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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

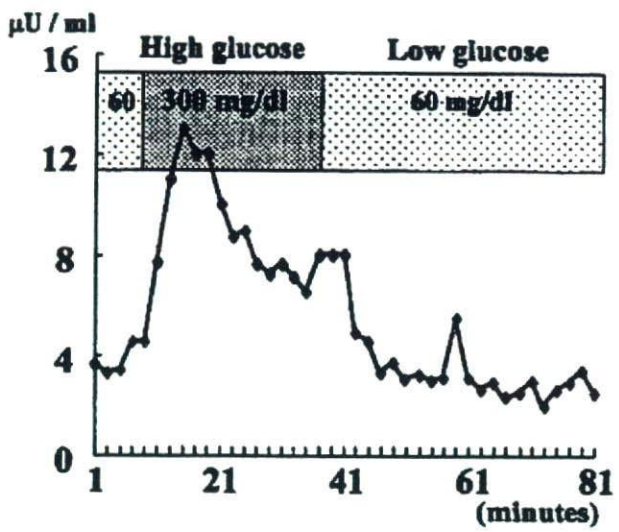

Figure 1. Insulin secretion from the frozen–thawed beagle islets was assessed by dynamic perifusion system. Prompt insulin release with two peaks showed the preservation of endocrine function of the frozen–thawed islets.

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.

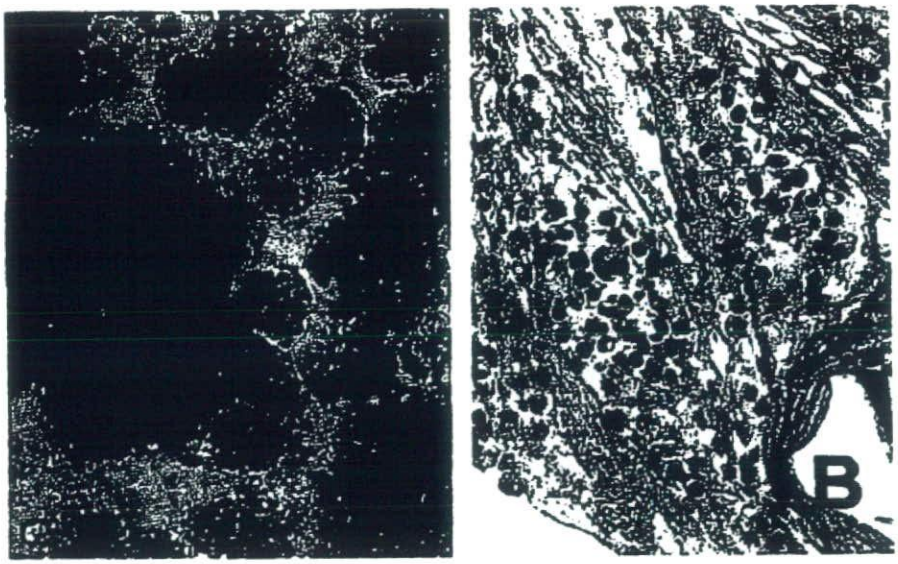

Figure 2. Microscopic findings of the frozen–thawed beagle islets stained with diphenylthiocarbazone (A) and hematoxylin-eosin (B). The normal morphology was preserved without fragmentation.

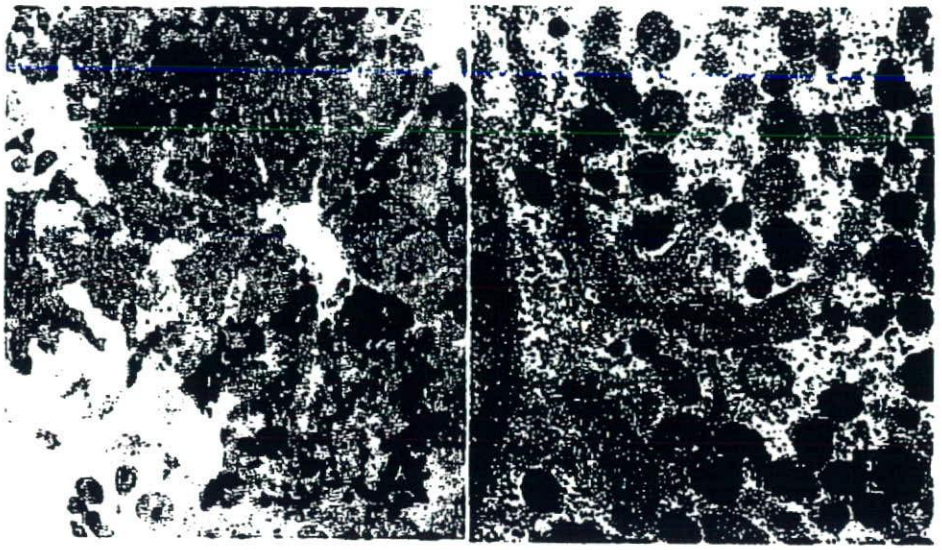

Figure 3. Frozen–thawed beagle islets stained with anti-insulin antibody (A) and electron microscopy (B). Positive staining of insulin as detected on the islets and the normal structure of organelles of the islet cell demonstrated the good preservation of the frozen–thawed islets.

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

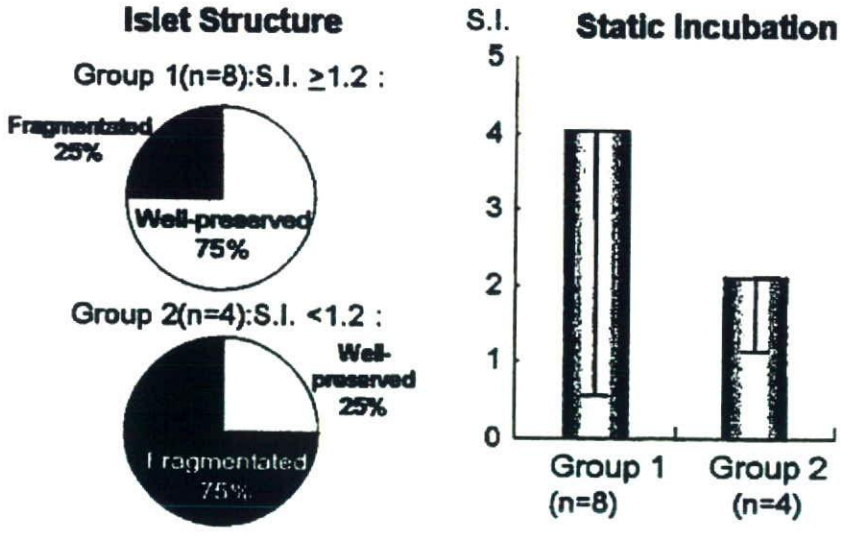

Figure 4. Human islet structure and stimulation index of static incubation at precryostorage. In group 1, islet structure was better preserved (75%) and the stimulation index was higher than those in group 2.

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

T. Kenmochi, K. Saigo, M. Maruyama, N. Akutsu, C. Iwashita, K. Otsuki, T. Ito, A. Suzuki, and M. Miyazaki

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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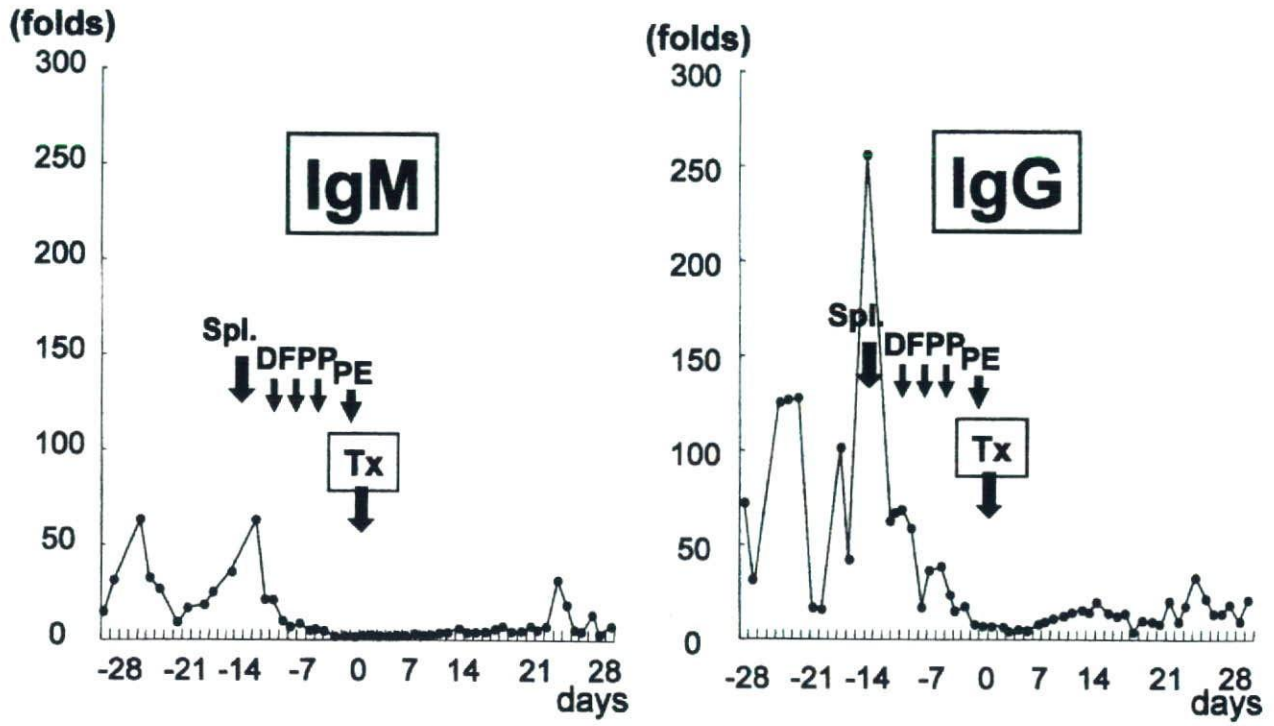

Fig 1. Changes in anti-A or -B antibodies.

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.

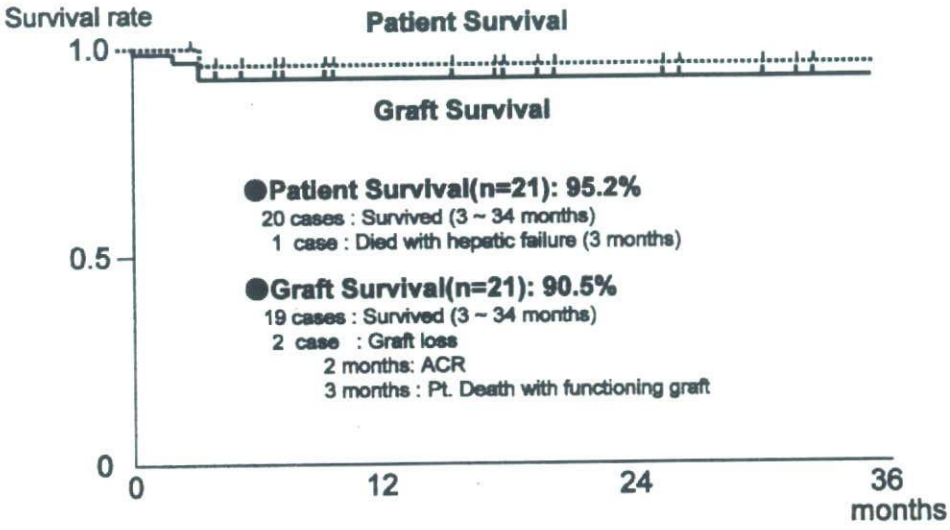

Fig 2. Patient and graft survival (ABO incompatible kidney transplantation, n = 21).

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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A Newly Developed Immunoisolated Bioartificial Pancreas With Cell Sheet Engineering

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The term "immunoisolation" refers to the encapsulation of a graft in a selectively permeable membrane. Encapsulation of cellular grafts may provide a way to protect the graft from immune attack without the need for immunosuppressive agents. Although numerous types of artificial materials have been used for encapsulating membranes, their incomplete biocompatibility causes foreign body reaction against the membranes. A new technique has been developed, called cell sheet engineering using temperature-responsive culture dishes, that allows the use of living cells as an immunoisolating membrane in this study. Using this method, the cultured cells can be easily harvested in the shape of a sheet by a simple change of the temperature without the use of proteolytic enzymes. A cell sheet can be created with three-dimensional structure by making multiple cell sheet layers. In this study, a new technique of macroencapsulation (bioartificial organs) has been developed using chondrocyte sheets. Among the various candidate cells, pancreatic islet cells were selected for a bioartificial organ in this study. A chondrocyte sheeting immunodelusive immunoisolated bioartificial pancreas (CSI-BAP) was manufactured by means of cell sheet engineering. An auricular cartilage, which is a histologically elastic cartilage from dogs (beagle), was used as a source of immunoisolating membrane. CSI-BAP was made by multilayering the chondrocyte sheets, and the donor's islets were located between each sheet. Islets were isolated and prepared from the dog (ALLO-model) and Brown Norway (BN) rat (XENO-model). The CSI-BAP was cultured for 83 days and the cultured medium was collected every 24 h to measure the insulin concentrations. The CSI-BAP was examined histologically using hematoxyhin and eosin (H&E), and azan dye staining. In addition, immunohistochemical staining was performed to demonstrate the insulin production of CSI-BAP. Insulin secretion of CSI-BAP on day 16 was reduced to 21.4% of the insulin secretion level of day 10, which was the start point of measurement. Although a gradual reduction was observed, insulin secretion was maintained for 3 months. The CSI-BAP was capable of secreting insulin to the culture medium during the observation period. Histological evaluations demonstrated the good viability of the islets, and immunohistochemistry showed the positive staining of insulin. This novel technology may be used for other kinds of endocrine cells or hepatocytes, which may become the models for immunoisolated bioartificial organs in the near future.

Key words: Bioartificial organ; Auricular chondrocyte; Islet; Cell sheet engineering; Maroencapsulation; Immunoisolation

INTRODUCTION

Immunoisolation has been achieved by encapsulating the graft cells using selectively permeable membranes. Such membranes only allow the permeation of smaller molecules, such as oxygen, CO_2, glucose, amino acids, and hormones, but prevent the penetration of immunocytes and larger immune molecules, such as antibodies and complements. Although many kinds of artificial materials have been used for the membranes during the past few decades (4,5,13,15,16,18,23), they are not sufficiently biocompatible and thus cause a foreign body reaction and fibrosis (13,15). If the immunoisolating method of macroencapsulating is properly used, bioartificial organs can be the technological key to transplantation without immunosuppression (24).

In contrast, a cartilage is a natural tissue that lacks blood vessels, lymph vessels, and nerves, and uses diffusion for allowing adequate exchanges of nutrients and waste as well as oxygenation. So it is impossible for leukocytes to penetrate into the normal cartilage (6). Chondrocytes in cartilage are surrounded with extracel-

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lular matrix (ECM; e.g., collagens and proteoglycan), which is produced by the chondrocyte itself. Moreover, because the auricular cartilage is an elastic cartilage, it has elasticity property and is easy to collect from patients. This suggests that cartilage can be used as an ultimate and versatile membrane for immunoisolation, if chondrocytes can be harvested in the shape of a membrane and cover the graft cells completely.

As a result of recent progress in tissue engineering (10), researchers can now use many techniques for the in vitro handling of vital tissues and cells. Cell sheet engineering, which is based on the technique of nanobiointerface, is one of the prospective methods among the newly developed tissue engineering technologies (1,7, 20,22,29,30). It could be a great source of immunoisolating membrane, if chondrocyte sheets are manufactured and harvested by this technique. For the first application of the immunoisolation using the chondrocyte sheet method, pancreatic islets were used in this study.

Islet transplantation has been clinically performed for patients with insulin-dependent type 1 diabetes mellitus (IDDM) instead of a conventional insulin injection treatment. However, there are many problems to be solved including a shortage of donor organs. Another factor that may contribute to a lower success rate of islet transplantation in type 1 diabetic patients is the side effects of the immunosuppressive drugs. Although every recipient has to take these expensive drugs after islet transplantation to avoid rejection by the attack of their own immune system, these drugs have some serious side effects (19). Furthermore, the function of islet cells may be diminished by the direct toxicity of immunosuppressive drugs to the beta cells (26).

The purpose of this study was to prospectively evaluate long-term function of CSI-BAP in vitro, and to investigate the structure of CSI-BAP and its relationship between chondrocyte sheets and islets.

MATERIALS AND METHODS

Islet Isolation and Purification Procedure

Pancreatic islets were prepared using the technique described previously (25) with some modifications. Pancreata from Brown Norway (BN) rats (males, 250 ± 50 g, SLC Japan Co., Hamamatsu, Japan) and dogs (beagle, 12–24 months old, body weight 9–13 kg) were expanded with Hank's balanced salt solution (HBBS) containing 10 mmol/L HEPES plus 2.0 mg/ml cold collagenase (Collagenase P, Roche Applied Science Co., Germany) and 5% heat-inactivated fetal bovine serum (FBS: JRH Biosciences, Lenexa, KS, USA) by the ductal injection. Subsequently, the distended pancreas was mixed with a Vortex Genie 2 (Scientific Industries, Bohemia, NY, USA) for a while , and then it was digested by shaking in a water bath at 37°C for 16 min (for rat) or 45 min (for dog), respectively. The resulting cell suspensions were passed through a 600-μm steel mesh filer. Islets were purified by Histopaque (Sigma Chemical Co., St. Louis, MO, USA) density gradients. Rat islets were collected and hand-picked twice for purification. The islet number was determined by counting the triple samples of each batch of islet preparations. Islets were counted and their diameters were measured after staining with diphenylthiocarbazone (dithizone: Sigma Chemical Co.). Islets were cultured in the mixed culture medium (MCM) containing 50% Ham's F-12 medium (Invitrogen Co., Carlsbad, CA, USA) and 50% RPMI-1640 without glucose (Invitrogen Co.) with plus 25 mmol/L HEPES, 1% antibiotic/antimycotic mixture (ABAM; 10,000 U/ml penicillin G, 10,000 μg/ml streptomycin sulfate, and 25 μg/ml amphotericin B as Fungizone®; Invitrogen Co.), and 50 μg/ml ascorbic acid (Sigma Chemical Co.). The final concentration of heat-inactivated FBS was adjusted to 10% and the glucose concentration was adjusted to 100 mg/dl by adding D-(+)-glucose (Sigma Chemical Co.).

Primary Culture of Chondrocytes From Auricular Cartilage

Auricular cartilage was obtained from dogs. The specimens, which were stripped of their surrounding tissue, including the skin, subcutaneous fat tissue, muscles, and perichondrium, were cut into small pieces. The cartilage was cut using scissors with curved blades on a special glass dish (12 cm diameter, Toshin-Riko Co., Japan). Cartilage tissue was cut into small pieces (~0.5-mm cubes) in a short time. Thereafter, the chondrocytes were digested in the cocktail solution of Ham's F-12 medium (Invitrogen Co.) plus 0.3% collagenase (Collagenase class II: Worthington, Biochemical Co., Lakewood, NJ, USA), 0.25% trypsin (Invitrogen Co.), 4% ABAM, and 50 μg/ml ascorbic acid in a shaking water bath at 37°C overnight. The cell suspension was passed through a 70-μm and a 40-μm nylon cell strainer (BD Falcon™; BD Biosciences, Bedford, MA, USA), and the isolated cells were washed twice with phosphate-buffered saline (PBS; Invitrogen Co.) containing 4% ABAM, and the viable cells were counted on Burker-Turk hematocytometer with a trypan blue staining. These chondrocytes were seeded and expanded by the sequential passages (passage 1–3) in monolayer culture in CBM™ medium (Cambrex Bio Science, Walkersville, MD, USA) supplemented with CGM SingleQuot® (Cambrex Bio Science), and 50 μg/ml ascorbic acid, and maintained at 37°C in humid 5% CO_2. The chondrocytes at passage 1 were plated at a cell density of $0.5 \times 10^4/cm^2$. Passages 2–3 were subsequently reseeded at a density of $0.25 \times 10^4/cm^2$. The culture medium was changed twice a week. To manufacture CSI-BAP, only cells recovered from

passage 3 cells were used as a macroencapsulating source of sheets.

Manipulation of Chondrocytes Sheets Into Layered Constructs for CSI-BAP

Cells recovered from passage 3 of the chondrocyte culture were used for constructing the cell sheets. Expanded to passage 3, the elastic chondrocytes were plated onto 60-mm plates made of the temperature-responsive polymer, poly(*N*-isopropylacrylamide) (PIPAAm), pattern-grafted dishes (Upcell™; Cellseed, Co., Tokyo, Japan) at a seeding density of $1.5–2.0 \times 10^4/cm^2$, and cultured in the growth medium until they reached 100% confluency. The culture medium was changed twice a week for 3–6 weeks until the cell sheets were harvested. At 100% confluency, the chondrocytes were detached from the dish and multilayered using the previously described technique (7,20,21) with a little modification. The procedure for three-dimensional manipulation of cultured chondrocyte sheets and the process that produces CSI-BAP is shown in Figure 1. Briefly, to release confluent cells as a cell sheet, the culture dishes were located in a clean bench at room temperature around 24°C, and the sheets were detached spontaneously from the dish surfaces within 30 min. In order to preserve cell sheet morphology without shrinking, hydrophilically modified poly(vinylidene difluoride) (PVDF) membranes (Millipore, Bedford, MA, USA) were used as supporting membranes. The supporting membranes, which were cut to fit Upcell™, were placed directly over the confluent chondrocytes. The lifting cell sheet edges promptly attached to the overlaid PVDF membranes, and the membrane–cell sheet films gently were peeled off Upcell™ with forceps. These cell sheets physically attached to the PVDF membranes were transferred onto other confluent chondrocytes sheets. Double-layered cell sheets were then incubated at 37°C for 30 min in a minimum amount of culture medium to attach the newly transferred cell sheets to the basal cell sheets. Then additional culture medium was added and the dish was incubated again at 37°C. By repeating this procedure, the chondrocyte multilayer sheets were finally obtained. From three to five layered chondrocyte sheets were used to fabricate a CSI-BAP. Subsequently, CSI-BAP was made by unifying the two multilayered chondrocyte sheets and layering the donor's islets between each multilayer sheet. The CSI-BAP was then incubated at 37°C for 30 min in a small amount of medium to attach the opposite side of chondrocyte sheets to cover basal sheets on which isolated islets were placed. Then culture medium was added and the culture dishes were incubated again at 37°C. To obtain firm and perfect integration of the cells in the CSI-BAP including pancreatic islets and chondrocyte sheets, the CSI-BAP was incubated under ordinary culture conditions (37°C and 5% CO_2) with MCM for 7–10 days. Islets prepared from dog (ALLO-model) and the BN rat (XENO-model) were used for CSI-BAP. The ALLO-model of CSI-BAP was kept in culture for 3 months, and the XENO-model was used for histological analysis.

Measurement of Insulin Secretion

CSI-BAP was maintained in the culture media at 37°C in humid 5% CO_2 using MCM for 83 days. MCM was changed every 24 h and the medium samples were immediately frozen at –80°C until measurement. The supernatants were collected and the insulin content was determined using a microparticle enzyme immunoassay (MEIa) (IMx Insulin, Abbot Laboratories, Tokyo, Japan).

Histological Studies

The CSI-BAP was fixed in 4% paraformaldehyde. After washing with PBS (Invitrogen Co.) it was embedded in paraffin. Subsequently, the CSI-BAP was sectioned and stained with hematoxylin and eosin (H&E) and azan staining for histological evaluation. The samples were also analyzed by immunohistochemistry for insulin using the avidin-biotin-peroxidase complex technique according to the manufacturer's protocol (LSAB 2 kit/HRP, DAKO Japan Co., Ltd.) using 3-amino-9-ethylcarbazole (AEC) substrate-chromogen solution (DAKO Japan Co., Ltd.).

RESULTS

Islet Yield

Dog islets were obtained from one fourth of the whole pancreas, which resulted in an approximately 7-ml pellet after digestion. The final average of yield was 32,447.56 islet equivalent (25,920 raw number) and the purity was approximately 85%. The average number of islets isolated from one pancreas per BN rat was 1792.11 islet equivalent (1464.38 raw number) and the purity was 99%.

Primary Culture of Chondrocytes From Auricular Cartilage

The number of chondrocytes from 1 g of auricular cartilage was an average of 351.25×10^4. The chondrocytes expanded from 15,046 to 129,377 during three passages. The chondrocytes were cryopreserved at every passage and they were thawed to use for the experiment. The recovery rate of frozen–thawed chondrocytes was $51.01 \pm 4.44\%$.

Morphological Studies of CSI-BAP

Chondrocyte sheets were prepared and combined to form three to five layers (Fig. 1). The confluent chondrocytes could be harvested noninvasively as the contig-

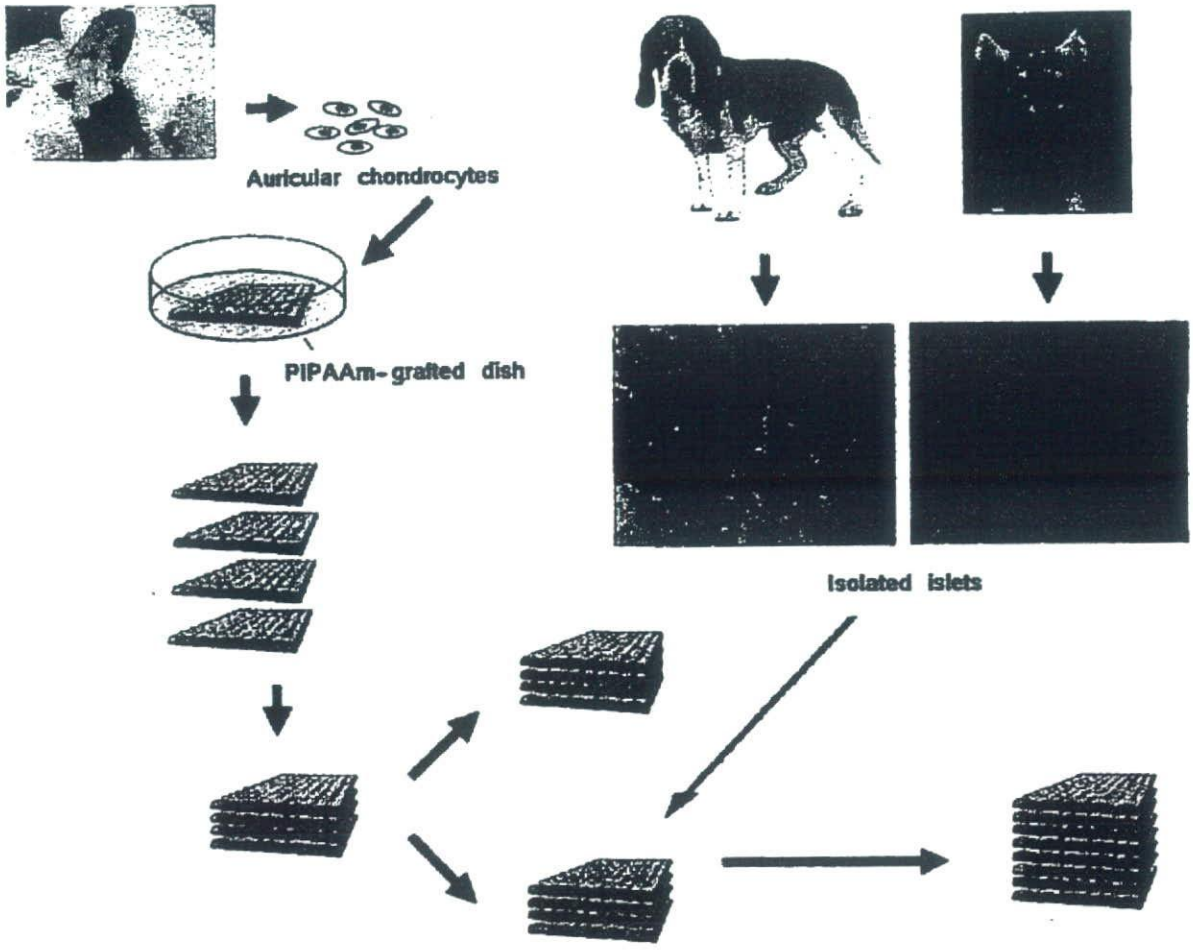

Figure 1. Schematic illustration of the manufacture of chondrocyte sheeting immunoisolated bioartificial pancreas (CSI-BAP). Confluently cultured chondrocytes were incubated at room temperature around 24°C, and the sheets began to detach spontaneously from the dish surfaces within 30 min. The detached chondrocyte sheet was transferred and piled onto another chondrocyte sheet and incubated at 37°C, yielding bilayer chondrocyte sheets. By repeating this process, multilayer sheets (3–10 layers) could be formed. Subsequently, CSI-BAP was made by unifying two multilayered chondrocyte sheets and then transferring the donor's islets in between the multilayer sheets.

uous cell sheets with intact cell–cell junctions and deposited extracellular matrix (ECM). Because the ECM associated with the basal side of the cell sheets shows adhesion, the harvested cell sheets could be stratified to reconstruct thicker or more complex tissue architectures. Confluent chondrocytes cultured were incubated at room temperature, and the sheets began to detach spontaneously from the dish surfaces. The detached chondrocytes sheet was transferred and piled onto another chondrocyte sheet, yielding bilayer chondrocyte sheets. Technically, it was possible to stack up to 10 sheets of chondrocytes (Fig. 2).

To fabricate CSI-BAP, it was necessary to unify the two multilayered chondrocyte sheets and to place donor islets between each multilayer sheet. For the XENO-model of CSI-BAP, two three-layered sheets and islets obtained from 1 to 6 rats were used. In contrast, in the ALLO-model, two five-layered sheets were used with the islets (46,000 islet equivalent, 64,800 raw number). Figure 3 shows the histological appearance of CSI-BAP with H&E (Fig. 3A) and azan staining (Fig. 3B). The well-formed and round-shaped islets were observed in the sheet, which demonstrated the preservation of the islet structure in the sheet in this study. In addition, there was positive staining of insulin with immunohistochemistry (Fig. 3C), thus suggesting a good endocrine function of the islets. Using azan staining, the islets were clearly detected in the multilayered chondrocyte sheets (Fig. 3B). The application of the "azan" staining to mammalian islet differentiates sharply the type of cells, including A, B, and D cells. A cells were shown as red granules, B cells as orange-gray, and D cells as blue (2).

From these data, it was demonstrated that completely viable pancreatic islets were maintained in the layered chondrocytes sheets.

Insulin Measurement

The insulin secretion from CSI-BAP was positively detected during the entire observation time of 83 days (Fig. 4). The amount of insulin gradually decreased from the first day of the insulin measurement, which was at day 10 in culture. Insulin release of CSI-BAP on day 16 decreased to 21.4% of insulin secretion level of day 10 (Table 1). After day 16, the insulin secretion was comparatively preserved until 83 days. Islets in CSI-BAP were capable of secreting insulin through the observation period, indicating that insulin was released from the islets in CSI-BAP by diffusion.

DISCUSSION

An immune reaction begins upon recognition of self and nonself. Allogenic or xenogenic grafts are recognized to be nonself and will be rejected in the case of transplantation. Immunosuppression is, thus, necessary to maintain the transplanted grafts. Various problems still exist regarding immunosuppression. One of the major problems of immunosuppressive drugs is that these agents focus not only on the transplanted grafts but also on the entire host immune system of the recipients. The patients must take drugs to prevent the rejection of the

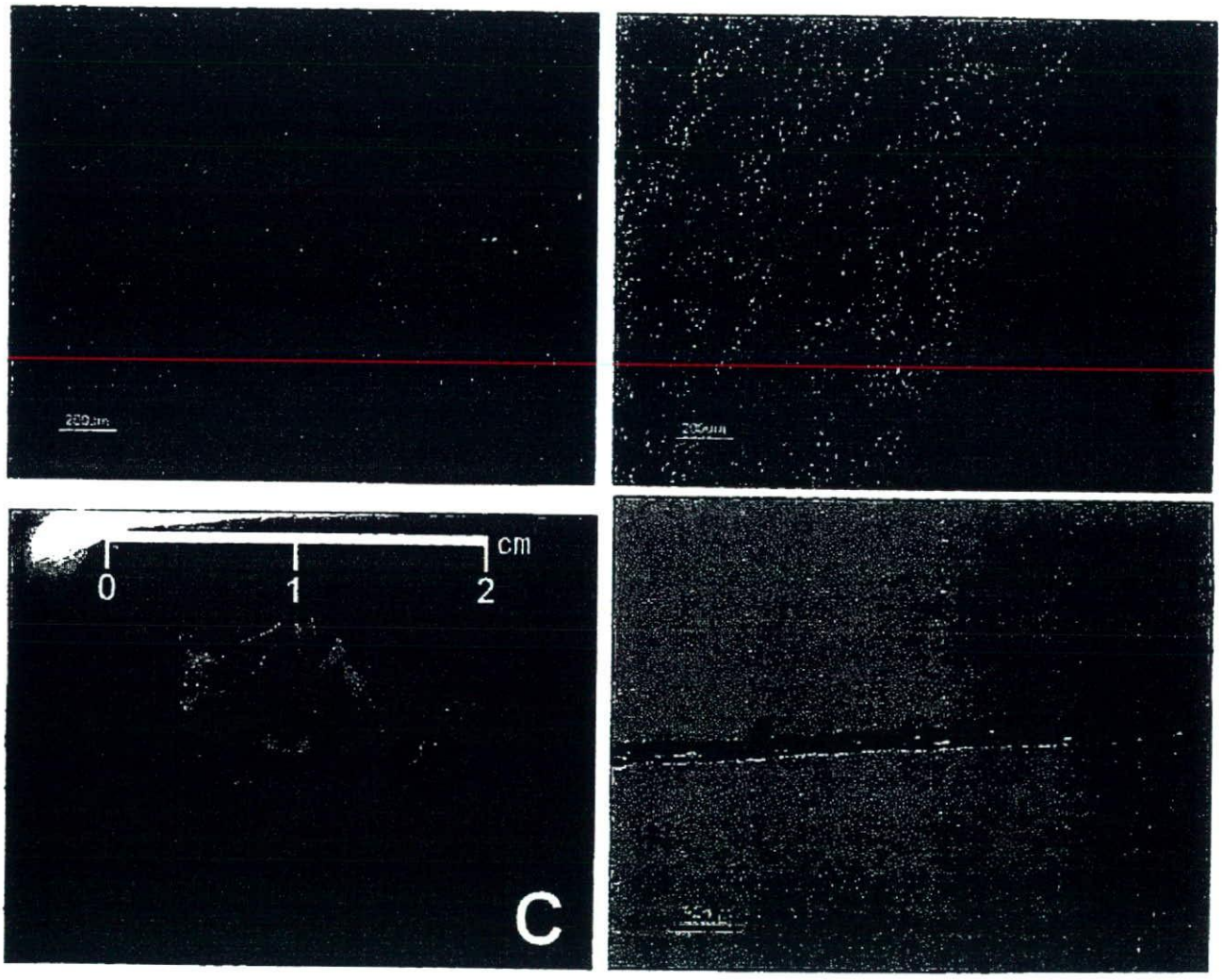

Figure 2. Chondrocyte sheet. Phase-contrast micrograph (A, B) of auricular chondrocyte sheet that is now being detached from temperature-responsive culture dishes (Upcell™; PIPAAm-grafted cell culture dishes). (A) Confluently cultured chondrocytes are spontaneously and gradually detached (B) when the temperature is reduced below 32°C, without the need for proteolytic enzymes. (C) The confluent cells are noninvasively harvested as single, contiguous cell sheets with intact cell–cell junctions and deposited ECM. Because the ECM associated with the basal side of the cell sheets shows adhesion, the harvested cell sheets can be stratified to construct thicker or more complex tissue architectures. (D) Four-layered chondrocyte sheet stained with hematoxylin and eosin.

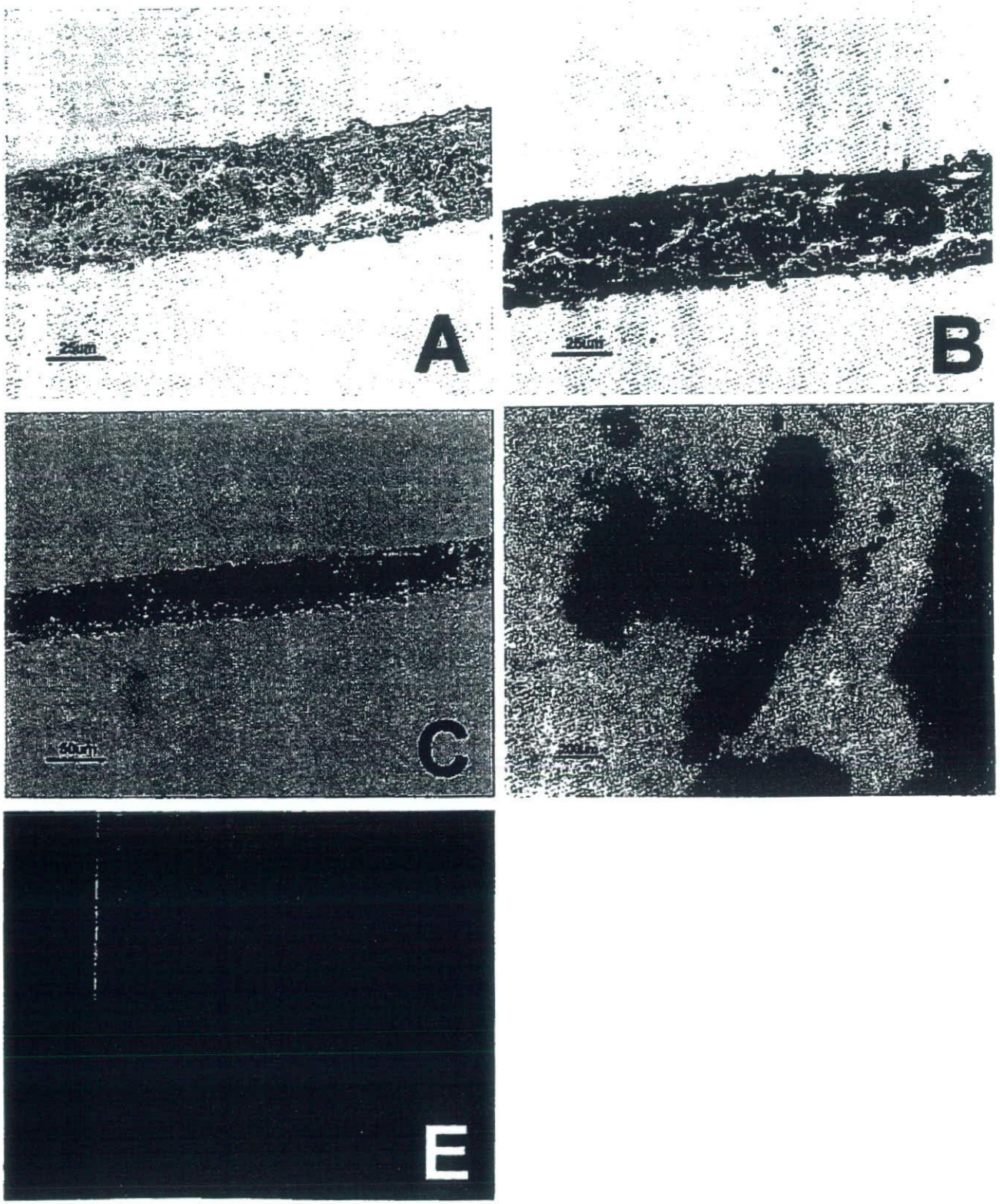

Figure 3. CSI-BAP. A histological evaluation with hematoxylin and eosin stain (A), azan dye (B), and immunohistochemistry for insulin (C) confirmed insulin secretion within the beta cells of pancreatic islets, showing that the islets of CSI-BAP were functional and viable. Phase-contrast micrograph of CSI-BAP was examined (D); dark areas stand for the existence of islets between chondrocytes sheets. The CSI-BAP was fixed with 4% paraformaldehyde solution for the histological studies (E).

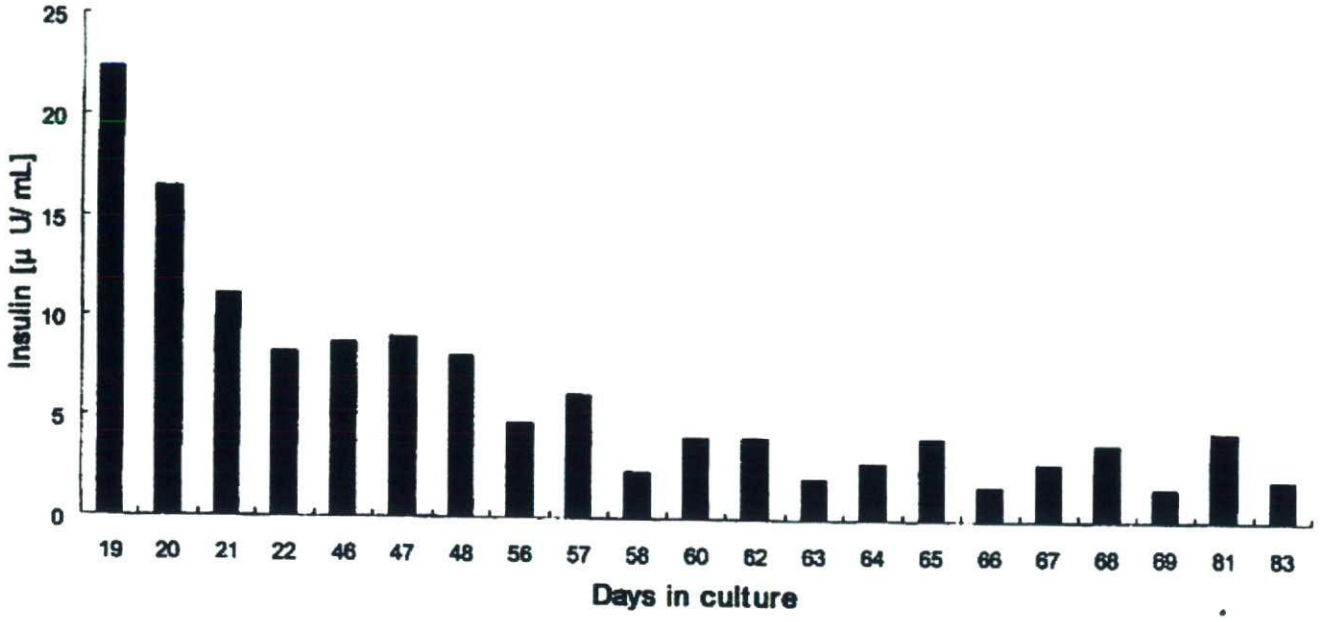

Figure 4. Insulin accumulation in the mixed culture medium of CSI-BAP over 83 days. The insulin concentration showed a tendency to decrease, but the decline was mild. Islets in the CSI-BAP were capable of secreting insulin throughout the observation period, indicating that insulin was released from the islets in CSI-BAP in diffusion manner.

implanted islet cells in islet transplantation. These drugs have serious side effects, including myelosuppression, nephrotoxicity, increased risk of infection, and increased risk of development of certain malignant diseases (e.g., lymphomas) (19). Furthermore, studies indicate that the function of islet cells may deteriorate because these treatments are directly toxic to beta cells (26).

If the donor islets can be fabricates covered with the recipient's tissue, the transplanted islets may not be recognized as nonself. This concept is called immunodelusion, namely the concept of recognizing tissue as if it is the recipient's own tissue. Although immunodelusive bioartificial pancreas (BAP) belongs in the category of immunisolated BAP, it is a new concept of BAP. The CSI-BAP in the present study is different from the conventional BAP because of the use of the chondrocytes (recipient cells) instead of artificial materials. Although both devices allow the passage of smaller molecules by means of diffusion, this CSI-BAP is recognized as self by the host immune cells, and prevents the penetration of immunocytes.

Table 1. Insulin Secretion Into the Mixed Culture Medium Over 24 h Measured by a Microparticle Enzyme Immunoassay

Days in Culture	Insulin Secreation (μU/ml)	Rate of 100% Conversion
10	279.7	100.0%
11	185.7	66.4%
12	141.2	50.5%
13	98.1	35.1%
14	74.1	26.5%
15	77.1	27.6%
16	59.9	21.4%

Insulin release of CSI-BAP on day 16 markedly decreased to 21.4% of the insulin secretion level of day 10, which was the starting day of the measurements.

In order to create the immunodelusive BAP, a cell sheet engineering technique (9,29,30) is utilized in this study. With this technology, the cell sheets are prepared using temperature-responsive polymer (PIPAAm) pattern-grafted dishes. Temperature-responsive polymers are covalently grafted onto the dishes, allowing various types of cells to adhere and proliferate at 37°C. The cells spontaneously detach from the plate when the temperature decreases to below 32°C without the need for proteolytic enzymes. The confluent cells are noninvasively harvested as contiguous cell sheets with intact cell–cell junctions and deposited ECM. Because the ECM associated with the basal side of the cell sheets shows adhesion, the harvested cell sheets can be stratified to reconstruct thicker or more complex tissue architectures. Using this cell sheet engineering, it is possible to manufacture the totally different concept of chondrocytes sheeting immunodelusive immunoisolated bioartificial pancreas.

Cartilage tissue, which lacks blood vessels, lymphatic

tissue, and nerves, exchanges nutrients (e.g., glucose and amino acids) and waste as well as gas (e.g., O_2 and CO_2) by diffusion. Therefore, it is impossible for leukocytes to invade normal cartilage (6). Chondrocytes in cartilage are surrounded with ECM, which is produced by the chondrocyte itself. Moreover, because an auricular cartilage is an elastic cartilage, it is easily collected from the patient. In addition, deformation of the ear after the excision of the auricular cartilage is rarely observed (12) in clinical plastic surgery field. Therefore, the collection of auricular cartilage as the source of immunoisolation material is acceptable for clinical application cosmetically. In addition, the chondrocytes can vastly expand and a sufficient number of cells can be obtained by continuous culture. Moreover, it was found that cell yield per gram of an ear cartilage is twice that of an articular cartilage (27), and chondrocytes of ear cartilage proliferated faster than articular cartilage. Although, articular cartilage has been frequently used for cartilage tissue engineering research, ear cartilage was used in this study because it is easy to harvest with less donor site morbidity (27). These results demonstrate that elastic cartilage can be used as a perfect and versatile membrane for immunoisolation, if the chondrocytes can be harvested as a shape of membrane and cover the graft cells perfectly.

There was only one previous study that used living cells for encapsulation of islets (17). This study used articular chondrocytes and polyglycolic acid polymer. They harvested chondrocyte membranes physically with a cell scraper when the chondrocyte culture reached confluency, which may weaken the membrane itself and its ECM. They observed that some of the single layered chondrocyte capsules were imperfect and were damaged by penetrating polymer fibers, which may cause host immune attack. The macroncapsulated materials used in their study were not simply living cells from recipients, because they utilized artificial polymer with the recipient cells.

In the current study, the CSI-BAP was developed by using cell sheet engineering to create a multilayer chondrocyte membrane, and its long-term function was evaluated. Histological analysis indicated that islets within the CSI-BAP produced insulin and were completely encapsulated with the chondrocyte sheets. It is possible that the reduction of insulin secretion was due to contamination of the exocrine cells in an early stage after the CSI-BAP was manufactured. Proteolytic enzymes released from the contaminated exocrine cells may impair the islet structure and/or chondrocytes of the CSI-BAP and diminish the endocrine function of CSI-BAP.

The major advantage of this CSI-BAP may be the interaction of the islets and the chondrocytes. The chondrocytes protect the pancreatic islets by providing ECM and avoiding disaggregation, which diminishes its function. This may provide an environment similar for the islets in the pancreas before isolation. In the normal pancreas, a complex network of collagen, proteoglycans (e.g., glycosaminoglycans, glycoproteins), and elastin (28) is present, which is very similar to auricular cartilage. Insulin released from the islets directly acts on chondrocytes as a growth factor. The importance of the ECM for islet function was confirmed in a previous study (3,8,14). It was found that the islets did not survive more than 14 days in the standard culture media (11). By contrast, when overlaid with collagen, monolayers of human islet cells underwent a gradual and complete reorganization into a three-dimensional islet-like structure with striking reinforcement of their secretory activity (11). It is thought that the islets in a CSI-BAP were capable of secreting insulin for a prolonged period compared to the standard culture conditions.

The application of CSI-BAP technology to other endocrine cells, such as parathyroid tissue and thyroid tissue, is also possible. Furthermore, genetically engineered cells that provide a source of erythropoietin, dopamine, or human growth factor, and the creation of an artificial liver using hepatocytes covered with chondrocytes sheets are being considered.

This study is the first report of a CSI-BAP, and there are several factors that require further investigation. The optimal number of layers of chondrocyte sheets must be determined. It is possible to layer the chondrocyte sheets to more than 10 sheets, because of extremely low metabolism of chondrocytes (data not shown). And an evaluation of an immunoisolation test of CSI-BAP both in vivo and in vitro should be performed in the near future. Furthermore, the optimal transplant site is a major issue. Because the CSI-BAP is extremely thin, the surface of the liver, the surface of the pancreas, or the surface of the abdominal wall are therefore all considered to be possible transplant sites.

The results of this study may therefore lead to a new strategies of allo- and xenotransplantion without using immunosuppressive drugs in islet transplantation.

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ELSEVIER

Rapamycin Induces Autophagy in Islets: Relevance in Islet Transplantation

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ABSTRACT

Islet transplantation can provide insulin independence in patients with type 1 diabetes mellitus. However, islet allograft recipients exhibit a gradual decline in insulin independence, and only 10% do not require insulin at 5 years. This decline may reflect drug toxicity to islet β cells. Rapamycin, a central immunosuppressant in islet transplantation, is a mammalian target of rampamycin inhibitor that induces autophagy. The relative contributions of autophagy in transplanted islets are poorly understood. Therefore, in the present study we sought to evaluate the effects of rapamycin on islet β cells. Rapamycin treatment of islets resulted in accumulation of membrane-bound light chain 3 (LC3-II) protein, an early marker of autophagy. In addition, rapamycin treatment of isolated islets elicited not only reduction of viability but also downregulation of in vitro potency. To further examine the occurrence of autophagy in rapamycin-treated islets, we used GFP (green fluorescent protein)–LC3 transgenic mice that express a fluorescent autophagosome marker. The GFP-LC3 signals were markedly increased in rapamycin treated islets compared with control islets. In addition, to show improvement by blockade of autophagic signaling, islets were treated with rapamycin in the presence of 3-methyladenine, which inhibits autophagy. Thereafter, both islet viability and islet potency were dramatically improved. The number of GFP-LC3 dots clearly increased after 3-MA treatment. Thus, rapamycin treatment of islets induces autophagy in vitro. This phenomenon may contribute to the progressive graft dysfunction of transplanted islets. Therapeutically targeting this novel signaling may yield significant benefits for long-term islet survival.

CLINICAL ISLET TRANSPLANTATION in patients with type 1 diabetes mellitus has recently increased because of the results of the Edmonton protocol, a rapamycin-based, glucocorticoid-free, immunosuppressive regimen that led to insulin independence at 1 year in 90% of treated patients.[1] However, long-term follow-up indicated marked reduction in graft function; only 10% of islet recipients maintained insulin independence at 5 years.[2] While the causes of decline in insulin independence rates seen in

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