasL (Mean Fluorescence Intensity by FACS)	c-FLIP$_L$/GAPDH Ratio (Arbitary Units)
Parental PEC	Not detected	0.4 (endogenous)
MOCK	Not detected	0.38 (endogenous)
PEC-FasL (single, high)	127	0.35 (endogenous)
PEC-FLIP$_L$ (single, high)	Not detected	1.8
Double 1: FasL (high) + FLIP$_L$ (low)	80.6	0.8
Double 2: FasL (low) + FLIP$_L$ (middle)	33.1	1.2
Double 3: FasL (middle) + FLIP$_L$ (middle)	62.0	1.1

Note: The expression level of membrane-bound FasL on the PEC surface was examined using FACS analysis. The intracellular expression of c-FLIP$_L$ protein was assessed using Western blotting. Image analyzer profiles were used to quantify the expression level of c-FLIP$_L$ protein in parental PEC, MOCK, and PEC transfectants. The relative protein expression of c-FLIP$_L$ in PEC transfectants was normalized to that of pig GAPDH and expressed as the c-FLIP$_L$/GAPDH ratio.

The single overexpression of either membrane-bound FasL or pig c-FLIP$_L$ in PEC resulted in marked cytoprotection from CD8$^+$ CTLs (Fig 1A). Inhibition of cytotoxicity by 64% to 73% was observed among single high expression clones of either FasL or c-FLIP$_L$ transfectants at effector-to-target ratios of 50:1. The double-overexpression of these molecules in PEC effectively reduced CD8$^+$ CTL-mediated cytotoxicity (Fig 1A). From the result for the double-3

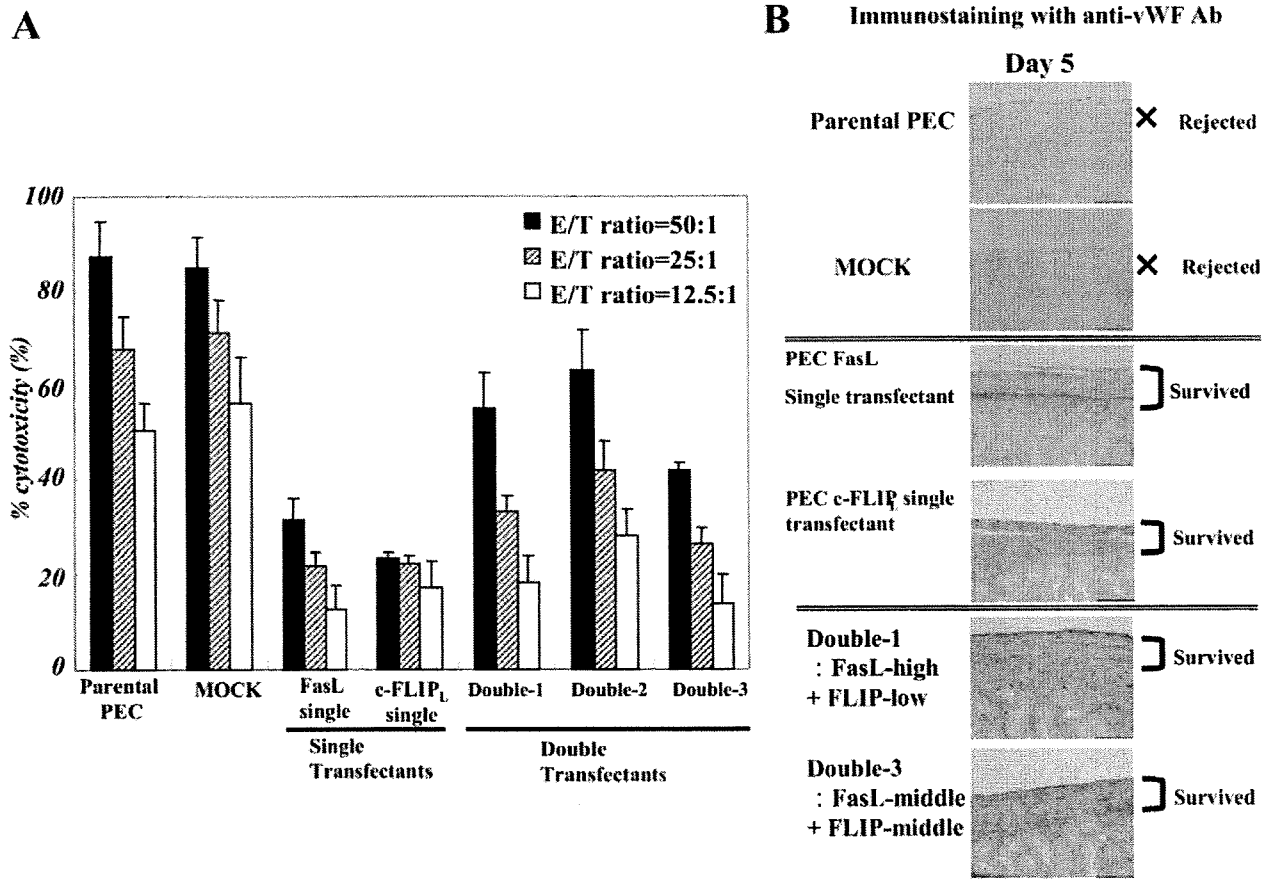

Fig 1. ^{51}Cr release assay of PEC transfectants and immunohistological findings of PEC xenografts transplanted under rat kidney capsule. Amelioration of human CD8$^+$ CTL-mediated cytotoxicity by the PEC transfectants, MOCK, and control parental PEC was estimated at the effector:target ratio of either 12.5:1, 25:1, or 50:1. (**A**) The percentages of CTL-killing by PEC transfectants. Each value is expressed as the mean ± SD from 5 independent experiments. (**B**) Immunohistological findings of rat kidney tissue of PEC transplanted rats. Immunostaining with anti-vWF Ab for transfected PEC of kidney specimens obtained at day 5 posttransplantation. Pictures are representative of immunostaining of kidney sections obtained from 5 animals per each transfectant group. The black bars in each picture indicated 100 μm.

transfectant, the inhibition level of CTL killing by this clone was similar to that of either the FasL or the c-FLIP$_L$ single high expression clones at an effector-to-target ratio of either 25:1 or 12.5:1. In contrast, the expression levels of these 2 molecules were almost half that of the single high transfectants, as judged by either c-FLIP$_L$/GAPDH ratio of western blot analysis or mean fluorescence intensity of FACS analysis. These findings indicated that additional effects to inhibit CD8$^+$ CTL-mediated xenocytotoxicity can be obtained by double-expression of both FasL and c-FLIP$_L$ in PEC.

Double-Overexpression of FasL and c-FLIP$_L$ can Prolong Xenograft Survival

To prove that the double-overexpression of both FasL and c-FLIP$_L$ molecules in PEC was effective to prolong xenograft survival, we transplanted PEC transfectants under the rat kidney capsule. The results of the immunohistochemical analysis are summarized in Fig 1B. At day 3 posttransplantation, large numbers of well-preserved both parental PEC, MOCK, and PEC transfectants were observed under the kidney capsules (data not shown). By day 5 posttransplantation, parental PEC and MOCK had been completely rejected (Fig 1B). In contrast, both single high and double transfected PEC xenografts survived intact at day 5 posttransplantation (Fig 1B). Accordingly, we observed beneficial effects for in vivo prolongation of xenografts with double PEC transfectants.

DISCUSSION

Cellular immunity toward xenograft rejection, including human NK cells, macrophages, and CD8$^+$ CTLs, seems to be an important obstacle to prolonged graft survival in pig-to-human xenotransplantation. We have previously developed methods to prevent immune attack of human CD8$^+$ CTLs toward pig xenograft cells by means of both extracellular remodeling of the death receptor using membrane-bound human FasL and intracellular blocking of death receptor-mediated apoptotic signals, such as Fas/FasL pathway by the use of pig c-FLIP$_L$ molecule. In the present study, we assessed the amelioration of cytoprotective effects from human CTL-mediated killing by combined overexpression of both membrane-bound human FasL and pig c-FLIP$_L$ genes.

Both in vitro and in vivo analyses revealed the cooperative effects of intracellular and extracellular remodeling with these molecules to inhibit CTL xenocytotoxicity. Our final goal was to generate a double transgenic pig with both membrane-bound FasL and c-FLIP$_L$ genes. Consequently, pig islets obtained from this double transgenic pig may prove to be beneficial to prolong xenograft survival by a high resistance to the immune attack of human CTLs. However, from the embryological view, the birth of a double transgenic pig, which highly expresses both FasL and c-FLIP$_L$ molecules, respectively, may be difficult. It takes a long time to select the high expression clone of these molecules. The cooperative effects and compatibility of multi-transgenes must be understood to efficiently generate transgenic pigs with multi-genes. Thus, our findings in the present study demonstrated that double remodeling with both membrane-bound human FasL and c-FLIP$_L$ may well be compatible to generate transgenic pigs and can elicit cooperative effects for the inhibition of human CTL xenocytotoxicity.

REFERENCES

1. Galili U: Interaction of the natural anti-Gal antibody with α-galactosyl epitopes: a major obstacle for xenotransplantation in humans. Immunol Today 14:480, 1993

2. Yamada K, Yazawa K, Shimizu A, et al: Marked prolongation of porcine renal xenograft survival in baboons through the use of α 1,3-galactosyltransferase gene-knockout donors and the cotransplantation of vascularized thymic tissue. Nat Med 11:32, 2005

3. Tanemura M, Chong AS, DiSesa VJ, et al: Direct killing of xenograft cells by CD8+ T cells of discordant xenograft recipients. Transplantation 74:1587, 2002

4. Itescu S, Kwiatkowski P, Artrip JH, et al: Role of natural killer cells, macrophages, and accessory molecule interactions in the rejection of pig-to-primate xenografts beyond the hyperacute period. Hum Immunol 59:275, 1998

5. Kawamoto K, Tanemura M, Ito T, et al: Significant inhibition of human CD8$^+$ cytotoxic T lymphocyte-mediated xenocytotoxicity by overexpression of the human decoy Fas antigen. Transplantation 81:789, 2006

6. Tanemura M, Saga A, Kawamoto K, et al: In vitro and in vivo prevention of human CD8$^+$ CTL-mediated xenocytotoxicity by pig c-FLIP expression in porcine endothelial cells. Am J Transplant 8:288, 2008

7. Miyagawa S, Shirakura R, Iwata K, et al: Effects of transfected complement regulatory proteins, MCP, DAF, and MCP/DAF hybrid, on complement-mediated swine endothelial cell lysis. Transplantation 58:834, 1994

8. Goto Y, Matsuda-Minehata F, Inoue N, et al: Porcine (Sus scrofa) cellular FLICE-like inhibitory protein (cFLIP): molecular cloning and comparison with the human and murine cFLIP. J Reprod Dev 50:549, 2004

糖尿病に対する膵島移植によるベータ細胞補充療法

膵島移植は糖尿病に対するベータ細胞補充療法であり，血糖値の安定化とそれに伴う合併症の予防効果が得られる．今後，バイオ人工膵島移植への発展によるドナー不足解消，免疫寛容などの重要な研究により，さらにこの治療は発展すると考えられている．

Baylor All Saints Islet Cell Laboratory, Baylor Institute for Immunology Research Islet Cell Transplantation
松本　慎一
MATSUMOTO, Shinichi

東京大学医科学研究所先端医療社会コミュニケーションシステム社会連携研究部門
畑中　暢代
HATANAKA, Nobuyo

Key Word
ベータ細胞　膵島移植　1型糖尿病
バイオ人工膵島　患者の自律　QOL

1．背景

糖尿病とは，膵臓にある膵島を構成するベータ細胞から分泌されるインスリンの分泌あるいは作用不足にて血糖値が上昇する病気である．21世紀に入りその数は世界的に急増し，わが国でも予備軍を含めると1000万人を超える患者がいる．症状として，口渇，多飲，多尿があるが，初期では無症状なことが厄介なところである．また慢性合併症があり，腎症，網膜症，壊死による下肢切断，虚血性心疾患，脳卒中などがある．この中でも特に糖尿病性腎症は，透析導入の一番の原因であり，わが国の医療費を圧迫しているため，対策が急がれている．

糖尿病はその発症の機序から1型糖尿病と2型糖尿病に分類される．1型糖尿病は自己免疫の機序によりベータ細胞を破壊する病気で，インスリン注射が不可欠になる症例が大半となる．さらに，ベータ細胞が完全に破壊されてしまうと，インスリンの補充は完全に注射のみに頼るようになり，血糖値を安定させることが困難となる．特に，血糖値が下がりすぎると意識喪失や昏睡やけいれんといった症状を呈するため，QOLはきわめて不良となる．2型糖尿病は生活習慣病の1つとされ，肥満などによるインスリンの作用不足がおもな原因である．ただし，2型糖尿病でも病歴が長くなると，ベータ細胞からのインスリン分泌能が下がり，インスリン依存状態になることもある．

膵島移植は，1型糖尿病を代表とするインスリン分泌が不足するタイプの糖尿病に対するベータ細胞補充療法として位置付けられている[1), 2)]．特に，インスリン分泌が枯渇しているために血糖値のコントロールが困難な患者に対し，血糖値を安定化させるうえで効果が高い．膵島移植の工程は，臓器提供者（ドナー）から膵臓摘出，摘出された膵臓の膵島分離施設への運搬，cGMPに準拠した膵島分離施設での膵島

cGMP: current Good Manufacturing Practice
QOL: quality of life

POINT

幹細胞：細胞分裂を経ても同じ分化能を維持する細胞で，受精卵由来の胚性幹細胞は理論上すべての種類の細胞に分化することができる．また，胚性幹細胞以外に分化することができる細胞が限られている組織幹細胞，体性幹細胞がある．

分離，レントゲン室での移植からなる．この中で，膵島分離の工程には高度の技術が求められるが，移植手技そのものは全身麻酔も手術も不要であり，きわめて低侵襲である．

2. 膵島移植の現状

糖尿病に対するベータ細胞補充療法としては，膵島移植以外に膵臓移植がある．膵臓移植は，ドナーから膵臓を摘出し，摘出した膵臓そのものを糖尿病患者に移植する方法である．この治療は1970年代に米国ミネソタ大学で開始され，現在では糖尿病性腎症の患者に対して，膵臓と腎臓を同時に移植する膵腎同時移植が一般治療とされている．

膵臓移植の利点は，成功するとインスリン注射が高率で不要になり，血糖値の安定化および2次合併症の予防が可能なことである．ただし，移植手技による侵襲が大きく，また，膵臓という自己融解しやすい臓器そのものを移植するため，外科的合併症が致命的にもなるというリスクがある．このため，膵臓移植は糖尿病性腎症の患者に対して腎臓と膵臓を同時に移植し，人工透析とインスリン注射の両方から解放を得るメリットの大きい，いわゆる膵腎同時移植として行われる．しかし，膵臓の単独移植は手術侵襲が大きく，一般には移植することで生命予後がかえって悪化するために症例を慎重に選択する必要があり，通常は行われない．

膵島移植も，1970年代に米国ミネソタ大学で最初の臨床応用が実施された．しかしながら，移植した膵島が機能することは難しく，臨床応用は限られた施設で限られた症例にのみ実施されていた．ところが，2000年にカナダのエドモントン市にあるアルバータ大学が膵島移植を行った7例すべてが移植後1年以上インスリン注射から解放されたと報告したことから，世界的に臨床応用が広がった．この方法は現在「エドモントンプロトコール」と呼ばれている[3]．

3. 膵島移植の臨床応用

エドモントンプロトコールが臨床膵島移植の原点となっているが，その特徴の1つとして，拒絶反応を抑えるための薬剤の中からステロイド剤を省いたことがあげられる．ステロイド剤は拒絶反応を抑えるために有効な薬剤であるが，副作用として血糖値を上昇させ，糖尿病を誘引する．このため，膵島移植を行っても薬剤性の糖尿病が起こり，移植した膵島が高血糖のために機能し続けられなくなる．エドモントンプロトコールでは，ステロイド剤を省き，新しい免疫抑制剤であるシロリムスを導入することでこの副作用を予防した．

もう1つの特徴は，複数の膵臓を利用したことである．通常の臓器移植であれば，提供された臓器は貴重であるために1つの臓器を少なくとも一人の患者，可能であれば2人の患者へと移植する．しかしながら，膵島を膵臓から分離する工程は高度の技術を要し，当時は半数以下しか分離できず，さらに膵島移植後に半数以上の膵島が死滅するため，一人のドナーからの膵臓では移植の効果が十分得られなかった．このため，膵島移植を2回あるいは3回行うことで，膵臓移植をしのぐ効果を出すことに成功した．エドモントンプロトコールは，2000年に発表された時点では，拒絶反応を抑える薬剤の副作用が少なく，さらに侵襲が低い糖尿病の治療として世界的に大きく取り上げられ，その後臨床応用が世界的に急増した．

エドモントンプロトコールによる膵島移植では，移植後5年経過してもおよそ80%の患者に膵島の機能が維持できており，これらの患者は血糖の安定化に伴うQOLの改善が得られて

POINT

万能細胞：体細胞へ数種類の遺伝子あるいは転写因子を導入することにより，胚性幹細胞に似た分化万能性をもたせた細胞のこと．胚性幹細胞のように受精卵を利用する必要がないことから，倫理的に利用しやすい．

いる．ただし，インスリン離脱率は年々低下し，2～3年でおよそ半減し，5年で10%以下となる．

エドモントンプロトコールの国際マルチセンタートライアルが実施され，その結果が2006年に発表された[4]．この報告では，1型糖尿病患者36名に膵島移植が実施され，1年後のインスリン離脱は16名，膵島の機能維持は10名，膵島が機能しなかった例は10名であった．移植後1年目のインスリン離脱率は0%の施設と100%の施設があり，施設間格差の大きさを認めた．これにより膵島移植には施設としての経験も重要であることが認識される結果となった．特に，膵島分離技術と免疫抑制剤のコントロールに経験が重要と考えられている．膵島分離は高度な技術と経験が必要とされており，実際に膵島分離を行っても移植に至らない膵島分離失敗がいまだに高率に起こる．そのため，安定した膵島分離技術の確立が急務である．

膵島分離が膵島移植の最も重要な過程であるが，我々は4～5名のチームで行い4～5時間を要する．チームは医師と技術者で構成されることが多く，我々も外科医および内科医と技術者にてチームを構成している．

現在，膵島移植は，経験のある施設で複数回の移植を行えば，まずインスリン離脱が得られ，その後インスリンは再開されるが，比較的長期にわたり良好な血糖コントロールが得られるという状況である．このため，血糖値のコントロールが難しい患者にとって最も有効な治療とされている．

4．わが国における膵島移植

膵島移植は，一般に脳死ドナーからの膵臓を利用して実施される．ところがわが国では脳死ドナーの数が極端に少なく，さらに膵島移植は臓器移植ではないために臓器の移植に関する法律の対象外となり，脳死ドナーを利用することはきわめて困難である．このため，我々はわが国で膵島移植を実施するためには，いわゆる心停止ドナーあるいは生体ドナーを利用する必要があると考えた．

心停止ドナーからの膵島移植は，脳死ドナーを用いる場合よりさらに膵島分離が困難であるため，膵島分離の技術を高めることが必須と考えられた．そこで我々は，心停止ドナーからの膵島移植を可能にすべく，大動物を用いて膵島分離方法の改善の研究を進めた．おもな改良点として，心停止後の膵臓を直ちに冷却する *in situ* cooling 方法の開発，膵臓を摘出してから膵島分離施設に運搬するまでの膵臓保存方法の改良，膵島分離方法の改良があげられる[1]．

in situ cooling 方法とは，ドナーに対して大腿動脈よりカニューレを挿入し，腎臓とともに膵臓を心停止後直ちに冷却する方法である．この方法を行うことで，心停止後の膵臓の自己融解を有効に防ぐことができる．

膵臓の運搬は，従来は膵臓や肝臓などの臓器保存と同じウイスコンシン溶液が使われていたが，我々は，酸素運搬能力が高いパーフルオロカーボンを用いた保存方法を採用した．この方法を用いると，保存中に，膵臓はパーフルオロカーボンより酸素を供給されるため良好な状態が保たれる[5]．また我々は，膵管から逆行性に保存液を注入することで膵管を良い状態に保てることを発見し，通常の臓器保存に加えてこの方法にて膵管保存を行っている．

膵島の分離は，膵臓の消化および膵島の純化の2つの行程からなる．膵臓の消化は，膵管から逆行性にコラーゲンの分解酵素であるコラゲナーゼを注入する方法がとられている．このため，前述の膵管保存は重要となる．膵島の純

POINT

バイオ人工膵島：ブタなどの動物由来の膵島や，幹細胞などから作られた膵島細胞を特殊な容器に入れた移植用の膵島．ブタ膵島を用いたバイオ人工膵島移植はすでに臨床応用が実施されている．

化は，膵島が膵臓のほかの組織より密度が軽いことを利用する．消化された膵臓を密度勾配溶液を用いて遠心分離することで，密度の軽い膵島が浮いてくる．従来，密度勾配を作る溶液は粘度が高く，遠心分離をする際に物理的な障害が発生していた．そこで我々は粘度が低い溶液を用いた密度勾配溶液を開発し，膵島の純化時の障害の軽減に成功している．これらの技術開発により，2004年に我々は，心停止ドナーを用いてわが国で初めての膵島移植に成功した[6]．この膵島分離方法は，心停止ドナーからの膵島分離方法として特許申請中である．

5. 生体ドナー膵島移植

心停止ドナー膵島移植の成功後は，わが国での膵島移植希望者が増加し，もう1つの可能性である生体ドナー膵島移植の可能性を追求した．生体ドナー膵島移植は，懸念材料として，①ドナーのリスク，特に糖尿病発症について，②確実な膵島分離方法，③部分膵臓からの分離膵島数の確保，などの検討が必要であった．糖尿病発症のリスクについては，ミネソタ大学で実施されてきた生体膵臓移植のドナーデータを解析し，さらに日本人の糖代謝を加味して生体膵ドナーのクライテリアを決定した．なお，2005年にはバンクーバーで生体ドナー移植の国際会議があり，生体膵臓ドナーの基準が定められた[7]．また，部分膵臓からの膵島分離では，膵の尾部は膵島分離に適しており，尾部のみで膵臓全部を利用した場合に近い数の膵島が分離できることが示された．

このようにさまざまな課題を解決し，2005年に生体膵島移植が実施された．移植後，インスリン離脱に成功し，世界で初めての生体ドナー膵島移植成功例となった[8]．また，移植後約1カ月目の経口ブドウ糖負荷試験では，ドナーおよびレシピエントともに正常型を示し，移植後1年の時点では，ドナーおよびレシピエントとも経過良好であった[9]．ただし，生体ドナー膵島移植は，レシピエントが重症であること，ドナーのインスリン分泌能が十分であることなど厳しい制限があるため，2例目はいまだ実施されていない．

6. 膵島移植における患者の自律の重要性

膵島移植のおもな対象となる1型糖尿病は，標準治療方法がインスリン注射であり，通常は生命の危機が迫っている疾患ではない．このため，膵島移植は救命的な治療ではなく，腎臓移植のようにQOLを改善する移植である．ここで重要なのは，QOLが改善したかどうかは患者自身が実感するものであり，本来医療者が外から計れるものではないということである．たとえば1型糖尿病患者にとって，頻回なる低血糖で外出がままならない場合は，膵島移植後に血糖値が安定することはQOLを向上させることにつながる．しかし，血糖の不安定がQOLに支障をきたしていない患者にとっては，血糖値が安定してもQOLの向上を認めることは少ないといえる．また，移植後は拒絶反応を抑えるために免疫抑制剤の内服が必要となる．この免疫抑制剤には，口腔内潰瘍，下肢の浮腫，タンパク尿，腎障害，骨髄抑制などの副作用があり，この副作用の苦痛が，血糖値が安定することよりもQOLを悪化させる可能性があることも軽視できない．

つまり，QOL改善を目的とする治療は，医療者が，患者にとって移植が適した治療だと勝手に判断し決定してはならないのである．医療者にとっては患者に正確な情報を提供することが重要で，患者にとってはその情報をもとに自分自身の受ける治療のこと，その後に起こり得

POINT

患者の自律(オートノミー)：複数の治療選択肢がある場合，医療者ではなく患者自身が治療を選択すること．この際，医療者は患者に対して選択肢を十分に説明する必要がある．治療方針が患者の価値観によって大きく変わる慢性疾患では特に重要である．

ることのすべてを考え，自分自身で治療を選択することが重要なのである．これは，他の治療でも基本的には同じであるが，特にQOL改善を目的とする場合，日常の生活そのものが個人個人で大きく異なるため，本当にその人にとってのQOLが向上するかを患者以外が判断することはきわめて難しいのである．膵島移植に限らず多くの治療の選択肢がある場合，患者自身がその選択肢から治療を自己決定するために，患者の自律(オートノミー)の力をサポートすることが重視されるべきである．

正確な情報伝達を十分に行うことが医療者にとって重要な責務となるが，膵島移植のような最先端医療では，年々治療成績が改良されていくため，情報のアップデートが重要である．さらに，患者に十分理解してもらうために，時間を割いて丁寧に説明することが不可欠である．このため，膵島移植のような専門性の高い先進医療には，その治療に精通したコーディネーターの存在が不可欠と考えている．

7. 膵島移植の役割と期待

7-1 膵島移植の現在の役割

膵島移植は，現在，血糖値が不安定なインスリン分泌が枯渇した糖尿病患者の治療として実施されている．おもな効果は，血糖値の安定化とそれに伴う低血糖の回避である．最近では，糖尿病性腎症による腎臓移植後に，血糖値の安定化による移植腎機能の維持を目的に腎移植後膵島移植が実施されている．腎臓移植後膵島移植は，腎臓移植後にすでに免疫抑制剤を内服していることで，膵島移植のために新たに免疫抑制剤を開始するというリスクが回避される．膵島単独移植と違い新たに免疫抑制剤を内服するというリスクがないために，すでに欧州では腎臓移植後の膵島移植が標準治療となっているところもある．以上のように，膵島移植の目的は血糖値を安定させ，その結果，2次合併症の予防と遅延が期待できることである．また2次合併症の予防は透析医療への移行を予防でき，医療経済への影響も期待できる．

7-2 膵島移植への期待

1)糖尿病の根治

膵島移植に対する期待は多岐にわたる．まず，身近な期待として，インスリン分泌が低下するタイプの糖尿病の根治である．現在，膵島移植は血糖値が不安定な患者に対する血糖値安定化をおもな目的としている．しかし，臓器が十分にあり，免疫抑制剤の副作用がインスリン注射の副作用を十分に下回れば，インスリン注射からの解放を目的とした治療法になり得る．特に日本人の場合，2型糖尿病であってもインスリン分泌が低下していることが原因であることが多いことから，膵島移植はインスリン治療に代わる治療として期待されている．これにより，臓器の十分な提供および免疫抑制剤の副作用を最小限にするということが重要な研究課題であることがわかる．

2)バイオ人工膵島移植の確立と異種移植への道

臓器不足の解決策として，ブタの膵臓を利用したバイオ人工膵島移植に大きな期待が寄せられている(図1)．これは，ブタの膵島を特殊な容器に入れ，皮下などに移植する治療法である．実際にメキシコで臨床応用され，インスリン離脱が得られたことから注目されている[10]．ただし，メキシコの臨床例は成功率が低く再現性が疑問視されているために，より効果の高いバイオ人工膵島移植の開発が望まれる．ここで重要なことは，ブタという異種の細胞を臨床応用できるという事実である．現在，心臓移植，肺移植，肝臓移植，腎臓移植などすべての臓器移植は，ヒトの臓器に依存している．このため，

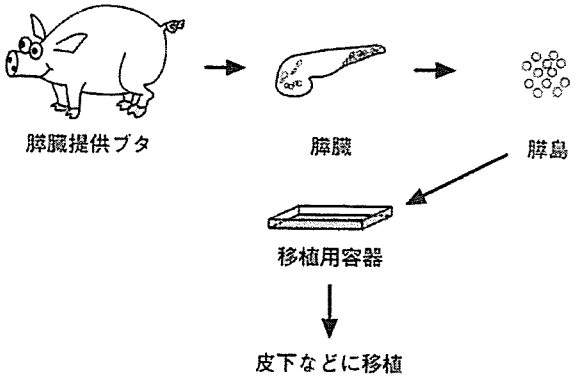

図1　バイオ人工膵島
バイオ人工膵島は，ブタから膵臓を摘出し，摘出膵臓から膵島を分離する．分離された膵島は特殊な移植用容器の中に入れられ，皮下などに移植される．

常に提供者(ドナー)不足が問題となる．もしバイオ人工膵島移植が一般化すれば，ブタを用いた臓器移植への基礎的なデータとなるため，バイオ人工膵島移植は異種移植への重要な布石と考えられている．

3)再生医療の臨床応用

バイオ人工膵島移植は，現在，ブタの膵島を利用したものの開発が一番臨床応用に近いとされている．しかし，細胞の供給源として幹細胞や万能細胞の応用も考えられる．つまり，幹細胞や万能細胞からインスリン分泌細胞を作り出すことができれば，それらを特殊な容器に入れ皮下などに移植することができる．幹細胞や万能細胞の利点は，自分の細胞からこれらの細胞を作り出すことができれば，いわゆる拒絶反応が起こらず免疫抑制剤が不要なことである．特に膵島移植の成功から細胞治療が臨床に有意義であるということが証明されているため，幹細胞や万能細胞の臨床応用の1つがバイオ人工膵島移植として期待されている．

4)免疫寛容の確立

臓器移植の場合，移植後臓器が生着している間は免疫抑制剤を飲み続けることが一般的であり，免疫抑制剤の内服をやめると拒絶反応が起こる．ただし，肝臓移植では，何らかの原因で免疫抑制剤を完全に中止しても拒絶反応が起こらない症例が実際にある．このように，免疫抑制剤を中止しても拒絶反応が起こらない状態を免疫寛容という．しかし，現在，免疫寛容を導き出す方法は確立されていない．心臓移植などの救命的な移植の場合，免疫寛容を誘導しようと免疫抑制剤を減量あるいは中止して拒絶反応が起こると致命的になるため，免疫寛容導入の臨床応用が難しい．一方で，膵島移植は救命的な治療でないために，仮に拒絶反応が起こっても患者の死亡にはつながらず，また，膵島の機能が低下しても追加移植が行える．このため，膵島移植は免疫寛容の臨床応用に最も適していると考えられている．さらに，異種細胞を使ったバイオ人工膵島移植で免疫寛容が達成できれば，免疫抑制剤の副作用という移植での大きな欠点がなくなるために，バイオ人工膵島移植が一気に広がる可能性がある．

8. おわりに

膵島移植はすでに臨床応用が進んでおり，将来的に糖尿病治療の重要な位置を占めることが予測できる．バイオ人工膵島移植が完成すればドナー不足が解消されるため，2型糖尿病への応用も開始されるであろう．さらに膵島移植は，異種移植，再生医療，免疫寛容に関する研究にとって原点として重要と考えられており，この分野での研究の重要性はいよいよ増すと考える．

■著者連絡先メールアドレス
ShinichM@BaylorHealth.edu

■文　献

1) Matsumoto S, Noguchi H, Yonekawa Y, et al: Pancreatic islet transplantation for treating diabetes, Expert Opinion on Biological Therapy 6(1): 23-37, 2006
2) Matsumoto S, Noguchi H, Naziruddin B, et al: Improvement of pancreatic islet cell isolation for transplantation, Baylor University Medical Center Proceedings 20(4): 357-362, 2007

3) Shapiro AMJ, Lakey JR, Ryan EA, et al: Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen, N Engl J Med 343(4): 230-238, 2000
4) Shapiro AMJ, Ricordi C, Hering BJ, et al: International trial of the Edmonton protocol for islet transplantation, N Engl J Med 355(13): 1318-1330, 2006
5) Matsumoto S, Rigley TH, Qualley SA, et al: Efficacy of the oxygen-charged static two-layer method for short-term pancreas preservation and islet isolation from nonhuman primate and human pancreata, Cell Transplant 11(8): 769-777, 2002
6) Matsumoto S, Okitsu T, Iwanaga Y, et al: Successful islet transplantation from non-heart-beating donor pancreata using modified Ricordi islet isolation method, Transplantation 82(4): 460-465, 2006
7) Barr ML, Belghiti J, Villamil FG, et al: A report of the Vancouver Forum on the care of live organ donor: lung, liver, pancras and intestine data and medical guidelines, Transplantation 81(10): 1373-1385, 2006
8) Matsumoto S, Okitsu T, Iwanaga Y, et al: Insulin independence after living-donor distal pancreatectomy and islet allotransplantation, Lancet 365(9471): 1642-1644, 2005
9) Matsumoto S, Okitsu T, Iwanaga Y, et al: The follow up study of the first successful living donor islet transplantation, Transplantation 82(12): 1629-1633, 2006
10) Valdes-Gonzalez RA, White DJG, Dorantes LM, et al: Three-yr follow up of a type 1 diabetes mellitus patient with an islet xenotransplant, Clinical Transplantation 21(3): 352-357, 2007

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Estimation of Donor Usability for Islet Transplantation in the United States With the Kyoto Islet Isolation Method

Shinichi Matsumoto,* Hirofumi Noguchi,* Nobuyo Hatanaka,† Masayuki Shimoda,‡ Naoya Kobayashi,§ Andrew Jackson,* Nicholas Onaca,* Bashoo Naziruddin,* and Marlon F. Levy*

*Baylor All Saints Medical Center, Baylor Research Institute, Fort Worth, TX 76104, USA
†The Institute of Medical Science, The University of Tokyo, Tokyo, 108-8639, Japan
‡Baylor University Medical Center, Dallas, TX 75246, USA
§Department of Surgery, Okayama University, Okayama, 700-8530, Japan

The quality of donor pancreata is important for successful islet isolation. However, in some countries like Japan, the number of donor pancreata is very low; therefore, marginal donors have been used with less restrictive donor criteria. In order to use marginal donor pancreata, we established the Kyoto islet isolation method (KIIM). According to United Network for Organ Sharing (UNOS) in 2005, more than 6,000 pancreata were not clinically used in the US. In this study, we applied the KIIM for brain-dead donors and reevaluated donor usability based on the Japanese islet donor criteria. Islets were isolated with the Ricordi method using pancreata stored in University of Wisconsin (UW) solution (UW group) or by the two-layer method (TLM group) or the TLM combined with ductal injection (DI group). We implemented the KIIM (KIIM group) to confirm the effect of the KIIM on brain-dead donors. Donor charts in Texas from 2005 to 2006 were reviewed. If pancreata were not used clinically, the reason was reviewed and donors were reevaluated based on Japanese criteria. There were no significant differences of islet yield, viability, and purity between the UW and TLM groups. The DI group significantly improved islet yields and isolations were further improved in the KIIM group [UW: 251,663 ± 60,217 islet equivalent (IE); TLM: 243,738 ± 54,170 IE; DI: 498,639 ± 28,853 IE; KIIM: 678,286 ± 55,853]. The KIIM provided high-quality islets in high numbers from islet isolations from brain-dead donors. A total of 236 donor charts were reviewed and 194 pancreata (82%) were not used. Of these, 185 cases identified the reasons that the pancreata were not used. When we applied the Japanese criteria, an additional 82 cases out of 185 (44%) seem to be suitable for islet isolations. With the KIIM, more than 2,500 additional donor pancreata can be used for islet isolation in the US every year when the Japanese criteria are applied.

Key words: Donor; Ductal injection; Islet transplantation; Kyoto islet isolation method; Kyoto solution; Two-layer method

INTRODUCTION

Islet transplantation is an option for the treatment of type 1 diabetic patients who maintain hypoglycemic unawareness despite maximal care (20,21). Successful islet isolation is the key to successful islet transplantation; the quality of the donor pancreas is important for successful islet isolation (1–3,5,7,15,22). Highly restricted criteria were made in order to assure high quality of donor pancreata for islet isolation (20). However, in some countries like Japan, the number of donor pancreata is very low; therefore, marginal-quality donor pancreata have been used. In order to use marginal donor pancreata, especially from non-heart-beating donors (NHBDs), we modified the Ricordi islet isolation method and developed the Kyoto Islet Isolation Method (KIIM) (11,14,16,17). Major implementations of this modification are pancreatic ductal preservation, modified two-layer pancreas preservation with Kyoto Solution and density-adjusted continuous density gradient purification (10). Using the KIIM, we successfully isolated islets and transplanted into type 1 diabetic patients in 17 out of 21 cases (81%) (8). This transplantation rate is remarkably high compared with previously published data (4,6,9). In fact, the transplantation rate of the majority of islet centers is less than 50%. Despite this low percentage, the majority of pancreata in the US, even from brain-dead heart-beating donors, are not clinically

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Address correspondence to Shinichi Matsumoto, M.D., Ph.D., Baylor All Saints Medical Center, Baylor Research Institute, 1400 8th Avenue, Fort Worth, TX 76104, USA. Tel: 817-922-2570; Fax: 817-922-4645; E-mail: shinichi41@mac.com

used. According to UNOS in 2005, only 1,466 pancreata out of 7,593 cases (19%) were clinically used, meaning that more than 6,000 pancreata were not used. However, we postulated that if we implemented the KIIM for islet isolation from brain-dead donors, we should be able to use marginal donor pancreata for islet isolation, meaning that more pancreata could be clinically used.

In this study, we first implemented the KIIM for islet isolation from brain-dead donors. Then we evaluated pancreas donors in our local area by reviewing well-documented donor charts and estimated how many pancreata in the US could be used for islet isolation when we apply the Japanese islet donor criteria in combination with an improved islet isolation technique, such as the KIIM.

MATERIALS AND METHODS

Pancreas Procurement and Islet Isolation

From January 2005 to August 2007, we performed 27 islet isolations using brain-dead donor pancreata. In all cases, pancreata were stored less than 8 h. All 27 islet preparations were manipulated according to Current Good Manufacturing Practice (cGMP) in the cell processing facility at Baylor Institute for Immunology Research.

The first protocol included the regular Ricordi islet isolation method (19,20) and pancreata were preserved in UW solution ($N = 6$, UW group) or by the oxygen static charged two-layer method ($N = 13$, TLM group) (13). For the next protocol, the islet team participated in pancreas procurement and pancreatic ductal injection was introduced at the donor site ($N = 3$, DI group) (10,11,16). Then pancreata were preserved by the oxygen static charged TLM and islets were isolated by the Ricordi method. For the final protocol, we implemented the KIIM ($N = 3$, KIIM group) (10,11,17). The key procedures of the KIIM are described as follows. We procured pancreata in conjunction with multiorgan procurement. We removed the duodenum and spleen from the pancreas at the procurement site. A cannula was immediately inserted into the procured pancreas through the main pancreatic duct from the direction of the pancreatic head and M-Kyoto solution was administered intraductally (ductal injection). M-Kyoto solution is 100,000 U/L of ulinastatin (Mochida Pharmaceutical Co. Ltd., Tokyo, Japan) in ET-Kyoto solution (Otsuka Pharmaceutical Co., Naruto, Japan).

For pancreas preservation during transportation, we use an oxygenated perfluorocarbon/M-Kyoto solution-based two-layer method. Before islet purification, we checked the density of the isolation aggregates with a test tube density adjustment method as follows (10). Six discontinuous density test tubes were prepared with 5 ml each of purification solution of different densities (1.085, 1.090, 1.095, 1.100, 1.105, and 1.110 g/cm^3). The different densities were achieved by adjusting the ratio of iodixanol (OptiPrep, Axis-Shield PoC AS, Norway) to M-Kyoto solution. Samples were taken from the isolation aggregates and added to the discontinuous density test tubes. The tubes were spun at 1,000 rpm for 5 min and the densities of aggregates were determined by whether they pelleted or floated in the different density solutions. Islets were purified with a continuous density gradient of iodixanol/M-Kyoto solution in an apheresis system (COBE 2991 cell processor, Gambro Laboratories, Denver, CO). The heavy density solution was chosen according to the test tube density adjustment method described above and the gradient was achieved by varying the ratio of iodixanol to M-Kyoto solution.

Islet Evaluation

Islet evaluation was independently judged by two investigators. Islet yield was determined with dithizone staining (2 mg/ml; Sigma Chemical Co., St. Louis, MO) under an optical graticule and converted into a standard number of islet equivalents (IE, diameter standardizing to 150 μm) (12,18). Purity was assessed by comparing the relative quantity of dithizone-stained tissue to unstained exocrine tissue. Islet viability was evaluated using fluorescein diacetate (FDA) and propidium iodide (PI) staining to visualize living and dead cells simultaneously (12,18). The recovery rate after purification was determined by dividing IE before purification by IE after purification. For qualification of transplantation, we used the original Edmonton protocol criteria (20). For a qualified transplantation, islet yield should be more than 5,000 IE/kg patient body weight (we used 60 kg as a default body weight, which means that the total islet yield should be more than 300,000 IE), viability above 70%, purity of more than 30%, and a tissue volume of less than 10 ml (20). In addition, the final product needs to have negative gram staining and have endotoxin levels below 5 EU/kg patient body weight (we used 60 kg as a default so that the total endotoxin level should be less than 300 EU).

Isolated islets from all three cases of the DI group were transplanted into two type 1 diabetic patients. One patient received two islet infusions and the other patients received one. We submitted the data obtained from the KIIM group to Food and Drug Administration (FDA) for the approval of clinical use of the KIIM.

Donor Chart Review

Two hundred and thirty-six donor charts from well-documented multiorgan procurements in Texas (Southwest Transplant Alliance; Dallas, TX and LifeGift Organ Donation Center; Fort Worth, TX, USA) from 2005 to 2006 were reviewed. Twenty-nine pancreata were

used for whole pancreas transplantations and 13 pancreata were used for islet isolation. Therefore, 194 (82%) pancreata were not used. In the unused 194 cases, the reasons that they were not used were also reviewed based on donor-specific inclusion and exclusion criteria at Baylor. Then unused donors were reevaluated based on Japanese islet specific donor inclusion and exclusion criteria.

Statistics

Values for the data collected represent means ± SE. Four groups were compared by means of ANOVA followed by Fisher's Protected Least Significant Difference post hoc test. The ratios between the two groups were compared using Fisher's exact test. Values of $p < 0.05$ were considered significant.

RESULTS

Donor Characteristics

In terms of donor characteristics among the four groups (UW group, TLM group, DI group, and KIIM group), there were no significant differences in donor age, body mass index (BMI), pancreas weight, or cold ischemic time (CIT) (Table 1).

Islet Isolation Outcomes

There was no significant difference in islet yields between the UW group and TLM group (251,663 ± 60,217 IE UW group vs. 243,738 ± 54,170 IE TLM group) (Fig. 1). Islet yields were significantly increased when we introduced ductal injection to the TLM method (498,639 ± 28,853 IE) (Fig. 1). The use of the KIIM further improved islet yields compared to the DI groups (678,286 ± 55,853 IE) (Fig. 1). There were no significant differences of viability or purity of isolated islets among the four groups (Table 2). The recovery rate after purification was significantly higher in the KIIM group compared with the UW and TLM groups (Table 2). Qualifying the transplantation criteria was 2/6 (33%) in the UW group and 4/13 (31%) in the TLM group. After the addition of ductal injection, both DI and KIIM groups had a 100% success rate for qualification of transplantation. The success rate of islet isolation was significantly improved with ductal injection (DI and KIIM groups) [6/19 (32%) without ductal injection (UW, TLM) vs. 6/6 (100%) with ductal injection (DI, KIIM), $p < 0.01$].

The clinical results in the DI group are shown in Figure 2. The daily insulin dose was gradually decreased after the first islet transplantation and the patient became insulin independent after the second islet transplantation (Fig. 2, top). Fasting glucose levels became well controlled after the first islet transplantation and further improved after the second islet transplantation (Fig. 2, middle). HbA_{1C} levels gradually decreased after the first islet transplantation and reached normal range (Fig. 2, bottom).

Donor Chart Review

Donor chart review revealed that out of 194 unused cases, the reasons that the pancreata were not used were identified in 185 cases, based on Baylor islet-specific donor inclusion and exclusion criteria (Table 3). Forty cases (21.6%) were aborted during procurement, 37 pancreata (20.0%) were not used due to donor age, 29 pancreata (15.7%) were not recovered due to high glucose, 17 pancreata (9.2%) could not be used due to diabetes, in 15 cases (8.1%) the family did not consent to the procurement, 14 cases (7.6%) had infectious disease, in nine cases (4.9%) no specific reasons were mentioned, eight cases (4.3%) were not attempted due to cardiac arrest events, seven cases (3.8%) were not performed for social reasons, five pancreata (2.7%) were not used due to fatty pancreata, and elevated creatinine levels prevented pancreas procurement in four cases (2.2%) (Fig. 3).

Next, unused donors were reevaluated based on Japanese islet-specific donor inclusion and exclusion criteria (Table 3). Infection, lack of family consent, diabetic pancreata, and social reasons were considered as not

Table 1. Donor Characteristics

	UW	TLM	DI	KIIM
N	6	13	3	3
Age (years)	48.0 ± 3.5	43.6 ± 3.1	37.5 ± 14.5	34.3 ± 9.2
BMI (kg/m^2)	28.7 ± 2.0	29.5 ± 1.6	36.1 ± 1.2	35.7 ± 2.5
Pancreas weight (g)	115.0 ± 21.6	99.1 ± 7.2	121.4 ± 31.1	95.9 ± 4.4
CIT (h)	6.0 ± 0.6	4.8 ± 0.6	3.0 ± 0.6	3.3 ± 0.9
Gender (F/M)	2/4	3/10	2/1	1/2

There were no significant differences in all categories among all groups. UW, University of Wisconsin solution; TLM, Two-layer method; DI, TLM + ductal injection; KIIM, Kyoto islet isolation method; BMI, body mass index; CIT, cold ischemic time.

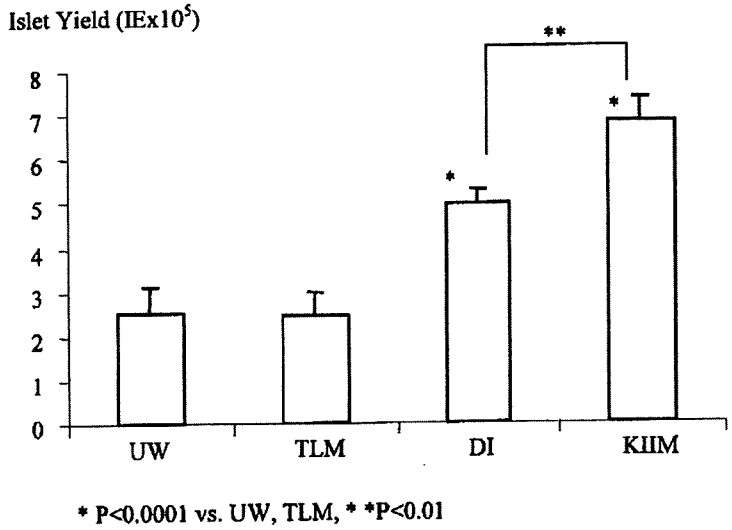

Figure 1. Islet yields of four different groups, including the Ricordi method using UW-stored pancreata (UW), the Ricordi method using TLM-stored pancreata (TLM), the Ricordi method using ductal preservation (DI), and the KIIM (KIIM). Islet yields were significantly higher in the DI group compared with the UW and TLM groups (*$p < 0.0001$ UW vs. DI and TLM vs. DI). Islet yields were significantly higher in the KIIM group compared with all other groups (*$p < 0.0001$ UW vs. KIIM and TLM vs. KIIM, **$p < 0.01$ DI vs. KIIM).

qualified cases (63 cases, 34%). Based on the Japanese criteria, high glucose without diabetes, fatty pancreas, or elevated creatinine levels are acceptable for islet donors; therefore, we counted those as qualified pancreata (47 cases, 25%). Ages less than 25 years old and up to 70 years old and cardiac arrest events with less than 30 min of warm ischemia are also acceptable. In some cases where the procedure was aborted, there were no contraindications. Out of 85 of those cases (age, cardiac arrest, aborted cases), 25 cases were qualified for islet donation. A total of 82 cases (44%) were qualified for islet donation.

When we extrapolated the 44% qualification rate that we identified here, but of the currently unused 6,000-plus pancreata, more than 2,500 pancreata could be used for islet isolation in 1 year in the US.

DISCUSSION

Currently, we have established the KIIM for islet isolation with marginal donor pancreata (8,10,11,14,17). With the KIIM, we successfully isolated transplantable islets from NHBDs in 17cases out of 21 isolations (81%) (8). All transplanted islets secreted insulin and all of the patients improved glycemic control without hypoglycemic unawareness. Three out of five multiple transplantation cases (60%) became insulin independent (8).

In this study, we first compared UW and TLM storage. We then tested ductal injection (DI group) and KIIM (KIIM group) to confirm the effect of DI and KIIM on islet isolation from brain-dead donors. We did not see any significant differences between the UW group and the TLM group. Previously, we demonstrated

Table 2. Islet Isolation Outcomes for the Different Groups

	UW	TLM	DI	KIIM
Viability (%)	87.3 ± 6.2	92.3 ± 0.8	95.7 ± 2.7	93.9 ± 0.9
Purity (%)	53.7 ± 7.9	67.8 ± 6.0	49.3 ± 3.8	43.0 ± 5.0
Recovery rate after purification (%)	53.9 ± 10.9	45.4 ± 6.1	61.2 ± 9.0	90.0 ± 6.9*
Qualified for transplantation	2/6 (33%)	4/13 (31%)	3/3 (100%)	3/3(100%)

*$p < 0.01$ versus TLM and $p < 0.05$ versus UW.

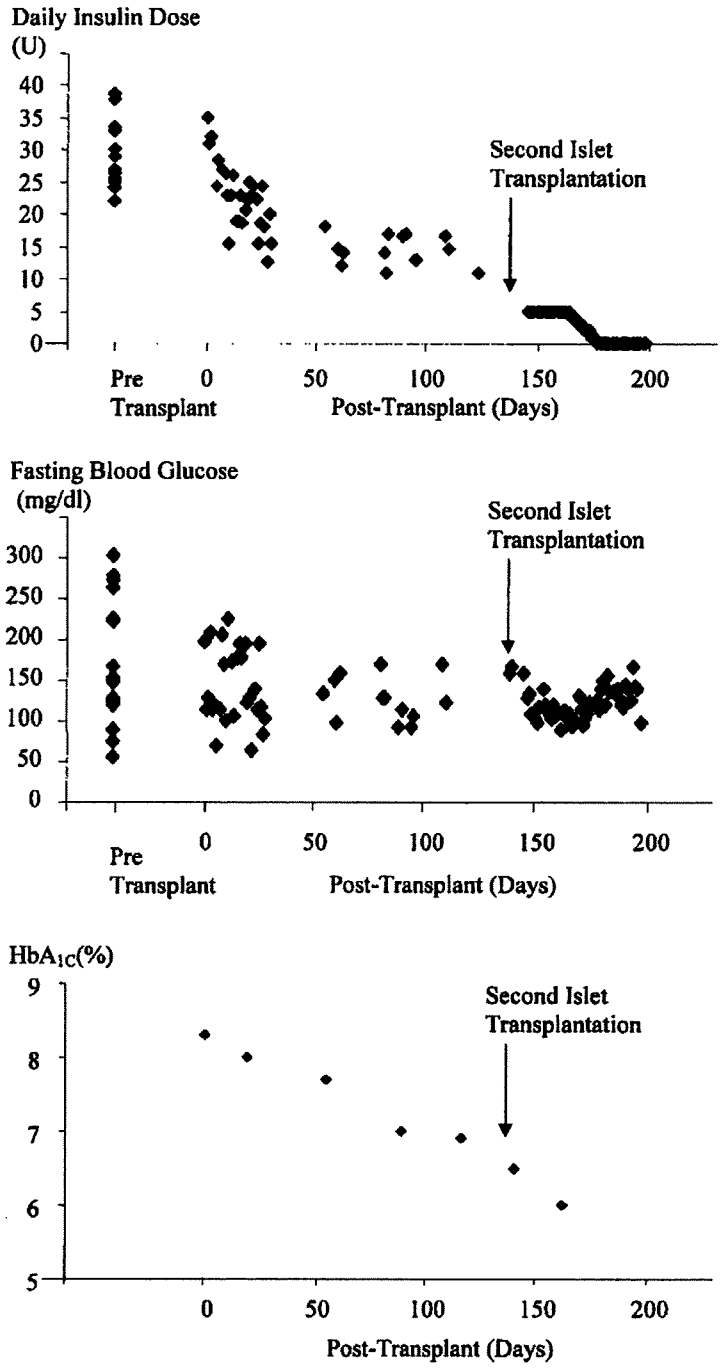

Figure 2. Daily insulin dose (top), fasting blood glucose (middle), and HbA_{1C} (bottom) before and after two islet transplantations with the islets from the DI group. Daily insulin dose decreased after the first islet transplantation and the patient became insulin independent after the second islet transplantation (top). Fasting blood glucose became more stable after the first islet transplantation and became more stable after the second transplantation (middle). HbA_{1C} continuously decreased after the first islet transplantation and achieved normal range after the second transplantation (bottom).