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Functional and histological evaluation of transplanted pancreatic islets immunoprotected by PEGylation and cyclosporine for 1 year

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Abstract

Pancreatic islet transplantation is one of the most promising strategies for patients suffering from type 1 diabetes mellitus, but several therapeutic immunosuppressive medications must be administered simultaneously to protect transplanted islets in the long-term, and these expose patients to the risk of serious complications. Thus, we developed chemically modified islets with a protective poly(ethylene glycol) (PEG) layer, which reduces immunogenicity by preventing cellular immune reactions. We report here that PEG-based chemical immunomodulation can provide a semi-permanent effective therapy that protects transplanted islets at least for 1 year when accompanied by cyclosporine. Moreover, this combinatorial approach appears to avoid the toxicities associated with immunosuppressive medications because of the reduced amounts of medication required. Also, the conjugated PEG molecules were found to be continuously present at the transplanted islets. However, unmodified islets (control) were completely eliminated within 2 weeks even when CsA was administered. These results strongly suggest that this new combinatorial therapy provides a semi-permanent, effective clinical means of attenuating transplanted islet immunogenicity for a long time, whilst avoiding the toxicities associated with therapeutic levels of immunosuppressants owing to the minimized immunosuppressant.
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Keywords: Diabetes; Islet; Cyclosporine; Immunomodulation; PEGylation; Transplantation

1. Introduction

Type 1 diabetic mellitus (T1DM), an autoimmune disease that targets immunologic destruction of insulin-producing pancreatic β-cells, is an important and increasing health problem. Current therapy for T1DM, based on the replacement of insulin or on islet transplantation and immune suppression, has substantial limitations. Exogenous insulin is used for the control of blood glucose; however, hypoglycemic episodes are unavoidable. Presently, pancreatic islet transplantation is one of the most promising strategies for patients suffering from T1DM, as it can achieve strict control of blood glucose and offers a potential cure for T1DM [1,2]. After islet transplantation, however, several kinds of immunosuppressant therapies, i.e. the Edmonton protocol, should be continuously followed to inhibit immune rejection [3–5]. Since immunosuppressants are known to induce complications, this reminds us that the final goal of islet transplantation remains the elimination of chronic recipient immunosuppression [6–10].

Currently, to immunologically protect transplanted islets from the immune system, a strategy for surface modification of islets has been proposed whereby biocompatible poly(ethylene glycol) (PEG) molecules are conjugated onto islet surfaces (Fig. 1(A)) [11–19]. This immunologically protective strategy was first applied to the surface modification of red blood cells (RBC) using PEG to chemically camouflage the antigenic determinants of RBC [20–22]. Monomethoxy-PEG-succinimidyl propionic acid (PEG-SPA) molecules are conjugated with amine groups of collagen matrix composing of islet surface, thereby forming a stable amide bond. The hypothesis of PEG conjugation, or PEGylation, is that the conjugated PEG molecules protect islets from immune cell attack based on its low

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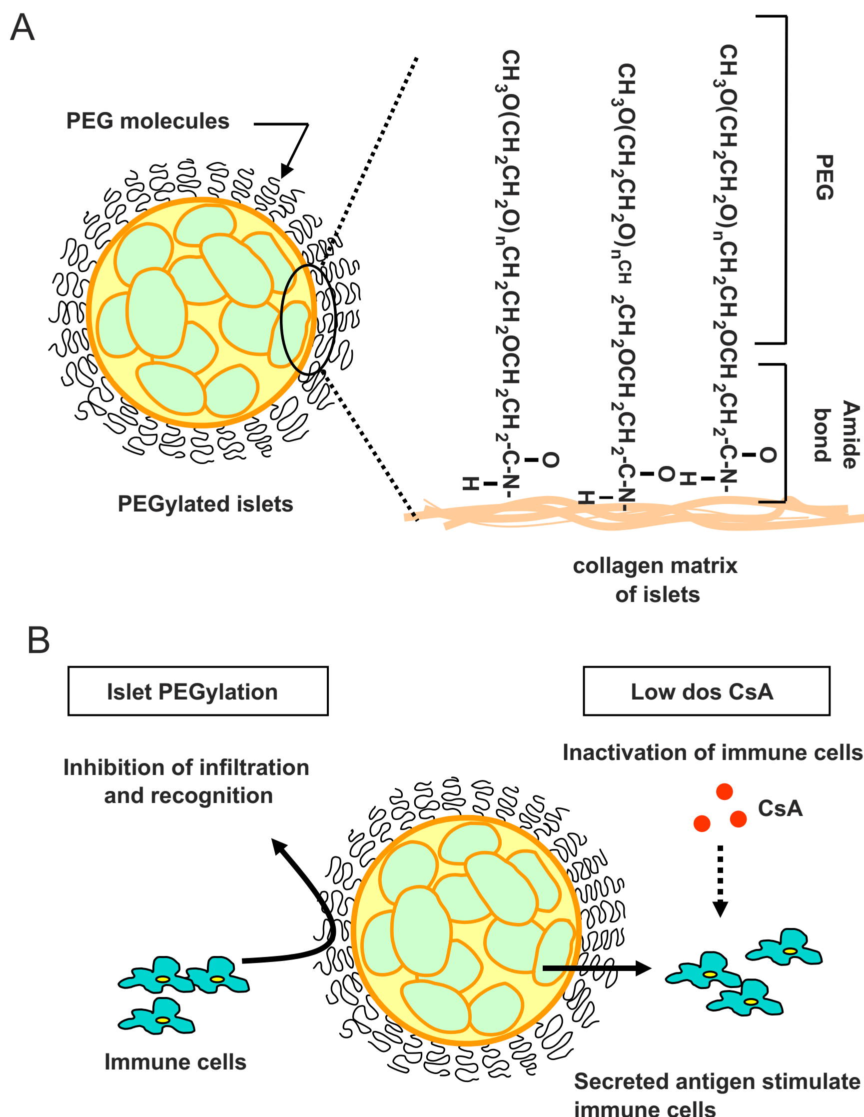


Fig. 1. The concept of pancreatic islet PEGylation. (A) Scheme for the surface modification of islets with PEG. PEG conjugated with the amine groups of the collagen matrix of islets by the formation of a stable amide bond. (B) Schematic illustration of immunological protection of transplanted islets using PEGylation and CsA based immunosuppressant therapy. The synergistic effect of the PEGylation/CsA combination effectively prevented immune cell infiltration into transplanted islets, and inactivated immune cells that were stimulated by shed antigens secreted from transplanted islets.

interfacial free energy with water, its unique properties in aqueous solutions, its high surface mobility, and its substantial steric stabilization effects (Fig. 1(B)) [23]. We have previously demonstrated the therapeutic potential of the PEGylation strategy for the immunologic protection of transplanted islets from immune cells such as lymphocytes, macrophages, and splenocytes *in vitro* [15,16]. In addition, the conjugated PEG molecules could block immune cells from infiltrating into the transplanted islets that had stably functioned in the diabetic recipients for several weeks [17,18]. However, transplanted PEG-conjugated islets (PEGylated islets) did not function long term despite the fact that PEG prevented cellular immune reactions by blocking the infiltration of immune cells. The suggested reason for this is that immune cells surrounding islets are stimulated by islet secreted antigens, and then the immune cells secrete cytotoxic chemokines that adversely affect islet functionality, which was already confirmed by culturing PEGylated islets with soluble cytotoxic cytokine *in vitro* [16]. It is known that endocrine cells such as pancreatic islets can indirectly stimulate immune reactions by the secreted proteins or cellular components [24–26]. Therefore, this result suggested that the PEGylation technique is not sufficient for immunological protection *in vivo*.

Based on these earlier findings and hypotheses, we evaluated the clinical potential of a new combinatorial therapy based on PEGylation and immunosuppressant administration in an effort to minimize the dosages required. When we administered a low dose of cyclosporine A (CsA), a widely used immunosuppressant, after PEGylated islet transplantation, the survival time of the transplanted PEGylated islets was strongly improved. This

study indicated the possibility that the new combinatorial therapy might be used to reduce immunogenicity of PEGylated islets. However, we are wondering whether transplanted islets could be immunologically protected from immune reaction by the combinatorial therapy for a long time and whether the continuously administered CsA affected the diabetic recipients or the transplanted islets. To clearly answer those questions, we transplanted PEGylated islets in a streptozotocin-induced diabetic rat model and evaluated the possible causes of the long-term functioning of PEGylated islets for 1 year, which is the average life span of the rat used [27].

2. Materials and methods

2.1. Animals

Inbred F344 male rats (7–8-week old) and outbred Sprague–Dawley (SD) male rats (8-week old) were used as recipients and donors, respectively. Animals were purchased from Japan SLC (Hamamatsu, Japan) and housed in ventilated cages under specific pathogen-free conditions at our institution. All animal procedures were approved by our Institutional Animal Care and Use Committee, which is certified by the Institute of Laboratory Animal Resources of Seoul National University.

2.2. Synthesis of PEG-SPA

PEG-SPA was synthesized and characterized as described previously [14]. Fifty grams of monomethoxy-PEG (MW 5000; Fluka Chemical Co., Switzerland) was reacted with potassium butoxide (3.65 g; Fluka) in toluene at 80 °C for 6 h. After cooling to room temperature, 5 ml of ethylbromopropionate (Aldrich Chemical Co. Milwaukee, WI) was added to the solution and stirred for 20 h. After removing the potassium bromide salt, the reacted PEG was precipitated and dissolved in 250 ml of 1 N NaOH solution and stirred for 2 h. HCl (6 N) was then added to the solution slowly to pH 3.0, and a volume of chloroform equivalent to that of the added HCl was used to extract the reacted PEG. In order to decolorize the product and remove water, silica gel (Merck, Darmstadt, Germany) and magnesium sulfate were added to the solution. Finally, the reacted PEG was precipitated with cold ethyl ether, filtered and dried in vacuum. We refer to the product obtained as PEG-propionic acid (PEG-PA). Ten grams of PEG-PA and 0.52 g of *N*-hydroxy succinimide (HOSU, Sigma, St. Louis, MO) were then dissolved in 50 ml methylene chloride under nitrogen; 0.74 g of *N,N′*-dicyclohexylcarbodiimide (DCC, Sigma) was added to this solution and stirred in ice bath for 20 h. After removing the precipitated dicyclohexyl urea (DCU), the product was precipitated and dried in vacuum overnight. The final product obtained is referred to as PEG-SPA, and its end group (SPA) can rapidly react with the amines of collagen matrix. All products were characterized by FT-IR (SPECTRUM 2000, Perkin Elmer Co., England) and 300 MHz NMR (JEOL JNM-LA 300WB FT-NMR, Japan), and stored under vacuum at −20 °C until used.

2.3. Isolation of pancreatic islets

Pancreatic islets were isolated from the pancreases of outbred male SD rats. Briefly, SD rats were anaesthetized with 50 mg/kg of pentobarbital, and pancreases were exposed by laparotomy. The common bile duct was ligated, cannulated with a 25-G needle, and then 10 ml of Ca^{2+} and Mg^{2+}-free HBSS containing 1.5 mg/ml collagenase (Sigma) was injected. Distended pancreases were removed and incubated at 37°C for 10 min. After incubation, digested tissues were washed with cold HBSS and filtered through a tissue-collecting sieve (40 mesh). Islets were then purified by discontinuous Ficoll™ PM Density gradient (Amersham Biosciences AB, Uppsala, Sweden) and centrifuged at 200*g* for 25 min. Isolated islets were cultured for 3 days in RPMI-1640 (Sigma) containing 10% FBS (Sigma) at 37 °C in a humidified 5% CO_2 atmosphere. Islet purity was assayed by dithizone staining (purity > 98%).

2.4. Islet surface modification with PEG

Isolated islets were washed with HBSS, and resuspended in 8 ml of HBSS (pH 8.0). 2 ml of HBSS (pH 8.0) containing 25 mg PEG-SPA was added to this islet suspension and incubated at 37 °C in a humidified 5% CO_2 atmosphere for 1 h. The final concentration of PEG-SPA obtained was 0.25% wt/vol. After incubation, PEGylated islets were washed twice and cultured for one more day prior to transplantation. Unmodified islets were cultured for the same period as normal controls. On the other hand, to confirm the existence of the conjugated PEG molecules onto islet surface *in vivo*, biotin-labeled PEG molecules were conjugated onto islet surface, and then the islets were allotransplanted in the diabetic F344 rats. Thirty days after post-transplantation, the existence of biotin-labeled PEG molecules on the transplanted islets was visualized by using the avidin–biotin complex (ABC) stain method. The streptavidin-horseradish peroxidase (Research Genetics, Huntsville, AL) detection system was used to react with 0.02% 3,3′-diaminobenzidine as chromogen (Dako, Carpinteria, CA).

2.5. Allotransplantation of PEGylated islets

Inbred male F344 rats were rendered diabetic with a singly intraperitoneal injection of 45 mg/kg of streptozotocin (Sigma), freshly dissolved in citrate buffer (pH 4.5) 5 days before transplantation. Only rats with stable nonfasting blood glucose levels of > 350 mg/dl over 5 consecutive measurements were considered diabetic and used for islet allotransplantation. The blood glucose levels were measured in tail vein blood using a portable Gluco-card (Super glucocard II, Arkray, Kyoto, Japan). Streptozotocin-induced diabetic recipients were anaesthetized by injection of 50 mg/kg of intraperitoneal pentobarbital. Left kidneys were exposed through a lumbar incision, and capsulotomy was performed on the caudal outer surface of the left kidney, and unmodified islets or PEGylated islets (1200 islets/recipient) were injected. After allotransplantation, the glucose levels and body weights of nonfasting recipients were measured daily between 1:00 and 3:00 p.m. Transplantations were considered successful if blood glucose levels returned to normal- < 120 mg/dl for two consecutive days after islet transplantation, and islet rejection was deemed to have occurred if two consecutive blood glucose readings exceeded 200 mg/dl. To confirm that blood glucose was controlled by the transplanted islets alone, kidneys containing islets were nephrectomized and blood glucose levels were measured on the following two consecutive days.

2.6. Minimized immunosuppressive therapy using CsA

To evaluate synergism between PEGylation and CsA administration (Sandimmun®, Novartis International AG, Basle, Switzerland), CsA was daily injected into animals transplanted with nonPEGylated or PEGylated islets. Initially, 3 mg/kg of CsA was freshly diluted with 100 μl of Hanks solution, and administered daily through a tail vein from the day of transplantation for 2 weeks. Then, 1 mg/kg/day of CsA was administered for up to 1 year.

2.7. Intraperitoneal glucose tolerance

To evaluate long-term blood glucose responsiveness in PEGylated islet transplanted animals, intraperitoneal glucose tolerance testing (IPGTT) was performed at 100 and 200 days, and at year 1 after allotransplantation. For this purpose, glucose was injected intraperitoneally at 1.5 g/kg; blood glucose levels were then determined in tail blood at 0, 15, 30, 60, 90, and 120 min after injection.

2.8. Immunohistochemical analysis

In cases where transplanted islets were rejected or survived for 1 year, left kidneys were removed and immediately immersed in 4% paraformaldehyde-phosphate-buffered saline. Immunohistochemical staining was performed using the ABC method. Briefly, representative paraffin blocks were sectioned at 4 μm and immunostained using a microprobe immuno/DNA stainer (Fisher Scientific International Inc., Pittsburgh, PA). Sections were then deparaffinized in xylene and treated with 0.3% hydrogen peroxide in methanol for 20 min to block endogenous peroxidase activity. Sections were then stained with primary anti-insulin (1:200; Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-glucagon (1:200; DAKO, Carpinteria, CA), anti-somatostatin (1:50; Biomeda, Foster City, CA), anti-CD34 (1:10; Abcam, Cambridge, UK), anti-$CD4^{+}CD8^{+}$ T cells (1:50; Chemicon International, Temecular, CA), anti-$CD4^{+}$ T cells (1:50; Chemicon), anti-$CD8^{+}$ T cells (1:50; Chemicon), or anti-$CD79a^{+}$ B cells antibodies (1:50; Chemicon). The streptavidin–horseradish peroxidase (Research Genetics) detection system was used to be reacted with 0.02% 3,3′-diaminobenzidine as chromogen (Dako). Sections were counter-stained with hematoxylin and mounted in Universal Mounts (Research Genetics). Negative controls were treated similarly but not treated with primary antibodies.

2.9. Biochemical analyses

To evaluate transplanted PEGylated islets functions, several kinds of physiological markers were measured at 100 and 200 days, and at 1 year. Insulin concentrations were determined by radioimmunoassay (DPC, Los Angeles, CA), and glycated hemoglobin (HbA1c) using HLC723-G7 kits (Tosoh, Tokyo, Japan). Plasma glucose, creatinine, triglyceride, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) concentrations were determined using an automated biochemical analyzer HITACHI-7180 (Hitachi, Tokyo, Japan).

2.10. Statistical analysis

Islet survival data are expressed as median ± s.d. and analyzed using log-rank statistics. Other analyses were conducted using the Student's *t*-test by the SigmaStat (Window version 3.1; Systat Software, Inc., San Jose, CA). *P* values of less than 0.05 were considered statistically significant. Area under curve (AUC) value was calculated by using SigmaPlot software (Windows version 9.01; Systat Software, Inc.).

3. Results

3.1. Prolongation of PEGylated islet allograft survival with cyclosporine administration

To investigate long term therapeutic potential of a new combinatorial protocol using PEGylation and CsA on the transplanted islets, we allotransplanted PEGylated islets or unmodified islets (control) under the caudal outer surface of the left kidney in diabetic rats and blood glucose levels were measured daily. On the day following islet allotransplantation, the blood glucose levels of recipients returned to normal in both cases. The median survival times of unmodified and PEGylated islets were 5 ± 0.9 and 11 ± 0.9 days (median ± s.d., $n = 7$), respectively, and this difference between the two groups was significant (Table 1). Low dose CsA, initially 3 mg/kg CsA for 2 weeks followed by 1 mg/kg for up to 1 year, was also administered daily to recipients after islet transplantation. During CsA administration, unmodified islet survival times improved slightly, but all transplanted islets were completely rejected within 2 weeks (median survival time: 12 ± 2.6 d, $n = 7$). However, in animals that received PEGylated islet transplantation, all the islets survived for more than 1 year. Moreover, transplanted PEGylated islets firmly controlled recipient blood glucose levels in the normal range without the evidence of severe fluctuation observed in animals that received unmodified islets (Fig. 2(A)). To confirm that the blood glucose levels of recipients were controlled by the transplanted PEGylated islets, left kidneys containing the transplanted islets were nephrectomized at 1 year (Fig. 2(A), arrow). All recipients so treated became hyperglycemic on the day following nephrectomy, indicating that insulin independence in these animals was due to transplanted PEGylated islets. On the other hand, the pattern of body weight gain of rats receiving PEGylated islets was similar to that of nondiabetic healthy rats although the body weight gain of rats receiving PEGylated islets was a little lower than that of normal rats (Fig. 2(B)).

3.2. Intraperitoneal glucose tolerance test

To evaluate the blood glucose responsiveness of PEGylated islets that had survived long term, IPGTT was carried out on days 100 and 200 and 1 year after post-transplantation (Fig. 3). The blood glucose responsivenesses of transplanted PEGylated islets was found to be similar to those of nondiabetic normal rats for 1 year, although $AUC_{0-120\,min}$ values of normal and recipient animals were statistically different after 1 year. The

Table 1
Survival date of transplanted islets without or within low dose CsA treatment

Transplantation ($n = 7$)	Survival time (day)	Median (±s.d.)
Unmodified islets		
(−) CsA	4, 4, 5, 5, 6, 6, 6	5 ± 0.9
(+) CsA	8, 8, 11, 12, 13, 14, 14	12 ± 2.6
PEGylated islets		
(−) CsA	7, 8, 9, 11, 11, 11, 13, 14	11 ± 0.9[a]
(+) CsA	>1 year (all)	1 year

CsA was administered daily i.v. in recipients according to the followed protocol: first 2 weeks 3 mg/kg/day and then 1 mg/kg/day for up to 1 year. Data are expressed as median ± s.d.

[a] $P < 0.05$ compared with unmodified islets without CsA.

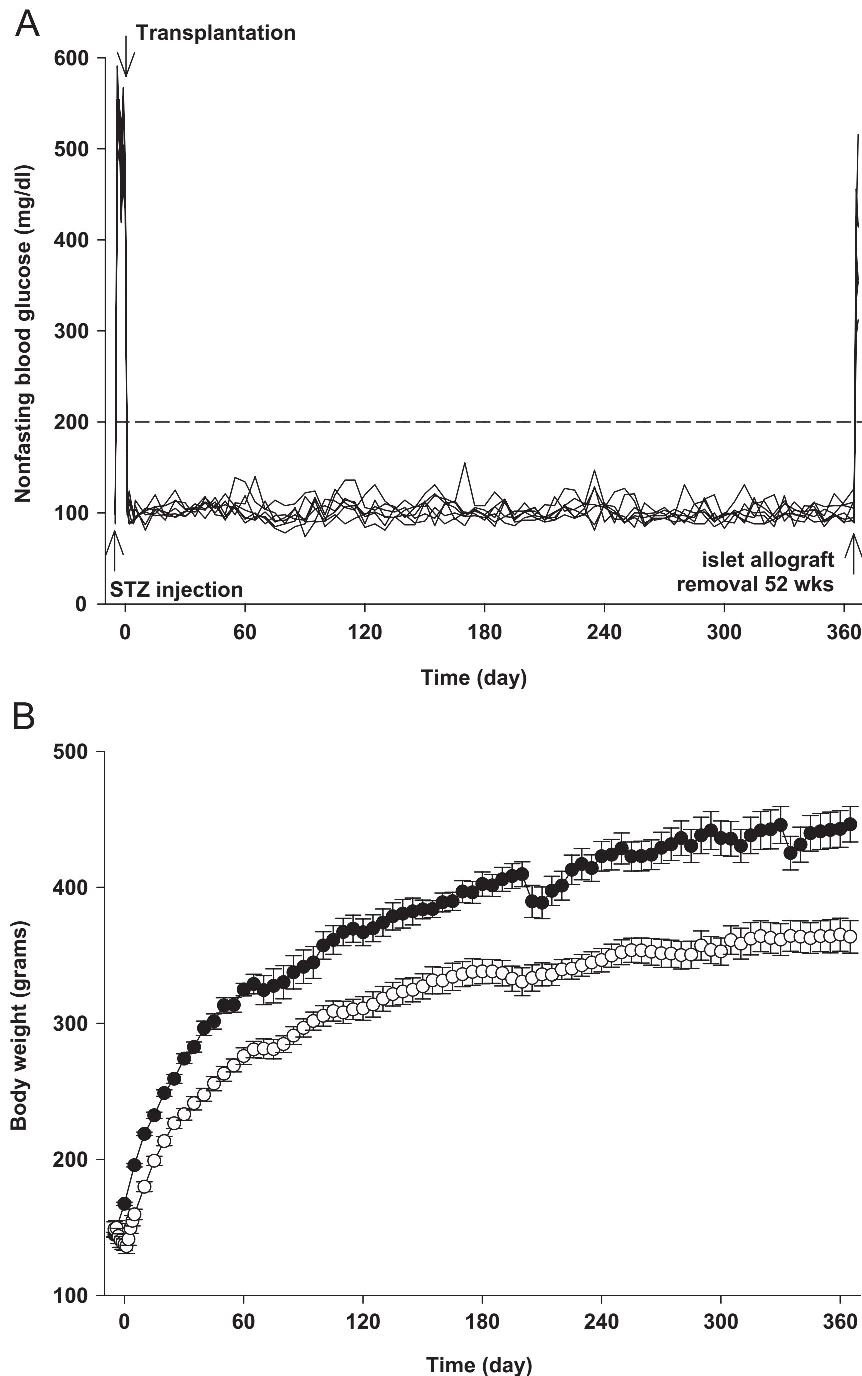


Fig. 2. Monitoring of blood glucose levels and body weights of recipients. (A) Long-term monitoring of blood glucose levels of recipients receiving PEGylated islets after allotransplantation for 1 year ($n = 7$). On the day following nephrectomy (islet allograft removal at 52 weeks), all recipients became hyperglycemic. (B) Body weights of nondiabetic healthy (●, $n = 5$) and recipients receiving PEGylated islets (○, $n = 7$).

$AUC_{0-120\,min}$ value of healthy, normal, nontransplanted diabetic, and PEGylated islet recipients at 1 year was 293.2 ± 41.2, 1092.3 ± 82.4 and 364.5 ± 40.3 mg h/dl, respectively. These results suggested that the long-functioning PEGylated islets could rapidly respond to the change of blood glucose levels and normalize those levels by secreting islet hormones.

3.3. Immunohistochemical analysis to transplanted islets

Long-functioning transplanted PEGylated islets were immunohistochemically analyzed after islet removal at 1 year (Fig. 4(A)). The long-functioning PEGylated islets firmly expressed insulin, glucagon and somatostatin hormone. Furthermore, a small number of anti-CD34

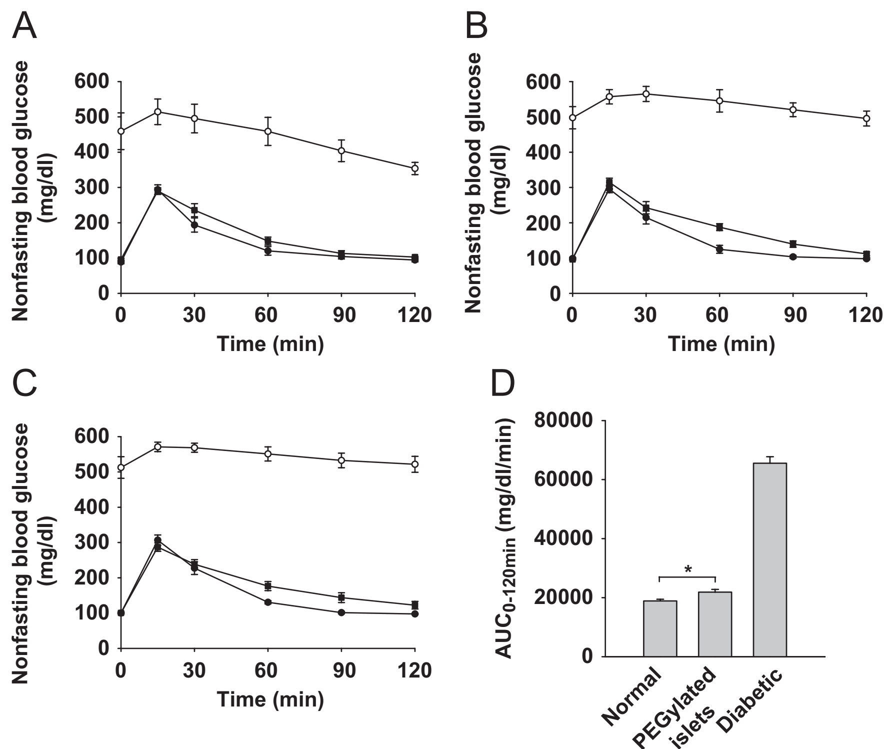


Fig. 3. Intraperitoneal glucose tolerance test (IPGTT) to healthy normal (●), nontransplanted diabetic (○) and PEGylated islet transplanted recipients (■) at day 100 (A), day 200 (B), and 1 year (C), respectively, after transplantation. (D) Area under curve $(AUC)_{0-120\,min}$ values of healthy normal ($n = 5$), nontransplanted diabetic ($n = 5$) and PEGylated islet transplanted recipients ($n = 5$) after IPGTT at 1 year. Data were expressed as mean ± s.d. *$P<0.05$.

immunostained microvessels were observed around the transplanted PEGylated islets. To confirm that PEG molecules conjugated onto islet surfaces can exist *in vivo*, biotin-labeled PEG molecules were conjugated onto islet surfaces and transplanted under the caudal outer surface of the left kidney in diabetic rats. One month later, biotin-labeled PEG molecules were strongly detected by streptavidin–biotin staining (anti-biotin immunostain). On the other hand, the pancreas of recipients that were transplanted with PEGylated islets for 1 year were not immunostained with anti-insulin antibody (Fig. 4(B)).

3.4. Immunohistochemical analysis to infiltrated immune cells

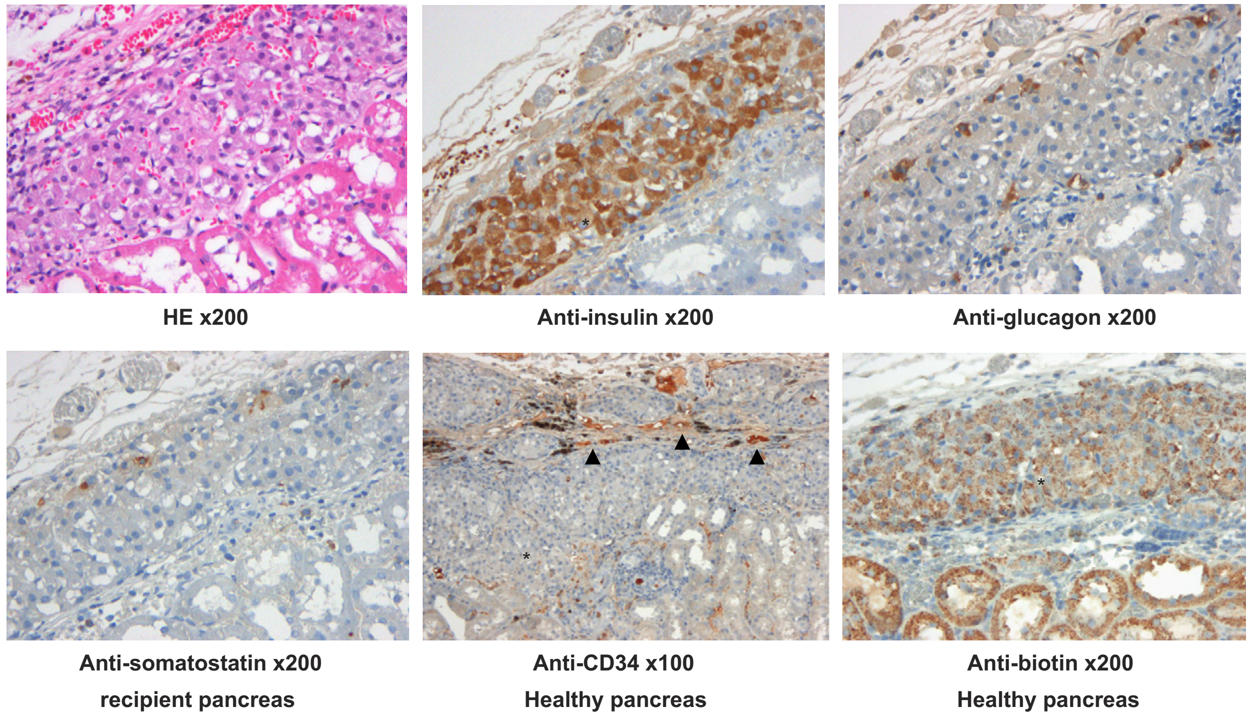
Host's immune cells were stained with each specific antibodies to lymphocytes subtypes (Fig. 5). In the case of unmodified islets rejected within 2 weeks, host's T lymphocytes were severely infiltrated into the islets. Specially, $CD8^+$ T lymphocytes were highly detected around and into the unmodified islets. However, $CD4^+$ T or $CD79a^+$ B lymphocytes were rarely detected at the transplanted islets. On the other hand, in the case of PEGylated islets surviving for 1 year, T and B lymphocytes were rarely observed around transplanted PEGylated islets but did not infiltrate into the islets.

3.5. Physiological recuperation of diabetic recipients after PEGylated islet allotransplantation

After PEGylated islet allotransplantation, several physiological markers were analyzed at days 100 and 200, and at 1 year (Table 2). Compared with healthy normal recipients, nontransplanted diabetic recipients became hyperglycemic and hypoinsulinemic, and HbA1c contents were significantly elevated at all time. In addition, the levels of hepatic functional parameters, such as AST, ALT and ALP, were highly elevated. On the other hand, in the recipients receiving PEGylated islets and continuous long-term administration of low dose of CsA, the recipients maintained a normoglycemic normoinsulinemic state and blood HbA1c contents were normal at 1 year. In addition, creatinine levels and levels of hepatic functional parameters were similar to those of healthy normal recipients. Therefore, the long-term administration of low dose CsA did not show any toxicity to recipients and it did synergistically protect transplanted PEGylated islets for 1 year without damage to islet insulin secretion.

4. Discussion

In this study, we established a strategy for modifying the surfaces of islets with PEG molecules with the aim of minimizing immunosuppressive therapy and improving



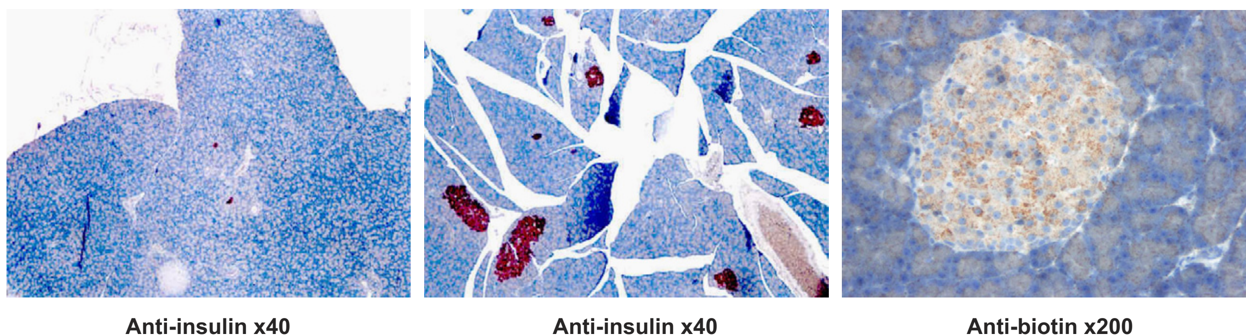


Fig. 4. Immunohistochemical analysis of transplanted PEGylated islets (A) or recipient pancreas (B) at 1 year after transplantation using hematoxylin & eosin (HE) stain, anti-insulin, anti-glucagon, anti-somatostatin, anti-CD34 microvessel immunostain, and anti-biotin immunostain, respectively. Anti-biotin immunostain was carried out by using streptavidin–biotin complex at day 30 after biotin-PEGylated islet allotransplantation. Asterisk: PEGylated islets, arrow head: anti-CD34 positive microvessels.

long-term islet function after allotransplantation without adverse effects. Using the described approach, PEG molecules were firmly conjugated with amines of the collagen matrix; an islet surface component. We previously found that conjugated PEG molecules do not cause damage to either structural integrity or functional activity of islets [14,16], and that PEGylated islets do not stimulate co-cultured immune cells, e.g., lymphocytes, macrophages, and splenocytes *in vitro* [15,16]. Furthermore, we found that PEGylated islets can produce normal levels of blood glucose in recipients within a day of islet allotransplantation, indicating that the conjugated PEG molecules do not affect islet glucose responsiveness or insulin secretion *in vivo*. In these previous studies, transplanted PEGylated islets were found to be immunologically protected from recipient immune cells, which were observed in surrounding of PEGylayed islets without infiltrating. However, the PEGylated islets were not able to function for protracted period of time although the PEGylated islets were not destroyed by immune reactions [17,18]. This impairment of the PEGylated islets may have been caused by immune cell stimulation by cellular components released from the transplanted islets. In general, transplanted islets can be rejected by direct cellular immune reaction and also by indirect immune reaction that is caused by the secreted shed antigens of islet [25,26]. We found in a previous study that PEGylated islets could be impaired by cytotoxic cytokines during culturing of the islets with soluble cytokine *in vitro* [16]. Therefore, we concluded that although conjugated PEG molecules can block direct cellular immune reactions, meaning that the immunogenicity of islets can only be attenuated by PEGylation, another means should be found to produce long-functioning PEGylated islet allografts.

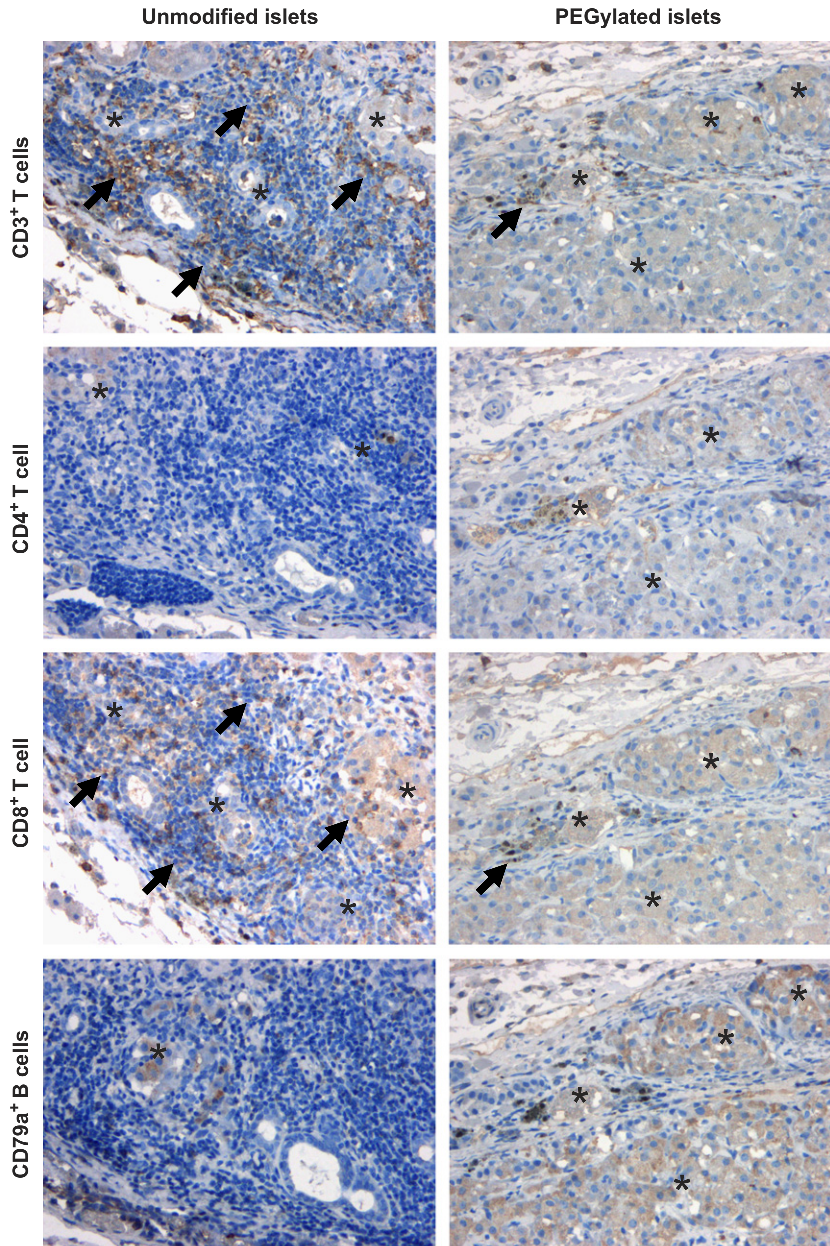


Fig. 5. Immunohistochemical analysis to host's immune cells around transplanted unmodified islets rejected within 2 weeks or PEGylated islets surviving for 1 year. Host's immune cells were stained with antibodies against anti-CD3$^+$ T cell, anti-CD4$^+$ T cell, anti-CD8$^+$ T cells, or anti-CD79a$^+$ B lymphocytes. Asterisk: transplanted islets, arrow: each immune cells (dark brown). Magnification: $\times 200$.

In clinical practice, several kinds of immunosuppressive medications, such as sirolimus, tacrolimus, and monoclonal antibodies against interleukin-2 receptor, are used simultaneously to protect transplanted islets from immune reactions [2,3,5]. However, the required therapeutic doses can induce several serious adverse effects, e.g., nephrotoxicity, neurotoxicity, and hepatotoxicity. Thus, we considered that the PEGylation strategy could be used to reduce the dosage and frequency of immunosuppressive medication and to provide long-term functionality due to the attenuated immunogenicity of PEGylated islets. When a low dose of CsA was daily administered in the recipients, the survival time of the PEGylated islets could be significantly improved [17]. The improvement of survival time of the PEGylated islets was contributed by the synergistic effect of the conjugated PEG molecules and the treated CsA; that is, the conjugated PEG molecules could block the infiltration of immune cells into the islets and the treated CsA could inactivate the infiltrated immune cells around the islets. Actually, T and B lymphocytes were rarely observed around the PEGylated islets surviving for 1 year. On the other hand, unmodified islets (control) were completely rejected despite continuous administration of CsA. Mostly, cytotoxic CD8$^+$ T lymphocytes severely infiltrated into the transplanted unmodified islets. Therefore, this PEGylation technique could be used as a new

Table 2
Physiological markers of normal, diabetic, and PEGylated islet transplantation groups

Physiological markers	Normal[a] ($n = 5$)	Diabetic[b] ($n = 5$)	PEGylated islet transplantation ($n = 7$)		
			Day 100[c]	Day 200[c]	Year 1
Glucose (mg/dl)	91.4±4.4	424.4±21.1[d]	108.1±9.2	99.6±4.4	99.0±4.7
Insulin (μU/ml)	9.9±1.0	0.3±0.0[d]	9.9±1.3	9.0±0.8	8.7±1.0
HbA1c (%)	4.4±0.1	9.6±0.1[d]	4.9±0.3	5.5±0.4[d]	5.4±0.4[d]
Creatinine (mg/dl)	0.9±0.1	0.9±0.1	0.9±0.0	0.9±0.0	0.9±0.1
Triglycerides (mg/dl)	139.0±15.5	150.8±21.8	145.8±21.2	145.4±23.8	132.4±17.4
AST (U/l)	76.8±5.0	497.6±35.5[d]	109.4±14.9	115.4±13.5[d]	102.8±10.7
ALT (U/l)	55.6±7.4	313.6±19.3[d]	66.6±7.8	67.0±9.0	59.6±7.8
ALP (mg/dl)	271.2±13.6	1153.0±37.7[d]	228.0±17.0	218.0±18.7	241.8±14.9

Amounts of physiological markers in serum or plasma were analyzed at each time interval. Data are expressed as mean±s.d.
[a]Normal: nondiabetic healthy recipients at 1 year.
[b]Diabetic: streptozotocin-induced type 1 diabetic recipients at day 30.
[c]These groups were separately prepared for the biochemical analyses.
[d]$P < 0.05$ compared with the normal group.

therapeutic remedy to minimize immunosuppressant medications, thereby reducing the adverse effects of the medications.

However, we wondered whether this new combinatorial therapy could stably protect transplanted islets from immune reactions for a long time. In the present study, we clearly demonstrated that the islet PEGylation/low dose CsA regimen can protect transplanted islets from immune system with no apparent deficit for more than 1 year without adverse effect. At 1 year after continuous administration of low dose CsA, biochemical analyses showed that the low dose CsA regimen had no toxic effect in recipients after long-term treatment as the dosage used was substantially lower than clinical levels. Moreover, CsA is clinically used in combination with other immunosuppressants to bolster its immunosuppressive effect, which offers an obvious means of further enhancing the benefits of PEGylated islet transplantation. In the present study, we had to terminate experimental observations at year one because of the limited life spans of the recipients [27]. For 1 year after transplantation, PEGylated islets firmly controlled blood glucose levels, and enabled normal blood glucose responsiveness, hormone synthesis, and the existence of PEG molecules at transplanted islets, suggesting that PEGylation/CsA combinatorial therapy could semi-permanently protect transplanted islets from immune reactions at least in the rodent model.

In terms of the synergistic effects between PEGylation and low dose CsA, we suggest the following. First, immune cells cannot infiltrate transplanted PEGylated islets. Instead, they surround islets even at 1 year post-transplantation, indicating that PEG molecules remain attached throughout this period, as detachment would probably be followed by rapid rejection. Second, whereas immunosuppressants such as CsA, tacrolimus and sirolimus can affect islet functionality in general, at the levels administered, CsA could not have affected expression and secretion of islet hormones such as insulin, glucagon, and somatostatin, and thus, allowed normal blood glucose level balances to be achieved without incidents of hypoglycemia or hyperglycemia. Furthermore, long-term treatment with low dose CsA proved nontoxic in terms of measured physiological parameters, i.e. creatinine, AST, and ALT levels. Finally, microvessels formed around transplanted islets undoubtedly promoted islet survival, and probably allowed islets to respond faster to glucose fluctuations by facilitating the systemic dissemination of islet-produced hormones.

5. Conclusion

We found that chemically conjugated PEG molecules not only inhibit direct cellular immune reactions, but also synergistically with CsA, improve the functionalities and viabilities of transplanted islets at 1 year post-transplantation. These findings could present an exciting new approach to immunological prevention of transplanted islets for curing T1DM.

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