Table 2. Summary of the immunohistochemical staining (as depicted in Fig. 4) of the islet grafts in recipient monkeys receiving LMW-DS or heparin

Treatment	CD41	C3c	C9	Neutrophil elastase	CD68	MAC 387	CD56	CD3	CD20	IgG	IgM
LMW-DS	(−)-(++)	(−)-(++)	(−)-(+++)	(−)-(++)	(−)-(++)	(−)-(++)	(−)-(+)	(−)-(++)	(−)-(+)	(−)	(−)-(+)
(n = 21)	0.59 ± 0.19[a]	0.80 ± 0.37	1.50 ± 0.31	0.42 ± 0.23	1.31 ± 0.21	0.90 ± 0.28	0.10 ± 0.10	0.63 ± 0.20	0.20 ± 0.13	0	0.25 ± 0.25
Heparin (n = 18)	(−)-(+++)	(−)-(++)	(−)-(+++)	(−)-(++)	(++)-(+++)	(+)-(+++)	(−)-(+)	(−)-(+++)	(−)-(++)	(−)	(−)-(++)
	1.60 ± 0.51	0.63 ± 0.26	1.67 ± 0.33	1.08 ± 0.23	2.17 ± 0.11	2.11 ± 0.26	0.22 ± 0.15	1.90 ± 0.35	0.40 ± 0.27	0	0.60 ± 0.24
P value	0.056	0.69	0.71	0.04	0.002	0.01	0.48	0.006	0.83	–	0.36

[a]Values are expressed as mean ± SEM. LMW-DS, Low molecular weight dextran sulfate.

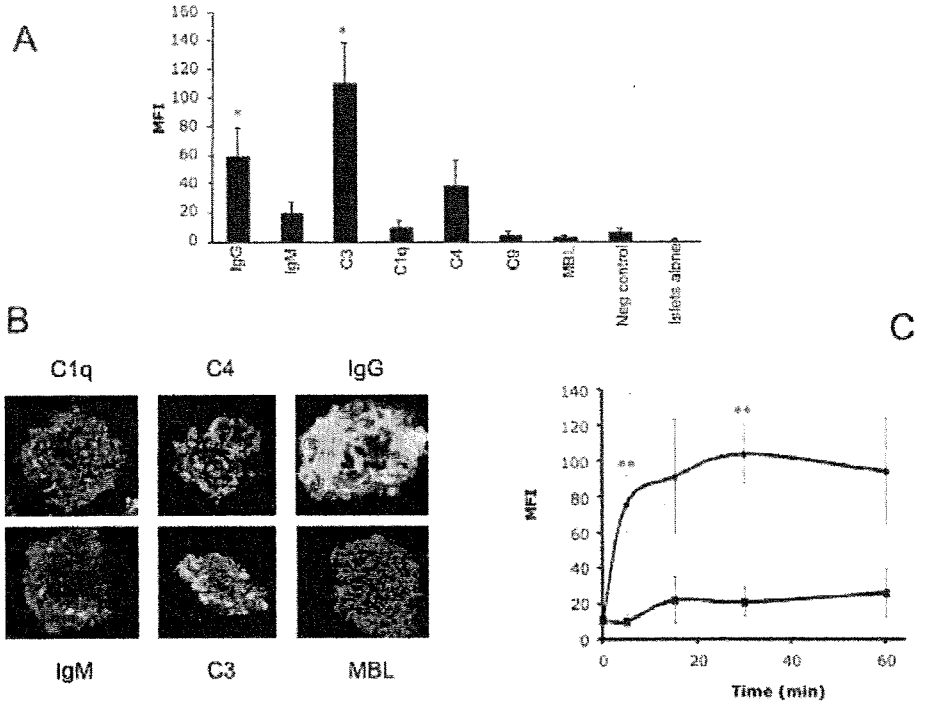

Fig. 5. Porcine islets incubated in hirudin-treated plasma for 30 min. The islets were stained for IgG (n = 5), IgM (n = 5), C3b/iC3b (n = 5), C1q (n = 3), C4 (n = 5), C9 (n = 3), and MBL (n = 3). As negative control, an antibody recognizing mouse IgG was used (n = 5). The islets were analyzed by (A) large particle flow cytometry and (B) confocal microscopy. In (C), the deposition of C3b/iC3b on the islet in the absence and presence of Compstatin is presented after analysis by large particle flow cytometry (n = 5; statistical evaluation was performed at 5 and 30 min where n = 7; *P < 0.05 and **P < 0.01).

far more efficient in inhibiting the IBMIR than heparin. These data confirm those of Rood et al. [19] who recently demonstrated longer porcine islet survival in non-human primates treated with LMW-DS.

In this study, both the morphological findings and the measurements in the plasma were similar to those in our previous studies, in which APIs were surrounded by clots and infiltrated by numerous leukocytes immediately after contact with fresh blood seen in the tubing loop model and our small animal model [9]. Most parameters reflecting the IBMIR, i.e. both coagulation and complement cascades, platelet deposition, and infiltration of macrophages and neutrophils, were attenuated in the monkeys treated with LMW-DS compared to the controls. There was also a tendency that increases in granulocyte count and liver enzymes were attenuated. One control monkey (M6) died of hypoglycemia, suggesting a strong IBMIR. Notably, T-cell infiltration observed in some of the transplanted islet grafts was also effectively suppressed, demonstrating that also the adaptive immune responses are attenuated by LMW-DS.

The effects of LMW-DS on the adaptive immune system may be explained by the effects on complement activation as complement is also of great importance in bridging innate immunity and specific immune responses. In allogeneic whole organ transplantation, C3 is one of the essential factors that trigger rejection in mice [20–22] and humans [23]. It is therefore reasonable to expect that

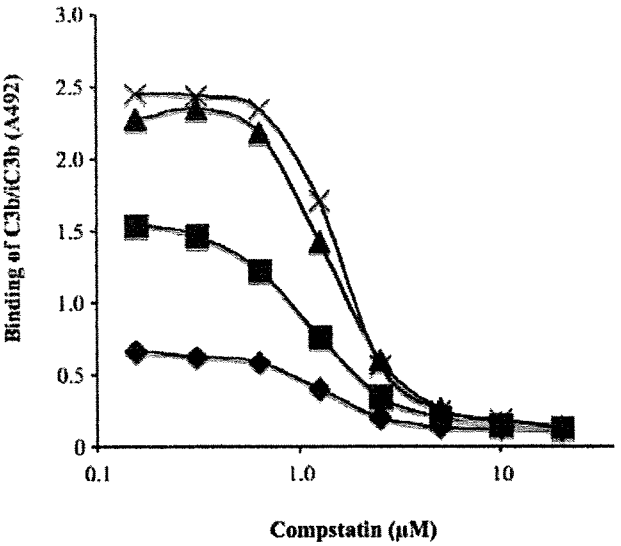

Fig. 6. Binding of C3b/iC3b to the surface of microtiter wells after incubation with 10% serum in the presence of increasing doses of Compstatin for 30 min at 37 °C. 0 (cross), 10 (triangle), 100 (squares), and 1000 (diamond) mg/l of LMW-DS was present in the wells.

complement activation will trigger a profound adaptive immune response raised against the graft, necessitating an unwarrantedly heavy immunosuppressive regimen. Previous studies support such a hypothesis [4,5].

As shown in Table 1, the islet grafts in M9 that reached an APTT of 107 s 24 h after transplantation, demonstrated well-preserved morphology suggesting that this dose of LMW-DS would be preferable. In a recently performed phase I study in normal individuals, we have shown that this concentration can be reached without an increased risk of bleeding or side effects (manuscript under preparation). This makes treatment with LMW-DS during xenogeneic islet transplantation an attractive alternative. It should be noted that a specific concentration of LMW-DS gives different APTT in blood from different individuals both in vitro and in vivo, probably due to that different allotypes of certain coagulation factors interact with LMW-DS differently.

In our previous studies, we showed that complement activation induced in xenogeneic IBMIR occurs secondarily to coagulation activation; a reaction which is also seen in allogeneic IBMIR [9,24] and which is elicited by chondroitin sulfate released by activated platelets [25]. This explains why complement activation in this study was suppressed in parallel with the reduction of coagulation activation at substantially lower concentrations (15–35 mg/l) of LMW-DS than used in other studies aiming for a inhibition of hyper acute rejection [26,27]. However, unlike complement activation in the fluid phase, immunohistochemical analyses showed that complement deposition was still seen on the islet grafts in the monkeys treated with LMW-DS. These reactions were analyzed in detail in vitro using large particle flow cytometry and confocal microscopy to be able to clarify the mechanism of activation. The experiments were performed using human plasma to directly translate the findings to clinical islet xenotransplantation. Pig islets incubated in human plasma revealed an almost instantaneous binding of IgM and IgG antibodies and complement components already after 5 min. This rapid activation was completely inhibited by Compstatin. It is possible that the instantaneous insulin dumping in a non-human primate model previously reported by Bennet et al. [3] and also observed in monkey M6 is explained by this antibody-mediated reaction. The severity of this reaction, which was totally abrogated by the recombinant complement inhibitor CR1, is reflected in the fact that the release of insulin corresponded to about 40% of the insulin in the transplanted islets.

One way to fully inhibit complement activation is to increase the dose of LMW-DS, but as shown in our in vitro experiments, doses between 10 and 100 mg/l have only minor effects on complement activation alone. Moreover, higher doses of LMW-DS are likely to give side effects. It is therefore obvious that LMW-DS must be combined with a specific complement inhibitor such as Compstatin to block the immediate destructive immunoglobulin-triggered complement activation found both in vitro and in vivo [10,28]. The in vitro studies show that LMW-DS and Compstatin do not interact in human serum.

Taken together, it possible to propose a model of how the different components of IBMIR interact in xenogeneic combinations: (1) Immediately when porcine islets come in contact with human blood there is an instantaneous binding of IgG and IgM antibodies to the islet surface which triggers a deleterious complement activation; (2) This is followed by a clotting reaction with accompanying complement activation. Based upon the experimental data presented, LMW-DS combined with a specific complement inhibitor is an attractive alternative to control the detrimental innate immune responses that are postulated to occur in forthcoming intraportal pre-clinical and clinical islet xenotransplantation trials. We are at the moment in progress to produce sufficient amounts of Compstatin to perform studies in the NHP model with Compstatin combined with LMW-DS.

Acknowledgments

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ELSEVIER

Optimization of a Prominent Oxygen-Permeable Device for Pancreatic Islets

M. Goto, Y. Yoshikawa, K. Matsuo, A. Shirasu, N. Ogawa, H. Takahashi, Y. Saitoh, M. Goto, K. Fujimori, Y. Kurokawa, M. Tamai, and S. Satomi

ABSTRACT

Background. We have demonstrated a culture bag system that is useful for pancreatic islet transplantation. To improve and simplify islet transplantation procedures from culture to transplantation, we developed a novel device specific for both islet culture and transplantation (TUBERO Device [TD]) using an oxygen-permeable material.

Materials and Methods. Porcine islets with 30 minutes warm ischemia time were cultured for 24 hours at 37°C in 5% CO_2 and humidified air under three different procedures: (1) ordinary culture flask, (2) culture bag suitable for platelets, and (3) TD. Loss of islets during culture, glucose-stimulated insulin release as an islet functional test, and ADP/ATP ratio as an index of islet viability tests were evaluated to compare the devices. TD was further applied in two clinical islet transplantations using non–heart-beating donors in Japan.

Results. The loss of islets during culture was considerably lower in the TD group. The stimulation index upon glucose challenge tests was significantly higher in the TD group than the others. The ADP/ATP ratio in TD group was significantly lower than that in the ordinary flask group, suggesting that the apoptotic islets were relatively lower among TD. Most importantly, TD was successfully applied both in the clinical islet cultures and in transplantation, resulting in excellent graft function.

Conclusions. We propose that the TD, a novel product, not only simplifies islet transplantation procedures, but also maintain the quality of isolated islets.

WE HAVE demonstrated a culture bag system that is useful for pancreatic islet transplantation.[1] To improve and simplify the islet transplantation procedures, we have described a novel device for both islet culture and transplantation (*T*UBERO *D*evice [TD]) using a prominent oxygen-permeable material.

MATERIALS AND METHODS

The TD was made from polyethylene film. The oxygen permeation coefficient of the polyethylene film is 3,000 cm^3/m^2 atm. Porcine islets with 30 minutes warm ischemia time were cultured for 24 hours at 37°C in 5% CO_2 and humidified air under three different procedures: (1) ordinary culture flasks, (2) culture bags suitable for platelets, and (3) TD. The loss of islets during culture, the glucose-stimulated insulin release as a islet functional test, and the ADP/ATP assay[2] as an islet viability test were evaluated to compare the devices. TD was further applied in two clinical islet transplantations using non–heart-beating donors in Japan.

RESULTS

The loss of islets during culture tender to be lower among the TD group: (1) 22.9 ± 4.4, (2) 23.9 ± 10.0, and (3) 38.9 ± 6.0: (P = .09, Fig 1). The stimulation index on glucose challenge tests was significantly higher in TD group than

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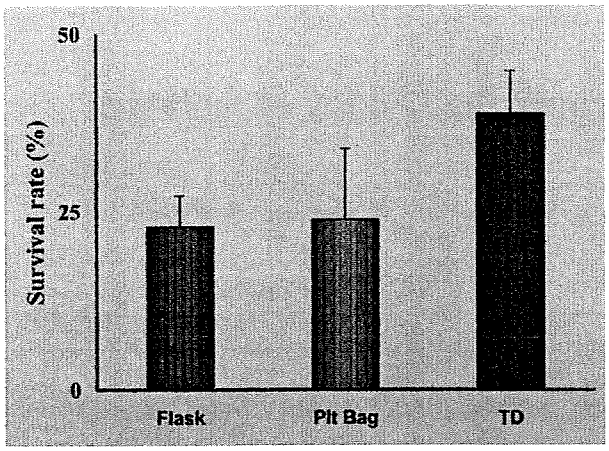

Fig 1. The loss of porcine islets during 24-hour culture was evaluated as the survival rate.

the other methods: (1) 1.46 ± 0.15, (2) 1.67 ± 0.17, and (3) 2.09 ± 0.18 (P = .03; Fig 2). The ADP/ATP ratio in the TD group was significantly lower than that in the ordinary flask

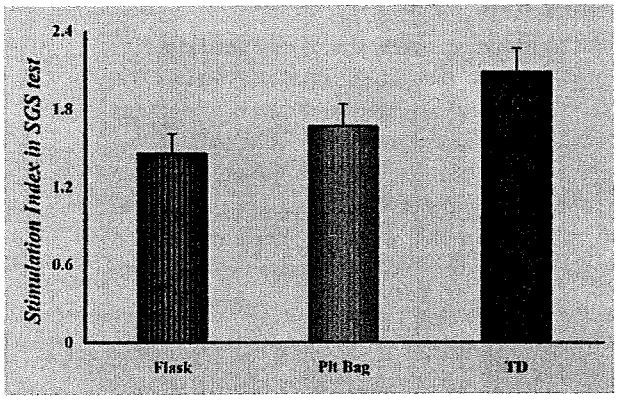

Fig 2. Glucose-stimulated insulin release was evaluated as a functional test.

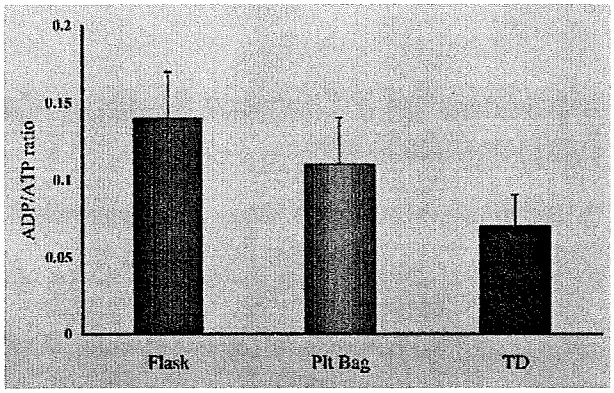

Fig 3. The ADP/ATP assay was evaluated as a viability test.

group (TD 0.07 ± 0.02 vs. flask 0.14 ± 0.03; P = .07; Fig 3), suggesting fever apoptotic islets with TD. Most importantly, TD was successfully applied in transplantation, resulting in excellent graft function: fasting C-peptide levels during 6 months after transplantation: 1.2 to 2.7 ng/mL and secretory units of islets in transplantation (SUIT) index[3]: 15 to 45.

In conclusion, the TD simplified islet transplantation procedures and maintained the quality of isolated islets.

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ELSEVIER

Influence of a Current Style of Culture on the Quality of Isolated Pancreatic Islets

H. Takahashi, M. Goto, N. Ogawa, Y. Saitoh, K. Fujimori, Y. Kurokawa, H. Doi, and S. Satomi

ABSTRACT

Objective. Comparable outcomes of islet transplantation with short periods of culture may be achieved with various culture media. To clarify the influence of a style of culture on isolated pancreatic islets, islet quality of fresh islets was compared with those cultured in several different fashions including not only for viability but also for inflammatory mediators.

Materials and Methods. Wistar rat islets were cultured for 48 hours with CMRL including 10% allogeneic serum; CMRL including 0.5% human serum albumin (HSA); and Miami medium including 0.5% HSA. The influence of culture conditions on islet integrity was evaluated by survival rate of islets during culture and visual scoring. The influence of culture conditions on islet function and viability was examined by ADP/ATP tests, insulin/DNA content, and glucose stimulation tests.

Results. Although the survival rates were similar for all groups, the visual scoring was lower in Miami medium. The stimulation index in glucose challenge tests was higher for fresh islets than the media ($P = .02$). Insulin/DNA ratios revealed the same tendency as glucose challenge tests ($P = .0005$). ADP/ATP ratio was lower in both the fresh and serum groups than in the others ($P = .38$), suggesting that apoptotic islets are relatively fewer in both fresh and serum groups. Most importantly, the expression of tissue factor (TF) on the islets was considerably lower in the fresh group, suggesting that a current style of culture could enhance TF-dependent instant blood-mediated inflammatory reactions after transplantation.

Conclusion. In conclusion, Isolated islets without prior culture shows characteristics beneficial for transplantation using current modes of culture.

ALTHOUGH ONE KEY FACTOR of the Edmonton protocol is transplantation of fresh islets just after isolation, it has recently been reported that the outcomes are comparable with short periods of culture. This observation may be based upon specific culture media used in an institution. To clarify the influence of culture media on isolated pancreatic islets, the present study compared islet quality including not only viability but also expressed inflammatory mediators of fresh islets with those cultured in several different fashions.

MATERIALS AND METHODS

Wistar rat islets cultured for 48 hours with CMRL including 10% allogeneic serum or 0.5% human serum albumin (HSA), and Miami medium including 0.5% HSA are the most established current media. The influence of culture on islet integrity was evaluated by the survival rate of islets during culture and by visual scoring.[1] The influence of culture conditions on islet function and viability was examined by glucose stimulation tests[1] (Fig 1), insulin/DNA ratios (Fig 2), and ADP/ATP assays[2] (Fig 3). The influence of culture on the expression of inflammatory mediators in the islets

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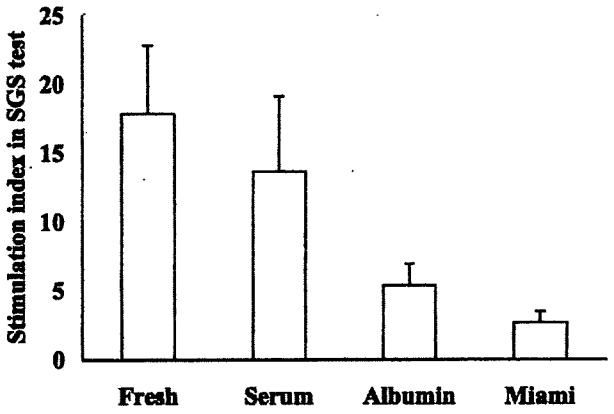

Fig 1. The influence of culture conditions on islet function was examined by static glucose stimulation tests (SGS).

was examined by Western blotting assays for tissue factor (TF), which is the initiator of detrimental instant blood-mediated inflammatory reactions (IBMIR). Statistical analyses were performed by using ANOVA.

RESULTS

Although the survival rates were similar for all groups, the visual scoring was lower in Miami medium. The stimulation index in glucose challenge tests was higher among fresh than the other islets: fresh = 17.89 ± 4.93; serum = 13.69 ± 5.44; HSA = 5.36 ± 1.60; Miami = 2.69 ± 0.82 (P = .02; serum vs Miami; n = 5). Insulin/DNA ratios revealed the same tendency as glucose challenge tests: fresh = 1.02 ± 0.07; serum = 0.83 ± 0.11; HSA = 0.52 ± 0.07; Miami = 0.37 ± 0.08 (P = .0005; fresh vs HSA, fresh vs Miami; n = 5). ADP/ATP ratios were lower among both fresh and serum than the other groups: fresh = 0.047 ± 0.021; serum = 0.054 ± 0.017; HSA = 0.086 ± 0.004; Miami = 0.084 ± 0.026 (P = .38), suggesting a relatively lower content of apoptotic islets in both fresh and serum groups. Most importantly, the expression of TF on the islets was considerably lower in the fresh group, suggesting that the current style of culture enhanced tissue factor-dependent IBMIR after transplantation.

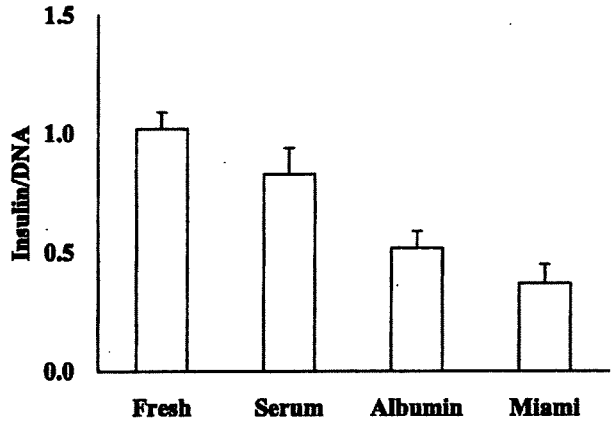

Fig 2. The influence of culture conditions on islet function was examined by insulin/DNA assays.

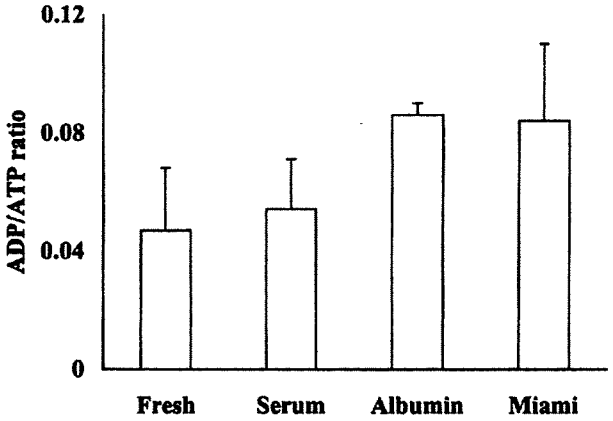

Fig 3. The influence of culture conditions on islet viability was examined by ADP/ATP assays.

DISCUSSION

Isolated islets without prior culture were beneficial for transplantation using current modes of culture. Further improvements are required to optimize a substitute for the serum supplement.

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Cryopreservation of Human Pancreatic Islets From Non-Heart-Beating Donors Using Hydroxyethyl Starch and Dimethyl Sulfoxide as Cryoprotectants

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Although widely used, DMSO is toxic for pancreatic islets. We combined hydroxyethyl starch (HES) with DMSO to simplify the procedure of freezing and thawing, and to decrease the toxicity of DMSO. A preclinical study was performed using islets from beagle dogs. After storage for 4 weeks, the islets were thawed and examined. The islet structure was well maintained after thawing. Although the number of the islets decreased to 71.2 ± 20.1%, the function of the islets was evaluated by static incubation after thawing and showed a 1.80 ± 0.78 stimulation index. We have introduced this technique for the cryopreservation of human islets from non-heart-beating donors. Twelve cases of human islet cryopreservation were performed. The sample tube of each human cryopreservation was thawed to evaluate the morphology, contamination, and endocrine function. Although fragmentation was observed in five samples (41.6%), the other seven (58.4%) showed a normal structure when evaluated by microscopic and electron microscopic study. The stimulation index (SI) of static incubation deteriorated from 3.37 ± 3.02 to 1.34 ± 0.28 after thawing. We divided the thawed islets into two groups: group 1 ($n = 8$), SI >1.2; group 2 ($n = 4$), SI <1.2. The group 1 islets showed a higher rate of normal structure (87%) than did group 2 (25%). Moreover, the SI before cryopreservation was 4.01 ± 3.57 in group 1, which was higher than the SI of 2.11 ± 0.72 in group 2. Based on the good results from the preclinical study using a large-animal model, this method was introduced for clinical application. Even from the pancreata of non-heart-beating donors, a successful islet cryopreservation was achieved. However, the isolated islets with poor function should not be cryopreserved for transplantation.

Key words: Cryopreservation; Human islets; Hydroxyethyl starch; Islet transplantation

INTRODUCTION

Pancreatic islet transplantation is considered to be the most physiologically advantageous procedure for the treatment of type 1 diabetes mellitus. The introduction of the Edmonton protocol, with a highly improved rate of insulin independency, encouraged us to promote clinical islet transplantation (18,20). In Japan, clinical islet transplantation was first performed by Kyoto University in 2004 (10). Thirteen type 1 diabetic patients, thus far, have undergone one or more islet transplantations. According to the social circumstances of Japan, the pancreata were able to be harvested only from non-heart-beating donors for islet isolation. The non-heart-beating donors in Japan are usually of higher age and the most frequent cause of death is a cerebrovascular disorder. Moreover, the withdrawal of respirator support is not commonly performed and, thus, a cannulation of the aorta before a cardiac arrest is not necessarily performed. Therefore, the viability of the pancreata may deteriorate due to various factors that include aging, a prolonged warm ischemia, and a damaged microcirculation. In Japan, the criteria for fresh islet transplantation have been determined based on the Edmonton protocol (20). According to the rules of the Japanese Islet Transplant Team, the islets must be cryopreserved even if the results (yield, purity) do not reach the criteria for fresh islet transplantation.

The cryopreservation of human pancreatic islets offers many advantages for clinical transplantation. Cryostorage allows for the accumulation of a large amount of donor tissue so that a sufficient number of islets with

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a desired HLA tissue type can be provided for transplantation. The frozen islets can be shipped to other institutions worldwide. In addition, an accurate pretransplant evaluation, in terms of safety and efficacy, is possible during cryopreservation. However, the major problem with the cryopreservation of islets is a decreased number and function of the frozen–thawed islets compared with the fresh islets. According to the Edmonton protocol, only fresh islets can be used for clinical islet transplantation.

In the present study, we have designed a simple technique of cryopreservation using hydroxyethyl starch (HES) and dimethyl sulfoxide (DMSO) as cryoprotectants for the purpose of protecting the islets from the toxic effect of DMSO. Based on the preclinical study using a large-animal model, this technique was introduced for the cryopreservation of human islets from the pancreata of non-heart-beating donors.

MATERIALS AND METHODS

Preclinical Study Using Beagle Dogs

Animals and Islet Preparation. Five beagle dogs, weighing from 7.5–12.5 kg, were used. The islets were isolated from the pancreata of the dogs by an automated two-step digestion method that we have developed, followed by a Ficoll purification as previously described (5,6).

Cryopreservation and Thawing of the Islets. After an overnight culture, a known number of islets were suspended in RPMI-1640 containing 5% DMSO, 6% HES, and 4% FBS on ice, then transferred into a 75-ml cryogenic storage container (7005-2, CharterMed Inc, Lakewood, NJ). The container was cooled using a programmed freezing system, Cryomed Model 1010 (Forma Med Inc., Marietta, OH). After 4 weeks of storage in liquid nitrogen, the container was rapidly thawed in a 37°C water bath. The islets were sedimented and resuspended with RPMI-1640 containing 10% FBS.

Static Incubation. To assess the function of the thawed islets, static incubation was performed. Briefly, five aliquots of 10 islets were placed into 12-well transwell microplates with 1 ml RPMI-1640 containing 3.3 mmol/L D-glucose and 0.1% BSA as the basal medium. After 60 min, the culture transwells were transferred into new 12-well microplates with RPMI-1640 containing 20 mmol/L D-glucose and 0.1% BSA (glucose stimulation). After 60 min, the culture transwells were transferred to new 12-well microplates to add basal medium again for an additional 60-min culture. Each medium was centrifuged and immediately frozen for a later assay of the insulin concentration by ELISA. The stimulation index was calculated by comparing the insulin content in the glucose stimulation medium with the second basal medium.

Cryopreservation of Human Pancreatic Islets From Non-Heart-Beating Donors

Harvesting of the Pancreata. Twelve pancreata were harvested from the non-heart-beating donors under an informed consent from September 2003 to July 2006. The characteristics of donors are shown in Table 1. Half of the non-heart-beating donors were over 50 years of age and, thus, the major cause of death was a cerebrovascular disorder. Five of the cases were not permitted a cannulation of the aorta and a systemic heparinization before cardiac arrest, which resulted in the prolonged warm ischemic period. In addition, the durations of hypotension and anuria were extremely prolonged because the withdrawal of a respirator is not commonly performed in Japan.

Islet Preparation. The pancreata were preserved by the two-layer method (9,11) in eight cases and by simple cold storage in University of Wisconsin solution in four cases, and were transferred to the Cell Processing Center located in the Clinical Research Center of our hospital. The islet isolation was performed according to the Edmonton protocol with some modifications. Briefly, the pancreas was distended with cold Liberase solution (Liberase HI™, Roche Diagnostics, IN) by a ductal injection. Thereafter, the distended pancreas was cut into several pieces and put into a Ricordi chamber and digested using a closed automated system at 37°C. The shaking of the Ricordi chamber was performed either by hand or by a shaker. The pancreatic digests were collected in a flask on ice and were purified on a Euro-Ficoll discontinuous solution using a COBE 2991 cell processor.

Cryopreservation and Thawing of the Islets. After an overnight culture with serum-free medium [1% L-glutamine, 1% antibiotic antimycotic solution, 16.8 mM zinc sulfate, 1% ITS (6.25 mg/ml insulin, 6.25 mg/ml transferrin, 6.25 ng/ml selenious acid, 5.35 mg/ml linoleic acid, 1.25 mg/ml albumin; Collaborative Biomedical Products), CMRL1066], the islets were cryopreserved. The cryopreservation method used is the same procedure as described above in the preclinical study except for the use of human albumin in place of fetal bovine serum. In addition to the container, eight sample tubes containing 500–1000 islets were cryopreserved for the purpose of a sample check during cryostorage. From 2 weeks to 3 months after cryostorage, the sample tube was thawed using the rapid thawing technique as described above to evaluate the islet morphology, to check for the presence of bacterial or fungal contamination, and to evaluate the islet function.

Table 1. Characteristics of the Non-Heart-Beating Donors Used for the Cryopreservation of the Islets

Age (years): 43.2 ± 20.2 (10–69) (>50 years: 6 cases)
Male/female: 6/6
Cause of death
Cerebrovascular disorder: 7 cases (58.3%)
Hanging (suicide): 6 cases (50%)
Brain tumor: 1 case (8.3%)
Cannulation into the aorta before cardiac arrest: Yes—7 cases (58.3%), No—5 cases (41.7%)
Warm ischemic time (min) 10.5 ± 10.5 (1–30)
Total ischemic time (min) 325 ± 54.3 (231–436)
Duration of hypotension* (min) 333 ± 332 (0–840)
Duration of anuria (min) 447 ± 620 (0–1800)

*Maximum blood pressure: <60 mmHg.

Evaluation of the Islets. The frozen–thawed islets in the sample tube were cultured overnight in serum-free medium and were evaluated to determine if it was possible to use the islets for transplantation, by the following examinations. The morphology of the islets was microscopically examined by a staining with dithizone, and by an immunostaining with anti-insulin and antiglucogon antibodies. In addition, an electron microscope was used for further study of the islets' morphology. The supernatant of the cryopreservation solution was checked for contamination by bacteria, including the acid-fast bacteria and the tubercle bacillus, as well as fungal organisms, to assure the safe use for transplantation. As a functional assay, a static incubation was performed and the stimulation index was calculated as mentioned above.

RESULTS

Preclinical Study Using Beagle Dogs

The yield and purity of the islets of the five isolations, which were evaluated after an overnight culture and before cryostorage, were 80,349 ± 37,164 IEq and 87.0 ± 5.7%, respectively. Although the purities improved to 96.2 ± 1.6% after thawing, the number of the islets decreased to 57,595 ± 31,027 IEq (recovery rate: 71.2 ± 20.1%). In the morphologic study, however, the shape of the frozen–thawed islets was well maintained and the fragmentation or clumping of the islets was hardly observed.

The simulation index (SI) calculated from static incubation was 1.80 ± 0.78. In addition, the islets of isolate #2 were examined by a perifusion study and a prompt release of insulin with two peaks was observed (Fig. 1).

These preclinical data demonstrated the effective preservation of both the structure and the endocrine function of the frozen–thawed islets in our cryopreservation method.

Cryopreservation of Human Pancreatic Islets From Non-Heart-Beating Donors

Yield and Purity of the Islets. The results of the islet isolation are shown in Table 2. Because the high-yield (5,000 IEq/kg recipient body weight) viable islets with over 30% purity are used for fresh islet isolation, the results are from isolations that did not fulfill the criteria for fresh islet isolation.

Check for Contamination. The supernatant of the cryopreservation solution of all sample tubes was examined by a direct staining, and by a culture, for the contamination check. The direct staining and the culture for bacteria, including the acid-fast bacteria and the tubercle

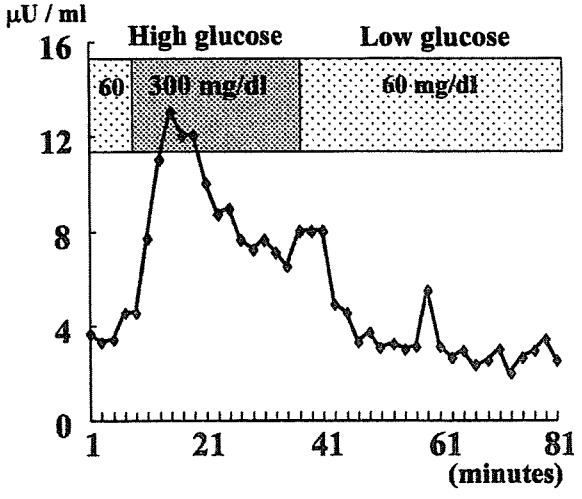

Figure 1. Insulin secretion from the frozen–thawed beagle islets was assessed by dynamic perifusion system. Prompt insulin release with two peaks showed the preservation of endocrine function of the frozen–thawed islets.

Table 2. Results of Islet Isolation From Non-Heart-Beating Donors*

Number: 12
Yield (IEq†): 108,656 ± 45,803 (37,840–177,800)
Weight of pancreas: 75.3 ± 23.6 (37–108)
Yield/g pancreas (IEq†): 1,546 ± 699 (394–2857)
Purity (%): 40.0 ± 18.1

*Department of Surgery, Chiba-East National Hospital; the cases not used for fresh islet transplantation (September 2003 to July 2006).
†IEq: the number of the islet equivalent to 150 μm.

bacillus, as well as fungal studies, were all negative for the presence or the growth organisms. The data showed that the frozen–thawed islets in the present study were safe for transplantation.

Morphological Study of Frozen–Thawed Islets. A severe fragmentation was observed in five samples (5/12; 41.6%) and the other seven samples (7/12; 58.4%) showed a normal morphology evaluated by the microscopic examination with a dithizone staining and a hematoxylin-eosin staining (Fig. 2). The well-preserved islets showed a positive insulin staining and a normal fine structure by an electron microscopic study (Fig. 3).

Static Incubation. The SI of the static incubation deteriorated from 3.37 ± 3.02 at precryostorage to 1.34 ± 0.28 after thawing. We divided the thawing islets into the following two groups: group 1 ($n = 8$)—the SI after thawing was >1.2, and group 2 ($n = 4$)—the SI after thawing was <1.2. The group 1 islets showed a higher rate of good preservation of the islets' structure at the point of precryostorage (7/8; 87%) than did the group 2 islets (1/4; 25%) (Fig. 4). Moreover, the SI of the static incubation performed at precryostorage was 4.01 ± 3.57 in the group 1 islets, which was higher than the 2.11 ± 0.72 determined in the group 2 islets (Fig. 4).

DISCUSSION

Islet transplantation is considered to be the most physiological treatment for type 1 diabetic patients. Although clinical islet transplantations have been successful in a limited number of patients (4) before 2000, the Edmonton protocol, which was introduced by an Alberta University group, drastically improved the results of clinical islet transplantation (18,20). In the Edmonton protocol, only fresh islets were used, immediately after isolation, for transplantation to achieve insulin independency in the diabetic patients. Cryopreserved islets were not used in this protocol in spite of the previous clinical experience with a cryopreserved islet allograft (19,22).

Encouraged by the successful results of the Edmonton protocol, the Japanese Islet Transplant Team prepared for the start of clinical islet transplantation, and the first human islet isolation was performed by our group on September 12, 2003. The first human islet transplantation for a type 1 diabetic patient was performed by the Kyoto group (10). In Japan, however, the pancreata from non-heart-beating donors are indicated for use in islet isolation because the pancreata from brain-dead donors are usually used for pancreas or pancreas/kidney transplantation.

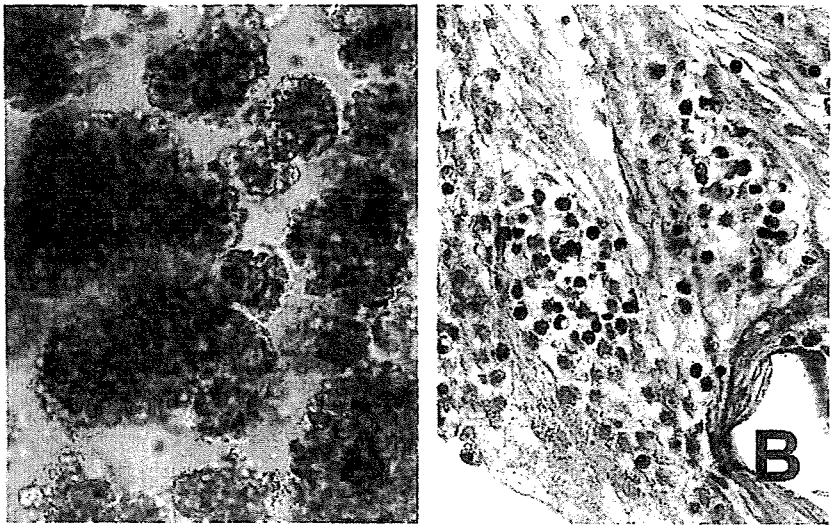

Figure 2. Microscopic findings of the frozen–thawed beagle islets stained with diphenylthiocarbazone (A) and hematoxylin-eosin (B). The normal morphology was preserved without fragmentation.

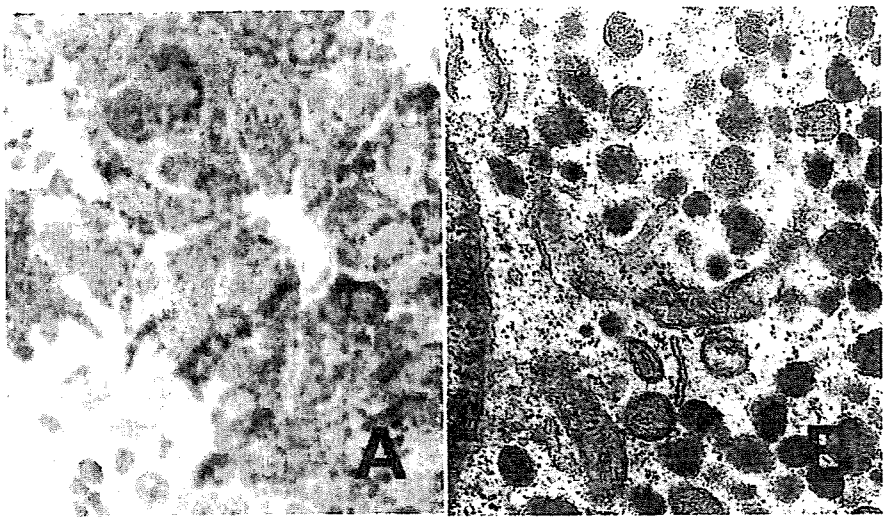

Figure 3. Frozen–thawed beagle islets stained with anti-insulin antibody (A) and electron microscopy (B). Positive staining of insulin as detected on the islets and the normal structure of organelles of the islet cell demonstrated the good preservation of the frozen–thawed islets.

In Japan, the withdrawal of a respirator is rarely performed even though the donor is diagnosed to be suffering from brain death. Moreover, the donors frequently are not given the examination to diagnose brain death, and thus, a cannulation of the abdominal aorta with a double balloon catheter via a femoral artery and a systemic heparinization are not indicated for the donors before cardiac arrest. Out of 12e non-heart-beating donors in the present study, we could not perform cannulation and heparinization before cardiac arrest in five cases (41.7%). Under the present conditions that exist in Japan, the viability of the pancreata may decrease before harvesting, thus often resulting in a poor yield and a low purity of the islet isolation. According to the rules of the Japanese Islet Transplant Team, the islets must be cryopreserved even if the results do not reach the criteria required for fresh islet transplantation.

Cryopreservation is thought to be an ideal method for

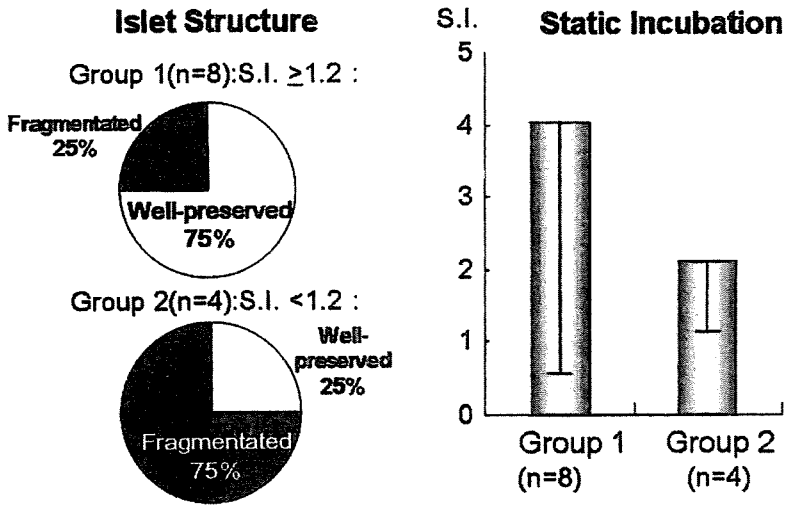

Figure 4. Human islet structure and stimulation index of static incubation at precryostorage. In group 1, islet structure was better preserved (75%) and the stimulation index was higher than those in group 2.

the long-term storage of human pancreatic islets, and many investigations concerned with the use of islet cryopreservation have been performed (3,7,12,15,16,21). The cryopreservation of islets may benefit many aspects of clinical islet transplantation. The islets are susceptible to contaminations by bacteria and fungi during the procedure of islet isolation and the check for contamination is impossible except for a gram staining in the fresh islet transplantation. The endocrine function does not necessarily correlate to the islet appearance immediately after isolation. The evaluation of the endocrine function, including a static incubation and a perfusion study, is also impossible in a fresh islet transplantation. For the safety and efficacy of the islets, contamination check and the evaluation of the endocrine function are primary, and these data can be obtained during the cryostorage of the islets. The cryostorage allows the accumulation of a large amount of donor tissue so that a sufficient number of islets with a desired HLA tissue type can be provided for transplantation. Furthermore, the frozen islets can be shipped to other institutions worldwide. The immunogenicity of the islets may deteriorate during cryostorage according to the reduction of the MHC antigen (2,13).

The major disadvantage of cryopreservation, however, is the deterioration of the number and function of the islets after thawing (17). One of the major causes is the toxicity of the DMSO, which is widely used for cryoprotection. DMSO has been used as an intracellular cryoprotectant. However, because of its toxicity against islet cells, the DMSO must be added stepwise and also diluted stepwise with sucrose. Several materials, including trehalose (1), ethylene glycol (8), and polyethylene glycol (14), were used as a cryoprotectant for the cryostorage of the islets in previous studies. In the present study, we combined hydroxyethyl starch (HES) as an extracellular cryoprotectant with the DMSO to simplify the freeze–thawing procedure. By adding HES, the final concentration of the DMSO can be reduced from 10–12% to 6%, which reduces the direct toxicity of the DMSO and simplifies the freeze–thawing procedure. This modification may contribute to a preservation of the islets during the cryopreservation procedure and result in a higher yield and an improved function after thawing. Furthermore, the simplification of the procedure is important to reduce the opportunity for contamination. In the present preclinical study, although the islet number decreased to 71.2% after thawing, the structure was well maintained and a positive insulin secretion against the glucose challenge was seen both in the static and the dynamic perifusion study, thereby demonstrating the ability of our method to achieve good preservation of the islets during cryostorage.

From the results of the present study using human islets isolated from non-heart-beating donors, the safety of our method was confirmed because no contamination was detected in any of the 12 cryopreservations. As for the function of the islets, the SI decreased by cryostorage from 3.37 ± 3.02 to 1.34 ± 0.28. It is questionable, however, that the levels of SI directly reflect the islet function, and the cutoff level of the stimulation index is used to decide the use for transplantation. In the present study, we divided the frozen–thawed islets into two groups using 1.2 as the cutoff level of the SI. The SI for group 1 was >1.2, which can be used for transplantation, and the SI for group 2 was <1.2, which should not be used for transplantation. A normal structure and positive insulin staining, as evaluated both by light microscopy and by electron microscopy, were observed in group 1, and the fragmentation of islets and a negative insulin staining were detected in group 2. These data may indicate that the cutoff level of 1.2 for the static incubation that we used is reasonable to decide the fate of the frozen–thawed islets (i.e., whether or not to use them for transplantation).

Another important finding from the present study is the possibility of the evaluation of fresh islets to determine which should be cryopreserved and which should not. In group 1, both the structure and function were already well maintained after isolation. Especially the fragmented islets immediately after isolation reflected the poor viability of the pancreas from the donors with poor conditions, including old age, a prolonged warm ischemia, and/or a cold ischemia. Therefore, we can select the fresh islets for cryopreservation if the criteria is determined, which is an important consideration from the both the economic and the academic points of view.

In conclusion, we developed a new cryopreservation method by the combined use of HES with DMSO to simplify the procedure of cryostorage of pancreatic islets and to decrease the toxicity of the DMSO. Based on the good results of our preclinical study using a large-animal model, our method has been introduced to clinical islet cryopreservation. The human islets were able to be cryopreserved using our method with good preservation of both the structure and the function. The islets with a poor viability, however, should not be used for cryopreservation and transplantation. Further studies are needed to accomplish a high recovery and a higher function of the frozen–thawed islets.

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ELSEVIER

Results of Kidney Transplantation From ABO-Incompatible Living Donors in a Single Institution

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ABSTRACT

ABO-incompatible kidney transplantation has become a popular alternative to kidney transplantation in Japan because of the severe shortage of cadaveric donors. In our institution, 21 cases of ABO-incompatible kidney transplantation were performed from April 2004, to October 2007. Recipient age was 42.8 ± 14.5 years old; there were 9 men and 12 women. Duration of hemodialysis was 1,914 ± 2,343 days. Donor operation was performed using a complete laparoscopic procedure. Recipient's splenectomy was performed using a hand-assisted laparoscopic procedure and kidney transplantation was performed with a standard method using an extraperitoneal approach. Pretransplant immunosuppressive protocol includes an administration of mycophenolate mofetil, tacrolimus, predonisolone, splenectomy, double filtration plasmapheresis (DFPP), and plasma exchange (PE). All patients showed an immediate graft function and their serum creatinine levels promptly decreased to 1.48 ± 0.99 mg/dL on day 7 and 1.21 ± 0.72 mg/dL on day 30. Both immunoglobulin (Ig)M and IgG titers were maintained at much lower levels for 7 days after transplantation in all patients. Cytomegalovirus antigenemia was observed in 11 patients (52.4%). One patient (4.8%) developed a *Pneumocystis Carinii* pneumonia and the formation of lymphocele was observed in one patient (4.8%). Total patient survival at 3 years was 95.2%, and graft survival at 3 years was 90.5%, which were almost equal to those in the patients who underwent ABO-matched, compatible kidney transplantation.

ALEXANDRE ET AL[1] were the first to design ABO-incompatible kidney transplantation in 1985 using pretransplant plasma exchange and splenectomy to reduce anti-A and -B antibodies. Because of the severe shortage of cadaveric donors in our country, >80% of kidney transplantations are from living donors. Since ABO-incompatible kidney transplantation was introduced in Japan by Takahashi et al[2] in 1989, this procedure has become a popular alternative to kidney transplantation, reaching >15% of the all living-donor kidney transplantations. At our institution, ABO-incompatible kidney transplantation was performed in 21 cases (>20% of living donor kidney transplantation) from April 2004 to October 2007.

However, a protocol for suppressing B lymphocytes and removal of anti-A and -B antibodies is still variable depending on the transplant centers. In this paper, we report our protocol and results, including graft survival and complications.

PATIENTS AND METHODS

One hundred seventeen patients with end-stage renal disease underwent kidney transplantation from cadaveric donors (25 cases) or living donors (92 cases) in between April 2004 and October 2007. Out of 92 living-donor kidney transplantations, 21 (22.8%) were from ABO-incompatible living-related (11 cases) or unrelated (10 cases) donors. Recipient age was 42.8 ± 14.5 years; there 9 men and 12 women. Duration of hemodialysis was 1,914 ± 2,343 days; five cases (23.8%) were preemptive transplantations. Pretransplant immunosuppressive protocol included a B-lymphocyte suppression with 4 weeks of mycophenolate mofetil (MMF), 10 days of tacrolimus and predonisolone, and removal of anti-A and -B antibodies by splenectomy (day −14), double filtration plasma-

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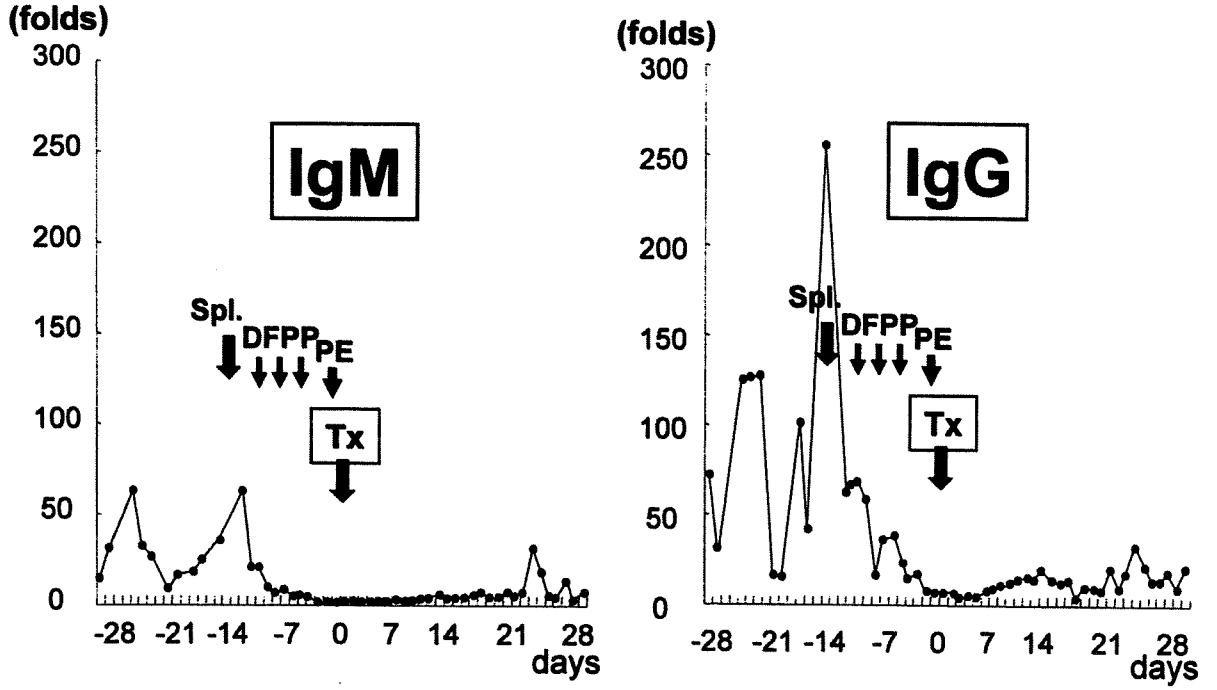

Fig 1. Changes in anti-A or -B antibodies.

pheresis (DFPP) on days −6, −4, and −2, and plasma exchange (PE) on day −1). In case of a rebound of anti-A and -B antibodies, rituximab was utilized. Posttransplant immunosuppression was achieved by quadruple therapy using MMF, tacrolimus, predonisolone, and basiliximab, which was the same protocol for ABO-matched, compatible kidney transplantation. Immunoglobulin (Ig)M and IgG anti-A and -B antibody titers were measured pre- and posttransplantation. Graft biopsies were performed at operation as a 1-hour biopsy, and at 4 weeks after transplantation as a protocol biopsy. The donor operation was performed using a complete laparoscopic procedure by a retroperitoneal approach. The recipient's splenectomy was performed using a hand-assisted laparoscopic procedure and kidney transplantation was performed with a standard method using an extraperitoneal approach.

RESULTS

IgM antibody titers decreased by eightfold and IgG titers decreased by 64-fold on day 0 from our pretransplant treatment (Fig 1). Both IgM and IgG titers were maintained at extremely low levels for 7 days after transplantation in all patients. IgM titers were maintained at less than twofold in 19 cases (90.4%) and IgG titers were less than eightfold in 17 cases (81.0%). All patients showed immediate graft function; their serum creatinine levels promptly decreased to 1.48 ± 0.99 mg/dL on day 7 and 1.21 ± 0.72 mg/dL on day 30, which was almost similar to ABO-matched or compatible kidney transplantation.

Antibody-mediated rejection or acute cellular rejection were not observed in clinical study or by graft biopsy within 30 days after transplantation. However, one patient lost the graft 2 months after transplantation with acute cellular rejection owing to interruption of tacrolimus, which induced severe encephalitis. Moreover, another patient died of hepatic failure from fulminant hepatitis. Cytomegalovirus antigenemia was observed in 11 patients (52.4%) and 1 patient (4.8%) developed a panperitonitis owing to jejunal ulcer perforation. She underwent partial resection of the jejunum. One patient (4.8%) developed *Pneumocystis Carinii* pneumonia 11 months after transplantation and needed 3 weeks treatment with intravenous trimethoprim-sulfamethoxazole. The formation of lymphocele was observed in one patient (4.8%) and she underwent laparoscopic fenestration. Total patient survival at 3 years was 95.2%; graft survival at 3 years was 90.5%, which were almost equal to those in the patients who underwent ABO-matched and compatible kidney transplantation (Fig 2).

DISCUSSION

ABO blood type used to be a major barrier in clinical transplantation. Since ABO-incompatible kidney transplantation has been successfully performed in Japan in 1989,[2] this procedure has become a popular alternative to living-donor kidney transplantation. Because of the severe shortage of cadaveric donors in our country, >80% of kidney transplantations are from living donors. In addition to living-related donors, the living-unrelated (spousal) donation has become popular in kidney transplantation in Japan. From this background, ABO-incompatible kidney transplantation is recently an important option.

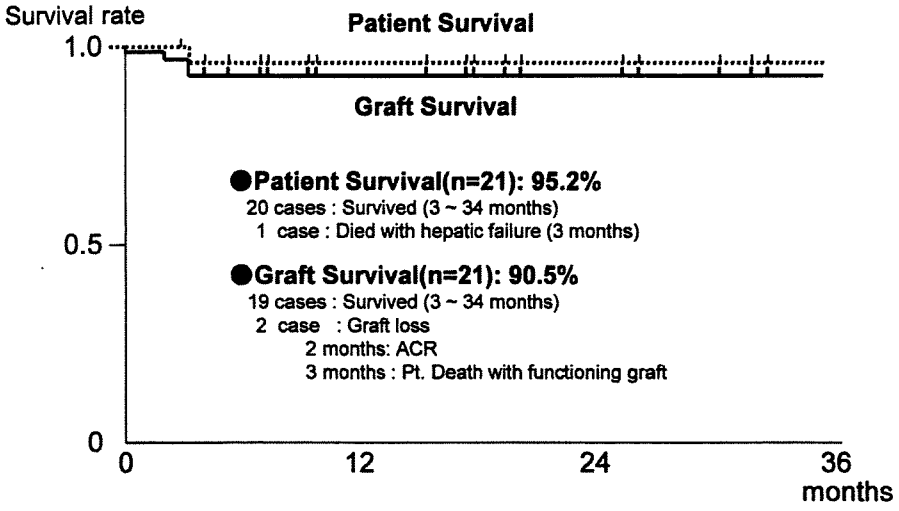

Fig 2. Patient and graft survival (ABO incompatible kidney transplantation, n = 21).

In our institution, we performed ABO-incompatible kidney transplantation for 21 patients with an end-stage renal disease from April 2004 to October 2007. Donor and recipient selection are the same as ABO-matched and compatible kidney transplantation in our institution. Positive T-cell cross-match by flow cross-match assay, and positive flow panel reactive antibodies (PRA) are no longer contraindications because of our improved results. Primary recipient diseases include glomelurar nephritis, IgA nephritis, membranoproliferative glomerulonephritis (MPGN), and diabetes. The immunosuppressive protocol includes pre-transplant removal of anti-A and -B antibodies using DFPP, PE, and splenectomy.

Patient and graft survival rates were high in the 21 patients who underwent ABO-incompatible kidney transplantation at our institution. These data indicated that the safety and the efficacy of ABO-incompatible kidney transplantation have reached the levels of those in ABO-matched and compatible kidney transplantation recipients. However, recipients have to endure pretransplant treatment, including splenectomy, DFPP, and PE. Splenectomy is particularly stressful, both physically and mentally before transplantation. The use of rituximab in place of splenectomy has been introduced and showed good results in many transplant centers.[3] Recently, we have also introduced rituximab for selected patients because of the advantages to the recipient. Further investigations are needed for the improvement of immunosuppressive protocols to realize better results with less stress for the recipient.

Cytomegalovirus infection is the most frequent complication after kidney transplantation. In the present study, 11 patients (52.4%) showed positive antigenemia. Although the frequency of antigenemia is not higher than ABO-matched or compatible kidney transplantation (60%; unpublished data), one patient developed a perforated ulcer secondary to cytomegalovirus infection and needed an additional operation. Furthermore, PC pneumonia was found in one patient. Infection is still a major problem after ABO-incompatible kidney transplantation.

Using our protocol, kidney transplantation from ABO-incompatible living donors was safely performed without severe complications. The early kidney graft survival was good, showing no difference from ABO-compatible kidney transplantation.

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ELSEVIER

Evaluation of Segmental Pancreatic Function Using ^{11}C-Methionine Positron Emission Tomography for Safe Operation of Living Donor Pancreas Transplantation

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ABSTRACT

For the safe operation of living donor pancreas transplantation, we investigated the utility of ^{11}C-methionine positron emission tomography (PET) to examine the function of the residual pancreatic head in patients with pancreatic disease undergoing distal pancreatectomy and in living donors of pancreas transplantation. After 6 hours of fasting, we intravenously injected 370 to 740 MBq ^{11}C-methionine. PET was scanned 30 minutes after injection. ^{11}C-methionine PET uptake by the pancreatic head versus body/tail was expressed as a standardized uptake value (SUV). The SUVs of the pancreatic head were compared before versus after surgery. The SUVs of the pancreatic head in patients before and after distal pancreatectomy were 15.3 ± 6.0 and 18.2 ± 2.4, respectively. The SUVs of the pancreatic head in donors before and after distal pancreatectomy were 16.1 ± 1.0 and 14.7 ± 1.4, respectively. Both patients and donors showed no significant difference in SUVs of the pancreatic head before and after surgery. However, the SUVs of the residual pancreatic head were elevated after distal pancreatectomy in 80% of patients and 50% of donors. These data indicated that the function of the pancreatic head may be maintained or improved after distal pancreatectomy. ^{11}C-methionine PET may become a potent modality to evaluate segmental pancreatic function for a safe living donor operation.

PANCREAS TRANSPLANTATION from living donors is one effective treatment for type 1 diabetes mellitus patients. Especially in Japan, this procedure is an important option because of the severe shortage of cadaveric donors. Donor safety is a major problem with this procedure. Although evaluation of normal glucose metabolism in the donor using oral or intravenous glucose challenge tests is important, segmental evaluation of the pancreas is impossible with these methods.

As previously reported, ^{11}C-methionine positron emission tomography (PET) is a potent tool for segmental evaluation of pancreatic function. ^{11}C-methionine PET may offer criteria for a safe living donor pancreas transplantation. In the present study, as a simulation of the donor operation, we evaluated the function of the residual pancreatic head using ^{11}C-methionine PET in patients who underwent distal pancreatectomy because of pancreatic disease. Furthermore, we evaluated the changes in the residual pancreatic head function of living donors at our institute.

PATIENTS AND METHODS

In the present study, we evaluated 16 healthy volunteers including 13 males and 3 females of mean age 60 ± 11 years as well as 5 patients including 3 males and 2 females of mean age 65 ± 10 years who underwent distal pancreatectomy because of pancreatic disease, and 6 donors, namely, 1 male and 5 females, of mean age 60 ± 4 years of living pancreas transplantation. The diseases in the 5 patients were intraductal papillary neoplasm (2 patients), serous cystadenoma (2 patients), and pancreatic cancer (1 patient). Six donors underwent distal pancreatectomy for living-related pancreas transplantation at our institute.

PET and PET/CT were performed with Headtome III (Shimadzu, Kyoto, Japan) for the healthy volunteers and patients, and Biograph Duo (Siemens, Munich, Germany) for the donors. ^{11}C-methionine PET was performed after 6 hours of fasting by intravenous injection of 370 to 740 MBq ^{11}C-methionine with PET performed 30 minutes after

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injection. From PET images, we measured ^{11}C-methionine PET uptake of the pancreatic head versus body/tail expressed as a standardized uptake value (SUV). The SUVs were compared between the head versus body/tail of the pancreas in healthy volunteers. The SUVs of the pancreatic head were compared before and after distal pancreatectomy, as the head and the body/tail were distinguished using the line of the left edge of a supramesenteric vein.

The statistical significance of differences was analyzed by paired Student t test with $P < .05$ considered significant.

RESULTS

No symptoms or complications were observed in the volunteers, patients and donors due to PET for this study. The SUVs of the pancreatic head and body/tail in the volunteers were 16.9 ± 4.9 and 15.3 ± 4.3, respectively, showing no significant difference. The SUVs of the pancreatic head in patients before and after distal pancreatectomy were 15.3 ± 6.0 and 18.2 ± 2.4, respectively (Fig 1). The SUVs of the donors before and after distal pancreatectomy were 16.1 ± 1.0 and 14.7 ± 1.4, respectively (Fig 2). Both the patients and donors showed no significant difference in SUVs of the pancreatic head before and after surgery. However, the SUVs of the residual pancreatic head were elevated after distal pancreatectomy in 4 patients (80%) and 3 donors (50%). The 6 donors did not develop any complications, including pancreatic fistula, cyst, or diabetes.

DISCUSSION

In living donor pancreas transplantation, donor safety must be guaranteed.[1] At our institution, donor pancreatic endocrine function was evaluated by oral glucose tolerance tests (OGTT) and intravenous glucose tolerance tests (IVGTT).[2] For the further safety of donors, we evaluated pancreatic function using ^{11}C-methionine PET.

The clinical use of ^{11}C-methionine for the pancreas was first reported in 1979 by Syrota et al.[3] The uptake of ^{11}C-methionine PET in chronic pancreatitis was lower

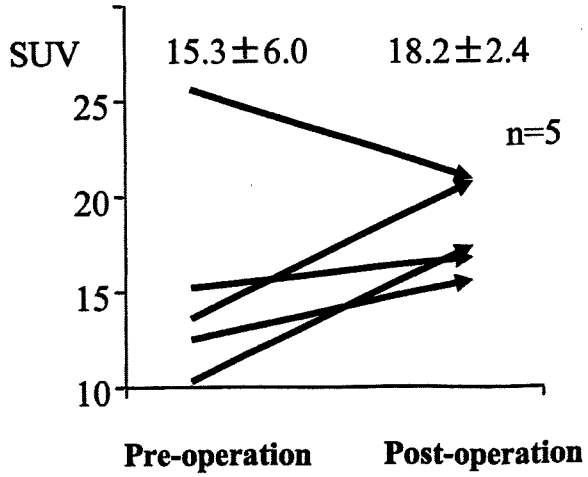

Fig 1. SUVs of the pancreatic head in patients before and after distal pancreatectomy.

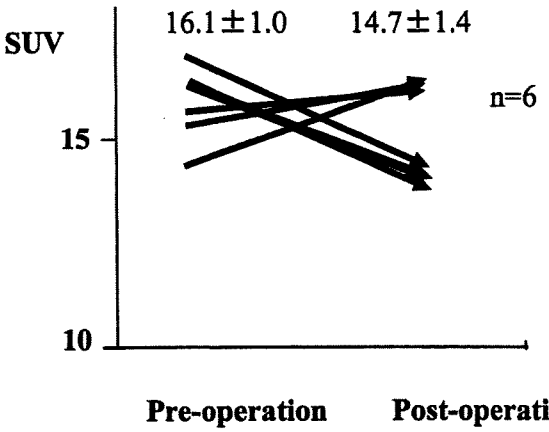

Fig 2. SUVs of the pancreatic head in living-related donors before and after distal pancreatectomy, as a donor operation.

than that of the normal pancreas correlating with exocrine function.[4] Okazumi et al[5] reported that the pancreatic uptake of ^{11}C-methionine was related to the endocrine function test. Moreover, Kono et al[6] demonstrated that postoperative ^{11}C-methionine PET uptake by the residual pancreas was highly maintained in the distal pancreatectomy cohort compared with the pancreaticoduodenectomy patients who underwent anastomosis to the jejunum. Those results demonstrated that although ^{11}C-methionine PET was able to evaluate both exocrine and endocrine functions, the data were influenced by the operative technique.

The present study demonstrated that the functions of the head and body/tail were almost equal in the normal pancreas. This result indicated that the function of the residual pancreatic head of the donor is equal to the function of the body/tail graft transplanted to the recipient. The SUVs of the pancreatic head showed no significant difference before and after surgery in both patients and donors. However, the SUVs of the residual pancreatic head were elevated after distal pancreatectomy in 4 patients and 3 donors. These data showed that the function of the pancreatic head is maintained or improved after distal pancreatectomy. ^{11}C-methionine PET may become a potent modality to evaluate segmental pancreatic function, providing safety for living donor operations. In conclusion, the results of this study demonstrated that pancreatic function evaluated with ^{11}C-methionine PET predicted the safety of the living donor of a pancreas transplantation.

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