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Mizoribine as Sole Immunosuppressive Agent in Islet Xenotransplantation Models: A Candidate Immunosuppressant Causing no Adverse Effects on Islets

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Mizoribine (MZ) inhibits the differentiation and proliferation of helper T and B cells after antigen recognition by suppressing the purine biosynthesis pathway and nucleic acid synthesis. MZ has been used in kidney transplantation, but distinct data are unavailable for islet transplantation. The present study investigated the efficacy of MZ for islet xenotransplantation. Immunosuppressive effects of MZ were determined by mixed lymphocyte reaction (MLR) assay in vitro. Toxicities for Wistar rat islets were determined by adenosine triphosphate (ATP) contents of islets during 3-day culture and stimulation index in response to glucose after culture. Immunosuppressive effects in vivo were tested in a Wistar-to-B6 islet xenotransplantation model. MZ was administered continuously for 28 days subcutaneously or intramuscularly. MZ inhibited MLR response by approximately 50% at 0.1 μg/ml. ATP contents decreased with MZ >100 μg/ml, while stimulation index was maintained. Continuous infusion of MZ at 10 mg/kg maintained blood concentrations at 0.13–0.19 μg/ml, while intramuscular injection of MZ at 100 mg/kg/day (peak 520 μg/ml at 1 h postinjection) resulted in below measurable levels (<0.03 μg/ml) within 24 h. Graft survival was significantly prolonged following continuous infusion of 10 mg/kg/day compared to controls (31.0 ± 9.5 vs. 13.2 ± 5.2 days; $p = 0.002$). Furthermore, animals with intramuscular injection at doses of 3.2, 10, or 100 mg/kg/day showed significantly longer graft survival (20.0 ± 7.5, 22.0 ± 7.31, and 24.5 ± 8.1 days, respectively; $p < 0.05$ each). Histological examination showed significant suppression of lymphocyte infiltration by MZ administration. MZ showed immunosuppressive effects in an experimental islet xenotransplantation model without adverse effects on endocrine function of islet grafts.

Key words: Islet xenotransplantation; Mizoribine (MZ); Immunosuppression

INTRODUCTION

Islet transplantation has spread worldwide since the successes of the Edmonton protocol (26,28,30). However, results from long-term follow-up have shown that most recipients revert to some level of insulin use, although islet transplantation can relieve glucose instability and problems with hypoglycemia by improving endogenous insulin secretion (28,31). Further progress in improving long-term results for islet transplantation will thus require immunosuppressive protocols (40,41). Sirolimus and tacrolimus were used in the Edmonton protocol, but have various adverse effects, including islet toxicity and suppression of both islet revascularization and islet regeneration (1,3,19,21,23). Immunosuppressive drugs used for islet transplantation should not only display minimal toxicity to transplanted islets, but also limited deleterious effects on renal function, since progression of renal dysfunction is one of the major complications in type 1 diabetes mellitus (1,3,9,19,21, 23,28,30,31).

Mizoribine (MZ) is an imidazole class nucleoside that was isolated from culture medium of the mold *Eupencillium brefeldianum* M-2166 (15,18). The drug has been found to inhibit both humoral and cellular immunity by suppressing DNA synthesis through the inhibition of purine biosynthesis via antagonistic blocking of inosine monophosphate (IMP) dehydrogenase (IMPDH) (15,18). Micophenolate mofetil (MMF), in the same class of drugs, has been used for immunosuppression following islet transplantation in cases requiring discontinuation of calcineurin inhibitors or sirolimus due to adverse effects (5). However, clinical studies of MMF have shown a high incidence of leucopenia and diarrhea (2,33). In contrast, MZ has fewer adverse effects, such as hematological and gastrointestinal events, in clinical usage

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(36). MZ has been widely used in human kidney transplantation, particularly in Japan (37). This evidence led us to examine the effects of MZ in islet transplantation.

A shortage of donor organs represents another serious problem in islet transplantation, as each recipient currently needs two to three transplantations under present methods (28,30,31). Use of xenoislet graft is one of the best options to resolve this problem (26). Recently, modification of α-galactosyl antigen expression in donor animals and new immunosuppressive drugs including induction therapy using CD25- and CD154-specific antibodies, FTY720 and everolimus, have been shown to significantly affect some islet xenotransplantation models (10,17). These findings indicate the necessity for new immunosuppressive drugs that are effective in xenotransplantation.

The present study assessed the applicability of MZ in a rat-to-mouse islet xenotransplantation model and found that MZ has potential as an immunosuppressive drug without causing adverse effects on islet endocrine function.

MATERIALS AND METHODS

Animals

Male C57BL/6 (B6; H-2b) mice and Wistar rats, approximately 8 weeks old, were purchased from Clea Japan (Osaka, Japan). Wistar rats were used as donors and B6 mice as recipients. Diabetes was induced by intraperitoneal injection of streptozotocin (STZ) (Sigma Chemical, St. Louis, MO) at 250 mg/kg body weight, and was diagnosed when nonfasting blood glucose levels increased to >400 mg/dl on two consecutive measurements. This experimental protocol was approved by the Ethics Review Committee for Animal Experimentation at Fukushima Medical University.

Islet Isolation and Transplantation

Islet isolation and transplantation were performed as described previously (7). Briefly, we injected Hank's balanced salt solution (Nissui, Tokyo, Japan) containing 2 mg/ml of collagenase (collagenase S-1; Nitta Gelatin, Osaka, Japan) into the common bile duct. The distended pancreas was removed and incubated at 37°C. Collagenase-digested islets were purified by centrifugation on gradients composed of three different Ficoll (type 400, Sigma) densities (1.120, 1.090, and 1.050). After centrifugation, the distinct layer of islets was collected and washed. Diabetic B6 mice acting as recipients were anesthetized with diethyl ether (Wako Pure Chemicals, Osaka, Japan). The left kidney was exposed through a lumbar incision under sterile conditions, and 350–400 islets were transplanted into the left renal subcapsular space of recipients.

Mixed Lymphocyte Reaction (MLR)

MLR was performed as previously described (38). Briefly, irradiated (2000 rads, cesium source) donor strain (Wistar rat) splenocytes were used as stimulators. A total of 8×10^5 cells isolated from the spleen of the recipient C57BL/6 were used as responders. Naive C57BL/6 splenocytes were used as controls. Responder cells and an equal number of irradiated stimulator cells were cultured with 0, 0.1, 0.5, 1, 2, or 5 μg/ml of MZ in 96-well round-bottom plates for 4 days at 37°C in 5% CO_2 and humidified air. All experiments were performed in triplicate. Cells were pulsed with [^{3}H]thymidime for the last 16 h of culture, then harvested on days 3, 4, and 5. Proliferation activity was calculated in triplicate microwells by incorporation of 1 μCi/well of [^{3}H]thymidime for 6 h. Cells were harvested and counted with a Betaplate (Pharmacia LKB Biotechnology, Uppsala, Sweden). Responses are reported as the mean cpm of triplicate measurements.

Measurement of Blood MZ Concentration

B6 mice were used as recipients for measurement of blood MZ concentrations after intramuscular or continuous subcutaneous injection of MZ. Blood samples were collected from the right ventricle of the heart at 1, 2, and 4 h after injection of the drug intramuscularly at doses of 1, 10, or 100 mg/kg/day, or at 3, 7, and 14 days after starting continuous administration of the drug subcutaneously at doses of 1, 10, 20, 30, or 60 mg/kg/day. Blood MZ concentration was measured by high-performance liquid chromatography (12).

Assessment of Islet Graft Function In Vitro

The insulin secretory capacities in response to low (50 mg/dl) and high (300 mg/dl) glucose, as glucose stimulation index, were evaluated using a modification of the method reported by Ricordi et al. after culture for 3 days following isolation with or without MZ (27). Doses of MZ were 0, 0.5, 1.0, 10, or 100 μg/ml. Briefly, after 6 h of culture in RPMI-1640 solution at 37°C under 5% CO_2 and 95% air in a humidified atmosphere, five sets of 20 islets of 150–200 μm in diameter were handpicked. These islets were placed in a 12-well transwell microplate (Corning Transwell 3403, pore size 12 μm; Corning, Lowell, MA, USA) with 50 mg/dl of glucose RPMI-1640 and 0.1% fetal calf serum (FCS) at 37°C for 60 min for stabilization. After preincubation, the transwell was placed in the second well cluster containing 3.3 mM glucose RPMI-1640 and 0.1% FCS and incubated for 60 min. The transwell was then placed in the third well cluster containing 300 mg/dl glucose for 60 min. Supernatants of the second and third well clusters were immediately collected and frozen at −20°C until assessment. Insulin content was measured using

ultrasensitive rat insulin enzyme-linked immunosorbent assay kits (Morinaga, Kanagawa, Japan). Stimulation index was calculated by dividing insulin secretion at high glucose by that at low glucose.

Evaluation of MZ Toxicity

Islets were cultured for 3 days in the presence of MZ at concentrations of 0, 1.0, 10.0, 100, or 1,000 μl/ml, and adenosine triphosphate (ATP) levels of 10 islet equivalent (IEQ) islets were measured. ATP contents of islets were measured using the bioluminescent enzymatic cycling assay as previously reported (14). Briefly, 33 μl of 0.5 N $HClO_4$ was added to the well containing islets with 300 μl of culture medium, and then this solution was frozen at −80°C until assayed. Next, 20 μl of sample and 80 μl of Tris buffer were mixed, then 100 μl of each bioluminescence reagent [firefly luciferase alone; firefly luciferase with pyruvate kinase (PK); or PK with pyruvate orthophosphate dikinase] was added. Bioluminescence intensity was measured within 90 s using a Lumitester C-100N (Kikkoman, Tokyo, Japan), MLX Microtiter plate luminometer (DYNEX, Chantilly, VA, USA), and Mithras LB940 (Berthold, Bad Wildbad, Germany).

Survival Study of Islet Xenografts

In the Wistar rat to B6 mice combination, recipient mice were divided into groups with MZ (MZ group) or without MZ (control group). In the MZ group, MZ was administered continuously at a dose of 10 mg/kg for 28 days subcutaneously using an osmotic pump (0.2 ml, Alzet 2001; Durect, Cupertino, CA, USA) implanted subcutaneously in the back of the mouse at the time of transplantation or intramuscularly at doses of 1.0, 3.2, 10, or 100 mg/kg/day on the medial aspect of the thigh once daily (29). The pump was exchanged using the same procedure after 14 days and was removed on day 28.

Nonfasting blood glucose levels of each recipient were measured daily to monitor islet graft survival during the first 3 weeks and then every other day. Graft rejection was defined by the day that blood glucose level exceeded 300 mg/dl on 2 successive days.

Histological Examination of Grafted Tissue

Grafts were removed at 7 days after transplantation for histological studies in the control group and continuous infusion of MZ at a dose of 10 mg/kg/day. Specimens containing the transplantation site were fixed in 10% buffered formalin, embedded in paraffin, and cut into 4.5-μm-thick sections. Sections were stained with hematoxylin and eosin (H&E) or stained immunohistologically with rabbit anti-human insulin polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit anti-human glucagon polyclonal antibody (Progen Bitechnik, Heidelberg, Germany). Cross-reactivity to mice of these antibodies has already been established by the supplying companies.

To detect CD4 and CD8 cells, cryostat sections were air dried, fixed in acetone for 10 min, and hydrated in Tris-buffed saline (pH 7.4) for 10 min. After fixation in formol calcium solution for 1 min, sections were washed three times with phosphate-buffered saline (PBS) and incubated overnight with biotin-conjugated rat anti-mouse CD8a monoclonal antibody (BD PharMingen, San Diego, CA, USA) or biotin-conjugated rat anti-mouse CD4 monoclonal antibody (BD PharMingen) at 4°C. After washing with PBS, slides were incubated with peroxidase-conjugated streptavidin for 30 min. Slides were washed with PBS and fixed with 1% glutaraldehyde solution for 3 min before staining for peroxidase activity with 3,3′-diaminobenzidine tetrahydrochloride (DAB; Wako Pure Chemicals) in 500 mM Tris-HCl buffer (pH 7.6) containing 0.01% H_2O_2.

Statistical Analysis

Data are expressed as mean ± SD. Graft survival in different experimental groups was compared using the log-rank test. All statistical calculations were performed using Statview-J5.0 system software (SAS Institute, Cary, NC, USA). Graft survival-prolonging effects were analyzed using the Kaplan-Meier method and log-rank test. Differences between groups in ATP levels and stimulation index (SI) were examined for statistical significance using Student's *t*-test with Bonferroni correction. All values of $p < 0.05$ were considered indicative of a significant statistical difference.

RESULTS

In Vitro MLR Assay

MLR assay was used to determine the immunosuppressive dose of MZ in vitro (Fig. 1). Maximum MLR responses were obtained on days 3 and 4 in this xenocombination. The lymphoproliferative reaction was inhibited by MZ in a concentration-dependent manner. The concentration of MZ at which the reaction was inhibited by approximately 50% was 0.1 μg/ml, while concentrations >0.5 μg/ml completely inhibited the reaction.

Pharmacokinetic Profile of MZ

MZ concentrations in blood after intramuscular administration at doses of 1, 10, and 100 mg/kg/day were 0.2, 1.5, and 500 μg/ml at 1 h, respectively ($n = 3$ in each group). In animals given 100 mg/kg/day of MZ, these concentrations rapidly decreased to 2.3 μg/ml after 4 h and were below the measurable level after 24 h. Blood concentrations in animals given intramuscular MZ at 1.0 or 10 mg/kg/day decreased more rapidly

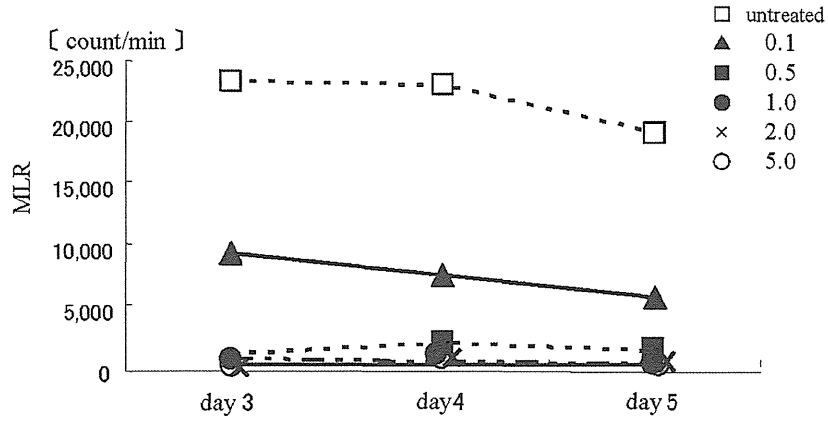

Figure 1. Mixed lymphocyte reaction (MLR). Responder cells (splenocytes of B6 mice) and an equal number of irradiated stimulator cells (splenocytes of Wistar rats) were cultured with 0, 0.1, 0.5, 1, 2, or 5 μg/ml concentrations of mizoribine (MZ; open square, filled triangle, closed square, filled circle, multiplication sign, and open circle, respectively).

below the measurable level within 4 h after administration (Fig. 2A).

In animals continuously infused with 1.0, 10, 20, 30, or 60 mg/kg/day, blood MZ concentrations on day 3 after starting administration were <0.03 (below the measurable limit): 0.17 ± 0.01, 0.20 ± 0.04, 1.00 ± 0.48, and 1.20 ± 0.42 μg/ml, respectively ($n = 3$ in each group).

These concentrations remained at measurable levels until 14 days after starting MZ, with the exception of 1.0 mg/kg/day. However, mice that received MZ at doses of 20, 30, and 60 mg/kg/day developed signs of severe anorexia, dehydration, and hypoglycemia, and died at 7–13 days after starting MZ (Fig. 2B).

Evaluation of MZ Toxicity on Islet ATP Content

ATP contents of islets cultured for 3 days with 0, 1.0, 10.0, 100, or 1,000 μl/ml of MZ were 25.0 ± 1.2, 28.2 ± 2.6, 22.3 ± 1.3, 15.2 ± 1.5, and 13.8 ± 1.0 pmol/10 IEQ, respectively (Fig. 3A). Groups of islets cultured with 100 or 1,000 μl/ml of MZ showed significantly lower ATP levels than islets without MZ ($p < 0.001$). These concentrations were thus considered as toxic levels.

Assessment of Islet Graft Function In Vitro

Glucose stimulation index after 3 days culture in the presence of MZ at doses of 0, 0.5, 1.0, 10, and 100 μg/ml were 2.3 ± 0.3, 2.4 ± 0.6, 2.4 ± 0.1, 2.7 ± 0.8, and 2.0 ± 0.4, respectively (Fig. 3B). No significant differences were noted between MZ-treated islets compared with untreated islets, indicating no immediate adverse effects of this drug with the doses and duration examined here on insulin secretory capacity in response to glucose.

Nonfasting Blood Glucose Levels and Graft Survival After Islet Transplantation

Nonfasting blood glucose levels in animals given islet xenograft without MZ treatment (Fig. 4A) or animals with MZ treatments, continuously at a dose of 10 mg/kg for 28 days subcutaneously (Fig. 4B) or intramuscularly at doses of 1.0 (Fig. 4C), 3.2 (Fig. 4D), 10 (Fig. 4E), or 100 (Fig. 4F) mg/kg/day were restored to normal within a couple of days following transplantation, indicating no toxic effects of MZ on glucose metabolism. Animals given continuous infusion of 10 mg/kg/day of MZ showed significantly longer graft survival compared to controls (control, 12.0 ± 0.9 days; continuous infusion of 10 mg/kg/day MZ, 31.0 ± 3.4 days; $p = 0.002$) (Fig. 5B). Furthermore, animals with intramuscular injection at doses of 3.2, 10, and 100 mg/kg/day showed significantly longer graft survival at 20.0 ± 3.4, 22.0 ± 3.1, and 24.5 ± 4.5 days, respectively ($p < 0.01$) (Fig. 5D–F). However, intramuscular administration at a dose of 1.0 mg/kg/day failed to prolong graft survival (Fig. 5C).

Histological Examination

Histologically, marked infiltration of inflammatory cells was observed at transplanted sites of grafts in the control group, whereas relatively fewer inflammatory cells were noted in MZ groups at 7 days after xenotransplantation. The microscopic structure of islets was maintained relatively well in MZ groups (Fig. 6B). Immunohistochemical study using anti-insulin antibody and anti-glucagon antibody revealed that many of the respective positive cells were observed in the MZ group compared with the control group (Fig. 6C–F).

Immunostaining of anti-CD4 and anti-CD8 revealed that marked CD4- and $CD8^+$ lymphocyte infiltration around transplanted islets was suppressed in the MZ groups in xenorecipients compared to controls (Fig. 6G–J).

DISCUSSION

MZ is an immunosuppressive drug showing antiproliferative effects by the blockade of IMPDH activity (15,18). This drug is widely used in clinical renal transplantation in Japan with a significant effect in preventing acute rejection and reducing the incidence of adverse effects, including leukopenia and diarrhea (13,36,37). However, MZ has not been applied for islet transplantation experimentally or clinically. To the best of our knowledge, this is the first investigation of the effects of MZ using in vitro assays and an islet xenotransplantation model. Our results from MLR showed that immunosuppressive effects of MZ were evident at concentrations ≥0.1 μl/ml. The immunosuppressive effects of MZ as evaluated by MLR in humans have been reported to be obtained with the same concentration used in our study (34). Furthermore, insulin secretory capacities were not

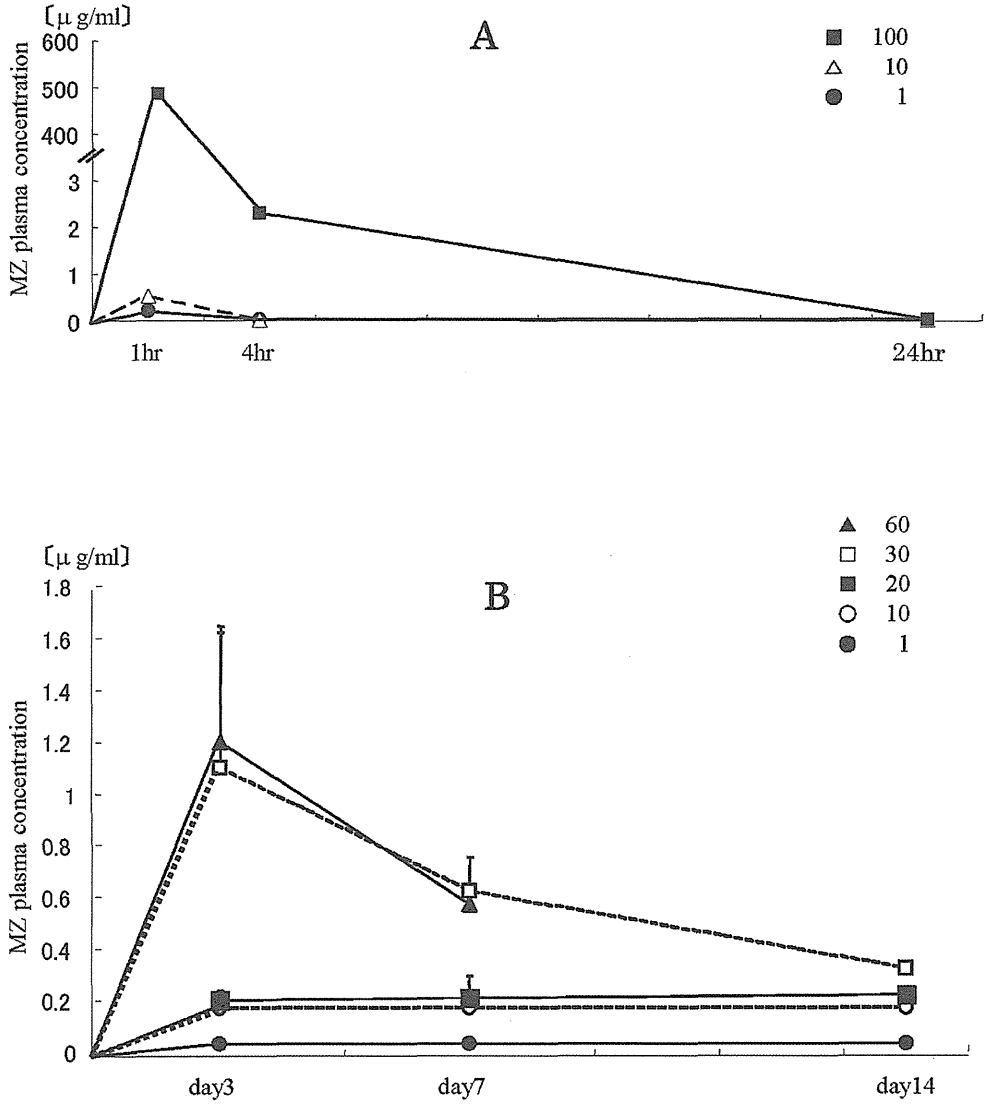

Figure 2. Blood MZ concentration after intramuscular (A) or continuous subcutaneous (B) administration. Blood MZ concentrations after intramuscular administration at doses of 1, 10, or 100 mg/kg/day rapidly decreased and were below the measurable level after 24 h (A). Blood concentrations of MZ, after subcutaneous administration by continuous pump infusion at doses of 1, 10, 20, 30, or 60 mg/kg/day, remained at measurable levels until 14 days after starting MZ (B).

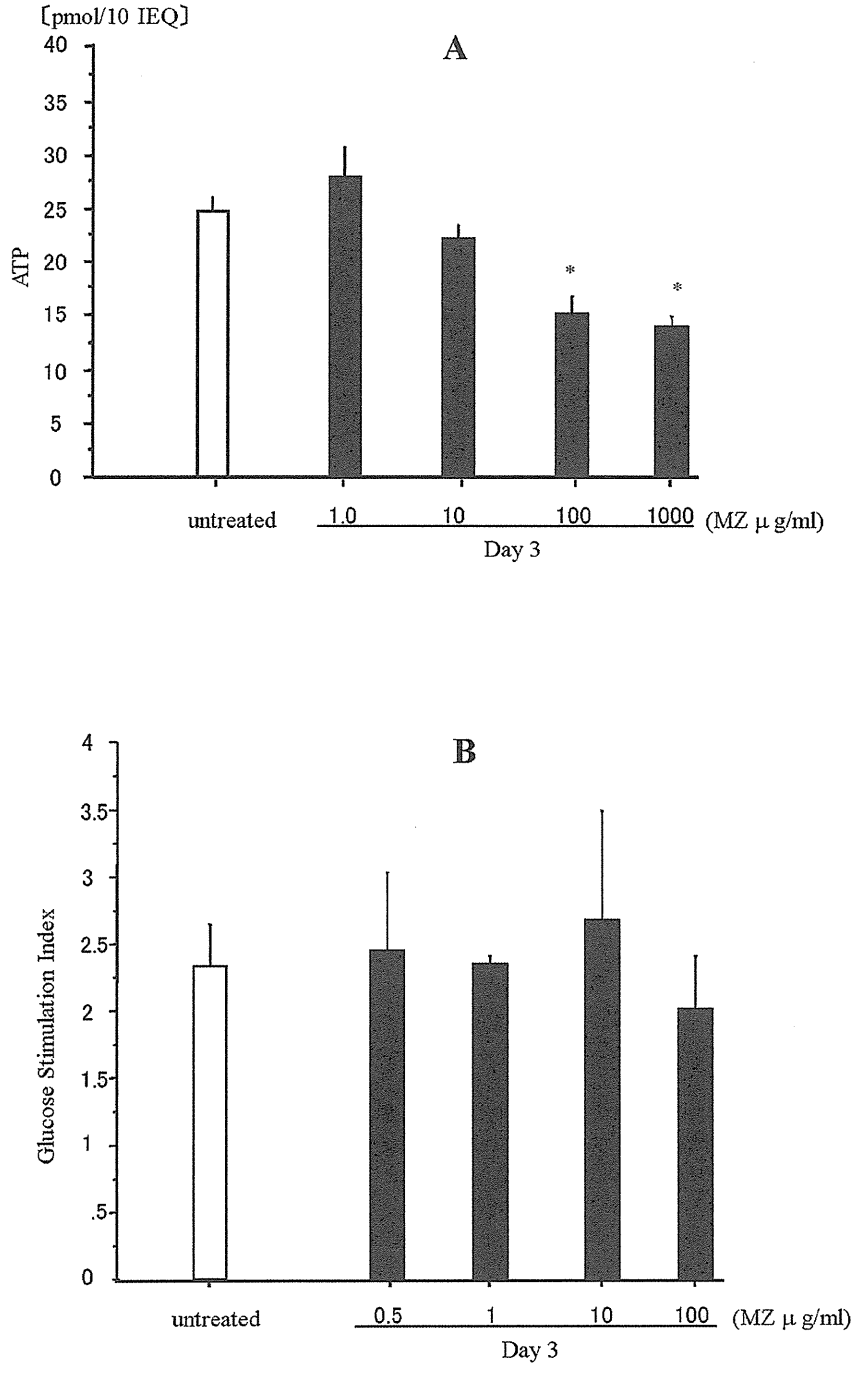

Figure 3. Evaluation of MZ toxicity by measuring of ATP content in islets cultured for 3 days (A) and glucose stimulation index at 3 days after isolation (B). Adenosine triphosphate (ATP) content of islets cultured with 100 and 1,000 μl/ml of MZ showed significantly lower levels than islets without MZ ($p < 0.001$) (A). Data represent mean ± SD. *$p < 0.001$ between groups. Glucose stimulation index showed no significant difference between control and MZ groups (B).

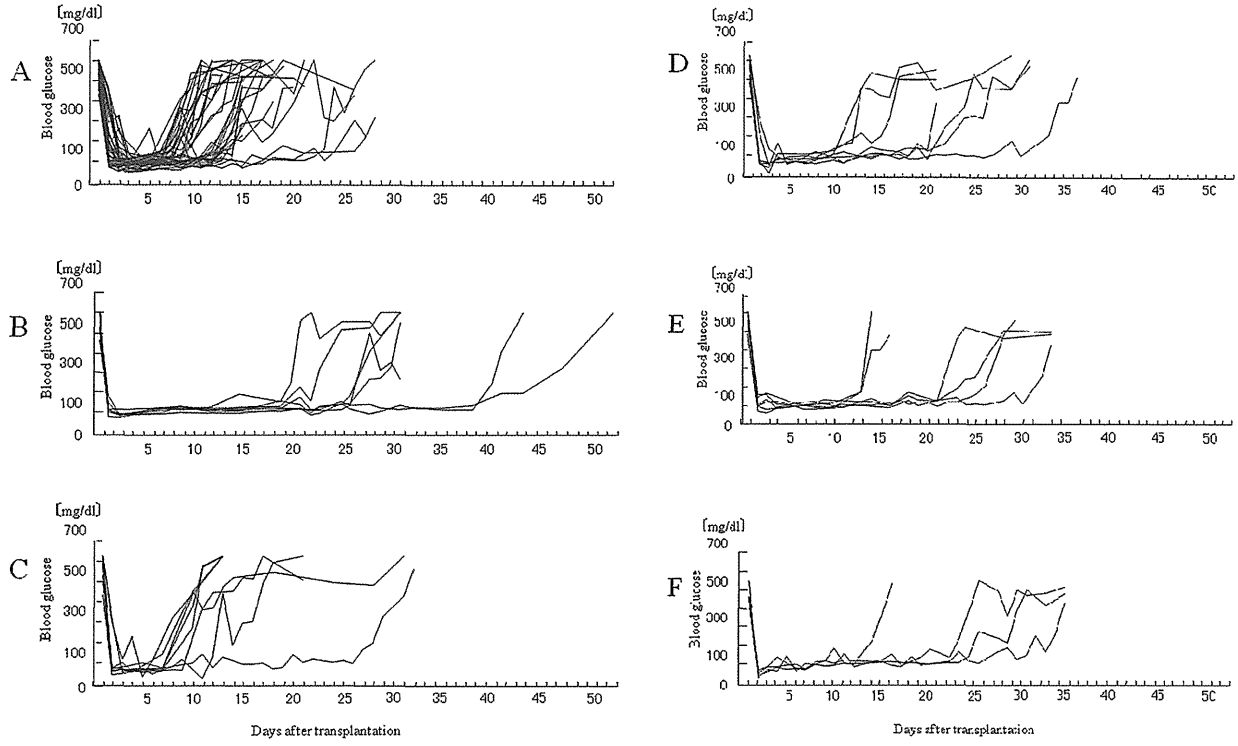

Figure 4. Nonfasting blood glucose levels after islet transplantation. Nonfasting blood glucose levels of mice undergoing islet transplantation with untreated (A: $n = 36$), treated with continuous infusion of MZ at 10 mg/kg/day (B: $n = 7$), and treated with an intramuscular once daily dose of 1 mg/kg/day of MZ (C: $n = 8$), 3.2 mg/kg/day of MZ (D: $n = 7$), 10 mg/kg/day of MZ (E: $n = 6$), or 100 mg/kg/day of MZ (F: $n = 4$).

suppressed with these concentrations. However, ATP content in islets significantly decreased with 100 and 1,000 μl/ml of MZ, suggesting some toxic effects at these doses. For this study, we therefore selected a target concentration for MZ of >0.1 μl/ml but <100 μl/ml.

In this rat-to-mouse xenotransplantation model, prolongation of graft survival was achieved by continuous subcutaneous administration of MZ at a dose of 10 mg/kg/day, with blood concentrations of MZ maintained within target levels during treatment. Furthermore, animals with intramuscular injection at doses of 3.2, 10, or 100 mg/kg/day showed significantly longer graft survival than untreated animals. Shimmura et al. reported that prolongation of graft survival in allocombination using a heart transplantation model was achieved when the drug was administered intramuscularly at >80 mg/kg/day (32). Our results indicate that prolongation of graft survival for islet xenotransplantation is achieved using a lower dose, unlike solid organ allotransplantation. Of note, prolongation was possible with both continuous infusion and intramuscular injection with an immediate peak concentration, although toxicity was more profound when given continuously. Murase et al. reported that MZ monotherapy at a dose of 7.5 mg/kg/day in hamster-to-rat heart transplantation had only marginal effects on prolongation of graft survival (22). MZ was administered by gastric instillation in that study, but no data were available for blood concentration. Our results demonstrate that MZ is effective either by continuous infusion or daily intramuscular injection for survival benefit in islet xenotransplantation.

Histological study of the xenografting model using Wistar rats to B6 mice revealed suppression of infiltration by $CD4^+$ and $CD8^+$ cells at transplanted islet sites in the subcapsular space of the kidney. We have previously reported that islet xenografts in the same combination using Wistar-to-B6 were rejected in a CD4-dependent pathway, since administration of anti-CD4 antibody significantly prolonged islet graft survival in this combination (8). MZ reportedly has an immunosuppressive effect in suppressing IL-2 production by $CD4^+$ T cells (35). The CD4 pathway could thus be dependently suppressed by MZ treatment, which might be a leading cause of prolonged survival of islet xenografts.

Histological study revealed that infiltration of $CD4^+$ and $CD8^+$ cells around transplanted islets was suppressed by MZ administration. The mechanism underlying immunosuppression by MZ is the inhibition of DNA

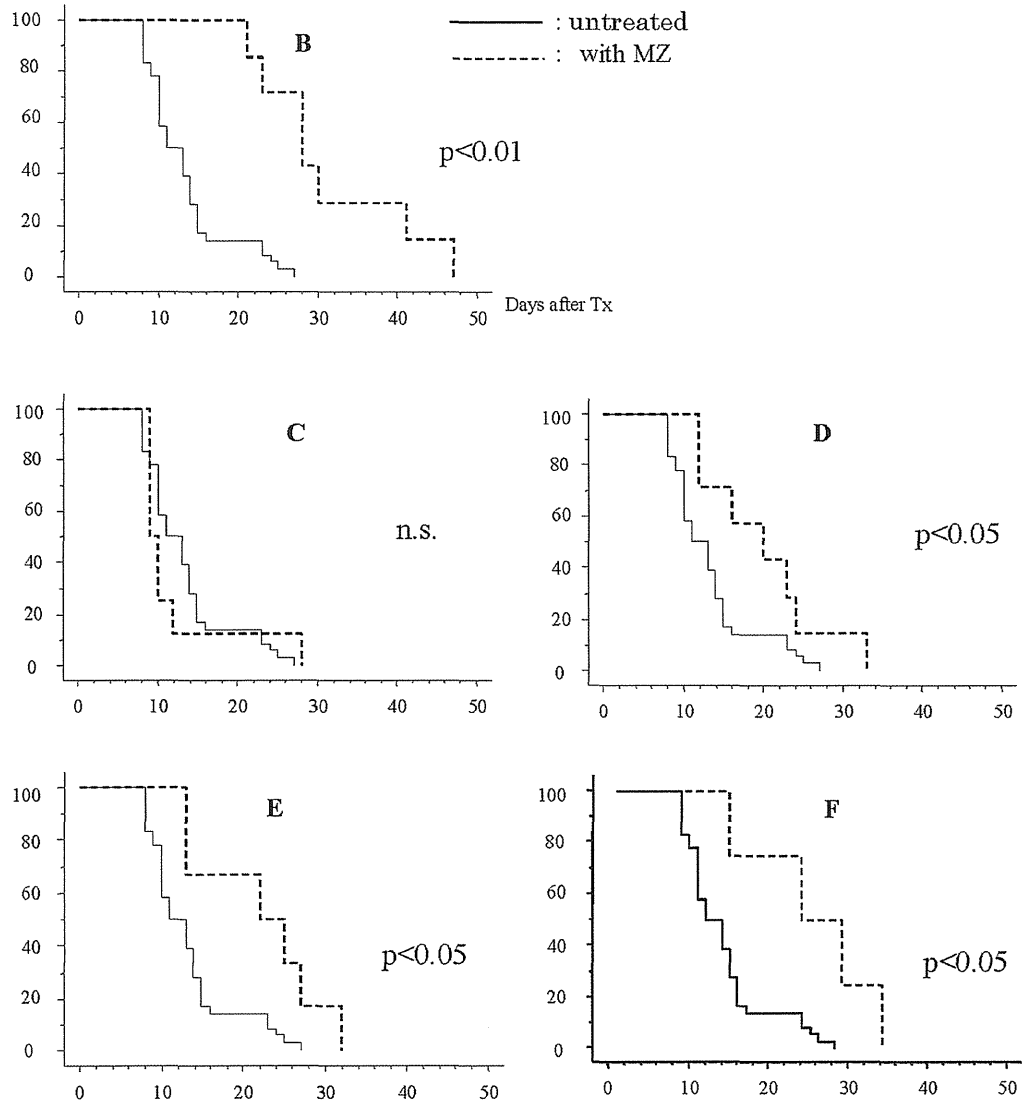

Figure 5. Graft survival after islet transplantation with or without MZ. Graft survival of animals given continuous infusion of 10 mg/kg/day of MZ was compared with that of control groups (B). Graft survival of animals with intramuscular injection of 1.0 (C), 3.2 (D), 10 (E), or 100 (F) mg/kg/day of MZ was also compared with that of controls, respectively.

synthesis in proliferative lymphocytes following antigen presentation (4,15,18). A similar mechanism has been reported for MMF, which is already used in clinical islet transplantation in combination with other immunosuppressive drugs (5). Recently, details have been reported of the mechanism underlying inhibition of IMPDH activity by MZ and mycophenolic acid (MPA), the active form of MMF (6). MPA inhibits the conversion of IMP to xanthine monophosphate (XMP) by binding to the nicotinamide adenine dinucleotide (NAD)-binding site of IMPDH and trapping E-XMP, an intermediate of IMP conversion to XMP. In contrast, MZ monophosphate inhibits IMPDH activity by binding to the IMP-binding site of IMPDH. Also, the strain of *Candida albicans* that shows MPA-resistant IMPDH is reportedly sensitive to MZ, since MPA and MZ have different mechanisms of IMPDH inhibition (16). MZ also shows other immunosuppressive activities, including inhibition of B-cell function and antibody production (11,25). Our results thus indicate that MZ could be one candidate for islet xenotransplantation in combination with other immunosuppressive drugs.

Although the Edmonton protocol has been spread worldwide, discontinuation of the immunosuppressive drugs, tacrolimus or rapamycin, represents one of the main problems, since these drugs have many adverse

effects, such as β-cell toxicity, aphtha, and renal dysfunction (1,3,19,21,23). In clinical situations, MMF has been used instead of these drugs, but clinical studies of MMF have shown a 12.1–37.8% incidence of leukopenia and a 13.9–38.4% incidence of diarrhea (2,5,33). Reports have described 18.1–36.3% of patients who received organ transplantation under MMF requiring reduction or discontinuation of MMF administration due

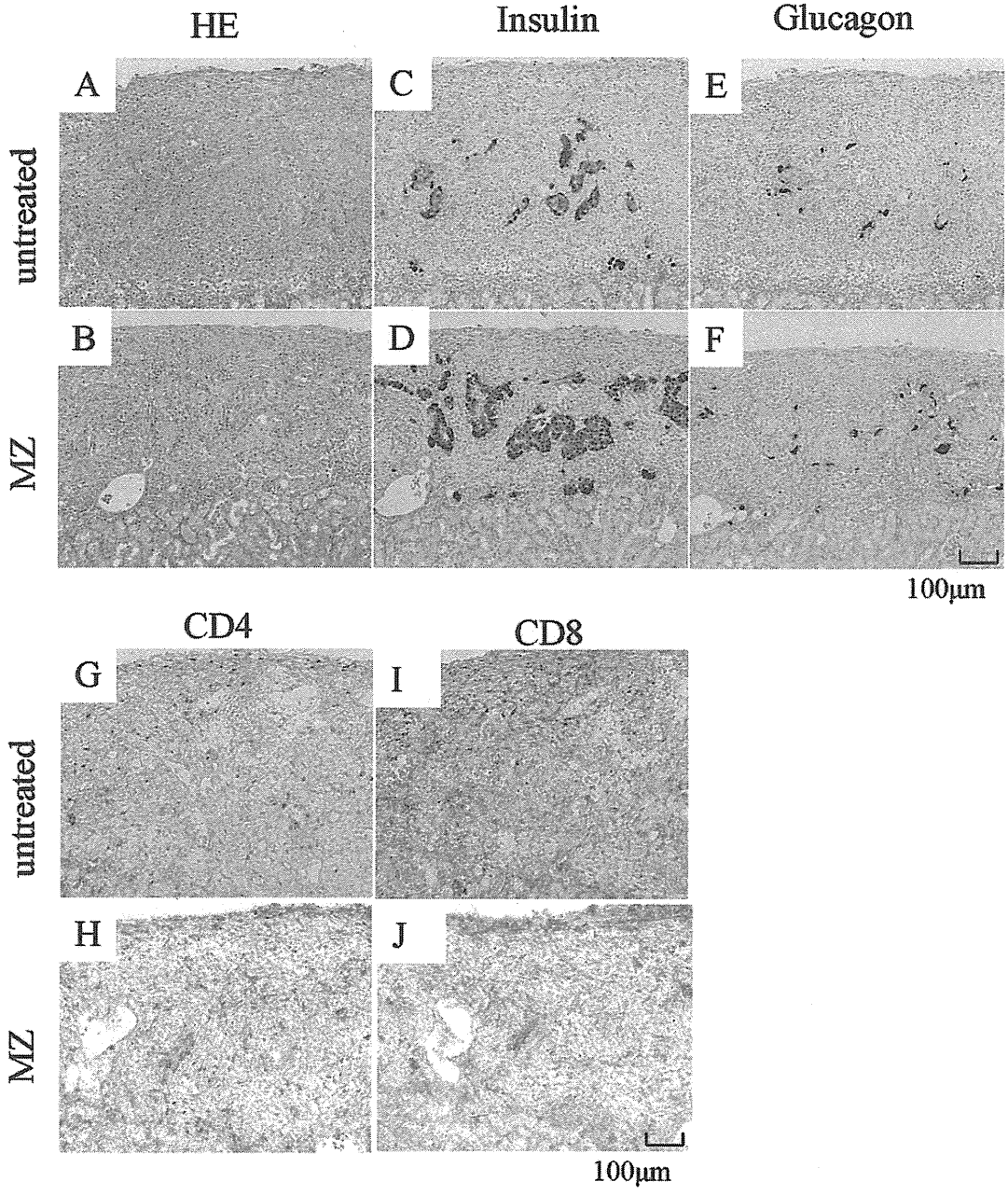

Figure 6. Histological findings 7 days after islet transplantation and immunohistochemical findings using anti-CD4 and anti-CD8 antibodies. Histology of the transplanted site in controls is expressed in upper rows, while findings from the MZ group are expressed in lower rows including H&E staining (A, B), immunohistochemistry with anti-insulin antibody (C, D), and anti-glucagon antibody (E, F) (100× magnification). Histology of the transplanted site in the control group is expressed in upper rows, while findings from the MZ group are expressed in lower rows including immunohistochemistry with anti-CD4 antibody (G, H) and anti-CD8 antibody (I, J) (100× magnification).

to adverse events (2,33). MZ might be one of the good candidates for resolving these problems. The low incidence of adverse effects and the relative safety of MZ are evident, because MZ has already been used clinically for kidney transplantation and treatment of relapsing steroid-dependent nephritic syndrome (24,36,37). In addition, high-dose MZ therapy was reported as experimentally effective for ongoing acute humoral rejection (20). In this study, the MZ dose was limited due to toxicities to mice, but human cells are reportedly more resistant than murine cells to both MZ and the aglycone (39). The present study shows that the function of isolated islet cells tolerates high-dose MZ, encouraging us to use higher doses of MZ in large animals (including nonhuman primates) before clinical trials in the future.

In conclusion, administration of MZ for islet xenotransplantation in mice is capable of suppressing rejection without affecting islet function. MZ has potential as an immunosuppressive drug for islet xenotransplantation.

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報告

膵島移植症例登録報告（2012）

日本膵・膵島移植研究会膵島移植班

Islet transplantations in Japan —Report from Japanese Islet Transplantation Registry—

The Japanese Pancreas and Islet Transplantation Association

【Summary】

The program of islet transplantations in Japan comprised 65 islet isolations mainly from nonheart-beating donors, and 34 transplantations into 18 patients with type 1 diabetes mellitus. Overall graft survival defined as C-peptide level more than or equal to 0.3 ng/ml was 76.5%, 47.1%, and 11.8% at 1, 2, and 5 years, respectively. Three of these recipients became insulin-independent transiently. Clinical islet transplantation has been resumed as a clinical trial using mammalian-free enzyme to assess the new immunosuppression protocol. The primary objective is to verify the effect of the immunosuppressants by confirming the existing islet graft function that can lead to glycemic stability.

Keywords: Japanese Pancreas and Islet Transplantation Association, clinical trial, islet transplantation, type 1 diabetes mellitus

I. はじめに

インスリン依存状態糖尿病に対する低侵襲移植療法である膵島移植は，心停止ドナー膵からの膵島を移植に供する特色を有し実績を重ねてきた。膵島分離用酵素の問題を機にその実施は一時停止したが[1]，その間，再開実施に向けた体制整備が行われ，現在，免疫抑制プロトコールの臨床効果と安全性の評価のため多施設共同臨床試験が実施されている。これまで実施された膵島移植の長期成績を報告し，進行中の臨床試験の進捗状況を踏まえて今後の展望を述べる。

II. 対象と方法

2007年から開始された日本移植学会への各臓器の症例登録作業に伴い，日本膵・膵島移植研究会膵島移植班で作成した膵島移植登録に関するフォーマットをもとに集計を行った。2007年4月以降，2012年9月現在まで，新規の膵島移植実施例はなかったが，膵島移植実施体制の現状と2012年9月までの膵島移植レシピエント候補者登録数，および，これまでに実施された膵島移植症例の2011年末までのフォローアップデータについて報告し，さらに膵島移植臨床試験の進捗状況と今後の展望について報告する。

1. 膵島移植施設認定および実施体制

日本膵・膵島移植研究会では，実際に膵島の分離・凍結・移植が可能であることを確認するために施設基準を設け，新たに膵島移植実施施設の申請があった場合はこの施設基準をもとに日本膵・膵島移植研究会内の施設認定委員会で検討し，施設認定を行っている[2]。2011年までに新鮮膵島分離・凍結・移植施設として，北から東北大学，福島県立医科大学，国立病院機構千葉東病院，京都大学，大阪大学，神戸大学，福岡大学の7施設が認定されている。このうち神戸大学から施設認定取り下げの申請があり，2009年3月以降2012年3月までは，上記の諸施設から神戸大学を除いた6施設による体制であった。さらに，2012年4月より，岡山大学，徳島大学，長崎大学が認定施設となり活動を開始している。膵臓摘出から移植までの時間を短縮するために，施設認定を受けた各施設は日本膵・膵島移植研究会内のシェアリング委員会における協議決定に従い，その施設が存在する地域（県）および隣接する地域を担当する形で地域を分担しブロック

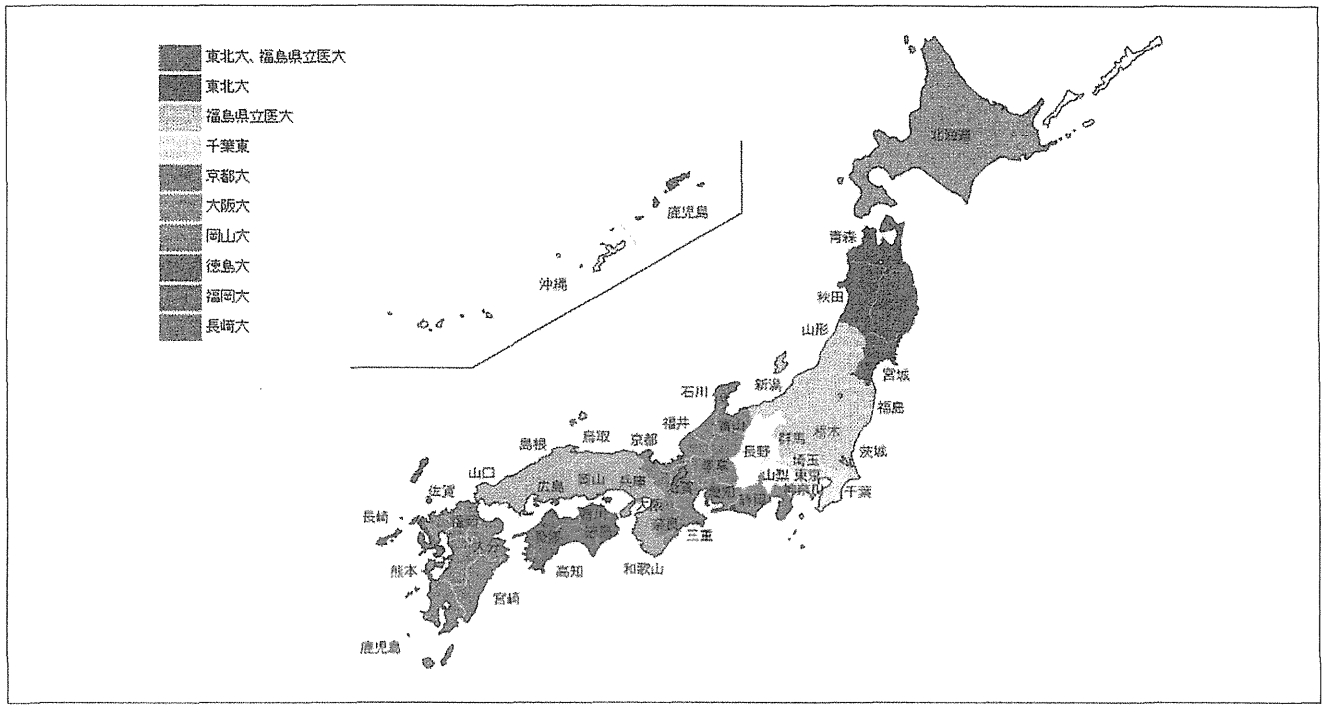


図1　膵島移植実施体制

※北海道は福島医大と東北大が交互に担当。茨城県は北部は福島医大，南部は千葉東，淡路島は徳島大が担当。長野・山梨・沖縄は，2012年9月現在担当施設なし。

体制を形成している（図1）。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脳死および心停止ドナーからの膵島移植），2011年までに認定されていた6施設においては膵島移植を高度医療として実施できる体制にある（なお，先進医療と高度医療の一本化のため，2012年10月より高度医療という名称は変更される見込みである）。

2. レシピエントの適応と選択基準

わが国での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，②1型糖尿病発症から5年以上経過し，③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，④原則として75歳以下の患者，としている。しかし，重度の心・肝疾患，アルコール中毒，感染症，悪性腫瘍の既往，重症肥満，未処置の網膜症などを認める場合は禁忌としている。糖尿病性腎症に関しては，第3期A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以下で，ステロイド内服量10 mg/dl以下，などの基準を満たす症例を移植の対象としている。これらの適応を満たした症例は，膵島移植班事務局へ登録され，レシピエント選択基準をもとに選択される[2]。また，現在実施されている臨床試験への参加者に対してはさらに，安全性および有効性への影響を考慮した適格基準，除外基準を定めている。年齢が20～65歳までで，糖尿病専門医によるインスリン強化療法を行っており，12カ月の間に1回以上の重症糖尿病発作の既往があることを主な適格基準としており，BMI 25 kg/m^{2}以上，インスリン必要量が0.8 IU/kg/日以上あるいは55 U/日以上，過去1年間に複数回測定したHbA1c値（NGSP値）の平均値が10.4%以上，eGFR 60 ml/min/1.73 m^{2}以下，といった項目を除外基準に挙げている（UMIN試験ID：UMIN000003977）。

III. 結果と考察

1. レシピエント候補者登録状況

膵島移植の適応基準に基づき[2]2012年9月末の時点で延べ177名が登録され，3回の移植を終了したものが6名，再判定にて判定保留となったものが2名，辞退者31名，待機中死亡9名あり，レシピエント候補者として129名が待機中である。この候補者に臨床試験の詳細を説明し，臨床試験参加の希望があれば臨床試験参加予定者となり，膵島移植の実施は臨床試験の

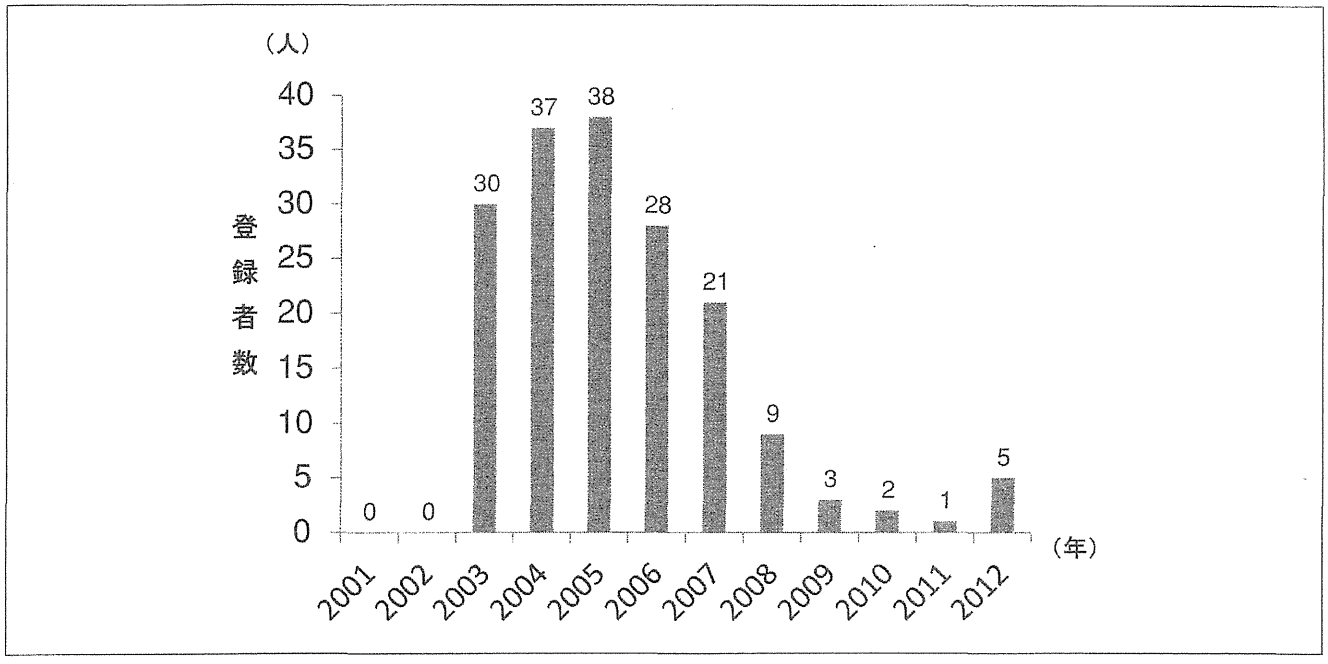


図２　新規登録者数の推移

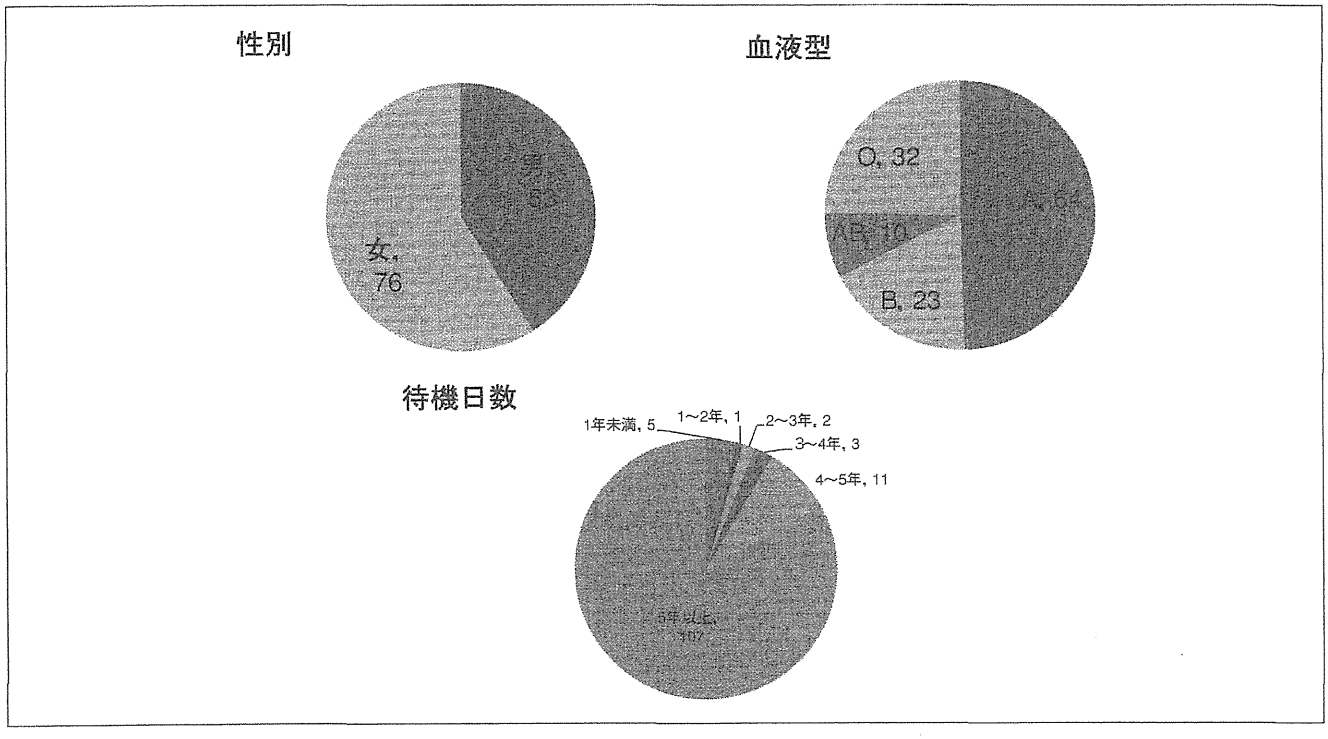


図３　レシピエント候補者情報

プロトコールに従って実施され，臨床試験参加の希望のない候補者および臨床試験参加の適応のない候補者は，臨床試験ではなく従来通りの形式にて膵島移植が実施される。2000年以降の新規登録者数の推移（図2）は，2007年3月以降の臨床膵島移植停止に伴い減少傾向にあったが，臨床試験開始に伴う膵島移植再開により，新規登録者が増加に転じている。レシピエント候補者の性別，血液型，待機日数を図3に示す。

2. 膵島移植フォローアップデータ

2007年12月までに65回の膵島分離が行われ，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，このうち34回で移植の条件を満たしていたため

18 症例（男性 5 例，女性 13 例）に対して膵島移植が行われた。膵島移植後の免疫抑制プロトコールはエドモントン・プロトコールに準じて実施された[1,3]。移植症例の平均年齢は 37.3 歳，糖尿病歴は 6～37（平均 20.8）年であった。本邦でのこれまでの膵島移植は 3 回までの移植を行うことを認めており，これらの 18 例に対する移植回数は 1 回 8 名，2 回 4 名，3 回 6 名であった。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 症例で一時的ではあるがインスリン離脱を達成した。インスリン離脱の最長期間は 214 日間であった。本邦における膵島移植症例にエドモントン・プロトコールによる膵島移植の多施設共同研究[3]における膵島生着の基準である，basal c-peptide level が 0.3 ng/ml 以上を当てはめると，初回移植後 1 年，2 年，5 年時における膵島生着率はそれぞれ 76.5%，47.1%，11.8% であった（**図 4**）。膵島生着率について，海外の成績と比較するにあたっては，膵島移植後の免疫抑制プロトコールだけでなく，本邦での移植実施例はすべて心停止ドナーからの提供であること，海外でエドモントン・プロトコールによる成績を報告する場合には，3 回の移植実施をされたレシピエントの成績を解析するが，本邦では移植を受けた 18 人のうち 3 回移植を受けられたレシピエントは 6 名に過ぎないこと，などの背景を考慮する必要がある。

重症低血糖発作の定義を，適切な血糖管理下においても，①自分以外の人(他人)による介助を必要とし，かつその際の血糖値が 60 mg/dl 以下である，②自分以外の人（他人）による介助を必要とし，かつ炭水化物の経口摂取，ブドウ糖の血管内投与，グルカゴン投与によって速やかに回復が認められたもの，のいずれかが認められること，とすると，膵島移植既往者の 2011 年末現在の重症低血糖発作の有無は**図 5** のような結果となり，膵島非生着と定義されたレシピエントにおいても重症低血糖発作が消失した症例も認められている。

膵島移植に関する有害事象は，免疫抑制剤に関連する有害事象と，移植手技に関連する有害事象が考えられるが，移植手技関連有害事象の報告は 1 例のみで，移植手術手技の低侵襲性を特色とする膵島移植の安全性を示唆する結果が示されている（**図 6**）。

3. 膵島移植臨床試験の進捗状況

これまで本邦で実施してきた膵島移植の成績より，膵島分離成績が不安定でドナーから膵の提供を受けても必ずしも移植が実施できない点，移植膵島障害により 1 人のレシピエントに複数回の移植が必要である点，および長期生着が困難であるという点が今後の一般医療化を阻む問題であると認識された。膵島移植停止の第一の要因となった膵島分離用酵素の問題に関しては，製造過程で哺乳類由来の材料を用いない安全性の高い酵素製剤（Liberase MTF，ロシュ社）が安定し

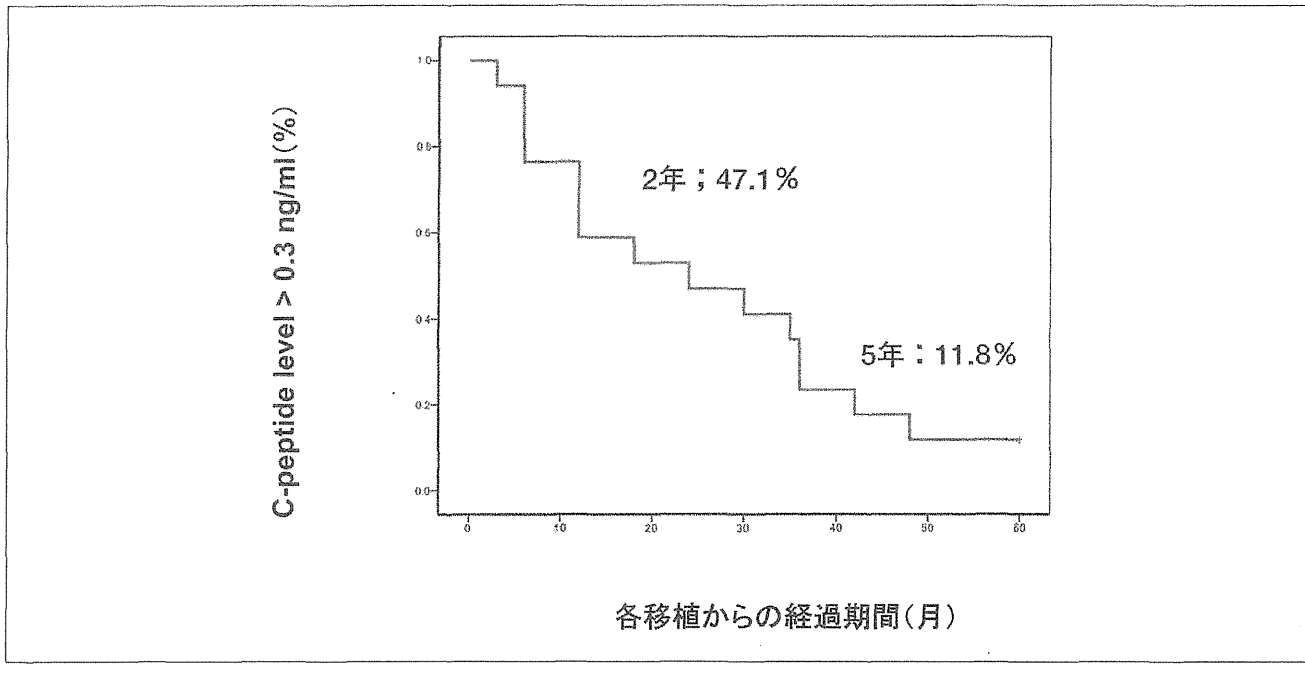


図 4　膵島移植後生着率

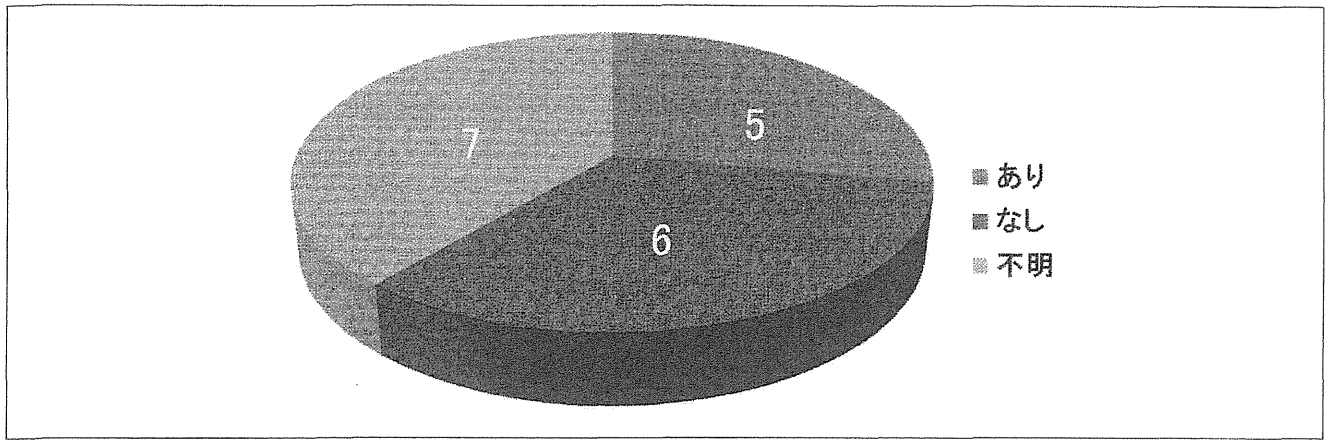
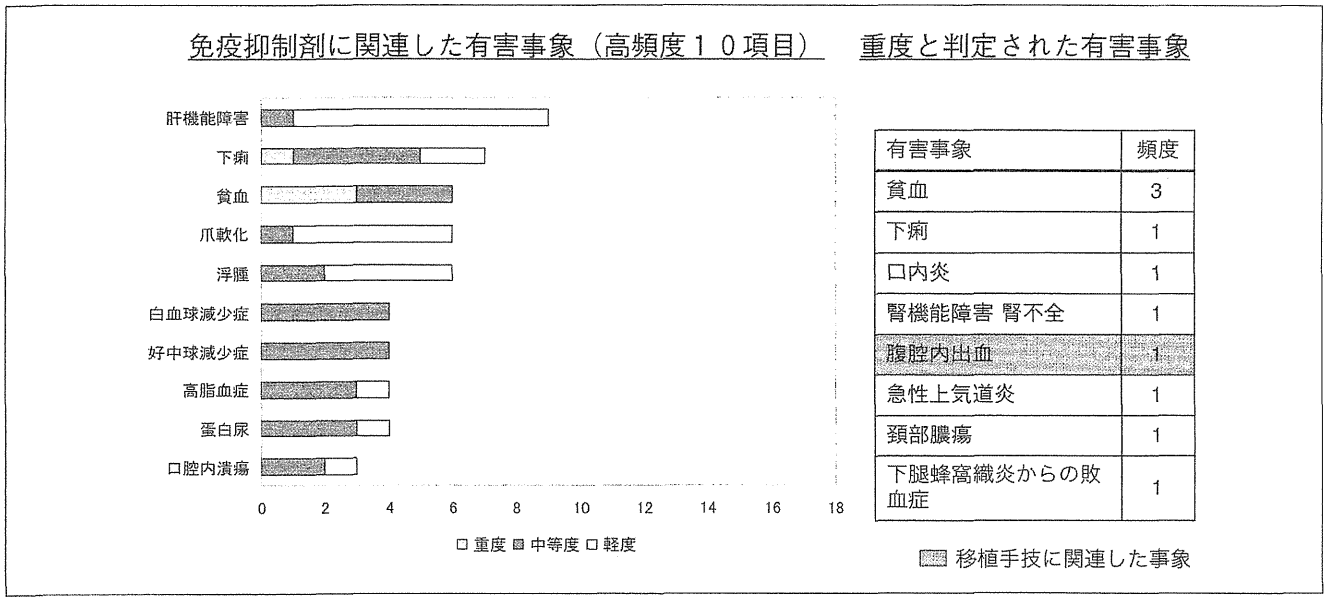


図５　重症低血糖発作の有無



重度と判定された有害事象

有害事象	頻度
貧血	3
下痢	1
口内炎	1
腎機能障害 腎不全	1
腹腔内出血	1
急性上気道炎	1
頚部膿瘍	1
下腿蜂窩織炎からの敗血症	1

▨ 移植手技に関連した事象

図６　膵島移植に関する有害事象

て入手可能となり，臨床膵島移植実施にあたっての分離用酵素に関する最大の懸念は解決された。この製剤は膵島分離に必要な組成に最適化され精製されており，今後の膵島分離成績の改善も期待される[4]。また，臨床再開にあたっては長期生着率を改善しうる免疫抑制プロトコールの導入が望まれた。欧米では anti-thymocyte globulin，抗 TNFα 抗体（etanercept），による導入療法に続いて，低容量 tacrolimus,sirolimus 主体の維持療法を行う方法が現時点でもっとも期待されているプロトコール[5]とされ，北米を中心に行われている多施設共同第Ⅲ相臨床試験が 2011 年末に症例登録が完了し，数年後にその結果が公表される見通しである。本邦でもこのプロトコールを踏襲し，本邦の薬剤入手の状況も踏まえ sirolimus を MMF に替えたプロトコールを作成し（図 7），多施設共同で臨床試験の実施体制を整えた。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。主要エンドポイントは，初回移植から 1 年後に HbA1c 値（NGSP 値）＜7.4% であり，かつ初回移植後 90 日から移植後 365 日にかけて重症低血糖発作が消失した患者の割合，としている。日本膵膵島移植研究会を中心に，膵島移植という医療技術を臨床試験として施行することについて，厚生労働省の創

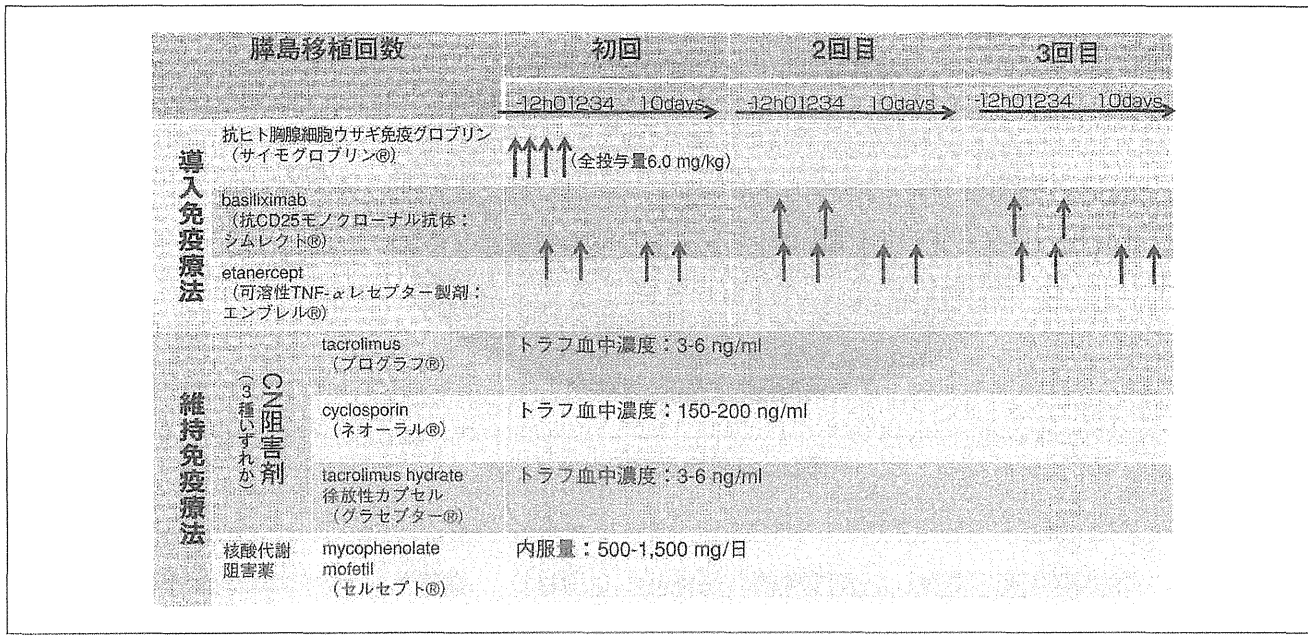


図7　膵島移植臨床試験の免疫抑制プロトコール

設した高度医療評価制度に申請，承認された。本制度の承認により，原則として保険との併用が認められていない医薬品・医療機器の使用を，先進医療の一類型として保険診療と併用することが可能となる。今後の薬事法上の申請につながるデータ収集を図るため，臨床試験推進拠点（東北大学病院臨床試験推進センターおよび先進医療振興財団）の支援を得て質の高い臨床試験体制が整備されている。

この臨床試験は2011年2月に実施施設の施設登録が開始され，その後，候補者に対する登録前検査の実施，コーディネーション体制の構築等が行われ，2012年6月より臨床膵島移植が再開となった。2012年6月から9月までに，2件の心停止ドナーからの膵提供があり膵島分離を施行したが，移植基準を満たさず移植には至っていない。

IV. 今後の展望

改正臓器移植法施行後，脳死下臓器提供数が増加した一方，脳死下に提供可能であった膵臓が，何らかの医学的理由により膵臓移植に用いることができない場合も散見されるようになった。これを受けて，膵臓移植に用いられない膵臓で，膵島移植には用いることができると判断された場合の膵臓を膵島移植に利用する体制が構築されてきている。先進医療の範疇であっても脳死下提供膵を用いることができるよう，再度高度医療評価会議での審議を受け承認された。先進医療専門家会議を経て，年内には脳死下提供膵から分離された膵島もこの臨床試験に用いることが可能になる見込みである。膵島移植が組織移植の範疇に分類されていることで複雑化しうるコーディネーション体制においては，人員の不足や連携体制などに課題が多い。また，移植の基準を満たさないヒト分離膵島の研究転用体制の構築も求められており，これらの課題について，日本膵・膵島移植研究会内や関係各所との間で検討が進められている。

V. おわりに

日本膵・膵島移植研究会が，膵島移植を1型糖尿病のひとつの治療選択肢として確立するために取り組んできたこれまでの過程を誌上で公にすることができた。日本膵・膵島移植研究会会員をはじめとする関係各位に対し，稿を終えるにあたり改めて感謝の意を表したい。

文責：日本膵・膵島移植研究会膵島移植班事務局
穴澤貴行，後藤満一

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VI 糖尿病の予防・管理・治療

糖尿病先進医療の現状と今後の展望

膵臓・膵島移植

Pancreas and islet transplantation

穴澤貴行　土屋貴男　後藤満一

Key words：膵臓移植，膵島移植，1型糖尿病，免疫抑制剤

はじめに

1型糖尿病患者の日常生活に著しい障害をきたす血糖不安定性からの解放を目指し，血糖値に応じてインスリン分泌を可能にする治療として，膵臓移植と膵島移植という移植医療が位置づけられている．臓器移植である膵臓移植は，1型糖尿病の治療の一選択肢として既に確立しているが，血管吻合を伴う難易度の高い開腹手術を必要とし，移植手術そのものに起因する合併症も少なくない．一方，組織移植に分類される膵島移植は，提供された膵臓から分離された膵島組織を，点滴の要領で門脈内に輸注する先進的な低侵襲治療である．しかし，複数のドナーが必要である点や長期成績の改善が望まれていることなど，解決すべき課題が多い．

1 膵臓移植の現況

膵臓移植は1型糖尿病の根治治療として位置づけられ，1人のドナーからでもインスリン離脱を達成することが可能で，長期のインスリン離脱も期待できる．新規免疫抑制剤の導入，臓器保存法の進歩，術式の改良，などに伴って移植症例数は増加し，現在欧米では年間1,600例前後の膵臓移植が行われている．移植成績も著しく向上し，移植膵の1年生着率は85％以上に達しており，1型糖尿病の治療の選択肢として定着しつつある[1]．一生涯免疫抑制剤の投与が必要となるが，血糖不安定性からの解放に加え，糖尿病性合併症の進行が阻止され，生命予後をも改善させうることが示されている[2]．

膵臓移植は腎移植との関係から，3つのカテゴリーに分けられる．糖尿病性腎症から慢性腎不全を呈した患者に対する膵腎同時移植(simultaneous pancreas and kidney transplantation: SPK)，先に生体腎移植を行い，その後に膵臓移植を行う腎移植後膵臓移植(pancreas after kidney transplantation: PAK)．そして腎機能が保たれた患者に対して行う膵単独移植(pancreas transplantation alone: PTA)である．我が国においては，2000年に第1例目のSPKが施行されて以降2009年末までに47例のSPK，9例のPAK，そして3例のPTAが脳死下の臓器提供により行われた．更に心停止下臓器提供で2例のSPK，20例の生体膵臓移植が行われている[3]．

脳死・心停止下臓器提供による膵臓移植症例61例の移植成績を**図1**に示す．膵臓の生着率に注目すると，1年，3年，5年生着率はそれぞれ88.4％，83.6％，73.3％であり，欧米と遜色ない成績を残している．しかし，登録日から移植日までの平均待機期間は約3年(1119.6日)と長

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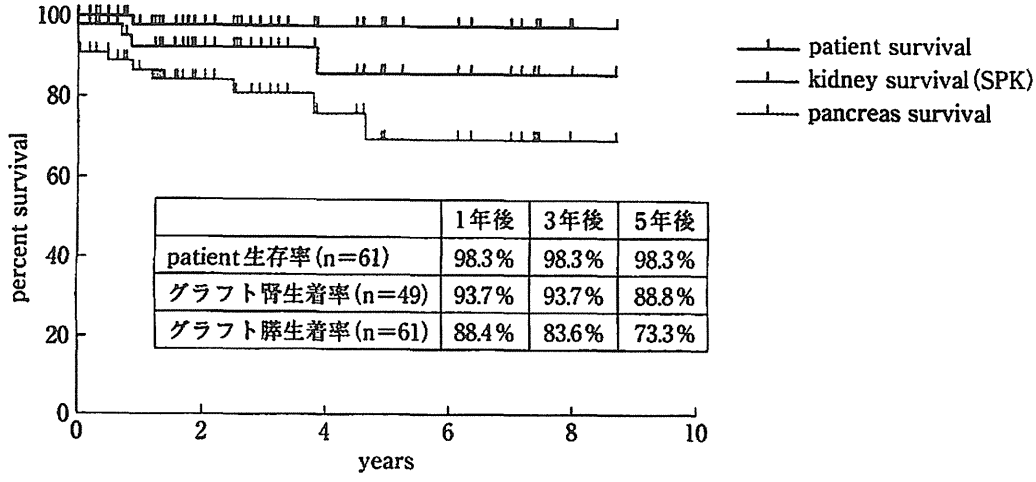

	1年後	3年後	5年後
patient生存率(n=61)	98.3%	98.3%	98.3%
グラフト腎生着率(n=49)	93.7%	93.7%	88.8%
グラフト膵生着率(n=61)	88.4%	83.6%	73.3%

図1 膵臓移植成績(2009年12月末集計)
(文献[3]より改変)

く，背景となるドナー不足が大きな問題であった．こうした中，2010年7月の臓器移植法改正により，家族承諾での脳死下臓器提供が可能となった．それ以降は2010年に23例の膵腎同時移植と2例の膵臓移植，2011年には9月末までに24例のSPKと3例の膵臓移植が実施されており，ドナー不足の問題に改善の傾向がみられる．

膵臓移植実施のためには，レシピエント候補者の主治医が地域の膵臓移植適応評価委員会にデータを添えて申請し，その結果が中央調整委員会へ送付される．中央調整委員会から移植施設に対して，移植可能の是非が確認され，日本臓器移植ネットワークへ登録となる．ドナー(脳死下，心停止下)発生時には，選択基準に従ってレシピエントが選択され，全国で18施設認定されている膵臓移植実施施設で移植が実施される[4]．

SPKの場合の手術手技としては，ドナーから全膵を十二指腸とともに摘出し，レシピエントの右腸骨窩に膵臓を移植し，腎臓は左側に移植する方法が一般的である．免疫抑制法は，抗IL-2レセプター抗体による導入療法に，タクロリムス，ミコフェノール酸モフェチル，ステロイドによる維持療法を行う4剤併用療法が主流である．

2 膵島移植の現況

膵島移植は，ドナーより提供された膵臓の膵管内に膵島分離用酵素を注入し，引き続き膵消化・膵島純化という過程を経て膵島組織のみを分離し，それを点滴の要領で門脈内に輸注する組織移植治療である．輸注移植された膵島は肝内に生着し，血糖感受性にインスリンを分泌し，1型糖尿病患者の重症低血糖発作を防ぎ生活の質を改善しうる[5]．低侵襲で，理想的な治療法として期待されてきたが，1990年代までのその移植成績は不良で，1型糖尿病患者の期待に応えるには不十分であった[6]．2000年代に入り報告された'エドモントン・プロトコール'[7]では，腎機能障害のない症例に膵島単独移植を行い，免疫抑制剤としてシロリムスを中心にdaclizumabと低用量のタクロリムスを組み合わせステロイドを使用せず，分離した膵島を直ちに移植し，移植膵島が十分量に達するまで異時性に複数回移植した．この方法でインスリン離脱の達成率は著明に改善された．その後多施設共同で臨床試験が行われ[8]，長期にわたる内因性インスリン産生と血糖安定性の回復に成功し，重症低血糖からの解放は得られるものの，長期的なインスリン離脱持続を得ることは難しいとされた．

ドナー膵から分離した膵島を移植に供するか