A combination therapy of PEGylation and immunosuppressive agent for successful islet transplantation

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Abstract

Several immunosuppressive medications are used simultaneously to protect transplanted islets. However, reports of severe side effects induced by immunosuppressive drugs have prompted attention on developing ways to reduce their toxicities. Toward attenuating the immunogenicity of islets, we studied a combination therapy of PEGylation and cyclosporin A (CsA) as a new immunoprotective strategy. This study aimed to find out whether PEGylation combined with cyclosporine and applied on islet surfaces could bring about a synergistic effect of reducing the dose of immunosuppressive medications as well as enhancing their medical effects. After islets were transplanted into the kidney of diabetic rats, different doses of CsA were administered daily. When 3 mg/kg of CsA was administered for 2 weeks, unmodified islets were completely rejected within 2 weeks, whereas the PEGylated islets survived for 32±14.6 days. When 1 mg/kg of CsA was further administered following the initial, 2-week CsA treatment of 3 mg/kg, the PEGylated islets in all recipients survived up to 100 days prior to nephrectomy and also rapidly responded to the fluctuation of blood glucose level. PEGylated islets were also present in large numbers in the transplantation site without causing the infiltration of immune cells. Therefore, these findings suggest that, when combined with an immunosuppressive drug, PEGylation of islets could have a dose reducing effect on the immunosuppressive medication and thus synergistically improve the survival time of islets.

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1. Introduction

Pancreatic islet transplantation is a promising method of cell therapy that can be used to treat type 1 diabetes mellitus. However, since transplanted islets are easily rejected by patients’ own immune reactions, patients receiving transplants have to simultaneously take several immunosuppressive medications such as tacrolimus, sirolimus or cyclosporine A (CsA) in order to protect the transplanted islets. At present, the use of immunosuppressive medications is the only clinically applicable method for preventing islet graft rejection [1,2]. Unfortunately, immunosuppressive medications can cause side effects such as nephrotoxicity, neurological complications, hepatotoxicity and abnormalities including abnormal glucose metabolism during long-term administration of the medications [3–6]. In this respect, there is a great need for a new immunoprotective strategy that can be applied to reduce toxicities of medications.

Recently, surface modification of islets using poly(ethylene glycol) (PEG) was proposed to prevent immunogenic reactions in transplantation [7–12]. This strategy is based on the unique properties of PEG in aqueous solutions, such as its low interfacial free energy, high surface mobility, steric stabilization effects, etc. [13,14]. The hypothesis of this method is that PEG molecules, which are chemically grafted onto islet surfaces, could protect islets from attacks by immune cells by virtue of its physical characteristics. We have previously demonstrated that the immunogenicity of islets was reduced when islet surfaces were modified with PEGylation, thereby attenuating the immune reactions in vitro [9,10]. In the animal study,
however, we found out that PEGylation to islets could not perfectly protect islets from immune reactions without using immunosuppressive medications [15]. This result suggested that PEGylation technique is not sufficient for immunological protection in vivo. Interestingly, the histological analysis revealed that the host’s immune cells remained around the transplanted PEGylated islets without having infiltrated into the islets, which have nevertheless lost their function. This study indicates the possibility that the simultaneous administration of immunosuppressive medication and PEGylated islets might be used to reduce immunogenicity of PEGylated islets.

In this study, we attempted to evaluate the gradual dose-reduction effect of CsA, when this immunosuppressive drug was administered after PEGylated islet transplantation. Dose and duration of the CsA administration regimen were varied since transplanted islets are in general severely rejected within 2 weeks after transplantation as a result of acute cellular immune reactions [16,17]. Also, a new regimen of CsA administration with PEGylated islet transplantation was proposed.

2. Materials and methods

2.1. Animals

Inbred male F344 rats, 7–8 weeks of age, and male Sprague–Dawley (SD) rats, 8 weeks of age, were used as recipients and donors, respectively. They were purchased from Japan SLC (Hamamatsu, Japan) and were housed under specific pathogen-free condition. Diabetes was induced in the recipient group of rats by a single intraperitoneal injection of 55 mg/kg of streptozotocin (STZ) (Sigma, St. Louis, MO). The diabetic rats (glycaemia >350 mg/dl) were used as recipients for islet transplantation after 5 days.

2.2. PEGylation onto islet surface

Pancreatic islets were isolated from outbred male SD rats by collagenase digestion of the pancreas and discontinuous Ficoll™ PM400 (Amersham Biosciences AB, Uppsala, Sweden) density gradient centrifugation and then hand-picked [7–9]. Isolated islets were then cultured for 3 days in the RPMI-1640 (Sigma) culture medium containing 10% fetal bovine serum (FBS; Sigma) at 37 °C in humidified atmosphere of 5% CO2 in air before PEGylation. Islet purity was assessed by dithizone (Sigma) staining (purity >95%) [18].

Active monomethoxy-PEG-succinimidyl propionic acid (PEG-SPA) was synthesized and characterized as described in the previous study [7–9]. The end group (SPA) of PEG-SPA can rapidly react with amine groups of collagen matrix that composes the outer surface of isolated islets. To chemically conjugate PEG onto the collagen matrix of isolated islets, the islets were first washed with Hank’s balanced salt solution (HBSS) and then incubated in the HBSS (pH 8.0) containing 0.25% w/v active PEG-SPA for 1 h. Finally, the PEGylated islets were washed twice and cultured in the culture medium for one more day until transplanted.

2.3. Islet transplantation and blood glucose monitoring

STZ-induced diabetic recipients were anaesthetized by intraperitoneal injection with 50 mg pentobarbital/kg. The left kidney of the recipient was exposed through a lumbar incision. A capsulotomy was performed on the caudal outer surface of the left kidney and unmodified islets or PEGylated islets were injected (1200 islets/recipient). The tail vein blood glucose level of these rats which were kept under free feeding condition was measured daily with a portable glucometer (Super glucocard II, Arkray, Kyoto, Japan) between 1:00 and 3:00 p.m. for 100 days. Transplantation was considered successful if the blood glucose level returned to normal (<120 mg/dl) and remained normal for the first 2 days after islet transplantation. Islet rejection was diagnosed when the blood glucose level became hyperglycemic (blood glucose >200 mg/dl) for 2 consecutive days.

2.4. Treatment of cyclosporine A (CsA)

We determined if there was dose-reducing effect on the medication owing to the chemically conjugated PEG. The immunosuppressive medication cyclosporine A (CsA) (Novartis International AG, Basle, Switzerland) was daily diluted with a volume of 100 μl HBSS (pH 7.4) and intravenously administered into different groups of recipients following transplantation of unmodified islets or PEGylated islets. For the first group, 3 mg/kg of CsA was intravenously administered once a day for 2 weeks and, for the second and the third groups, 0.5 and 1 mg/kg of CsA was intravenously administered once a day up to 100 days, respectively, after the initial administration of 3 mg/kg of CsA for 2 weeks.

2.5. Intraperitoneal glucose tolerance test

Intraperitoneal glucose tolerance test (IPGTT) was performed to evaluate the glucose responsiveness of transplanted islets at 100th day. Briefly, blood glucose levels were measured from the tail vein at 0, 15, 30, 60, 90 and 120 min after the intraperitoneal injection of glucose solution (2 g glucose/kg body weight). To confirm whether the blood glucose level of recipients was controlled only by the transplanted islets, the left kidney containing the transplanted islets was nephrectomized after IPGTT and the blood glucose of the recipients was measured the following day.

2.6. Histological analysis

The removed kidney containing transplanted islets was fixed in 4% paraformaldehyde-phosphate-buffered saline, embedded in paraffin and sectioned at 4 μm. Immunohistochemical stains for insulin were performed by the routine avidin–biotin complex (ABC) method. Briefly, the sections were deparaffinized in xylene and treated with 0.3% hydrogen peroxide in methanol for 20 min to block endogenous peroxidase activity. And then, the sections were incubated with anti-insulin primary antibodies (1:50; Santa Cruz Biotechnology Inc., Santa Cruz, CA) at room temperature for 2 h. After that, anti-mouse immunoglobulin G

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(Sigma) labeled with biotin was incubated for 7 min at 45 °C. The streptavidin–horseradish peroxidase (Research Genetics, Huntsville, AL) detection system was applied to capillary channels, followed by 10 min of incubation at 45 °C. After drainage, the tissue sections were ready for chromogen reaction with 0.02% 3-amino-9-ethyl carvazole (Zymed Laboratories Inc., San Francisco, CA). The sections were counterstained with hematoxylin and mounted in the Universal Mount (Research Genetics). Negative controls were treated similarly with the exception of primary antibodies.

2.7. Statistical analysis

Survival time of transplanted islets was expressed as median ± S.E. and analyzed by log-rank statistics. Other data analysis was performed using the nonparametric t-test. A P-value less than 0.05 was considered to be statistically significant.

3. Results

3.1. Short-term treatment of CsA after islets transplantation

Following the transplantation of PEGylated islets or unmodified islets, 3 mg/kg of CsA was daily administered for 2 weeks. Unmodified islets in all the recipients were completely rejected within 2 weeks although CsA was continuously administered up to 2 weeks (Fig. 1A). On the other hand, the PEGylated islets in four of the seven recipients were rejected. The remaining three recipients survived for 100 days although CsA treatment was stopped after 2 weeks (Fig. 1B). To check the glucose responsiveness of long-surviving PEGylated islets, intraperitoneal glucose tolerance test (IPGTT) was performed only for the three surviving recipients at 100th day. After injection of glucose solution, the blood glucose levels of the recipients were significantly increased higher than those of non-diabetic normal group and slowly returned to the normal state, as shown in Fig. 2. After performing IPGTT, the left kidney containing the islets was nephrectomized to confirm that the blood glucose of the three recipients was normally controlled only by the transplanted PEGylated islets. After nephrectomy, the blood glucose of the three recipients became rapidly hyperglycemic (Fig. 1B). The median survival times of unmodified islets and PEGylated islets were 10.0±0.7 and 32.0±14.6 days, respectively, and the difference was statistically significant (P<0.001, n=7).

To observe the recipients’ immune reactions against unmodified islets or PEGylated islets in the recipients, transplanted islets were histologically analyzed. In the case of unmodified islets, islets were severely destroyed (Fig. 3A, asterisk) and a great number of immune cells had infiltrated into the islets (Fig. 3A, arrow). In the case of the rejected PEGylated islets, they were well retained and immune cells did not infiltrate into the central area of the islets (Fig. 3B, asterisk); the immune cells were only recruited around the outer area of the islets (Fig. 3B, arrow). On the other hand, in the case of long-surviving PEGylated islets, the islets were well retained and immune cells did not infiltrate into the islets (Fig. 3C, asterisk). Furthermore, the anti-insulin-positive islet cells were well presented throughout the area where PEGylated islets were found (Fig. 3D).
3.2. Long-term treatment of a low dose CsA following the initial treatment of 3 mg/kg for 2 weeks

After PEGylated islet transplantation was followed by the administration of 3 mg/kg of CsA for the first 2 weeks, a lower dose of CsA was further administered continuously up to 100 days. When 0.5 mg/kg of CsA was used as a further treatment, the PEGylated islets survived for 100 days in five of seven recipients and the blood glucose levels of the recipients were maintained without severe fluctuation (Fig. 4A). In addition, when the recipients' kidney containing the PEGylated islets

Fig. 3. Histological analysis of transplanted islets. (A) H&E stain of unmodified islets rejected within 2 weeks, (B) H&E stain of PEGylated islets rejected at day 30, (C, D) H&E stain and insulin immunostain of PEGylated islets survived for 100 days, respectively. Asterisk: transplanted islets, arrow: immune cells.

Fig. 4. The blood glucose monitoring of recipients after PEGylated islets transplantation (TX) during further treatment of 0.5 mg/kg (A) or 1 mg/kg (B) of CsA after treatment of 3 mg/kg for 2 weeks (n=7). After nephrectomy (arrow), the recipients of those cases became hyperglycemic.

Fig. 5. Intraperitoneal glucose tolerance test on the only long-surviving PEGylated islets at 100th day. (●) Non-diabetic normal (n=5), (○) diabetic (n=5), (▼) PEGylated islets transplantation during further treatment of 0.5 mg/kg after treatment of 3 mg/kg for 2 weeks (n=5), (△) PEGylated islets transplantation during further treatment of 1 mg/kg after treatment of 3 mg/kg for 2 weeks (n=7). Results are expressed as mean±S.E. and statistically analyzed by nonparametric t-test (*p<.01).
that survived for 100 days was nephrectomized, the blood glucose of the recipients rapidly became hyperglycemic. In this case, the median survival time of the islets was 100.0 ± 11.3 days. In the case of further continuous treatment with 1 mg/kg of CsA, the PEGylated islets in all recipients completely survived for 100 days \( (n=7) \) (Fig. 4B) and the blood glucose levels of all recipients were maintained without severe fluctuation.

Prior to nephrectomy, IPGTT was also performed only on the recipients at 100th day. The results of IPGTT showed that the blood glucose responsiveness of the long-surviving islets of both cases was similar to that of non-diabetic normal group (Fig. 5). Upon completion of further treatment of lower doses of CsA, the PEGylated islets in the recipients were also histologically analyzed at 100th day. It was observed that, after both treatments of 0.5 and 1 mg/kg of CsA, the PEGylated islets were well retained and immune cells had not infiltrated into the islets (Fig. 6A and B, asterisk). In addition, the anti-insulin-positive islet cells were well presented throughout the area of the transplantation site (Fig. 6B and D).

4. Discussion

In this study, biocompatible PEG molecules were chemically grafted onto islet surfaces to prevent the recognition of islet cells by immune cells. In the previous study, we had demonstrated that the PEGylation did not affect blood glucose responsiveness or insulin secretion from the islets and that it could protect islets from immune cells in vitro by inhibiting the stimulation of immune cells. This finding led us to explore whether PEGylation of islets could attenuate the immunogenicity of islets in vivo. Here, we show that success of cell therapy with islets was contributed by the only one kind of immunosuppressive medication due to the attenuated immunogenicity of islets. This finding is clinically a matter of great import from a viewpoint of side effects of medication. Clinically, several different kinds of immunosuppressive medications have been used together to protect islets from the recipients’ immune reactions. However, because of their side effects, medication regimens such as “Edmonton protocol” and combined therapies of several medications have been currently developed to reduce the dosage and sorts of medications according to the different time period after islet transplantation.

Since most of transplanted islets were generally rejected within 2 weeks, 3 mg/kg of CsA was administered for the first 2 weeks in the recipients [16,17]. In the case of unmodified islet transplantation, islets were completely rejected within 2 weeks before the treatment of CsA was completed. This result indicates that 3 mg/kg of CsA was not sufficient for protection when unmodified islets are transplanted. On the other hand, when PEGylated islets were transplanted, islets survived for 100 days in three out of seven rats; the remaining four rats survived for 30 days on the average despite the fact that CsA treatment was stopped after 2 weeks. This interesting result is produced by employing the chemically conjugated PEG and the CsA treatment simultaneously, which produced a synergistic effect of attenuating the immunogenicity of islets. That is, the conjugated PEG blocked the infiltration of immune cells that would have initiated a major direct cellular immune reaction and the treatment of CsA inhibited the activation of immune cells.
We already investigated in vivo that long-term immunoprotection of transplanted islets was not sufficient by PEGylation of islets [15]. The reason is that endocrine cells such as pancreatic islets can initiate immune reaction by any kinds of secretion from the transplanted cells. Generally, the host’s immune cells directly act on the transplanted islets. Also, it is reported that immune cells can be activated by secretion from the transplanted islets. After the treatment of CsA was stopped, therefore, the PEGylated islets in all of recipients could not survive for a long time. This dysfunction of the PEGylated islets might be indirectly affected by the immune cells gathered around islets. In our histological analysis, immune cells were gathered around the PEGylated islets without infiltration. In this circumstance, immune cells might have been chronically activated by secretion from the islets even though they did not infiltrate into the islets. Therefore, CsA should be continuously administered even after the first 2 weeks.

In conclusion, the PEGylated islets in the recipients could survive for a long time even with only one kind of immunosuppressive medication, CsA and the dose of the medication could be significantly reduced. Therefore, we propose that this alternative immunoprotective remedy of combining PEGylation of islets with immunosuppressive medication would be highly effective in clinical islet transplantation.

References